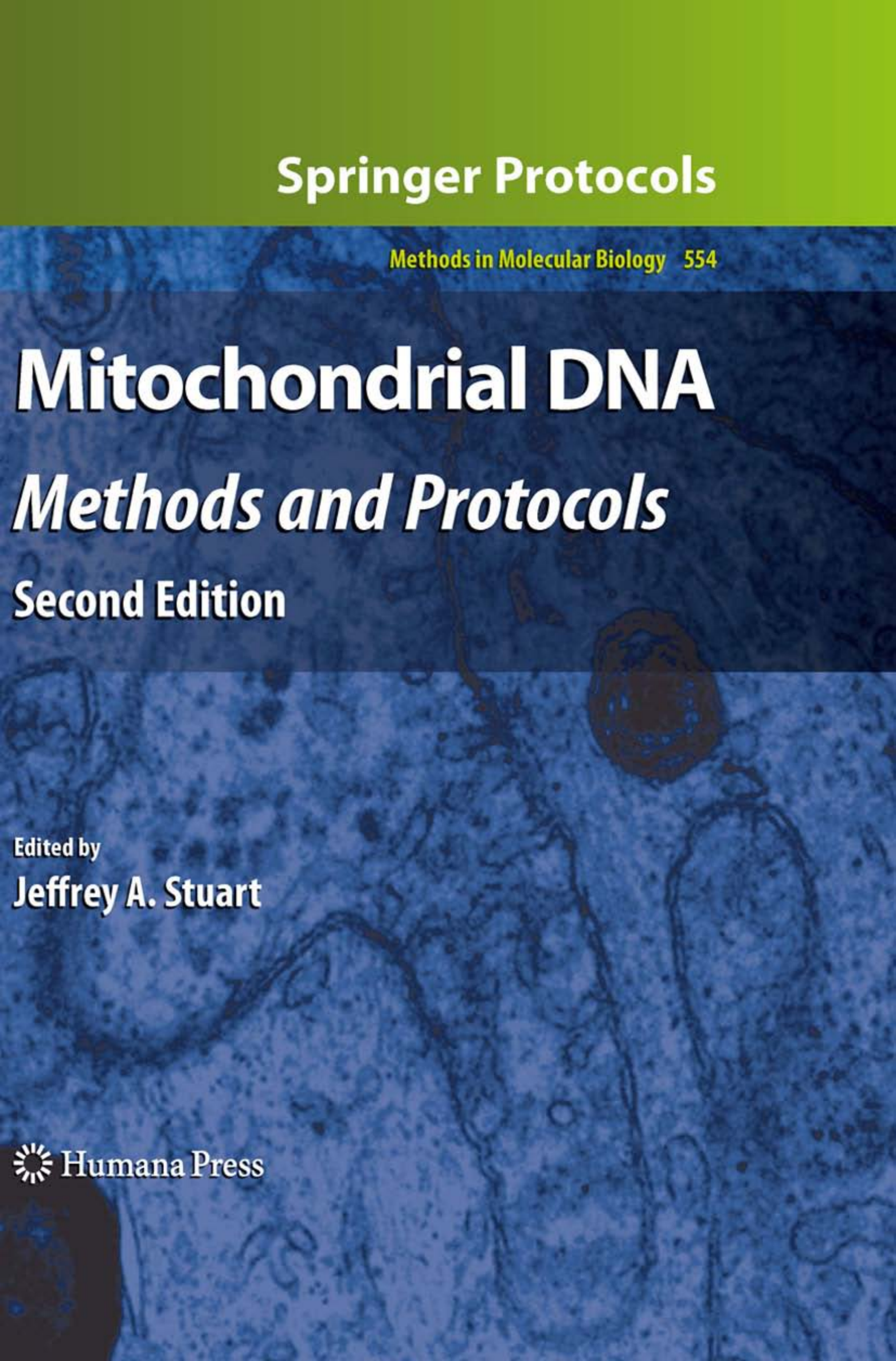
Springer Protocols

Methods in Molecular Biology 554

Mitochondrial DNA

Methods and Protocols

Second Edition



Edited by

Jeffrey A. Stuart

Humana Press

METHODS IN MOLECULAR BIOLOGY™

Series Editor
John M. Walker
School of Life Sciences
University of Hertfordshire
Hatfield, Hertfordshire, AL10 9AB, UK

For other titles published in this series, go to
www.springer.com/series/7651

Mitochondrial DNA

Methods and Protocols

Edited by

Jeffrey A. Stuart

Department of Biological Sciences, Brock University,
St. Catharines, ON, Canada

Humana Press

Editor
Jeffrey A. Stuart
Department of Biological Sciences
Brock University
500 Glenridge Avenue
Cpy Catharines ON L2S 3A1
Canada
jstuart@brocku.ca

Series Editor
John M. Walker
University of Hertfordshire
Hatfield, Herts
UK

ISSN 1064-3745 e-ISSN 1940-6029
ISBN 978-1-934115-60-2 e-ISBN 978-1-59745-521-3
DOI 10.1007/978-1-59745-521-3
Springer Dordrecht Heidelberg London New York

Library of Congress Control Number: 2009926583

Preface

Since the publication of the first edition of *Mitochondrial DNA: Methods and Protocols* in 2002, the number of unique heritable mtDNA mutations recognized as being associated with bioenergetic dysfunction, cell death, and disease has grown apace. At the same time, our understanding of the basic biology of somatic mtDNA mutations has improved. These ongoing advancements are due largely to the continuous development and improvement of techniques and approaches for studying the biology of mitochondria and their DNA. In this second edition of *Mitochondrial DNA: Methods and Protocols*, specialists from eight countries share their expertise by providing detailed protocols for studying many aspects of mtDNA.

This volume is divided into three sections. The first contains protocols that can be used to study the transduction of information from mtDNA to functionally active respiratory complexes. Included in this section are protocols for investigating the nucleoid proteome, mtDNA packaging, replication, transcription, and respiratory complex synthesis. In this section, methods for studying polymerase gamma mutations associated with mitochondrial disorders are also provided. The second section focuses on mitochondrial reactive oxygen species (ROS) production, mtDNA damage, and its repair. Included are descriptions of unique experimental systems for manipulating mtDNA repair capacities and evaluating the outcome. The application of such methods will improve our understanding of the basic biology of mtDNA damage, repair, and mutation. Finally, in the third section, in recognition of the observation that debilitating somatic mtDNA mutations underlie some of the bioenergetic deficits observed in age-associated disease, exciting new approaches for identifying and quantifying heteroplasmic mtDNA mutations are presented.

This volume contains detailed descriptions both of established techniques that continue to be usefully applied, and of some very recently developed approaches that hold great potential to improve our understanding of mtDNA biology. As such, graduate students, postdoctoral fellows, and established investigators should all find herein useful information presented in a straightforward manner with sufficient detail to be replicated in their own laboratories. I thank all of the authors who contributed their expertise and detailed protocols to this volume for their hard work, dedication, and patience.

Jeffrey A. Stuart

Contents

Contributors

Sylvie Bannwarth • *Department of Medical Genetics, Archet 2 Hospital, CHU Nice, France; IGMRC, FRE CNRS / UNSA 3086, Nice Sophia-Antipolis University, Nice, France*

Natalya Bodyak • *Cequent Pharmaceuticals Inc., Cambridge, MA, USA*

Daniel F. Bogenhagen • *Department of Pharmacological Sciences, University at Stony Brook, Stony Brook, NY, USA*

Martin D. Brand • *Buck Institute of Aging, Novato, CA, USA*

Palmiro Cantatore • *Dipartimento di Biochimica e Biologia Molecolare "Ernesto Quagliariello", Università degli Studi di Bari, Bari, Italy; Istituto di Biomembrane e Bioenergetica, CNR, Bari, Italy*

Sam W. Chan • *Department of Surgery, Division of Urology, McGill University Health Centre and Research Institute, Montreal, QC, Canada*

Sherine S. L. Chan • *Mitochondrial DNA Replication Group, Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC, USA*

Junjian Z. Chen • *Department of Surgery, Division of Urology, McGill University Health Centre and Research Institute, Montreal, QC, Canada*

William C. Copeland • *Mitochondrial DNA Replication Group, Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC, USA*

Stefania Deceglie • *Dipartimento di Biochimica e Biologia Molecolare "Ernesto Quagliariello", Università degli Studi di Bari, Bari, Italy*

Allison W. Dobson • *Department of Cell Biology and Neuroscience, College of Medicine, University of South Alabama, Mobile, AL, USA*

Paul W. Doetsch • *Emory University School of Medicine, Atlanta, GA, USA*

Nicole A. Doudican • *New York University School of Medicine, New York, NY, USA*

Konstantin Ebralidze • *Engelhard Institute of Molecular Biology, RAS, Moscow, Russia*

Atsushi Fukuoh • *Department of Clinical Chemistry and Laboratory Medicine, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan*

Maria Nicola Gadaleta • *Dipartimento di Biochimica e Biologia Molecolare "Ernesto Quagliariello", Università degli Studi di Bari, Bari, Italy*

Steffi Goffart • *Institute of Medical Technology, University of Tampere, Tampere, Finland*

Lyra M. Griffiths • *Emory University School of Medicine, Atlanta, GA, USA*

Tricia J. Cluett • *MRC Mitochondrial Biology Unit, Wellcome Trust/MRC Building, Cambridge, UK*

Richard H. Haas • *Department of Neurosciences and Pediatrics, University of California San Diego, La Jolla, CA, USA*

Kazunari Hashiguchi • *Laboratory of Radiation Biology, Graduate School of Science, Kyoto University, Japan*
Ian J. Holt • *MRC Mitochondrial Biology Unit, Wellcome Trust/MRC Building, Cambridge, UK*
Laurie S. Kaguni • *Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI, USA*
Dongchon Kang • *Department of Clinical Chemistry and Laboratory Medicine, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan*
Christopher A. Koczor • *Department of Cell Biology and Neuroscience, College of Medicine, University of South Alabama, Mobile, AL, USA*
Konstantin Khrapko • *Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA*
Yevgenya Kraytsberg • *Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA*
Adrian J. Lambert • *MRC Mitochondrial Biology Unit, Cambridge, UK*
Scot C. Leary • *Montreal Neurological Institute and Department of Human Genetics, McGill University, Montreal, QC, Canada*
Susan P. Ledoux • *Department of Cell Biology and Neuroscience, College of Medicine, University of South Alabama, Mobile, AL, USA*
Kok Seong Lim • *Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA*
Matthew J. Longley • *Mitochondrial DNA Replication Group, Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC, USA*
Christopher K. Mathews • *Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR, USA*
Susan H. Myerow • *Decision Biomarkers, Waltham, MA, USA*
Yusaku Nakabeppu • *Division of Neurofunctional Genomics, Department of Immunology and Neuroscience, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan*
Robert K. Naviaux • *Department of Pediatrics and Medicine, University of California San Diego, La Jolla, CA, USA*
Alexander Nicholas • *Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA*
Mizuki Ohno • *Division of Neurofunctional Genomics, Department of Immunology and Neuroscience, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan*
Sugako Oka • *Division of Neurofunctional Genomics, Department of Immunology and Neuroscience, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan*
Marcos T. Oliveira • *Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI, USA*
Melissa M. Page • *Department of Biological Sciences, Brock University, St. Catharine's, ON, Canada*
Véronique Paquis-Flucklinger • *Department of Medical Genetics, Archet 2 Hospital, CHU Nice, France; IGMRC, FRE CNRS / UNSA 3086, Nice Sophia-Antipolis University, Nice, France*

Paola Loguercio Polosa • *Dipartimento di Biochimica e Biologia Molecolare "Ernesto Quagliariello", Università degli Studi di Bari, Bari, Italy*
Vincent Procaccio • *Center for Molecular and Mitochondrial Medicine and Genetics, University of California, Irvine, CA, USA*
Aurelio Reyes • *MRC Mitochondrial Biology Unit, Wellcome Trust/MRC Building, Cambridge, UK*
Marina Roberti • *Dipartimento di Biochimica e Biologia Molecolare "Ernesto Quagliariello", Università degli Studi di Bari, Bari, Italy*
Florin Sasarman • *Montreal Neurological Institute and Department of Human Genetics, McGill University, Montreal, QC, Canada*
Gerald S. Shadel • *Yale University School of Medicine, New Haven, CT, USA*
Inna N. Shokolenko • *Department of Cell Biology and Neuroscience, College of Medicine, University of South Alabama, Mobile, AL, USA*
Leslie A. Smith • *Mitochondrial DNA Replication Group, Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC, USA*
Janet W. Snyder • *Department of Cell Biology and Neuroscience, College of Medicine, University of South Alabama, Mobile, AL, USA*
Hans Spelbrink • *Institute of Medical Technology, University of Tampere, Tampere, Finland*
Jeffrey A. Stuart • *Department of Biological Sciences, Brock University, St. Catharine's, ON, Canada*
Linda J. Wheeler • *Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR, USA*
Glenn L. Wilson • *Department of Cell Biology and Neuroscience, College of Medicine, University of South Alabama, Mobile, AL, USA*
Takehiro Yasukawa • *MRC Mitochondrial Biology Unit, Wellcome Trust/MRC Building, Cambridge, UK*
Qiu-Mei Zhang-Akiyama • *Laboratory of Radiation Biology, Graduate School of Science, Kyoto University, Japan*
Tawn D. Ziebarth • *Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI, USA*

Chapter 1

Biochemical Isolation of mtDNA Nucleoids from Animal Cells

Daniel F. Bogenhagen

Abstract

Mitochondrial DNA (mtDNA) in animal cells is organized into clusters of 5–7 genomes referred to as nucleoids. Contrary to the notion that mtDNA is largely free of bound proteins, these structures are nearly as rich in protein as nuclear chromatin. While the purification of intact, membrane-bound mitochondria is an established method, relatively few studies have attempted biochemical purification of mtDNA nucleoids. In this chapter, two alternative methods are presented for the purification of nucleoids. The first method yields the so-called native nucleoids, using conditions designed to preserve non-covalent protein–DNA and protein–protein interactions. The second method uses formaldehyde to crosslink proteins to mtDNA and exposes nucleoids to treatment with harsh detergents and high salt concentrations.

Key words: mtDNA, mitochondria, nucleoids, chromatin IP.

1. Introduction

The maintenance of mitochondria depends on the mitochondrial DNA (mtDNA) for synthesis of several protein components of the oxidative phosphorylation machinery. In mammals, 13 proteins are encoded in the mtDNA genome along with 12S and 16S rRNAs and a complete, albeit minimal, complement of 22 tRNAs. The 13 proteins synthesized on mitochondrial ribosomes are incorporated into respiratory complexes I, III, IV, and V along with approximately 67 nucleus-encoded subunits *(1)*. Cells typically maintain thousands of copies of mtDNA distributed among hundreds of organelles that exchange components through active cycles of fusion and fission *(2, 3)*. These mtDNA genomes are organized in nucleoids containing 2–10 genomes, as indicated in **Table 1.1** *(4)*.

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_1 Springerprotocols.com

Table 1.1
Number of nucleoids and mtDNA molecules per nucleoid in various cell types

Cell type	Nucleoids/cell	mtDNA/cell	mtDNA/focus	Reference
ECV304	480	3,500	~7	*(5)*
143B	553	4,126	7.5	*(7)*
HeLa	466	2,637	5.7	*(7)*

Using gold-labeled anti-DNA antibodies, Iborra et al. *(5)* have estimated that mtDNA nucleoids have an average diameter of 70 nm, indicating that several mtDNA genomes are very tightly packaged. CsCl density gradient analysis of crosslinked mtDNA nucleoid preparations yields a density of approximately 1.5 g/ml, consistent with an approximately equal content of protein and DNA. Thus, a nucleoid with 7 mtDNA genomes may be expected to have a mass of about 140 MDa, half of which is protein.

Time-lapse imaging has shown that nucleoids in mammalian cells are relatively stable structures rather evenly spaced within the mitochondrial reticulum *(6, 7)*. This has encouraged several laboratories to try to biochemically isolate nucleoids to identify proteins associated with the mtDNA genome. Such studies have routinely identified two well-established mtDNA-binding proteins as nucleoid markers. These are the mitochondrial single-stranded DNA-binding protein, mtSSB *(8)*, a tetramer of 16 kDa subunits homologous to *Escherichia coli* SSB, and TFAM, an abundant HMG-box protein that binds duplex DNA with limited sequence specificity. Kang's group has estimated that HeLa cells contain as many as 3,000 copies of mtSSB and 1,700 copies of TFAM per mtDNA molecule *(9)*. However, other groups have suggested that the TFAM content is considerably lower *(10)*. Both of these proteins occur with a pool of free proteins in equilibrium with the fraction bound to mtDNA. This is likely to be true for other proteins involved in mtDNA replication and transcription as well. For any individual nucleoid-associated protein, the fraction of protein retained in a biochemical preparation of nucleoids is expected to vary depending on the conditions used in the fractionation procedure. Investigators interested in studying mtDNA nucleoid proteins must be cognizant of this limitation.

A wide variety of proteins have been found in association with mtDNA nucleoids isolated from either yeast or animal cell sources. The list includes several proteins with known roles in mtDNA replication and transcription, which may be considered "positive controls," along with a number of chaperones and metabolic

proteins. Other reviews have summarized the results of such studies; it is not the goal of this methods' chapter to recapitulate or interpret these results. Instead, this chapter will present two alternative procedures for biochemical preparation of nucleoids. Kaufman et al. *(11)* have previously presented a protocol for formaldehyde crosslinking of proteins to mtDNA in this series. A comparison of the nucleoid protein composition obtained using gentle handling of native (non-crosslinked) complexes with that obtained using formaldehyde crosslinking coupled with harsh solution conditions provides significant insight into the structure of nucleoids. The results of this comparison suggest that nucleoids can be considered to contain a core of tightly associated proteins, including TFAM, mtSSB, mitochondrial DNA and RNA polymerases, surrounded by additional metabolic proteins and chaperones. At this juncture, it is not a simple matter to decide if a protein contained in a biochemical preparation enriched in nucleoids is an "authentic" nucleoid protein. It is hoped that these methods will contribute to further efforts to identify and characterize proteins important for the maintenance and expression of mtDNA.

Both methods described below begin with the preparation of highly purified mitochondria based on a protocol presented previously in this series *(2)*. This protocol is one of the many available in the literature, and it is not possible to review it in detail in the space provided. Methods for mitochondrial purification typically take advantage of the membrane-delimited nature of the organelle and its distinctive size and density. Following cell disruption, mitochondria must be handled in isotonic buffers like the mannitol–sucrose buffer described below to avoid organelle rupture. Detergents must be rigorously avoided during mitochondrial purification, although differential extraction with digitonin is often used to prepare mitoplasts lacking the outer membrane. In some cases, mitochondria can be purified by differential sedimentation, particularly if the source is enriched in mitochondria. However, it is always desirable to use a purification procedure that employs centrifugation in a gradient containing sucrose or other separation media in order to remove contaminating organelles of both higher and lower densities. Finally, it is critically important to recognize that even density gradient purified mitochondria are routinely contaminated with associated nuclear DNA. This contamination is well known to researchers with experience in biochemical isolation of mtDNA as ethidium bromide–CsCl gradients routinely show a heavy upper band of nuclear DNA *(2)*. Nuclear DNA contamination can be reduced, but not eliminated, by nuclease treatment of mitochondria at an intermediate stage in purification, as shown in the agarose gel analysis in (**Fig. 1.1**). The undigested DNA in a typical HeLa mitochondrial preparation contains a broad zone of high molecular weight DNA that appears to run as a band since it has been sheared by pipetting to a relatively uniform size of about 20–30 kb. When

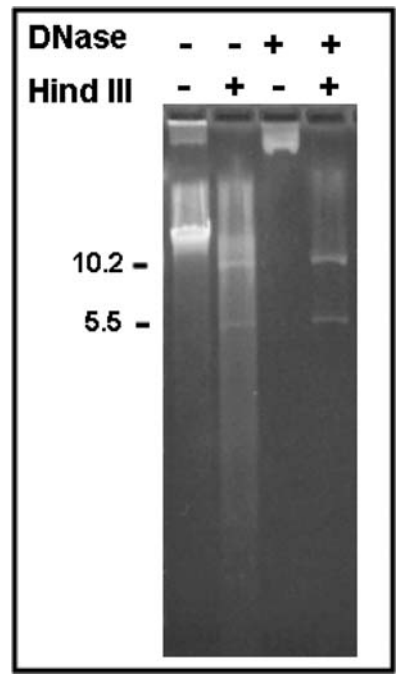


Fig. 1.1. Agarose gel analysis of mtDNA. Lanes 1 and 2 show the total DNA in a mitochondrial preparation without and with digestion by restriction endonuclease *Hin*dIII. Lanes 3 and 4 show a similar analysis of DNA from nuclease-treated mitochondria.

the DNA is digested with restriction endonuclease *Hin*dIII, the expected mtDNA fragments of 10.2 and 5.5 kb are resolved over a smear of nuclear DNA fragments. Treatment of mitochondria with DNase I at an intermediate step in purification reduces this background smear. However, low molecular weight fragments of nuclear DNA are retained in the mitochondrial fraction (not shown in **Fig. 1.1**). If the extent of nuclease digestion is sufficient, most of these fragments will be removed by a stringent sizing step that takes advantage of the large size of mtDNA nucleoids. This DNA contamination can introduce some non-mitochondrial proteins, such as histones, into nucleoid preparations.

2. Materials

2.1. MSH and Nuclease Treatment Buffer

Both buffers include protease inhibitors at final concentrations of 5 μg/ml leupeptin, 2 μg/ml E64, 1 μM pepstatin, and 0.2 μM PMSF.

1. MSH: 210 mM mannitol, 70 mM sucrose, 20 mM HEPES, pH 8, 2 mM EDTA, 2 mM DTT

2. Nuclease treatment buffer: 1X MSH containing 5 mM glutamic acid, 5 mM Na malate, 60 mM KCl, 10 mM $MgCl_2$, 1 mM K_2HPO_4, and 250 μg/ml BSA.
3. Nuclease wash buffer: 0.8 M sucrose, 20 mM HEPES, pH 8, 2 mM EDTA, 2 mM DTT.

2.2. Anti-TFAM and Anti-mtSSB Antibody Columns for Affinity Purification

1. Recombinant human TFAM and mtSSB are cloned in the pET22b+ vector and expressed as C-terminally His-tagged proteins in *E. coli* using standard techniques (*see* **Note 1**).
2. His-Trap columns or other immobilized Ni- or Co-affinity matrices.
3. MonoS columns for cation exchange chromatography.
4. MonoS elution buffer: 20 mM HEPES, pH 8, 5% glycerol, 1 mM EDTA, and 2 mM DTT plus protease inhibitors. Proteins are eluted with a gradient of KCl in this buffer.
5. Affigel 10 matrix (BioRad) for protein coupling. At least 1 mg of protein coupled per 1 ml of beads.
6. Antibodies directed against human TFAM or mtSSB (immunopurified from crude rabbit polyclonal antisera prepared in the Bogenhagen laboratory). The crude sera reacted specifically with the respective proteins in Western blots at 1:5,000–1:20,000 dilution.
7. Column wash buffer: PBS (10 ml).
8. Affigel 10 elution buffer: 150 mM NaCl, 100 mM glycine, pH 2.4.
9. Antibody collection buffer: 200 μl of 1 M Na_2HPO_4, pH 8.
10. Magnetic tosyl-activated Dynabeads (Dynal).
11. Immunoaffinity purification buffer: 30 mM HEPES, pH 8, 5% glycerol, 2 mM DTT, 70 mM NaCl, 1 mM EDTA with protease inhibitors.
12. Gradient buffer: 30 mM HEPES, pH 8, 2 mM EDTA, 2 mM DTT, 20 mM NaCl, 0.5% Triton X-100, and protease inhibitors.
13. Picogreen buffer: 10 mM Tris, pH 8, 1 mM EDTA.
14. Bead wash buffer: 20 mM HEPES, pH 8, 2 mM EDTA, 2 mM DTT, 70 mM NaCl, 0.5% Triton X-100.

2.3. Solutions for Sedimentation of Formaldehyde Crosslinked Nucleoids

1. Polyallomer tubes.
2. Beckman SW41 rotor.
3. Gradient solutions: 20 mM HEPES, pH 8, 2 mM EDTA, 100 mM NaCl, 0.5% sarkosyl, 2 mM DTT, and protease inhibitors. Upper gradient solution also contains 15% glycerol. Lower gradient solution also contains 30% glycerol and 30% Nycodenz.

4. 10% H_2CO.
5. 2 M Glycine, pH 7.3.
6. Nucleoid buffer: 20 mM HEPES, pH 8, 2 mM DTT.
7. Stock CsCl solution of density 1.71 g/ml.
8. TE buffer: 10 mM Tris, pH 8, 1 mM EDTA.
9. Tris–glycine sample loading buffer: 10% glycerol, 20 mM DTT, 62.5 mM Tris, pH 6.8, 2% SDS.

3. Methods

An overview of the native and crosslinked nucleoid preparations is shown in **Fig. 1.2**. The native immunoaffinity purification procedure has the practical disadvantage that it requires anti-TFAM or anti-mtSSB affinity reagents that are not commercially available in large quantities. He et al. *(12)* have used an alternative procedure using an immobilized prokaryotic DNA-binding protein to affinity-purify nucleoids. This procedure, however, did not recover a large fraction of known mtDNA interacting proteins reported by Wang and Bogenhagen *(13)* using anti-TFAM or anti-mtSSB columns.

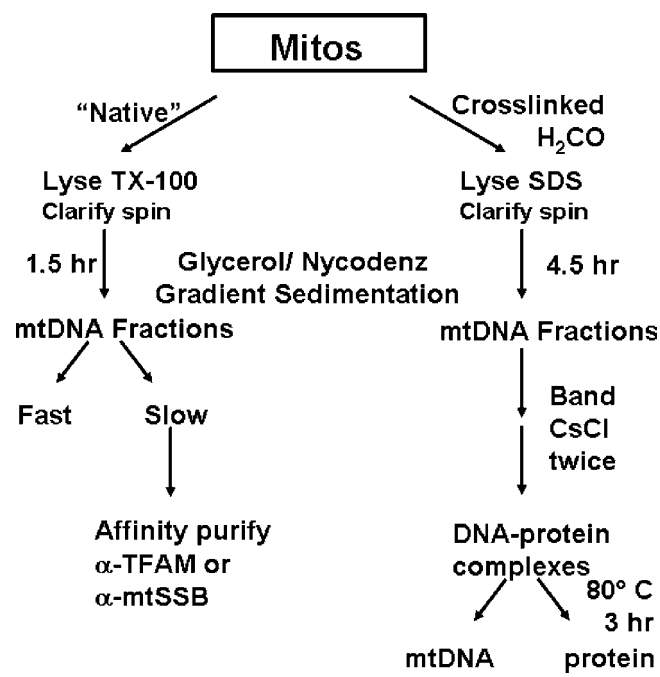


Fig. 1.2. Overview of schemes for purification of native and crosslinked nucleoids.

3.1. Nuclease Treatment of Mitochondria

1. Mitochondria are prepared as described by Bogenhagen *(14)* and as modified by Wang and Bogenhagen *(13)*. The principal changes are the use of a Percoll–Nycodenz gradient in place of a sucrose step gradient and the introduction of a nuclease treatment. Mitochondria are withdrawn from the gradient, diluted with three volumes of MSH buffer, and sedimented in a swinging bucket Sorvall HB-6 rotor at 10,000 rpm (16,000 g) for 10 min.
2. The mitochondrial pellet from 4 l of HeLa cells is resuspended in 5 ml of nuclease buffer (*see* **Section 2.1**, Step 1) and treated with 400 μg of DNase I (Sigma type II, 2,000 U/mg) plus 4 μl (1,000 U) of Benzonase (Novagen) for 15 min at 37°C (*see* **Note 2**).
3. EDTA is added to a final concentration of 20 mM to stop the nuclease.
4. The mitochondrial suspension is layered over 6 ml of nuclease wash buffer and centrifuged as above to pellet mitochondria.
5. The supernatant is carefully removed by suction to remove as much of the nuclease as possible.
6. Mitochondria are resuspended in 5 ml of MSH and repelleted as above.

3.2. Immunoaffinity Purification of Native Nucleoids

The goal of purification of native nucleoids is to lyse mitochondria with non-ionic detergent at moderate ionic strength in an attempt to preserve the structure of nucleoids. The method consists of sedimentation to select large complexes followed by immunoaffinity purification.

1. The mitochondrial pellet from **Section 3.1**, Step 1 is resuspended in 800 μl of immunoaffinity purification buffer.
2. Two hundred microliters of 6% Triton X-100 is added to achieve a final concentration of 1.2%.
3. The mitochondrial suspension clarifies quickly after the addition of detergent. The solution is incubated on ice with intermittent mixing for 5 min, then centrifuged for 3 min in a refrigerated microcentrifuge at 3,000 g to remove insoluble material.
4. Aliquots of 500 μl of the supernatant are layered over each of the two preformed 10 ml 17–45% glycerol gradients in gradient buffer.
5. The gradients are layered over a pad of 700 μl of 30% glycerol and 30% Nycodenz.
6. Gradients are spun at 33,000 rpm for 1.5 h at 4°C in a Beckman SW41 rotor (186,000 g).
7. A fraction of nucleoids sediment very rapidly to reach the pad (as described in Wang and Bogenhagen *(15)*), while the remainder is distributed in the lower half of the gradient.

Nucleoids in the rapidly sedimenting fraction were found to be associated with cytoskeletal proteins such as vimentin and actin and are not used for immunoaffinity purification.

8. Glycerol gradient fractions containing nucleoids are identified by fluorescent staining with the DNA-specific dye, Picogreen (Invitrogen).
9. Ten-microliter samples of each fraction are added to 100 µl of Picogreen buffer containing a 1:300 dilution of Picogreen (Invitrogen) in a 96-well microtiter dish.
10. Fluorescence is measured using a FluorImager 595 (GE Healthcare) with excitation at 488 nm and emission at 530 nm.
11. Glycerol gradient fractions containing mtDNA nucleoids are incubated with anti-TFAM or anti-mtSSB antibody columns (*see* **Section 2.2**) as described *(15)*.
12. Six hundred microliters of glycerol gradient is incubated with 5×10^8 antibody-coated beads for 90 min with continuous mixing on a rotator at 4°C.
13. Beads are collected on a magnet and washed thoroughly with three 1 ml changes of bead wash buffer.
14. Specifically bound proteins are eluted with successive 500 µl washes in bead wash buffer containing 0.5% SDS in place of Triton X-100.
15. Proteins are analyzed by standard SDS-PAGE and either in-gel digestion for peptide sequencing or immunoblotting.

3.3. Preparation of Formaldehyde Crosslinked Nucleoids

1. Nuclease-treated mitochondria are prepared through **Section 3.1**, Step 1 above and are resuspended in 900 µl of MSH.
2. Hundred microliters of 10% H_2CO is added and mitochondria are incubated at 4°C for 30 min.
3. Hundred microliters of 2 M glycine, pH 7.3, is added for an additional 5 min to react with excess H_2CO.
4. Mitochondria are lysed by the addition of 200 µl of 20% SDS to thoroughly denature proteins and disrupt non-covalent protein–protein interactions.
5. The lysate is clarified by centrifugation at 3,000 g for 5 min in a microcentrifuge.
6. Six hundred and fifty microliters of lysate is layered onto each of the two composite gradients containing 0.5% Na sarcosinate (Sarkosyl) (*see* **Section 2.1**, Step 3, and **Note 3**).
7. Gradients are centrifuged for 4.5 h at 4°C at 38,000 rpm (247,000 g) in the SW41 rotor.
8. Fractions are collected and DNA is assayed by Picogreen fluorescence as described in **Section 3.2** (*see* **Note 4**).

9. Gradient fractions containing mtDNA crosslinked to protein are pooled, diluted with three volumes of nucleoid buffer, layered over a 700 µl pad of CsCl solution at density 1.71 g/ml, and recentrifuged for 6–8 h at 4°C and 38,000 rpm (247,000 g) to concentrate the nucleoids, which sediment to the CsCl interface. This pelleting spin concentrates the sample and helps to remove free protein.
10. The bottom 1 ml of the final sedimentation to the CsCl interface is adjusted to density 1.5 g/ml and a final volume of 3 ml using a stock CsCl solution of density 1.71 g/ml and centrifuged to equilibrium in a Beckman SW60 Ti rotor at 40,000 rpm (215,000 g) for 40 h at 18°C. The CsCl gradient is overlayed with mineral oil to prevent evaporation during the run.
11. Fractions containing DNA are identified by Picogreen staining as above. It has been found to be desirable to repeat the equilibrium centrifugation step for higher purity (*see* **Note 5**).
12. At each stage, CsCl concentrations are determined by refractometry with reference to standard tables relating refractive index to density. Nucleoids are found in CsCl gradient fractions with a density of approximately 1.55 g/ml.
13. Fractions from the CsCl gradient are diluted with two volumes of TE buffer and are precipitated by the addition of ethanol to 70%.
14. Samples are stored at –20°C for 2 h and centrifuged at 16,000 g for 10 min in a refrigerated microcentrifuge. The DNA–protein complexes precipitate under these conditions in much the same way that free DNA is precipitated in ethanol. Gradient fractions at higher density may develop a substantial CsCl pellet under these conditions. If this is observed, the pellets are resuspended in 500 µl of TE and the precipitation with two volumes of ethanol is repeated.
15. The final precipitated fractions are rinsed with 70% ethanol to remove residual CsCl. Samples are dried briefly in a SpeedVac centrifugal lyophilizer and resuspended in the desired volume of 1X Tris–glycine sample loading buffer.
16. The samples are heated at 80°C for 3 h to reverse crosslinks (*see* **Note 6**).
17. Aliquots can now be analyzed by SDS-PAGE directly or extracted with phenol–chloroform to permit DNA analysis by restriction digestion or PCR. In our experience with a preparation beginning with 4 l of HeLa suspension cells, 10% of each fraction is sufficient for reliable detection of mtDNA following restriction digestion or of associated proteins following SDS-PAGE, in-gel digestion, and peptide analysis by LC–MS/MS.

Both the purification methods reported here have identified numerous proteins in HeLa cell mtDNA nucleoids. The native immunoaffinity purification outlined here has been reported to identify 21 proteins that were recovered when *both* anti-TFAM and anti-mtSSB antibody columns were used *(13)*. These have been grouped into a series of classes by Wang et al. *(13)*, the largest of which is a collection of 11 proteins that function in mtDNA maintenance or have DNA-binding activity. Among these are two helicases not previously known to reside in nucleoids, the Suv3-like helicase, which had been characterized as a mitochondrial helicase *(15)*, and the DHX30 helicase, which had not previously been identified as a mitochondrial protein. All of these DNA metabolic- and DNA-binding proteins were also identified in formaldehyde crosslinked proteins *(16)*. However, while the formaldehyde crosslinking method did lead to identification of a small number of metabolic proteins and chaperones, the majority of proteins in these classes identified in native nucleoids were not recovered in formaldehyde crosslinked nucleoids under harsh handling conditions. This suggests that the chaperones and metabolic proteins are bound to nucleoids as an outer protein layer not easily accessible to the DNA by formaldehyde crosslinking.

It maybe anticipated that further modifications to these protocols, coupled with more sensitive mass spectrometry methods, may further define the structure of mtDNA nucleoids and contribute to a better understanding of the organization and inheritance of mtDNA.

4. Notes

1. Expression plasmids for human TFAM and mtSSB are available from the author upon request as are small quantities of antibodies directed against these proteins. Due to limited stocks, quantities of antibodies sufficient for generation of immunoaffinity columns are not available.
2. Because DNase I is an endonuclease, very extensive digestion is required to substantially fragment DNA, rather than simply introducing nicks. Addition of a second nuclease enhances fragmentation. Benzonase is preferred to micrococcal nuclease since the latter requires Ca as a co-factor, and Ca is known to be toxic to mitochondria.
3. Sodium sarcosinate is used here as a detergent with ionic character and greater chaotropic nature than Triton X-100 and as an alternative to SDS, which is incompatible with CsCl centrifugation due to the insolubility of Cs-dodecyl sulfate.

4. Contaminating fragments of nuclear DNA crosslinked to protein are found at the trailing edge of the DNA distribution following sedimentation. Fractions from the top of the gradient should be excluded from the continued preparation. Analysis of gradient fractions for mtDNA using PCR or restriction digestion is recommended.
5. Contaminating fragments of nuclear DNA crosslinked to protein are found at lighter density in the CsCl gradient.
6. Crosslink reversal can also be performed for 5 h at 65°C.

Acknowledgments

We thank Yousong Wang for help in the development of the native nucleoid purification and Stephanie Burke for technical assistance. These methods were developed with support of NIH grants R01-GM029681 and R01-ES012039.

References

1. DiMauro, S., and E. Schon. (2003) Mitochondrial respiratory-chain diseases. New England J Med **348,** 2656–2668.
2. Bogenhagen, D., and D. A. Clayton. (1974) The number of mitochondrial deoxyribonucleic acid genomes in mouse L and human HeLa cells. J. Biol. Chem. **249,** 7991–7995.
3. Chan, D. C. (2006) Mitochondrial fusion and fission in mammals. Ann. Rev. Cell Dev. Biol. **22,** 79–99.
4. Chen, X. J., and R. A. Butow. (2005) The organization and inheritance of the mitochondrial genome. Nat Rev Genet **6,** 815–825.
5. Iborra, F., H. Kimura, and P. Cook. (2004) The functional organization of mitochondrial genomes in human cells. BMC Biology **2,** 9.
6. Garrido, N., L. Griparic, E. Jokitalo, J. Wartiovaara, A. M. van der Bliek, and J. N. Spelbrink. (2003) Composition and Dynamics of Human Mitochondrial Nucleoids. Mol. Biol. Cell **14,** 1583–1596.
7. Legros, F., F. Malka, P. Frachon, A. Lombes, and M. Rojo. (2004) Organization and dynamics of human mitochondrial DNA. J Cell Sci **117,** 2653–2662.
8. Pavco, P. A., and G. C. V. Tuyle. (1985) Purification and General Properties of the DNA-binding Protein (P16) from Rat Liver Mitochondria. J. Cell Biol. **100,** 258–264.
9. Takamatsu, C., S. Umeda, T. Ohsato, T. Ohno, Y. Abe, A. Fukuoh, H. Shinagawa, N. Hamasaki, and D. Kang. (2002) Regulation of mitochondrial D-loops by transcription factor A and single-stranded DNA-binding protein. EMBO Reports **3,** 451–456.
10. Cotney, J., Z. Wang, and G. S. Shadel. (2007) Relative abundance of the human mitochondrial transcription system and distinct roles for h-mtTFB1 and h-mtTFB2 in mitochondrial biogenesis and gene expression. Nucl. Acids Res **35,** 4042–4054.
11. Kaufman, B., S. Newman, P. Perlman, and R. Butow. (2002) Crosslinking of Proteins to mtDNA. Meth. Mol. Biol. **197,** 377–389.
12. He, J., C.-C. Mao, A. Reyes, H. Sembongi, M. Di Re, C. Granycome, A. B. Clippingdale, I. M. Fearnley, M. Harbour, A. J. Robinson, S. Reichelt, J. N. Spelbrink, J. E. Walker, and I. J. Holt. (2007) The AAA+ protein ATAD3 has displacement loop binding properties and is involved in mitochondrial nucleoid organization. J. Cell Biol. **176,** 141–146.
13. Wang, Y., and D. F. Bogenhagen. (2006) Human mitochondrial DNA nucleoids are linked to protein folding machinery and metabolic enzymes at the mitochondrial inner membrane. J. Biol. Chem. **281,** 25791–25802.

14. Bogenhagen, D. F. (2001) Purification of Mitochondria for Enzymes Involved in Nucleic Acid Transactions, p. 199–210. *In* W. Copeland (ed.), Mitochondrial DNA: Methods and Protocols, vol. 197. Humana Press, Totowa, NJ.
15. Minczuk, M., J. Piwowarski, M. A. Papworth, K. Awiszus, S. Schalinski, A. Dziembowski, A. Dmochowska, E. Bartnik, K. Tokatlidis, P. P. Stepien, and P. Borowski. (2002) Localisation of the human hSuv3p helicase in the mitochondrial matrix and its preferential unwinding of dsDNA. Nucl. Acids Res. **30,** 5074–5086.
16. Bogenhagen, D.F., Rousseau, D. and Burke, S. (2008) The layered structure of human mitochondrial DNA nucleoids. J. Biol. Chem. **283,** 3665–3675.

Chapter 2

Analysis of Mitochondrial DNA by Two-Dimensional Agarose Gel Electrophoresis

Aurelio Reyes, Takehiro Yasukawa, Tricia J. Cluett, and Ian J. Holt

Abstract

In higher vertebrates, the DNA of mitochondria takes the form of circular molecules of approximately 16 kbp. These circles are arranged in multigenomic nucleoprotein complexes or nucleoids. It is envisaged that nucleoid superstructure makes a critical contribution to the twin processes of replication and segregation of mtDNA. Replication intermediates can be isolated from cells or solid tissues and separated on agarose gels in two dimensions to reveal a wealth of data on mechanisms of DNA replication. Using this technique we have demonstrated that many molecules of replicating mtDNA have extensive regions of RNA: DNA hybrid in higher vertebrates. More recently, we have extracted mitochondrial nucleoprotein and analyzed it by the same method to derive information on the distribution of DNA-binding proteins on mitochondrial DNA. Here we describe the procedures used to isolate intact mitochondrial replication intermediates from liver and cultured cells of higher vertebrates and the process of separating DNA fragments on neutral two-dimensional agarose gels.

Key words: DNA replication, DNA isolation, humans, mammals, mitochondrial DNA, neutral/neutral two-dimensional agarose gel electrophoresis (N2D-AGE), replication intermediates, vertebrates, nucleoprotein complex, nucleoid.

1. Introduction

Mitochondria generate ATP via respiration; and this process of aerobic ATP production is defunct without the 13 proteins encoded in the mitochondrial genome. Mammalian mitochondrial DNA (mtDNA) is arranged as closed, circular molecules of approximately 16 kbp; mtDNA is not naked and floating free in the mitochondrial matrix, as often depicted in textbooks, but takes the form of multigenomic nucleoprotein complexes or nucleoids.

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_2 Springerprotocols.com

Maintaining a separate chromosome in mitochondria is grossly inefficient, as it requires dozens of gene products for its replication, organization, and expression; the mitochondrial ribosome alone comprises 80 proteins. The cost of maintaining a separate genome in mitochondria can be debilitating or even fatal, when mutations arise in nuclear genes whose products make a critical contribution to mtDNA metabolism *(1, 2)*. There are also numerous pathological mutations of mtDNA itself *(3, 4)*, and the mutant load of mtDNA depends on nuclear factors, at least in cell culture models *(5–7)*. The growing recognition of aberrant mtDNA as a cause of disease has fueled interest in how mtDNA is organized and replicated. The technique of two-dimensional agarose gel electrophoresis has advanced our understanding of both these processes.

1.1. Neutral Two-Dimensional Agarose Gel Electrophoresis

The study of DNA replication was transformed by the development of a method of separating branched molecules based on a combination of structure and mass *(8–16)*. Neutral/neutral two-dimensional agarose gel electrophoresis (N2D-AGE) has been used to map origins of replication in fragments of DNA *(8, 9, 11, 17)*, identify replication pause sites or termini *(18–20)*, determine the direction of replication fork movement *(12, 19, 21)*, and potentially distinguish multiple mechanisms of replication *(22)*. The technique is widely applicable, having been used successfully to study replication in eukaryotes, prokaryotes, and viruses.

N2D-AGE is technically straightforward. It entails separating fragments of DNA, first in a low percentage agarose gel, at low field strength; followed by separation at low temperature and high field strength in a higher percentage agarose gel containing a DNA intercalating dye, such as ethidium bromide (EB) (*see* **Fig. 2.1** and the step-by-step protocol for details). Separation in the first dimension is essentially on the basis of mass, whereas the conditions of second dimension electrophoresis mean that hydrodynamic volume becomes a key factor; consequently, replication intermediates RIs are more retarded than linear molecules during second dimension electrophoresis. The mobility of a particular RI depends on the position of the replication fork(s) within the fragment, i.e., the fraction of the fragment that has been replicated. Hence, the passage of a replication fork(s) through a fragment gives rise to a series of intermediates of different mobility, which form a characteristic arc.

The simplest example is a fragment where a replisome enters at one end and proceeds at a uniform rate to the other end of the fragment, creating a simple fork, or Y, arc (**Fig. 2.2, panel 1**). The arc reaches its apex when the fork is halfway through the fragment; thereafter the fragment becomes increasingly similar to a linear molecule. Immediately prior to separation of the

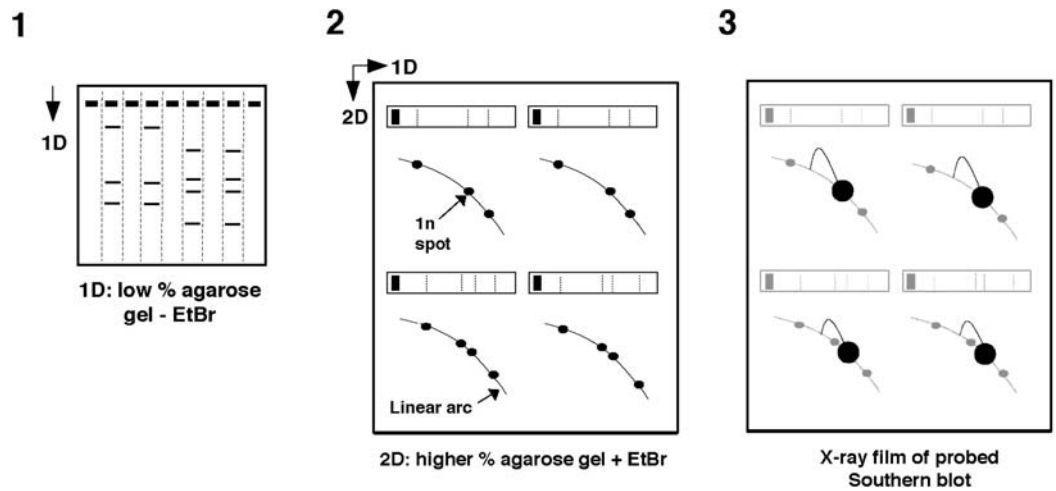


Fig. 2.1. **Schematic diagram of N2D-AGE.** Panel 1 depicts a gel after first dimension electrophoresis, filled black boxes represent wells of the gel, narrow horizontal lines represent linear fragments of double-stranded DNA, dotted vertical lines demarcate the lane to be excised. Panel 2, each 1D gel slice is rotated through 90° and a second gel cast around it, four 1D gel slices are shown on a single two-dimensional gel. After second dimension electrophoresis the linear double-stranded fragments (*black circles*) resolve on a defined arc (*narrow unbroken line*); the position of each fragment after the first dimension separation is shown by a broken faint line in the original 1D gel slice. After transfer to solid support and hybridization to a radiolabeled DNA probe that detects a specific fragment of (mt)DNA one of the 1*n* spots features prominently and its accompanying replication intermediates become visible (panel 3). UV illumination of an ethidium bromide stained two-dimensional gel will reveal only an arc of linear (nuclear) DNA fragments when total cellular DNA is used. In the case of DNA purified from mitochondria, individual fragments of mtDNA (1*n* spots) will be visible.

daughter fragments, the molecule is substantially linear, accordingly it migrates close to the arc of linear molecules, at a point equivalent to twice the mass of the unit length fragment (2*n*) (**Fig. 2.2**, **panel 1**).

If a fragment contains an origin of replication, a so-called bubble structure will form (**Fig. 2.2**, **panel 2**), whenever a replication fork exits the fragment, at either end, the restriction enzyme cleaves the bubble. Bubble arcs are readily distinguishable from Y arcs as they are more retarded in the second dimension and end abruptly. The extent of a bubble arc reveals the information about the location of the origin. However, it is essential to examine a series of overlapping fragments, as a single fragment cannot distinguish differences in the mode of replication (e.g., unidirectional from bidirectional replication); and short bubble arcs, due to origins located close to the end of a fragment, may be indistinguishable from simple Y arcs *(23)*. Composite or other more complicated patterns can also be obtained, depending on factors such as the number and distribution of origins (**Fig. 2.2**, **panel 3**) *(15, 24–26)*, the presence of ribonucleotides in replicating molecules (**Fig. 2.3**) *(27)*, or delayed second-strand synthesis *(28)*.

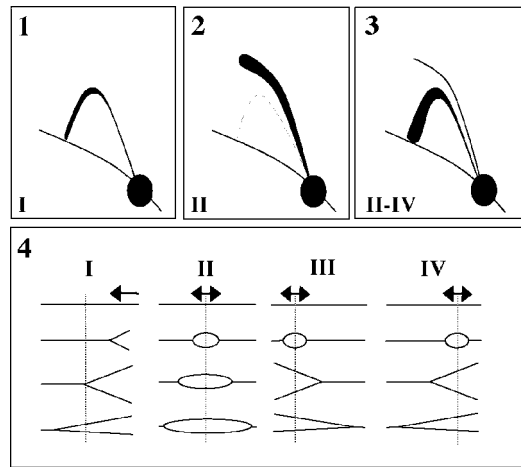


Fig. 2.2. **Different modes of replication produce distinct patterns of replication intermediates on N2D-AGE.** Panels 1–3: schematic patterns of RIs on N2D-AGE. Panel 4, line drawings of structures of RIs. Panel 1, a simple fork or Y-arc, replication initiates outside the fragment, interpreted in 4-I. Panel 2, initiation of bidirectional replication from a discrete origin located at the centre of a fragment giving rise to a complete bubble arc and no fork arc (the position of the fork arc is shown as a faint broken line for reference purposes), as the two forks exit the fragment simultaneously, interpreted in 4-II; note how the bubble arc increases in intensity as it reaches its apex, giving it a "clubheaded" appearance, due to compression. Panel 3, bidirectional initiation from multiple sites across a zone defined by the fragment; in this case initiation at the centre of the fragment is only one of many possibilities, at other initiation sites such as those depicted in 4-III and 4-IV one fork exits the fragment well before the other converting the bubble to a Y structure, thereafter the RIs contribute to the Y arc.

N2D-AGE is routinely combined with restriction endonuclease digestion, prior to electrophoresis, but a range of pretreatments with nucleic acid modifying reagents are also possible, which yield additional information on the nature of the intermediates and thus the mechanism of replication *(13)*. Such treatments have proved invaluable in deciphering the process of replication in mitochondria *(22, 27, 29)*. Graphic examples are the application of RNase H and single-strand nuclease to mtDNA preparations (**Fig. 2.4**). In some contexts, it may also be useful to alkali-denature RIs after N2D-AGE and apply a third electrophoresis step *(13)*.

1.2. Considerations Particular to Vertebrate Mitochondrial DNA

An analysis of mtDNA from mammalian cultured cells, conducted over 30 years ago, revealed that it contains sporadic ribonucleotides (up to 10 per molecule) *(30)*. A more recent study *(31)* suggested that the ribonucleotide content of mtDNA of solid tissues was ~fivefold greater than that of cultured cells (>30 per mtDNA molecule). Moreover, during vertebrate mtDNA replication RNA is initially incorporated throughout the lagging strand *(27)*.

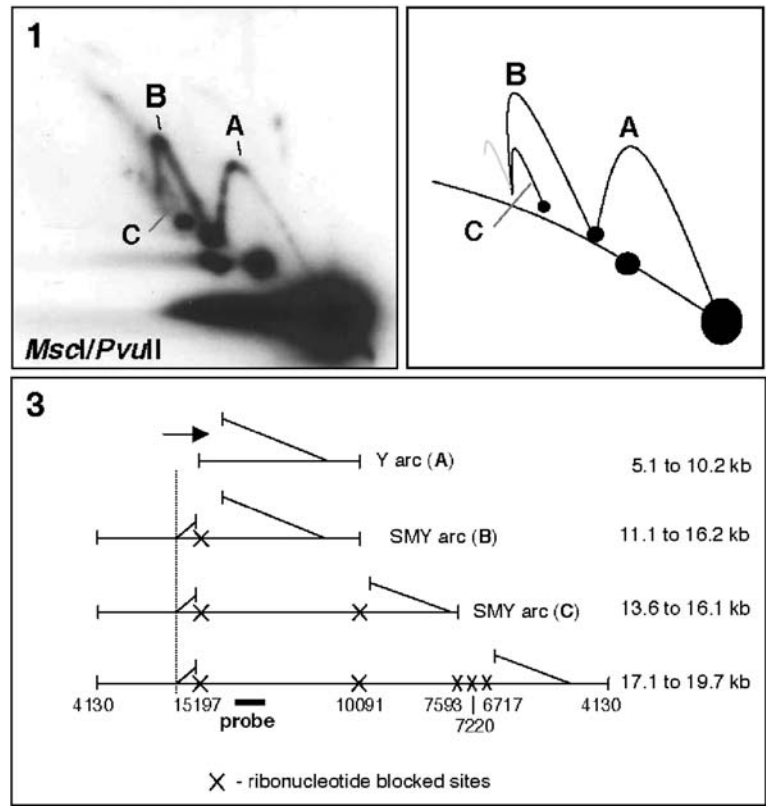


Fig. 2.3. **Novel arcs of mammalian mtDNA.** Mitochondrial DNA was isolated from sucrose gradient purified mitochondria of mouse liver and digested with *Msc*I and *Pvu*II. Restriction fragments were separated by N2D-AGE and after blot-transfer hybridized to a probe corresponding to nt 13,874–14,525 of the mouse mitochondrial genome. Vertebrate mtDNA prepared in this way yields slow-moving Y-like (SMY) arcs (arcs B, C and D in panel 1); SMY arcs arise due to the presence of ribonucleotides at one (or more) restriction site(s), thereby linking adjacent restriction fragments; interpreted in panels 2 and 3. SMY arcs are sensitive to RNase H, *see* Fig. 4 and *(27)*.

The presence of RNA in replicating and non-replicating mtDNA is problematic. Careful handling can minimize RNA degradation during isolation of mtDNA, but neutral/*alkaline* 2D-AGE *(32)* is inapplicable to mammalian mtDNA, because it results in RNA loss. We also find that atomic force microscopy is considerably harsher than N2D-AGE, often leading to fragmentation of mitochondrial replication intermediates (Yang and Holt, manuscript in preparation). Thus, while N2D-AGE is not the only means of dissecting DNA replication, it is undoubtedly one of the few methods appropriate to the study of replicating DNA with a high ribonucleotide content.

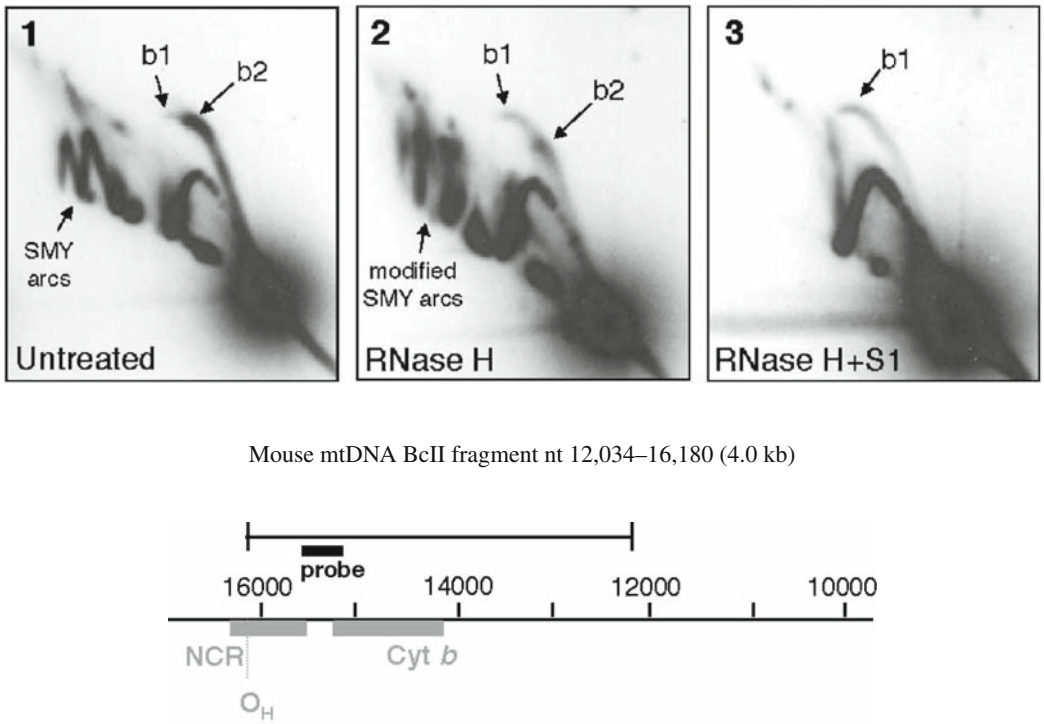


Fig. 2.4. **Slow-moving arcs are sensitive to RNase H and single-strand nuclease.** N2D-AGE of *Bcl*I digested mouse liver mtDNA reveals twin bubble arcs (b1 and b2) and SMY arcs, after hybridization to a probe detecting the 4-kb fragment nt 12,034–16,180 (panel 1). The more prominent of the two bubble arcs (b2) is sensitive to RNase H (panel 2) and single-strand (S1) nuclease (panel 3). The SMY arcs are modified by RNase H treatment, yet still resolve well above the arc of linear double-stranded DNA molecules (panel 2). S1 nuclease obliterates the SMY arcs (panel 3); *see (27)* for further details.

1.3. Analysis of Nucleoprotein Complexes by N2D-AGE

Our interest in mitochondrial DNA replication grew out of a desire to understand biased segregation of pathological mtDNA variants *(6, 7, 33)*, and so we are also intent on identifying and characterizing the apparatus of mtDNA segregation. To this end detergent-solubilized mitochondria were subjected to phenol–chloroform extraction without the usual protease treatment. When the mtDNA was digested with restriction enzymes and separated by N2D-AGE, the pattern produced was far from normal (**Fig. 2.5, panel 1**). The unusual fragment pattern was attributable to protein as the unexpected high molecular species were absent from equivalent samples treated with protease (**Fig. 2.5**, **panel 2**). Although mass spectrometry failed to identify the proteins associated with mtDNA (He & Holt unpublished experiments), we were able to infer that one of them is ATAD3p *(34)*. Efforts are underway to identify other components of these mitochondrial nucleoprotein complexes.

Here, we provide detailed protocols of our isolation and separation procedures as a guide to analyzing replicating mtDNA and mitochondrial nucleoprotein from solid tissues and cultured cells by neutral two-dimensional agarose gel electrophoresis.

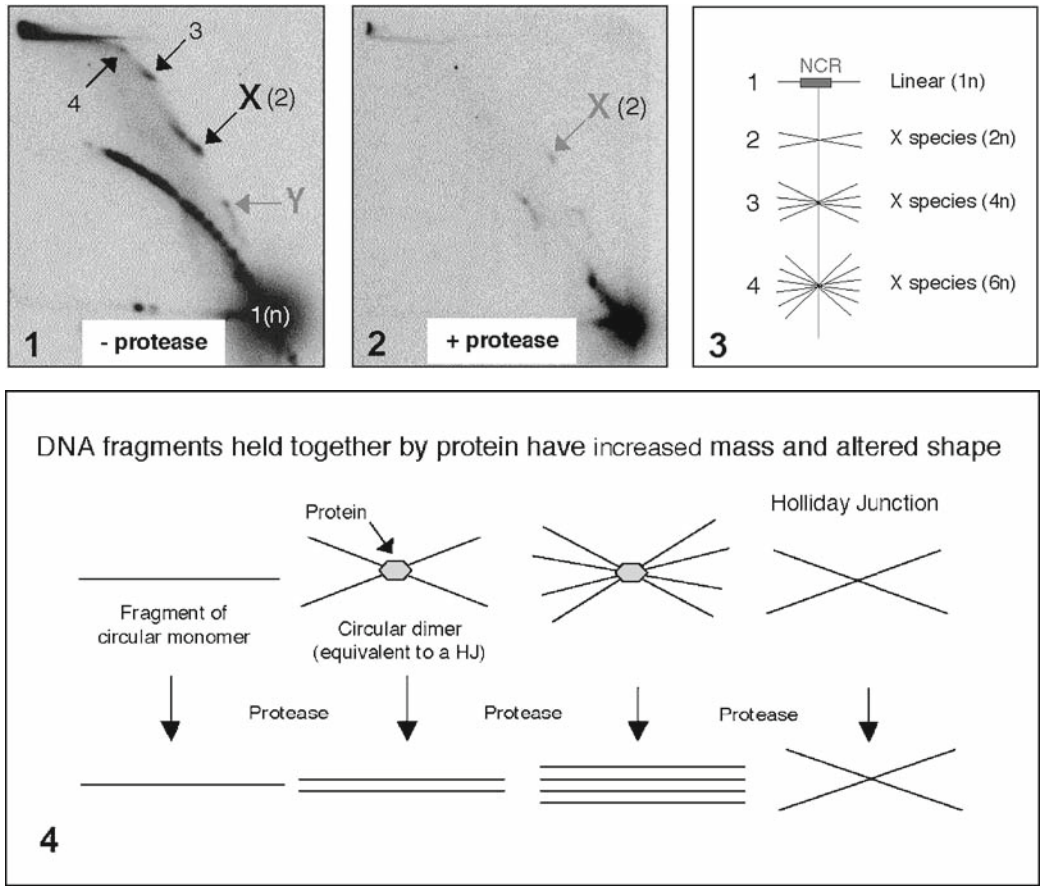


Fig. 2.5. **Multigenomic mitochondrial DNA held together protein can be resolved by N2D-AGE.** DNA extracted from human cells without the use of proteinase K (panel 1) or after proteinase K treatment (panel 2) was digested with *Acc*I, and the fragments separated by N2D-AGE and hybridized with a radiolabeled probe detecting a fragment (nt 15,255–1,504), which includes the major non-coding region of human mtDNA or NCR (panels 1 and 2). Key molecular species of panel 1 are interpreted in panel 3; 1(*n*) is the 2.8 kb linear fragment (nt 15,255–1,504); 2 is an X-like structure, which comprises two 2.8-kb fragments; species 3 and 4 (panel 1) are X-like structures with more copies of the 2.8-kb fragment of mtDNA than species 2. All the X-like species (2, 3, and 4) are considerably reduced in intensity after protease treatment (panel 2). The similarity in structure of two fragments joined at their centre by protein or through a four-way (Holliday) junction is illustrated in panel 4; protease treatment of such forms will have no effect on a Holliday junction, but will convert protein-dependent X-like structures to linear fragments (panel 4).

2. Materials

2.1. mtDNA Isolation from Solid Tissues

1. Phosphate-buffered saline: 10 mM phosphate buffer, pH 7.4, 2.7 mM KCl, 137 mM NaCl.
2. Homogenization buffer (HB): 225 mM mannitol, 75 mM sucrose, 10 mM HEPES–NaOH, pH 7.8, 10 mM EDTA, 0.1% (w/v) fatty acid-free bovine serum albumin (BSA), and 357.5 μM β-mercaptoethanol.

3. Gradient buffer (GB): 10 mM HEPES–NaOH, pH 7.8, 10 mM EDTA.
4. 1 M and 1.5 M Sucrose in GB.
5. Lysis buffer (LB): 20 mM HEPES–NaOH, pH 7.8, 75 mM NaCl, 50 mM EDTA.
6. Teflon homogenizer.
7. Proteinase K (PK): 20 mg/ml in water.
8. 20% (w/v) Sodium lauroyl sarcosinate (sarkosyl).
9. PCIA: equilibrated phenol:chloroform:isoamyl alcohol (25:24:1).
10. CIA: chloroform:isoamyl alcohol (24:1).
11. TE: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.
12. Isopropanol.
13. 70% (v/v) Ethanol.

2.2. mtDNA Treatment with Restriction and Modifying Enzymes

1. Restriction endonucleases.
2. Modifying enzymes: nuclease S1, RNase One, RNase H.
3. 3 M Sodium acetate, pH 5.2.
4. 10 mM Tris–HCl, pH 8.0.
5. TE: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.
6. 100% Ethanol.
7. 70% (v/v) Ethanol.
8. 0.5 M EDTA, pH 8.0.
9. Dry ice.

2.3. Neutral Two-Dimensional Agarose Gel Electrophoresis (N2D-AGE)

1. TBE: 89 mM Tris base, 87 mM boric acid, 2 mM EDTA, pH 8.0.
2. Low electroendosmotic (LEEO) agarose.
3. Ethidium bromide (EB): 10 mg/ml.
4. Gel electrophoresis tank with ports for buffer circulation.
5. Power supply.
6. Ultraviolet (UV) light box.
7. Peristaltic pump.
8. DNA molecular weight marker.
9. Loading buffer: 0.04% bromophenol blue (BPB), 0.04% xylene cyanol.

2.4. Southern Blot

1. Depurination buffer (DPB): 0.25 N HCl.
2. Denaturing buffer (DNB): 0.5 M NaOH, 1.5 M NaCl.

3. Neutralizing buffer (NB): 0.5 M Tris–HCl, pH 7.4, 1.5 M NaCl.
4. Genome-quality nylon membrane.
5. 3MM Whatman filter paper.
6. Paper towels.

2.5. Hybridization

1. Hybridization buffer: 0.25 M sodium phosphate, pH 7.2, 7% (w/v) sodium dodecyl sulfate.
2. 20X SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0.
3. Washing buffer 1 (WB1): 1X SSC.
4. Washing buffer 2 (WB2): 1X SSC/0.1% (w/v) sodium dodecyl sulfate.
5. X-ray film.

2.6. Total Cellular DNA Isolation

1. Phosphate-buffered saline (PBS): 10 mM phosphate buffer, pH 7.4, 2.7 mM KCl, 137 mM NaCl.
2. Lysis buffer: 20 mM HEPES–NaOH, pH 7.8, 75 mM NaCl, 50 mM EDTA, 0.5% (w/v) sodium dodecyl sulfate (SDS).
3. Proteinase K (PK): 20 mg/ml in water.
4. Phenol: equilibrated phenol.
5. CIA: chloroform:isoamyl alcohol (24:1).
6. 5 M NaCl.
7. Glycogen: 10 mg/ml.
8. Isopropanol.
9. 70% (v/v) Ethanol.
10. 10 mM Tris–HCl, pH 8.0.

3. Methods

3.1. mtDNA Isolation from Liver Tissue

1. Excise fresh liver and place it in cold phosphate-buffered saline on ice (*see* **Notes 1** and **2**).
2. Remove any blood and contaminating tissue and weigh (*see* **Note 3**).
3. Transfer to an ice-cold beaker containing 5 volumes of 1:10 diluted HB/g.
4. Mince the tissues finely with sharp scissors, changing the solution four or five times (*see* **Note 4**).
5. Wash diced tissue with 5 volumes of HB/g wet weight and discard as much solution as possible.

6. Add 9 volumes of HB/g and homogenize using a motorized tight-fitting Dounce homogenizer until the suspension is smooth (*see* **Notes 5** and **6**).
7. Centrifuge the homogenate at 600*gmax* for 10 min at 4°C to pellet nuclei and intact cells.
8. Transfer the supernatant to a clean tube and centrifuge at 5,000*gmax* for 10 min at 4°C.
9. Discard the supernatant and suspend the mitochondrial pellet in 5 volumes of HB/g (*see* **Note 7**).
10. Repeat Steps 7 and 8 once and after the last centrifugation suspend the mitochondrial pellet in 0.5 volumes of HB/g (*see* **Note 7**).
11. Prepare single-step sucrose gradients (*see* **Note 8**).
12. Load 2 ml of mitochondrial suspension per sucrose gradient and centrifuge in a swing-out rotor at 40,000*gmax* for 1 h at 4°C.
13. After centrifugation, mitochondria resolve at the interface of the 1 M and 1.5 M sucrose solutions.
14. Transfer the mitochondria to a 30-ml tube and add 5 volumes of GB slowly with gentle shaking of the mitochondrial solution (*see* **Note 9**). Centrifuge 9,900*gmax* for 10 min at 4°C.
15. Discard the supernatant and suspend the mitochondrial pellet in 1.6 ml LB/g (*see* **Note 7**). Transfer to a clean 15- or 50-ml disposable tube. Add 0.8 µl PK/g and incubate at 4°C for 45 min (*see* **Note 10**).
16. Add 80 µl of 20% sarkosyl/g, mix gently, and incubate at 4°C for 5 min (*see* **Notes 10** and **11**).
17. After incubation, mix thoroughly but gently with 1 volume of PCIA and spin at 6,000 × *gmax* for 10 min at room temperature (*see* **Note 12**).
18. Transfer the aqueous (upper) phase to a clean tube, mix carefully with 1 volume of CIA, and spin at 6,000*gmax* for 10 min at 4°C.
19. Recover the aqueous (upper) phase and aliquot in 500 µl in 1.5-ml Eppendorf tubes. Add 1 volume of isopropanol to each tube (*see* **Note 13**), mix gently, and keep at –20°C for at least 30 min (*see* **Note 14**).
20. Centrifuge the sample at 20,000*gmax* for 20 min at 4°C.
21. Discard the supernatant and wash the pellet with 70% ethanol.
22. Air-dry the pellet, suspend in TE, and determine the concentration by UV spectrometry (*see* **Note 15**).

3.2. DNA Treatment with Restriction and Nucleic Acid Modifying Enzymes

1. Digest 0.5–7.0 μg mtDNA with the appropriate restriction endonuclease following the conditions recommended by the manufacturer (*see* **Notes 16** and **17**).
2. Add 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol. Mix gently and keep at 20°C for at least 30 min.
3. Centrifuge the sample at 20,000*gmax* for 20 min at 4°C.
4. Discard the supernatant and wash the pellet with 70% ethanol.
5. Air-dry the pellet, suspend in TE or in 10 mM Tris–HCl, pH 8.0 if it is to be treated with modifying reagents (*see* **Note 18**). If no additional treatment is required then the sample is ready for loading on the first dimension agarose gel.
6. Treatments with modifying enzymes require the addition of 1/10 volume of the appropriate 10X buffer (*see* **Note 19**). Standard conditions for the different modifying enzymes are 1 unit of RNase H for 1 h at 37°C; 5 units of RNase One for 10 min at 37°C; and 1 unit of S1 nuclease for 1.5 min at 37°C.
7. Stop the reaction by flash-freezing the sample on dry ice/ethanol, after adding 1/10 volume of 0.5 M EDTA where appropriate (*see* **Note 20**).

3.3. Neutral Two-Dimensional Agarose Gel Electrophoresis

1. Prepare a 0.4% agarose gel in TBE buffer for first dimension gel electrophoresis (*see* **Notes 21** and **22**).
2. Submerge gel in a tank containing TBE.
3. Load sample(s) and a suitable size marker according to the length of the fragment of interest (*see* **Note 23**).
4. Run the first dimension at 0.7 V/cm for 20 h at room temperature (*see* **Note 24**).
5. After 20 h, cut out the lane(s) containing the sample with the aid of a razor blade and a ruler.
6. Prepare 1% molten agarose in TBE with 500 ng/ml EB (*see* **Note 25**).
7. Place gel slice(s) from the first dimension electrophoresis step in a gel-casting tray (*see* **Note 26**). Each slice is rotated 90° counter-clockwise, with respect to the first dimension run.
8. Remove excess TBE buffer around the gel slice with 3MM filter paper and adhere the gel slice to the tray with 1% molten agarose (*see* **Note 27**).
9. Carefully remove the excess of buffer from the well and fill with 0.4% molten agarose (*see* **Note 28**).
10. Pour the remaining 1% molten agarose from the opposite side of the gel slice and wait until it has solidified (*see* **Note 29**).

11. Place the gel in a tank containing cold TBE with 500 ng/ml EB (*see* **Note 30**).
12. Circulate the buffer from the positive to the negative electrode by means of a peristaltic pump (*see* **Note 31**).
13. Run the second dimension at 6 V/cm for 4 h at 4°C (*see* **Note 32**).

3.4. Southern Blot

1. After electrophoresis in the second dimension, remove the gel from the tank and invert into a glass dish (*see* **Note 33**).
2. Add 500 ml DPB for a dish measuring 300 × 400 mm, rock gently for 15–30 min, and discard solution (*see* **Note 34**).
3. Add 500 ml DNB and rock gently for 5 min. Pour off the solution and repeat the treatment for 15 min (*see* **Note 35**).
4. Add 500 ml NB and rock gently for 10 min. Discard the solution and repeat the treatment for 10 min (*see* **Note 36**).
5. Remove excess solution (*see* **Note 37**).
6. Cut a piece of membrane of the same dimensions as the gel and wet in water (*see* **Notes 38** and **39**).
7. Place the wetted membrane face down on the gel avoiding bubbles (*see* **Note 40**).
8. Soak two sheets of 3MM Whatman filter paper in water and place them on top of the membrane, again avoiding bubbles (*see* **Note 40**).
9. Stack 10–12 cm of paper towel on top of 3MM Whatman filter paper.
10. Place a glass plate or a tray on top and a weight to create some pressure to the stack (*see* **Note 41**). Blot overnight.
11. Remove and discard paper towels and 3MM Whatman filter paper. Place the membrane face up on 3MM Whatman filter paper and let stand for a few minutes.
12. Covalently link the DNA to the membrane in a UV cross-linker: total energy 1,200 × 100 μJ/cm^2 (*see* **Note 42**).

3.5. Hybridization

1. Place the membrane face up in a hybridization tube and add 15 ml pre-warmed hybridization buffer at 65°C (*see* **Note 43**).
2. Incubate in a hybridization oven for at least 30 min at 65°C.
3. Pour off the solution and repeat the incubation with fresh 15 ml pre-warmed hybridization buffer at 65°C.
4. Label an appropriate gel-purified mtDNA specific probe (*see* **Note 44**).
5. Denature the probe at 95°C for 5 min and chill on ice for 2 min (*see* **Note 45**).

6. Add the radiolabeled probe to the hybridization solution and incubate overnight at 65°C (*see* **Note 46**).
7. Discard the hybridization solution containing the probe and wash the membrane four times with 50 ml WB1 at 65°C for 20 min (*see* **Note 47**).
8. Wash the membrane twice with 50 ml WB2 at 65°C for 20 min (*see* **Note 48**).
9. Remove the membrane from the tube and place on top of paper towel, to blot excess liquid. Air-dry briefly (*see* **Note 49**).
10. Wrap the membrane in cling film (Saran Wrap). Expose to X-ray film for 0.5–7 days at –80°C.

3.6. Total Cellular DNA Isolation from Mammalian Cultured Cells

1. Grow cells in a 90-mm plate (*see* **Notes 50** and **51**).
2. Aspirate the medium and wash the cells with PBS (*see* **Note 52**).
3. Remove PBS and disrupt cells by the addition of 4 ml lysis buffer (*see* **Notes 53** and **54**).
4. Transfer the solution to a 15-ml tube containing 4 ml phenol, immediately.
5. Mix gently but thoroughly by rotation for 15 min at room temperature (*see* **Note 55**).
6. Centrifuge the sample at 6,000*gmax* for 15 min at room temperature.
7. Transfer the aqueous (upper) phase to a clean 15-ml tube (*see* **Note 56**). Add 1 volume of CIA and repeat Step 5.
8. Centrifuge the mixture at 6,000*gmax* for 15 min at 4°C.
9. Recover the aqueous (upper) phase and aliquot in 500 µl lots in 1.5-ml tubes (*see* **Note 57**).
10. Add 5 M NaCl to a final concentration of 175 mM (*see* **Notes 58** and **59**) and 1 volume of isopropanol. Mix gently and place on ice for 15 min (*see* **Note 60**).
11. Centrifuge the sample at 20,000*gmax* for 20 min at 4°C.
12. Discard the supernatant and wash the pellet with 70% ethanol.
13. Air-dry the pellet briefly (*see* **Note 61**), suspend in 10 mM Tris–HCl at pH 8.0 (*see* **Note 62**) on ice and determine the concentration by UV spectrometry.
14. Take 10 µg total DNA and add 10 mM Tris–HCl, pH 8.0, to 415 µl final volume (*see* **Note 63**).
15. Add 7.5 µl 5 M NaCl, 50 µl 0.5 M EDTA, 12.5 µl 20% sarkosyl, 10 µl 1 M HEPES–NaOH (pH 7.8), and 5 µl PK and incubate at 4°C for 30 min.

16. Mix thoroughly but gently with 1 volume of PCIA and spin at 20,000*gmax* for 5 min at room temperature.
17. Transfer the aqueous (upper) phase to a clean 1.5-ml tube, mix carefully with 1 volume of CIA for 2–3 min, and spin at 20,000*gmax* for 5 min at 4°C.
18. Follow Steps 10–13.

4. Notes

1. mtDNA isolation has to be done from fresh tissue, since the yield of replication intermediates is much lower from frozen material, and the replication intermediates tend to be more degraded.
2. All steps are carried out on ice, with ice-cold solutions, in a cold room.
3. It is recommended to start from 10 to 15 g of liver. More starting material yields more mtDNA; however, the increased time required for mtDNA isolation tends to result in poorer quality DNA.
4. The solution should be changed repeatedly until there is no significant trace of blood or fat, since both interfere with the isolation procedure.
5. Homogenization should be thorough (4–5 complete strokes) but not excessive since it will damage mitochondria, exposing the mtDNA to contaminating nucleases. We use an IKA Labortechnik RW 20 motorized homogenizer set at speed 5.
6. Depending on the amount of starting material, 30-ml tubes or 100–250-ml bottles can be used.
7. Suspension is achieved by gently swirling the buffer in the tube/bottle on ice or with the aid of a glass homogenizer. It is important to suspend the pellet completely before each centrifugation step.
8. For the sucrose step gradient, take a 25 × 89 mm tube and add 17.5 ml of 1.5 M sucrose. Then overlay, very slowly at first, an equal volume of 1 M sucrose taking care not to disturb the interface. To save time, sucrose gradients can be prepared during the earlier centrifugation steps.
9. Carefully remove most of the gradient above the mitochondrial layer. Then, transfer the mitochondria in the minimum volume to a clean tube, using a P1000 tip with the end

removed to avoid damaging the mitochondria. This step should be carried out quickly, yet carefully. The final sucrose concentration of the solution is 250 mM (isotonic).

10. Proteinase K is highly active across the range of 37–50°C; it is nevertheless able to digest protein at 4°C, albeit more slowly.
11. We prefer to incubate at 4°C rather than at 37–50°C after mitochondrial lysis, in order to limit the action of contaminating nucleases, before they are digested by proteinase K. This two-step proteinase K treatment is preferred to the one in which both proteinase K and sarkosyl are added together, as it better preserves replication intermediates.
12. After PCIA extraction, a white or cloudy interface indicates an excess of protein. Whenever this is the case, PCIA extraction of the aqueous (upper) phase should be repeated until the interface is clear.
13. Since lysis buffer contains 75 mM NaCl, there is no need to add extra salt to precipitate DNA.
14. Samples can be stored at this stage in the freezer for a long period of time. Indeed, unless they are to be used immediately, it is better to preserve DNA samples in this way.
15. Many DNA isolation procedures include an RNase treatment, it is imperative to avoid this when extracting mitochondrial replication intermediates, as they are highly sensitive to RNase. As RNA is preserved, UV spectrometry will measure total nucleic acid (DNA and RNA) present in the sample. Any protein remaining associated with mtDNA will also contribute to the absorbance measurement.
16. Wherever possible, avoid restriction endonucleases whose optimal temperature is greater than 37°C (50–65°C), as this may lead to strand separation of replication intermediates. If it is particularly advantageous to use such an enzyme then try incubating at 37°C. For example, 50°C is recommended for *BclI*; however, it has at least 50% activity at 37°C and is quite capable of giving complete digestion at the lower temperature, although it may be necessary to increase slightly the units of enzyme or the incubation time. Alternatively, try the intermediate temperature of 45°C.
17. Digestion with restriction endonucleases in small volumes can result in partial digestion. To avoid this problem perform digestions in a 200–400 µl reaction volume.
18. When analyzing mitochondrial nucleoids (*see* **Section 3**.6), restriction enzymes should be chosen such that the region of interest is close to the middle of the fragment. In the case of

human mtDNA, restriction enzyme *Acc*I proved ideal as it yields a 2.8-kb fragment containing the D-loop region in a central position *(34)*.

19. Most commercial modifying enzymes are supplied with 10X buffer. However, this is not always the case: RNase H buffer must be prepared by the user; 10X RH buffer: 200 mM HEPES–KOH (pH 7.8), 500 mM KCl, 100 mM $MgCl_2$, 10 mM DTT.
20. A rapid decrease in temperature effectively stops the reaction; hence, reaction times are readily reproduced from experiment to experiment. The addition of EDTA to RNase H and S1 reactions will sequester essential divalent cations, but this will have no effect on RNase One. Notwithstanding, results with RNase One are just as reproducible as those with RNase H and S1 nuclease, indicating the efficacy of rapid cooling.
21. The conditions listed here are designed to give optimal resolution of 3–4-kb fragments. In the case of fragments of >5 kb, first dimension electrophoresis is in a 0.35% agarose gel at 1.5 V/cm for 20 h, and second dimension electrophoresis at 3 V/cm for 18 h in a 0.875% agarose gel. Fragments shorter than 3 kb are separated in the first dimension at 0.9 V/cm for 20 h on slab gels containing between 0.55 and 1.0% (w/v) agarose, at room temperature. Second dimension electrophoresis is for 9 h at 260 mA in gels between 1.5 and 2.0% agarose, at 4°C. When analyzing nucleoprotein complexes (**Section 3**.6), the contribution of both DNA/DNA and protein/DNA complexes needs to be considered. For fragments of >3 kb with the non-coding region located in the middle, first dimension electrophoresis is in a 0.4% agarose gel at 0.7 V/cm for 20 h, and second dimension electrophoresis at 2.8 V/cm for 24 h in a 0.875% agarose gel.
22. For the first dimension, we routinely prepare 100 ml of agarose to fill a 110 × 140 mm tray, fitted with a 14-well comb with 4-mm wide teeth. It is recommended to keep a whole set (tray, comb, and tank) ethidium bromide-free for first dimension.
23. Leave two empty wells between samples, in order to avoid possible cross contamination and to facilitate gel slice excision after 1D-AGE.
24. Since the first-dimension gel lacks ethidium bromide, the rate of electrophoresis is estimated from the positions of bromophenol blue and xylene cyanol dyes contained in the loading buffer. The ability to accurately predict the position of fragments comes with experience. More than one lane containing size markers can be loaded, and one of them is excised and

stained in TBE containing 500 ng/ml ethidium bromide and visualized under UV light at intervals to confirm the position of fragments of interest.

25. Molten agarose should be prepared toward the end of the first dimension electrophoresis step, and cooled in a 50°C water bath, before adding ethidium bromide and casting.
26. For the second dimension gel, a small tray (110 × 140 mm) able to fit just one of the gel slices can be used. However, larger trays (200 × 240 mm) can accommodate up to four gel slices. In the case of analysis of nucleoprotein complexes, a large tray (200 × 240 mm) is needed since a longer run is required to resolve complex structures.
27. Pour some agarose at the back of the gel slice with the aid of a P1000 tip and allow it to solidify. Avoid agarose in front of the gel slice, as it may distort the migration of replication intermediates or even prevent them from entering in the second dimension gel if it is too dry.
28. Whenever the well has been kept for the second dimension, sealing it with molten agarose will prevent the loss of material that has not entered the gel in the first dimension, such as certain nucleoprotein complexes.
29. Agarose should completely cover the gel slices from the first dimension. Usually, 150 ml of agarose is enough for a 110 × 140 mm tray and 400 ml for a 200 × 240 mm tray.
30. The tank with TBE to be used for the second dimension should be pre-cooled to 4°C and ethidium bromide added immediately prior to electrophoresis.
31. Because ethidium bromide migrates to the negative electrode, under the electric field, recirculation of buffer ensures that it will be homogeneously distributed in the gel. Lack of recirculation may result in poor resolution in the second dimension since intercalation of EB into DNA is critical for separating DNA molecules on the basis of structure.
32. The second dimension gel contains ethidium bromide and hence DNA migration can be visualized under UV light.
33. 1% Agarose gels measuring 200 × 240 mm are inverted by hand routinely. Novices can employ a piece of X-ray film (typically an old unwanted autorad) to support the gel.
34. The low pH of DPB turns xylene cyanol greenish and BPB yellow.
35. After this treatment, both bromophenol blue and xylene cyanol should recover their original color.

36. It is important not to leave the gel longer than recommended in DPB or DNB. In contrast, the gel can be left in NB solution for up to an hour without any discernible effect. Notwithstanding, slightly longer treatments are appropriate for higher percentage agarose gels, as the solution requires longer time to permeate the gel.
37. Dry blotting is effective; transfer buffer is not required.
38. Do not touch the membrane without gloves. It is always useful to write down in pencil relevant information on a corner of the membrane, and it also aids orientation.
39. Use a hydrophilic positively charged nylon membrane with high DNA-binding capacity.
40. Air bubbles between the gel and the membrane are removed by rolling a wet Pasteur pipette or glass rod across the surface. The same applies to bubbles between the membrane and the 3MM paper.
41. The glass plate or tray should be as large as the gel, in order to transmit pressure evenly across the gel. As weights, empty bottles filled up with 0.5 l and 1 l of water can be used for 110 $\times$ 140 mm and 200 $\times$ 240 mm gels, respectively.
42. The crosslinked membrane can be kept at room temperature indefinitely.
43. Pre-warm hybridization tubes and solution in the hybridization oven at 65°C.
44. There are a variety of labeling kits available and no significant difference has been found between them.
45. Removal of unincorporated label is not essential; probes can be used directly after labeling and denaturation.
46. Add the probe to the hybridization solution, never directly onto the membrane. This avoids strong background in the area where the concentrated probe touched the membrane.
47. Probes can be stored at room temperature and re-applied to a different membrane.
48. Check the washing solution after each wash with a Geiger counter. If it remains hot after the second wash in WB2, continue washing until there are no appreciable counts in the wash solution. Also check the membrane with the Geiger counter after the full wash cycle: the unit length fragment, of say 4 kb, should be readily apparent as the hottest area of the membrane, recording 200–1,000 cps, whereas areas without DNA should record few (<5 cps) if any counts.
49. Do not over-dry the membrane. Frequently it is necessary to strip and re-probe membranes; over-drying impedes stripping of probes.

50. Cells can also be grown in 6-well plates, if required (e.g., in the case of RNA interference experiments); in which case, the volumes of the solutions in Steps 3 and 4 should be halved. Cells should be grown to 80–90% confluency.
51. A 90-mm plate yields 100–150 μg of total nucleic acids, whereas a single well from a 6-well plate yields 20–30 μg nucleic acid.
52. Trypsin–EDTA treatment is not required for adherent or semi-adherent cells. If working with cells in suspension, sediment cells at 180*gmax* for 2 min at room temperature, suspend in 1/10 volume of PBS, and re-pellet the cells again at 180*gmax* for 2 min at room temperature. Proceed from Step 3.
53. Lysis buffer should ideally be prepared fresh from stock solutions. However, if prepared in advance, add SDS immediately before use. No proteinase K is included in the buffer in order to preserve those proteins remaining bound to DNA.
54. Add 3 ml lysis buffer, mix thoroughly with a P1000 tip, to detach cells and ensure rapid lysis. The solution becomes very viscous. Transfer lysate to a 15-ml tube containing phenol as described in Step 4. Add another 1 ml lysis buffer to wash the plate and recover the remaining lysate. Lysis should be carried out as quickly as possible in order to minimize nuclease action.
55. Mixing should be thorough so that all the free protein partitions into the phenol phase, yet gently to limit DNA damage. We use a Roto-Shake Genie rotor (Scientific Instruments) set at speed 5. This treatment is not able to remove all proteins bound to DNA.
56. It is generally preferable to leave some of the aqueous phase in order to avoid transferring the protein-rich inter-phase.
57. We use 4 ml in 500 μl aliquots for a 90-mm plate and a single 4 ml aliquot for a well of a 6-well plate.
58. When adding additional NaCl bear in mind that the buffer already contains 75 mM NaCl. The increase in NaCl concentration from 75 mM to 175 mM facilitates DNA precipitation.
59. Glycogen (1/100 volume) can be added to the solution to make the pellet visible, and often increases the yield, when extracting DNA from small numbers of cells (e.g., a single well from a 6-well plate, 40–50% confluent).
60. Samples can be stored at this stage in the freezer for a long period of time. Indeed, unless they are to be used immediately, it is better to preserve DNA samples in this way.

61. Over-drying the samples makes it very difficult to get it back into solution due to the significant amount of protein.
62. The DNA must be completely in solution. It takes about 20 min to 1 h depending on the amount of DNA. Excessive pipetting trying to dissolve pellet should be avoided since it causes shearing of the DNA.
63. Treatment with PK (Steps 14–18) is optional and should be used when replication intermediates without attached protein are to be analyzed. It is obvious that this treatment should be avoided when analyzing nucleoprotein complexes.

References

1. Spelbrink, J.N., Li, F.Y., Tiranti, V., Nikali, K., Yuan, Q.P., Tariq, M., Wanrooij, S., Garrido, N., Comi, G., Morandi, L. et al. (2001) Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria. *Nat Genet,* **28,** 223–31.
2. Van Goethem, G., Dermaut, B., Lofgren, A., Martin, J.J. and Van Broeckhoven, C. (2001) Mutation of POLG is associated with progressive external ophthalmoplegia characterized by mtDNA deletions. *Nat Genet,* **28,** 211–2.
3. Holt, I.J., Harding, A.E. and Morgan-Hughes, J.A. (1988) Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. *Nature,* **331,** 717–9.
4. Wallace, D.C., Singh, G., Lott, M.T., Hodge, J.A., Schurr, T.G., Lezza, A.M., Elsas, L.J., 2nd and Nikoskelainen, E.K. (1988) Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. *Science (New York, N.Y.,* **242,** 1427–30.
5. Bourgeron, T., Chretien, D., Rotig, A., Munnich, A. and Rustin, P. (1993) Fate and expression of the deleted mitochondrial DNA differ between human heteroplasmic skin fibroblast and Epstein-Barr virus-transformed lymphocyte cultures. *J Biol Chem,* **268,** 19369–76.
6. Dunbar, D.R., Moonie, P.A., Jacobs, H.T. and Holt, I.J. (1995) Different cellular backgrounds confer a marked advantage to either mutant or wild-type mitochondrial genomes. *Proc Natl Acad Sci U S A,* **92,** 6562–6.
7. Holt, I.J., Dunbar, D.R. and Jacobs, H.T. (1997) Behaviour of a population of partially duplicated mitochondrial DNA molecules in cell culture: segregation, maintenance and recombination dependent upon nuclear background. *Hum Mol Genet,* **6,** 1251–60.
8. Brewer, B.J. and Fangman, W.L. (1987) The localization of replication origins on ARS plasmids in S. cerevisiae. *Cell,* **51,** 463–71.
9. Brewer, B.J. and Fangman, W.L. (1991) Mapping replication origins in yeast chromosomes. *Bioessays,* **13,** 317–22.
10. Brun, C., Dijkwel, P.A., Little, R.D., Hamlin, J.L., Schildkraut, C.L. and Huberman, J.A. (1995) Yeast and mammalian replication intermediates migrate similarly in two-dimensional gels. *Chromosoma,* **104,** 92–102.
11. Dijkwel, P.A. and Hamlin, J.L. (1997) Mapping replication origins by neutral/neutral two-dimensional gel electrophoresis. *Methods,* **13,** 235–45.
12. Friedman, K.L. and Brewer, B.J. (1995) Analysis of replication intermediates by two-dimensional agarose gel electrophoresis. *Methods Enzymol,* **262,** 613–27.
13. Kalejta, R.F. and Hamlin, J.L. (1996) Composite patterns in neutral/neutral two-dimensional gels demonstrate inefficient replication origin usage. *Mol Cell Biol,* **16,** 4915–22.
14. Kuzminov, A., Schabtach, E. and Stahl, F.W. (1997) Study of plasmid replication in Escherichia coli with a combination of 2D gel electrophoresis and electron microscopy. *J Mol Biol,* **268,** 1–7.
15. Little, R.D. and Schildkraut, C.L. (1995) Initiation of latent DNA replication in the Epstein-Barr virus genome can occur at sites other than the genetically defined origin. *Mol Cell Biol,* **15,** 2893–903.
16. Schvartzman, J.B., Martinez-Robles, M.L. and Hernandez, P. (1993) The migration

behaviour of DNA replicative intermediates containing an internal bubble analyzed by two-dimensional agarose gel electrophoresis. *Nucleic Acids Res,* **21,** 5474–9.

17. van Brabant, A.J., Hunt, S.Y., Fangman, W.L. and Brewer, B.J. (1998) Identifying sites of replication initiation in yeast chromosomes: looking for origins in all the right places. *Electrophoresis,* **19,** 1239–46.
18. Brewer, B.J. and Fangman, W.L. (1988) A replication fork barrier at the 3' end of yeast ribosomal RNA genes. *Cell,* **55,** 637–43.
19. Brewer, B.J., Lockshon, D. and Fangman, W.L. (1992) The arrest of replication forks in the rDNA of yeast occurs independently of transcription. *Cell,* **71,** 267–76.
20. Viguera, E., Hernandez, P., Krimer, D.B., Boistov, A.S., Lurz, R., Alonso, J.C. and Schvartzman, J.B. (1996) The ColE1 unidirectional origin acts as a polar replication fork pausing site. *J Biol Chem,* **271,** 22414–21.
21. Reyes, A., Yang, M.Y., Bowmaker, M. and Holt, I.J. (2005) Bidirectional replication initiates at sites throughout the mitochondrial genome of birds. *J Biol Chem,* **280,** 3242–50.
22. Holt, I.J., Lorimer, H.E. and Jacobs, H.T. (2000) Coupled leading-and lagging-strand synthesis of mammalian mitochondrial DNA. *Cell,* **100,** 515–24.
23. Linskens, M.H. and Huberman, J.A. (1990) Ambiguities in results obtained with 2D gel replicon mapping techniques. *Nucleic Acids Res,* **18,** 647–52.
24. Bowmaker, M., Yang, M.Y., Yasukawa, T., Reyes, A., Jacobs, H.T., Huberman, J.A. and Holt, I.J. (2003) Mammalian mitochondrial DNA replicates bidirectionally from an initiation zone. *J Biol Chem,* **278,** 50961–9.
25. Dijkwel, P.A., Vaughn, J.P. and Hamlin, J.L. (1994) Replication initiation sites are distributed widely in the amplified CHO dihydrofolate reductase domain. *Nucleic Acids Res,* **22,** 4989–96.
26. Trivedi, A., Waltz, S.E., Kamath, S. and Leffak, M. (1998) Multiple initiations in the cmyc replication origin independent of chromosomal location. *DNA Cell Biol,* **17,** 885–96.
27. Yasukawa, T., Reyes, A., Cluett, T.J., Yang, M.Y., Bowmaker, M., Jacobs, H.T. and Holt, I.J. (2006) Replication of vertebrate mitochondrial DNA entails transient ribonucleotide incorporation throughout the lagging strand. *EMBO J,* **25,** 5358–71.
28. Belanger, K.G., Mirzayan, C., Kreuzer, H.E., Alberts, B.M. and Kreuzer, K.N. (1996) Two-dimensional gel analysis of rolling circle replication in the presence and absence of bacteriophage T4 primase. *Nucleic Acids Res,* **24,** 2166–75.
29. Yasukawa, T., Yang, M.Y., Jacobs, H.T. and Holt, I.J. (2005) A bidirectional origin of replication maps to the major noncoding region of human mitochondrial DNA. *Mol Cell,* **18,** 651–62.
30. Grossman, L.I., Watson, R. and Vinograd, J. (1973) The presence of ribonucleotides in mature closed-circular mitochondrial DNA. *Proc Natl Acad Sci USA,* **70,** 3339–43.
31. Yang, M.Y., Bowmaker, M., Reyes, A., Vergani, L., Angeli, P., Gringeri, E., Jacobs, H.T. and Holt, I.J. (2002) Biased incorporation of ribonucleotides on the mitochondrial L-strand accounts for apparent strand-asymmetric DNA replication. *Cell,* **111,** 495–505.
32. Nawotka, K.A. and Huberman, J.A. (1988) Two-dimensional gel electrophoretic method for mapping DNA replicons. *Mol Cell Biol,* **8,** 1408–13.
33. Vergani, L., Rossi, R., Brierley, C.H., Hanna, M. and Holt, I.J. (1999) Introduction of heteroplasmic mitochondrial DNA (mtDNA) from a patient with NARP into two human rho degrees cell lines is associated either with selection and maintenance of NARP mutant mtDNA or failure to maintain mtDNA. *Hum Mol Genet,* **8,** 1751–5.
34. He, J., Mao, C.C., Reyes, A., Sembongi, H., Di Re, M., Granycome, C., Clippingdale, A.B., Fearnley, I.M., Harbour, M., Robinson, A.J. et al. (2007) The AAA+ protein ATAD3 has displacement loop binding properties and is involved in mitochondrial nucleoid organization. *J Cell Biol,* **176,** 141–6.
35. Reyes, A.Y., Yasukawa, T. Holt, I.J. (2007) Analysis of Replicating Mitochondrial DNA. *Methods in Molecular Biology: Mitochondria Practical Protocols* **372,** 219–32.

Chapter 3

Comparative Purification Strategies for *Drosophila* and Human Mitochondrial DNA Replication Proteins: DNA Polymerase γ and Mitochondrial Single-Stranded DNA-Binding Protein

Marcos T. Oliveira and Laurie S. Kaguni

Abstract

The mitochondrion is the eukaryotic organelle that carries out oxidative phosphorylation, fulfilling cellular requirements for ATP production. Disruption of mitochondrial energy metabolism can occur by genetic and biochemical mechanisms involving nuclear-encoded proteins that are required at the mitochondrial DNA replication fork, which often leads to human disorders and to animal lethality during development. DNA polymerase γ (pol γ), the mitochondrial replicase, and the mitochondrial single-stranded DNA-binding protein (mtSSB) have been the focus of study in our lab for a number of years. Here we describe the purification strategies that we developed for obtaining the recombinant forms of pol γ and mtSSB from both *Drosophila melanogaster* and humans. Despite the fact that similar approaches can be used for purifying the homologous proteins, we have observed that there are differences in the behavior of the proteins in some specific steps that may reflect differences in their structural and biochemical properties. Their purification in homogeneous, active form represents the first step toward our long-term goal to understand their biochemistry, biology, and functions at the mitochondrial DNA replication fork.

Key words: mitochondrial DNA replication, pol γ, mtSSB, *Drosophila*, human.

1. Introduction

Animal mitochondrial DNA (mtDNA) is a circular, compact, double-stranded molecule of about 16 kb, whose gene content is a remnant of the genome of the α-proteobacterium that was incorporated by a nucleated cell early in the evolution of the eukaryotes, according to the endosymbiont theory of mitochondrial origin *(1)*. Because

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_3 Springerprotocols.com

mitochondria retain a genome with genes essential for energy production in eukaryotic cells, they require enzyme systems for mtDNA replication and expression. So far, only three nuclear-encoded proteins have been identified at the mtDNA replication fork in animal cells: DNA polymerase γ, the mitochondrial single-stranded DNA-binding protein and the Twinkle DNA helicase. Together these proteins function to synthesize and proofread new DNA strands, destabilize the DNA helix and protect single-stranded DNA regions, and unwind the duplex DNA, respectively *(2, 3)*.

After the first identification in animal cells and through the development of purification strategies for the enzyme, the biochemical and structural properties of pol γ have been studied in a number of organisms, with an emphasis on *Drosophila melanogaster* and humans *(2, 4–6)*. The *Drosophila* pol γ holoenzyme consists of a heterodimer of a catalytic subunit, pol γ–α, and an accessory subunit, pol γ–β *(2)*. On the other hand, the human homolog comprises a catalytic core in a heterotrimeric complex with a dimer of the accessory subunit *(6)*. In both cases, pol γ–α retains 5′–3′ DNA polymerase and 3′–5′ exonuclease activities, whereas pol γ–β is responsible for stimulating pol γ–α activity and enhancing holoenzyme processivity and DNA-binding properties *(4)*. Recently, some studies have stressed the importance of pol γ in human diseases and animal development: functional defects due to mutations in human pol γ–α lead to mitochondrial disorders *(7, 8)*; a mutation in *Drosophila* pol γ–α causes mitochondrial and nervous system dysfunction and developmental lethality in the larval third instar *(9)*; site-directed mutagenesis of *Drosophila* pol γ–α and human pol γ–β alter enzyme activity, processivity, and DNA-binding affinity *(4, 10)*; and null mutations of *Drosophila* pol γ–β cause lethality during early pupation, concomitant with loss of mtDNA and mitochondrial mass, and reduced cell proliferation in the central nervous system *(11)*.

mtSSBs share similar physical and biochemical properties with *Escherichia coli* SSB *(12, 13)*, with which they exhibit a high degree of amino acid sequence conservation. Like *E. coli* SSB, mtSSBs are homotetramers of 13–16 kDa polypeptides. It has been demonstrated that *Drosophila* mtSSB can stimulate 15–20-fold in vitro DNA synthesis and the 3′–5′ exonuclease activity of pol γ, in an assay that mimics lagging DNA strand synthesis in mitochondrial replication *(13, 14)*. Furthermore, human mtSSB is able to stimulate specifically the unwinding activity of Twinkle helicase *(15)* and, along with pol γ and Twinkle helicase, reconstitute a minimal mtDNA replisome in vitro *(3)*. The biochemical data are consistent with an important role for mtSSB in initiation and elongation of DNA strands in mtDNA replication, which has been documented genetically by

the fact that an insertion in the third intron of the *Drosophila* gene (*lopo*) results in developmental lethality, concomitant with the loss of mtDNA and respiratory capacity *(16)*. It is important to note the striking conservation of some biological processes from flies to humans. When a *Drosophila* homolog of an essential but poorly understood mammalian gene is identified, as happens with a large number of mitochondrial genes, powerful genetic and molecular techniques available in *Drosophila* can be applied to its characterization *(17)*.

In this chapter, we review the strategies our lab has developed over the years for purifying the recombinant forms of *Drosophila* pol γ and mtSSB *(14, 18–21)*, and we include comparative schemes for purification of the human homologs *(4, 8)*. In Chapter 8, we also address the approaches we have taken to study the human Twinkle helicase *(22)*, the other major component of the mtDNA replication fork. With regard to the purification protocols presented, we have found that the chromatographic methods we employ are generally efficacious for proteins involved in nucleic acid metabolism. In addition, hydroxylapatite, double- and single-stranded DNA cellulose, ATP agarose, and protein affinity chromatography resins, as used for the purification of the native forms of *Drosophila* pol γ and mtSSB *(20, 21)*, are also generally useful. However, the production of a C-terminally histidine-tagged pol γ–α and consequently, the use of nickel-nitrilotriacetic acid (Ni-NTA) agarose chromatography have facilitated the purification of the recombinant protein by reducing the number of chromatographic steps required to achieve homogeneity. The order of use can be varied to good purpose but as a rule of thumb, phosphocellulose as a first step is effective to eliminate ~90% of bulk mitochondrial protein while retaining nucleic acid binding proteins, and velocity sedimentation is useful as a final step to remove adventitious nuclease activities that are invariably of low molecular mass and to remove any small ligands introduced in affinity chromatography steps. Furthermore, different from its *Drosophila* homolog because the human pol γ–β dimer does not require co-overexpression with pol γ–α to obtain a soluble form, we have benefited by use of *E. coli* as the overexpression system, as we have done for overexpression of both fly and human mtSSBs. In principle, after preparation of a soluble fraction, a single chromatographic step combined with a final step of velocity sedimentation is sufficient to obtain highly pure proteins. Velocity sedimentation is of course also useful to link activity and polypeptide profiles and to obtain an *S* value of the protein of interest to help in the determination of subunit structure. Again, these approaches can be used as general guidelines for proteins involved in mitochondrial nucleic acid metabolism. The authors are pleased to address any queries by electronic mail and wish all success in the development of new purification strategies.

2. Materials

2.1. Recombinant D. melanogaster DNA Polymerase γ Purification

The ionic strength of all buffers is determined using a Radiometer conductivity meter.

1. Construction of recombinant transfer vectors and baculoviruses encoding the two subunits of *D. melanogaster* pol γ is described in *Ref. (19)*. Briefly, the complete coding sequences were cloned into the baculovirus transfer vector pVL1392/1393 (PharMingen) and viruses were constructed using linearized wild-type baculovirus AcMNPV DNA (BaculoGold) (PharMingen). The recombinant baculoviruses used here, $\alpha_{\text{C-HIS}}$ (α with a C-terminal hexahistidine tag inserted between Ser1145 and the stop codon) and β may be obtained from the authors.
2. *Sf9* (*Spodoptera frugiperda*) cells (PharMingen).
3. TC-100 insect cell culture medium and fetal bovine serum (GIBCO-BRL).
4. Insect cell transfection buffer and Grace's medium (PharMingen).
5. Phosphate-buffered saline (PBS): 135 mM NaCl, 10 mM Na_2HPO_4, 2 mM KCl, 2 mM KH_2PO_4.
6. Phosphocellulose P-11 (Whatman), prepared according to the manufacturer's directions.
7. Ni-NTA agarose (QIAGEN).
8. 3 M Ammonium sulfate [$(NH_4)_2SO_4$], ultrapure.
9. 1 M Sucrose, ultrapure.
10. 1 M HEPES-OH, pH 8.0, stored at 4°C.
11. 1 M Tris–HCl, pH 7.5.
12. 0.5 M Ethylenediaminetetraacetic acid (EDTA), pH 8.0.
13. 1 M Dithiothreitol (DTT). Store aliquots at –20°C.
14. 2-Mercaptoethanol.
15. 1 M Imidazole.
16. 10% Triton X-100.
17. 2 M Potassium chloride (KCl).
18. 1 M Potassium phosphate (K_2HPO_4/KH_2PO_4), pH 7.6.
19. 5 M Sodium chloride (NaCl).
20. Phenylmethylsulfonyl fluoride (PMSF) (Sigma) prepared as a 0.2 M stock solution in isopropyl alcohol and stored at –20°C.
21. Sodium metabisulfite is prepared as a 1.0 M stock solution at pH 7.5 and stored at –20°C.

22. Leupeptin is prepared as a 1 mg/ml stock solution in 50 mM Tris–HCl, pH 7.5, and 2 mM EDTA, and stored at –20°C.
23. 7 ml Dounce homogenizer.
24. Collodion membranes (Schleicher and Schuell).
25. Polyallomer centrifuge tubes (14 × 89 mm, Beckman).
26. All potassium phosphate buffers are at pH 7.6.
27. All buffers used throughout the purification contain 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium metabisulfite, and leupeptin at 2 μg/ml. Where indicated, buffers also contain 0.015% Triton X-100.
28. Homogenization buffer (50 mM Tris–HCl, pH 7.5, 100 mM KCl, 280 mM ultrapure sucrose, 5 mM EDTA).
29. 10 mM Potassium phosphate buffer (10 mM potassium phosphate, pH 7.6, 20% glycerol).
30. 80 mM Potassium phosphate buffer – phosphocellulose equilibration buffer (80 mM potassium phosphate, pH 7.6, 20% glycerol).
31. 100 mM Potassium phosphate buffer – phosphocellulose wash buffer (100 mM potassium phosphate, pH 7.6, 20% glycerol).
32. 150 mM Potassium phosphate buffer – phosphocellulose elution buffer (150 mM potassium phosphate, pH 7.6, 20% glycerol).
33. 350 mM Potassium phosphate buffer – phosphocellulose elution buffer (350 mM potassium phosphate, pH 7.6, 20% glycerol).
34. 600 mM Potassium phosphate buffer – phosphocellulose elution buffer (600 mM potassium phosphate, pH 7.6, 20% glycerol).
35. Ni-NTA agarose equilibration buffer (20 mM Tris–HCl, pH 7.5, 500 mM KCl, 8% glycerol, and 5 mM imidazole).
36. Ni-NTA agarose elution buffer 1 (20 mM Tris–HCl, pH 7.5, 500 mM KCl, 8% glycerol, and 25 mM imidazole).
37. Ni-NTA agarose elution buffer 2 (20 mM Tris–HCl, pH 7.5, 500 mM KCl, 8% glycerol, and 250 mM imidazole).
38. Ni-NTA agarose elution buffer 3 (20 mM Tris–HCl, pH 7.5, 500 mM KCl, 8% glycerol, and 500 mM imidazole).
39. Glycerol gradient buffer (50 mM potassium phosphate, pH 7.6, 200 mM $(NH_4)_2SO_4$, 0.015% Triton X-100).
40. Stabilization buffer (25 mM HEPES, pH 8.0, 2 mM EDTA, 80% glycerol, 0.015% Triton X-100).

2.2. Recombinant Human DNA Polymerase γ–α Purification

1. Human $\alpha_{\text{C-HIS}}$ recombinant baculovirus encoding the catalytic subunit of human pol .γ was obtained from Dr. William C. Copeland, Laboratory of Molecular Genetics, National Institutes of Environmental Health Sciences, Research Triangle Park, NC.
2. All potassium phosphate buffers are at pH 7.6.
3. All buffers used throughout the purification contain 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium metabisulfite, and leupeptin at 2 μg/ml. Where indicated, buffers also contain 0.015% Triton X-100.
4. Homogenization buffer (50 mM Tris–HCl, pH 7.5, 100 mM KCl, 5 mM EDTA).
5. 10 mM Potassium phosphate buffer (10 mM potassium phosphate, pH 7.6, 20% glycerol).
6. 80 mM Potassium phosphate buffer – phosphocellulose equilibration buffer (80 mM potassium phosphate, pH 7.6, 20% glycerol).
7. 100 mM Potassium phosphate buffer – phosphocellulose wash buffer (100 mM potassium phosphate, pH 7.6, 20% glycerol).
8. 150 mM Potassium phosphate buffer – phosphocellulose elution buffer (150 mM potassium phosphate, pH 7.6, 20% glycerol).
9. 350 mM Potassium phosphate buffer – phosphocellulose elution buffer (350 mM potassium phosphate, pH 7.6, 20% glycerol).
10. 600 mM Potassium phosphate buffer – phosphocellulose elution buffer (600 mM potassium phosphate, pH 7.6, 20% glycerol).
11. Ni-NTA agarose equilibration buffer (20 mM Tris–HCl, pH 7.5, 500 mM KCl, 8% glycerol, 0.1% Tx-100, and 5 mM imidazole).
12. Ni-NTA agarose elution buffer 1 (20 mM Tris–HCl, pH 7.5, 500 mM KCl, 8% glycerol, and 25 mM imidazole).
13. Ni-NTA agarose elution buffer 2 (20 mM Tris–HCl, pH 7.5, 500 mM KCl, 8% glycerol, and 250 mM imidazole).
14. Ni-NTA agarose elution buffer 3 (20 mM Tris–HCl, pH 7.5, 500 mM KCl, 8% glycerol, and 500 mM imidazole).
15. Glycerol gradient buffer (30 mM Tris–HCl, pH 7.5, 100 mM KCl, 2 mM EDTA).
16. Stabilization buffer (25 mM HEPES, pH 8.0, 2 mM EDTA, 80% glycerol, 0.015% Triton X-100).
17. Other materials are as in **Section 2.1.**

2.3. DNA Polymerase Assay

1. Pol γ, approx. 0.1 unit/μl.
2. DNase-I activated calf thymus DNA, 2 mg/ml stock, prepared as described *(23)*.
3. 5X Polymerase buffer (250 mM Tris–HCl, pH 8.5, 20 mM $MgCl_2$, 2 mg/ml BSA).
4. 1 M DTT (Sigma).
5. 2 M KCl.
6. Stock mix of deoxynucleoside triphosphates containing 1 mM each of dGTP, dATP, dCTP, and dTTP (Amersham Pharmacia Biotech).
7. [^{3}H]-dTTP (ICN Biochemicals).
8. 100% Trichloroacetic acid (TCA).
9. 0.1 M Sodium pyrophosphate (NaPPi).
10. Acidic wash solution (1 M HCl, 0.1 M NaPPi).
11. 95% Ethanol.
12. Glass-fiber filter paper (Schleicher and Schuell).
13. 10 × 75 mm Disposable culture tubes (Fisher).

2.4. Recombinant Human DNA Polymerase γ–β Purification

1. pQESL encoding the human pol γ–β without the mitochondrial presequence was obtained from Dr. William C. Copeland, Laboratory of Molecular Genetics, National Institutes of Environmental Health Sciences, Research Triangle Park, NC.
2. *E. coli* XL-1 Blue.
3. Bacterial media (L broth): 1% tryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl, pH 7.5.
4. 100 mg/ml Ampicillin. Filter-sterilize with a 0.2-μm syringe filter, aliquot, and store at –20°C.
5. 10 mg/ml Tetracycline in 70% ethanol. Filter-sterilize with a 0.2-μm syringe filter, aliquot, and store at –20°C.
6. 100 mM Isopropyl-β-D-thiogalactopyranoside (IPTG). Aliquot and store at –20°C.
7. Ultrasonic Processor Model W-225 (Heat Systems, Ultrasonics, Inc.).
8. 1 M Tris–HCl, pH 7.5.
9. 0.5 M EDTA, pH 8.0.
10. 5 M NaCl.
11. 3 M KCl.
12. 1 M Potassium phosphate (K_2HPO_4/KH_2PO_4), pH 7.6.
13. 100% Glycerol, anhydrous (J.T. Baker).
14. 80% Sucrose, ultrapure.

15. 0.2 M Phenylmethylsulfonyl fluoride (PMSF) in isopropanol. Store aliquots at –20°C.
16. 1 M Sodium metabisulfite, prepared as a 1.0 M stock solution at pH 7.5 and stored at –20°C.
17. Leupeptin is prepared as a 1 mg/ml stock solution in 50 mM Tris–HCl, pH 7.5, 2 mM EDTA, and stored at –20°C.
18. 1 M Imidazole.
19. 10% Sodium dodecyl sulfate (SDS).
20. 5 X SDS loading buffer (50% glycerol, 2 M Tris base, 0.25 M DTT, 5% SDS, 0.1% bromophenol blue). Aliquots are stored at –20°C.
21. 10% SDS-polyacrylamide resolving gels (8 cm × 10 cm × 1 mm) with 4% stacking gels.
22. Polyallomer centrifuge tubes (14 × 89 mm, Beckman).
23. Ni-NTA agarose (QIAGEN).
24. All buffers used throughout the purification contain 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium metabisulfite, and leupeptin at 2 μg/ml.
25. Tris–sucrose buffer (50 mM Tris–HCl, pH 7.5, 10% sucrose).
26. Resuspension buffer (35 mM Tris–HCl, pH 7.5, 150 mM NaCl, 25 mM imidazole, 0.1% (v/v) Triton X-100).
27. Ni-NTA agarose equilibration buffer (35 mM Tris–HCl, pH 7.5, 500 mM KCl, 25 mM imidazole).
28. Ni-NTA agarose elution buffer 1 (35 mM Tris–HCl, pH 7.5, 500 mM KCl, 25 mM imidazole).
29. Ni-NTA agarose elution buffer 2 (35 mM Tris–HCl, pH 7.5, 500 mM KCl, 250 mM imidazole).
30. Ni-NTA agarose elution buffer 3 (35 mM Tris–HCl, pH 7.5, 500 mM KCl, 500 mM imidazole).
31. Glycerol gradient buffer (35 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA).

2.5. Recombinant Drosophila mtSSB Purification

1. pET-11a encoding *Drosophila* mtSSB without the mitochondrial presequence, available from this lab.
2. *E. coli* BL21 (λDE3) pLysS (Stratagene).
3. Bacterial media (L broth): 1% tryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl, pH 7.5.
4. 100 mg/ml Ampicillin. Filter-sterilize with a 0.2-μm syringe filter, aliquot, and store at –20°C.
5. 100 mM IPTG. Aliquot and store at –20°C.
6. 1 M Tris–HCl, pH 7.5.
7. 0.5 M EDTA, pH 8.0.

8. 5 M NaCl.
9. 3 M Sodium thiocyanate (NaSCN).
10. 1 M Potassium phosphate (K_2HPO_4/KH_2PO_4), pH 7.6.
11. 80% Sucrose, ultrapure.
12. 100% Glycerol, anhydrous (J.T. Baker).
13. 0.2 M Phenylmethylsulfonyl fluoride (PMSF) in isopropanol. Store aliquots at –20°C.
14. 1 M sodium metabisulfite, prepared as a 1.0 M stock solution at pH 7.5 and stored at –20°C.
15. Leupeptin is prepared as a 1 mg/ml stock solution in 50 mM Tris–HCl, pH 7.5, 2 mM EDTA, and stored at –20°C.
16. 1 M DTT. Store aliquots at –20°C.
17. 1 M Spermidine trihydrochloride ($SpCl_3$, Sigma). Store at –20°C.
18. 3 M Ammonium sulfate [$(NH_4)_2SO_4$], ultrapure.
19. 100% TCA. Store at 0–4°C.
20. 10% SDS.
21. 5 X SDS loading buffer (50% glycerol, 2 M Tris base, 0.25 M DTT, 5% SDS, 0.1% bromophenol blue). Aliquots are stored at –20°C.
22. 17% SDS-polyacrylamide resolving gels (8 cm × 10 cm × 1 mm) with 4% stacking gels.
23. 5% Tween 20.
24. Centricon-30 spin concentrators (Amicon/Millipore).
25. Polyallomer centrifuge tubes (14 × 89 mm, Beckman).
26. Blue Sepharose CL-6B (Amersham Pharmacia Biotech).
27. All buffers used throughout the purification contain 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium metabisulfite, leupeptin at 2 μg/ml, and 2 mM DTT.
28. Tris–sucrose buffer (50 mM Tris–HCl, pH 7.5, 10% sucrose).
29. 5 X Lysis buffer (1.25 M NaCl, 25 mM DTT, 100 mM $SpCl_3$, 10 mM EDTA, 1.5 mg/ml lysozyme).
30. Dilution buffer (30 mM Tris–HCl, pH 7.5, 10% glycerol, 2 mM EDTA).
31. Blue Sepharose equilibration buffer (30 mM Tris–HCl, pH 7.5, 10% glycerol, 100 mM NaCl, 2 mM EDTA).
32. Blue Sepharose wash buffer (30 mM Tris–HCl, pH 7.5, 10% glycerol, 800 mM NaCl, 2 mM EDTA).
33. Blue Sepharose elution buffer 1 (30 mM Tris–HCl, pH 7.5, 10% glycerol, 0.5 M NaSCN, 2 mM EDTA).
34. Blue Sepharose elution buffer 2 (30 mM Tris–HCl, pH 7.5, 10% glycerol, 1 M NaSCN, 2 mM EDTA).

35. Blue Sepharose elution buffer 3 (30 mM Tris–HCl, pH 7.5, 10% glycerol, 1.5 M NaSCN, 2 mM EDTA).
36. Glycerol gradient buffer (50 mM Tris–HCl, pH 7.5, 200 mM $(NH_4)_2SO_4$, 2 mM EDTA).

2.6. Recombinant Human mtSSB Purification

1. pET-11a encoding the human mtSSB without the mitochondrial presequence (beginning with the amino acid sequence: ESETTTSLV), available from this lab.
2. All buffers used throughout the purification contain 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium metabisulfite, leupeptin at 2 μg/ml, and 2 mM DTT.
3. Tris–sucrose buffer (50 mM Tris–HCl, pH 7.5, 10% sucrose).
4. 5 X Lysis buffer (1.25 M NaCl, 25 mM DTT, 100 mM $SpCl_3$, 10 mM EDTA, 1.5 mg/ml lysozyme).
5. Dilution buffer (30 mM Tris–HCl, pH 7.5, 10% glycerol, 2 mM EDTA).
6. Blue Sepharose equilibration buffer (30 mM Tris–HCl, pH 7.5, 10% glycerol, 100 mM NaCl, 2 mM EDTA).
7. Blue Sepharose wash buffer (30 mM Tris–HCl, pH 7.5, 10% glycerol, 400 mM NaCl, 2 mM EDTA).
8. Blue Sepharose elution buffer 1 (30 mM Tris–HCl, pH 7.5, 10% glycerol, 700 mM NaCl, 2 mM EDTA).
9. Blue Sepharose elution buffer 2 (30 mM Tris–HCl, pH 7.5, 10% glycerol, 1.5 M NaCl, 2 mM EDTA).
10. Blue Sepharose elution buffer 3 (30 mM Tris–HCl, pH 7.5, 10% glycerol, 3.0 M NaCl, 2 mM EDTA).
11. Glycerol gradient buffer (35 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA).
12. Other materials are as in **Section 2.5**.

3. Methods

3.1. Purification of Recombinant Drosophila DNA polymerase γ

3.1.1. Sf9 Cell Growth and Soluble Cytoplasmic Fraction Preparation

1. Grow *Sf9* cells (500 ml) in TC-100 insect cell culture medium containing 10% fetal bovine serum at 27°C to a cell density of 2×10^6, dilute to a cell density of 1×10^6 with TC-100 containing 10% fetal bovine serum, and then infect with recombinant *Drosophila* pol γ-$\alpha_{C\text{-}HIS}$ and -β baculoviruses at a multiplicity of infection of 5. Harvest 48 h postinfection.
2. Pellet the cells at 400 × g for 5 min, wash with an equal volume of cold PBS, repeat the centrifugation, and discard the supernatant.

3. Resuspend the cell pellet (approx. 1×10^9 cells) using 0.01 culture volume of homogenization buffer.
4. Lyse cells by 20 strokes in a Dounce homogenizer.
5. Centrifuge the homogenate at 1,000 × g for 7 min.
6. Resuspend the resulting pellet using 0.005 culture volume of homogenization buffer and rehomogenize and centrifuge as in Steps 4 and 5 (*see* **Note 1**).
7. Centrifuge the combined supernatant fractions at 8,000 × g for 15 min to pellet the mitochondria.
8. Remove the supernatant and centrifuge at 100,000 × g for 30 min to obtain the cytoplasmic soluble fraction (fraction I).

3.1.2. Phosphocellulose Chromatography and Ammonium Sulfate Fractionation

1. Adjust fraction I (70–90 mg protein) to an ionic equivalent of 80 mM potassium phosphate and load onto a phosphocellulose column equilibrated with 80 mM potassium phosphate buffer at a packing ratio of 6 mg protein per packed ml of resin, at a flow rate of 0.8 column volume (CV)/h (*see* **Note 2**).
2. Wash the column with 3 CV of 100 mM potassium phosphate buffer at a flow rate of 2 CV/h.
3. Elute proteins with a 3-CV linear gradient from 150 to 350 mM potassium phosphate buffer at a flow rate of 2 CV/h.
4. Follow the gradient with a 2-CV high-salt wash of 600 mM potassium phosphate buffer at the same flow rate.
5. Assay as described in **Section 3.3**., and pool active fractions (fraction II, *see* **Note 3**). Adjust to a final concentration of 10% sucrose.
6. Add solid ammonium sulfate (0.351 g/ml of fraction II) and incubate overnight on ice (*see* **Note 4**).
7. Collect the precipitate by centrifugation at 100,000 × g for 30 min at 3°C.
8. Resuspend the pellet in 0.05–0.1 volume of fraction II with 10 mM potassium phosphate buffer containing 45% glycerol, and store at –20°C (fraction IIb).

3.1.3. Ni-NTA Agarose Affinity Purification

1. Dialyze fraction IIb (3–5 mg protein) against 10 mM potassium phosphate buffer in a collodion bag (M_r cutoff 25 kDa) until an ionic equivalent of 100 mM KCl is reached.
2. Mix dialyzed fraction IIb with 500 µl of pre-charged Ni-NTA agarose (QIAGEN) equilibrated in Ni-NTA agarose equilibration buffer.
3. Incubate the suspension for 10 h on ice with gentle shaking.

4. Allow the beads to settle and then wash twice with 1 ml of equilibration buffer for 30 min with gentle shaking.
5. Pack washed beads into a column (0.5 ml), and elute protein retained on the beads successively at 5 CV/h with elution buffer containing 25 mM imidazole (2 CV), 250 mM imidazole (2 CV), and 500 mM imidazole (2 CV). Collect 1/4-CV fractions.
6. Assay and pool active fractions (fraction III, approx. 0.9 ml).

3.1.4. Glycerol Gradient Sedimentation

1. Layer fraction III onto two pre-formed 12–30% glycerol gradients prepared in polyallomer tubes for use in a Beckman SW 41 rotor.
2. Centrifuge at 140,000 × g for 60 h at 3°C and then fractionate by collecting five-drop (200–300 µl) fractions.
3. Assay and pool active fractions, then add an equal volume of stabilization buffer and store the enzyme (fraction IV) at –20°C, –80°C, or under liquid nitrogen.

3.2. Purification of Recombinant Human DNA Polymerase γ–α

3.2.1. Sf 9 Cell Growth and Soluble Cytoplasmic Fraction Preparation

1. Grow and dilute *Sf 9* cells (500 ml) as described in Step 1 of **Section 3.1.1**. and then infect with recombinant human pol γ–$\alpha_{\text{C-HIS}}$ at a multiplicity of infection of 5. Harvest 48 h postinfection.
2. Pellet the cells at 400 × g for 5 min and wash with an equal volume of cold PBS, repeat the centrifugation and discard the supernatant.
3. Resuspend the cell pellet (approx. 1×10^9 cells) using 0.03 culture volume of homogenization buffer.
4. Lyse cells by 20 strokes in a Dounce homogenizer.
5. Centrifuge the homogenate at 1,000 × g for 7 min.
6. Resuspend the resulting pellet using 0.01 culture volume of homogenization buffer, rehomogenize and centrifuge as in Steps 4 and 5 (*see* **Note 1**).
7. Follow Steps 7 and 8 of **Section 3.1.1**. to obtain the cytoplasmic soluble fraction (fraction I).

3.2.2. Phosphocellulose Chromatography and Ammonium Sulfate Fractionation

1. Adjust fraction I (140–240 mg protein) to an ionic equivalent of 80 mM potassium phosphate and load onto a phosphocellulose column equilibrated with 80 mM potassium phosphate buffer at a packing ratio of 6 mg protein per packed ml of resin, at a flow rate of 0.8 CV/h (*see* **Note 2**).
2. Wash the column and elute proteins as described in Steps 2–4 of **Section 3.1.2**.
3. Assay and pool active fractions (fraction II, *see* **Note 3**) and adjust to a final concentration of 10% sucrose.

4. Precipitate and pellet proteins as described in Steps 6 and 7 of **Section 3.1.2**.
5. Resuspend the pellet in 0.05–0.1 volume of fraction II in 10 mM potassium phosphate buffer containing 45% glycerol, and store at –20°C (fraction IIb).

3.2.3. Ni-NTA Affinity Chromatography

1. Dialyze fraction IIb (10–20 mg protein) against 10 mM potassium phosphate buffer in a collodion bag (M_r cutoff 10 kDa) until an ionic equivalent of approx. 300–400 mM KCl is reached.
2. Load dialyzed fraction IIb onto an equilibrated Ni-NTA resin at a ratio of approx. 7.5 mg protein per ml of packed resin, at a flow rate of 1–2 CV/h.
3. Wash the column with 2 CV of equilibration buffer and elute successively with elution buffers containing 25 mM imidazole (2 CV), 250 mM imidazole (2 CV), and 500 mM imidazole (2 CV), at a flow rate of 5 CV/h. Collect 1/4-CV fractions.
4. Assay and pool active fractions (fraction III, approx. 1.6–2.3 ml).

3.2.4. Glycerol Gradient Sedimentation

1. Layer fraction III onto two pre-formed 12–30% glycerol gradients prepared in polyallomer tubes for use in a Beckman SW 41 rotor.
2. Centrifuge at 140,000 × g for 60 h at 3°C, then fractionate by collecting five-drop (200–300 μl) fractions.
3. Assay and pool active fractions, then add an equal volume of glycerol gradient buffer containing 80% glycerol or stabilization buffer, and store the enzyme (fraction IV) at –20°C, –80°C, or under liquid nitrogen.

3.3. DNA Polymerase Assay

This section describes an assay of DNA synthesis that utilizes a multiply primed double-stranded DNA substrate and pol γ fractions. DNA polymerase activity can be measured at different salt concentrations to discriminate nuclear (low-salt stimulated) and mitochondrial (high-salt stimulated) DNA polymerase activities. The assay measures the incorporation of [^{3}H]-labeled dTMP into the DNA substrate, such that 1 unit of activity is the amount that catalyzes the incorporation of 1 nmol of deoxyribonucleoside triphosphate into acid insoluble material in 60 min at 30°C.

1. Adjust the water bath to 30°C.
2. Dry down the radioactive substrate by lyophilizing to less than half the original volume.
3. Prepare a master reaction mix using the stock solutions in **Section 2.3** in a microcentrifuge tube on ice such that each reaction (0.05 ml) contains 1 X polymerase buffer, 10 mM

DTT, 200 mM KCl (*see* **Note 5**), 30 μM each of dGTP, dATP, dCTP, and [^{3}H]dTTP (1,000 cpm/pmol), and 250 μg/ml DNase I-activated calf thymus DNA. Vortex and spin briefly in the microcentrifuge.

4. Dispense the mix, 49 μl, to pre-chilled and numbered 10 × 75-mm tubes on ice.
5. Add the enzyme, approx. 0.1 unit, to each tube avoiding bubbles and mix gently by flicking the tube three times.
6. Incubate the tubes for 30 min at 30°C, then transfer to ice.
7. Stop the reactions with 1 ml of 10% TCA, 0.1 M NaPPi, mix, and leave on ice ≥ 5 min.
8. Filter samples through glass-fiber filters. Wash the tube twice with acidic wash solution, then wash the filtration funnel three times with acidic wash solution and once with 95% ethanol.
9. Dry the filters under a heat lamp for approx. 5 min, then count in scintillation fluid in a liquid scintillation counter.
10. Spot 1–2 μl of mix directly onto filters, dry, and count in scintillation fluid without filtration, to calculate the specific radioactivity of the mix.

3.4. Purification of Recombinant Human DNA Polymerase γ–β

3.4.1. Bacterial Cell Growth and Protein Overproduction

1. Inoculate 2 l of L broth containing 15 μg/ml tetracycline and 100 μg/ml ampicillin with *E. coli* XL-1 Blue containing pQESL (complete human pol γ–β cDNA without the mitochondrial presequence) at A_{595} = 0.06 and grow with aeration at 37°C (*see* **Note 6**).
2. At A_{595} = 0.6:
   (a) Remove a 1.0-ml aliquot of uninduced cells to a microcentrifuge tube, pellet cells, aspirate supernatant, and resuspend cells in 200 μl of 1X SDS loading buffer. Use a 10-μl aliquot as control for SDS-PAGE.
   (b) Induce target protein expression by adding IPTG to 0.3 mM final concentration and continue incubation at 37°C with aeration for 2 h.
3. Harvest cells:
   (a) Save a 1.0-ml aliquot of induced cells as in Step 2a. Use a 5-μl aliquot in SDS-PAGE.
   (b) Harvest remaining cells in Sorvall GSA bottles by centrifugation at 3,600 × g for 5 min at 4°C. Decant supernatant.
4. Resuspend cells in 1/10 volume of original culture in Tris–sucrose buffer. First resuspend pellets in one-half of the total resuspension volume, transferring to a chilled plastic beaker. Wash GSA bottles with remaining one-half of volume and combine.

5. Centrifuge washed cells in a Sorvall SS-34 at 3,000 × g for 5 min at 4°C. Aspirate supernatant and freeze cell pellet in liquid nitrogen. Store at –80°C.

3.4.2. Cell Lysis and Soluble Fraction Preparation

The following protocol uses a 2-l induced cell pellet (A_{595}=1–1.2) as the starting material with an overall expression of approx. 200 μg pol γ–β/ml of induced cells. The ionic strength of buffers is determined using a Radiometer conductivity meter (*see* **Note 1**).

1. Thaw cell pellet (2 l) on ice for at least 30 min.
2. Resuspend cell pellet completely in 1/30 volume of original cell culture in resuspension buffer. Use one-half of the total volume to resuspend the cells and the remaining one-half divided to wash the tubes, combining all samples.
3. Lyse cells by sonication with an Ultrasonic Processor Model W-225 for 20 pulses using microtip at maximum output and 50% usage. Cool the samples for at least 1 min on ice. Repeat sonication and cooling (*see* **Note 7**).
4. Centrifuge in a Sorvall SS-34 rotor at 20,000 × g for 15 min at 4°C.
5. Collect supernatant by pipetting into a fresh tube. Resuspend resulting pellet in 1/80 volume of original cell culture in resuspension buffer, sonicate, and centrifuge as described in Steps 3 and 4.
6. Collect supernatant and combine with the previous one (fraction I).

3.4.3. Ni-NTA Chromatography and Glycerol Gradient Sedimentation

The following protocol uses a soluble bacterial fraction from 2 l of induced cells loaded onto an equilibrated Ni-NTA column.

1. Load fraction I onto an equilibrated Ni-NTA resin at a ratio of approx. 7.5 mg protein per ml of packed resin, at a flow rate of 2 CV/h.
2. Elute protein by successive washes with elution buffers containing 25 mM imidazole (3 CV), 250 mM imidazole (2 CV), and 500 mM imidazole (2 CV), at a flow rate of 2 CV/h. Collect 1/2-CV fractions for the first elution, 1/6-CV fractions for the second elution, and then 1/3-CV fractions for the last elution.
3. Analyze column fractions, 5 μl, by SDS-PAGE in 10% gels followed by silver nitrate staining.
4. Pool fractions containing pol γ–β (fraction II, approx. 1.6–2.3 ml).
5. Prepare two (10 ml) 12–30% glycerol gradients and chill on ice for at least 1 h.
6. Layer 1–1.2 ml of Ni-NTA pool of pol γ–β onto each gradient and spin in a SW41 rotor at 170,000 × g for 60 h at 4°C.

7. Fractionate gradients by collecting 200–300-µl aliquots. Analyze 5 µl per fraction by SDS-PAGE in 10% gels followed by staining with silver nitrate.
8. Pool peak fractions of pol γ–β (fraction III). Pol γ–β typically sediments in fractions 10–16.
9. Aliquot pol γ–β, freeze in liquid nitrogen, and store at –80°C (*see* **Note 8**).

3.5. Purification of Recombinant Drosophila mtSSB

3.5.1. Bacterial Cell Growth and Protein Overproduction

1. Inoculate 1 l of L broth containing 100 µg/ml ampicillin with *E. coli* BL21 (λDE3) pLysS containing pET11a–*Drosophila* mtSSB (complete cDNA without the mitochondrial presequence) at A_{595} = 0.06 and grow with aeration at 37°C (*see* **Note 6**).
2. At A_{595} = 0.6:
   (a) Remove a 1.0-ml aliquot of uninduced cells to a microcentrifuge tube, pellet cells, aspirate supernatant, and resuspend cells in 200 µl of 1X SDS loading buffer. Use a 10-µl aliquot as control for SDS-PAGE.
   (b) Induce target protein expression by adding IPTG to 0.3 mM final concentration and continue incubation at 37°C with aeration for 2 h.
3. Harvest cells:
   (a) Save a 1.0-ml aliquot of induced cells as in Step 2a. Use a 5-µl aliquot in SDS-PAGE.
   (b) Harvest remaining cells in Sorvall GSA bottles by centrifugation at 3,600 × g for 5 min at 4°C. Decant supernatant.
4. Resuspend cells in 1/10 volume of original culture in Tris–sucrose buffer. First resuspend pellets in one-half of the total resuspension volume, transferring to a chilled plastic beaker. Wash GSA bottles with remaining one-half of volume and combine.
5. Aliquot washed cells into Sorvall SS-34 tubes, 200 ml cell equivalent per tube, and centrifuge cells at 3,000 × g for 5 min at 4°C. Aspirate supernatant and freeze cell pellets in liquid nitrogen. Store at –80°C.

3.5.2. Cell Lysis and Soluble Fraction Preparation

The following protocol uses 400-ml induced cell pellets (A_{595}=1–1.2) as the starting material with an overall expression of 15 µg mtSSB/ml of induced cells. The ionic strength of buffers is determined using a Radiometer conductivity meter (*see* **Note 2**).

1. Thaw cell pellets (2 × 200 ml) on ice for at least 30 min.
2. Resuspend cell pellets completely in 1/25 volume of Tris–sucrose buffer. Use one-half of the total volume to resuspend the cells and the remaining one-half divided to wash the tubes, combining all samples.

3. Add 5X lysis buffer to 1X final concentration. Incubate for 30 min on ice, mixing every 5 min by inversion.
4. Freeze cell lysis suspension in liquid nitrogen. Thaw partially in ice water, then transfer to ice until thawed completely. Centrifuge in Sorvall SS-34 rotor at 17,500 × g for 30 min at 4°C.
5. Collect soluble fraction (supernatant) by pipetting into a fresh tube. Adjust the sample to 100 mM NaCl by the addition of dilution buffer.

3.5.3. Blue Sepharose Chromatography and Glycerol Gradient Sedimentation

The following protocol uses a soluble bacterial fraction from 400 ml of induced cells loaded onto 10 ml of equilibrated Blue Sepharose resin.

1. Load salt-adjusted soluble fraction (approx. 15–20 mg protein) at a flow rate of 1 CV/h onto a 10-ml column of Blue Sepharose equilibrated with 10-CV equilibration buffer.
2. Wash column with 4 CV of wash buffer at a flow rate of 2 CV/h, collecting 1-CV fractions.
3. Elute column with three steps of increasing NaSCN stringency at 2 CV/h:
   (a) 4 CV of elution buffer containing 0.5 M NaSCN, collecting 1/2-CV fractions.
   (b) 4 CV of elution buffer containing 1 M NaSCN, collecting 1/3-CV fractions.
   (c) 4 CV of elution buffer containing 1.5 M NaSCN, collecting 1/4-CV fractions.

   Drosophila mtSSB elutes in the 1.5 M NaSCN step.
4. Analyze column fractions, 5 µl, by SDS-PAGE in 17% gels.
5. Pool fractions containing mtSSB and spin-concentrate sample in a Centricon-30 concentrator (pre-treated with 5% Tween 20, *see* **Note 9**) to desalt. Exchange elution buffer for 50 mM potassium phosphate, pH 7.6, 8% glycerol, 200 mM $(NH_4)_2SO_4$, 2 mM EDTA.
6. Prepare three (10-ml) 12–30% glycerol gradients and chill on ice for at least 1 h.
7. Layer 1–1.2 ml of concentrated mtSSB pool onto each gradient and spin in SW41 rotor at 170,000 × g for 60 h at 4°C.
8. Fractionate gradients by collecting 200–300-µl aliquots. Analyze 30 µl per fraction by TCA precipitation followed by SDS-PAGE in 17% gels followed by staining with silver nitrate.

9. Pool peak fractions of tetrameric mtSSB, which typically sediments in fractions 25–31 (*see* **Note 10**). Concentrate gradient pool using a Centricon-30 spin concentrator (pre-treated with 5% Tween 20, *see* **Note 9**).
10. Aliquot concentrated mtSSB, freeze in liquid nitrogen, and store at –80°C (*see* **Note 8**).

3.6. Purification of Recombinant Human mtSSB

3.6.1. Bacterial Cell Growth and Protein Overproduction

1. Inoculate 2 l of L broth containing 100 μg/ml ampicillin with *E. coli* BL21 (λDE3) pLysS containing pET11a–human mtSSB (complete cDNA without the mitochondrial presequence) at A_{595} = 0.06 and grow with aeration at 37°C (*see* **Note 6**).
2. At A_{595} = 0.6:
   (a) Remove a 1.0-ml aliquot of uninduced cells to a microcentrifuge tube, pellet cells, aspirate supernatant, and resuspend cells in 200 μl of 1X SDS loading buffer. Use a 10-μl aliquot as control for SDS-PAGE.
   (b) Induce target protein expression by adding IPTG to 0.2 mM final concentration and continue incubation at 37°C with aeration for 3 h.
3. Harvest cells:
   (a) Save a 1.0-ml aliquot of induced cells as in Step 2a. Use a 5-μl aliquot in SDS-PAGE. Stain the gel with Coomassie Blue (*see* **Note 11**).
   (b) Harvest remaining cells in Sorvall GSA bottles by centrifugation at 3,600 × g for 5 min at 4°C. Decant supernatant.
4. Resuspend cells in Tris–sucrose buffer as described in Step 4 of **Section 3.5.1**.
5. Centrifuge washed cells into a Sorvall SS-34 tube at 3,000 × g for 5 min at 4°C. Aspirate supernatant and freeze cell pellet in liquid nitrogen. Store at –80°C.

3.6.2. Cell Lysis and Soluble Fraction Preparation

The following protocol uses 2-l induced cell pellet (A_{595}=1–1.3) as the starting material with an overall expression of approx. 250 μg mtSSB/ml of induced cells. The ionic strength of buffers is determined using a Radiometer conductivity meter (*see* **Note 2**).

1. Thaw cell pellet (2 l) on ice for at least 30 min.
2. Resuspend cell pellet in Tris–sucrose buffer as described in Step 2 of **Section 3.6.2**.
3. Follow Steps 3–5 of **Section 3.6.2**. to obtain soluble fraction (*see* **Note 12**). Adjust the sample to 100 mM NaCl by the addition of dilution buffer.

3.6.3. Blue Sepharose Chromatography and Glycerol Gradient Sedimentation

The following protocol uses a soluble bacterial fraction from 2 l of induced cells loaded onto 10 ml of equilibrated Blue Sepharose resin.

1. Load salt-adjusted soluble fraction (approx. 75 mg protein) at a flow rate of 1 CV/h onto a 10-ml column of Blue Sepharose equilibrated with 10-CV equilibration buffer.
2. Wash column with 4 CV of wash buffer at a flow rate of 2 CV/h, collecting 1-CV fractions.
3. Elute column with three steps of increasing NaCl stringency at 2 CV/h:
   (a) 4 CV of elution buffer containing 700 mM NaCl, collecting 1/2-CV fractions.
   (b) 4 CV of elution buffer containing 1.5 M NaCl, collecting 1/4-CV fractions.
   (c) 4 CV of elution buffer containing 3.0 M NaCl, collecting 1/4 CV-fractions.

   Human mtSSB elutes between the 1.5 M and 3 M NaCl steps.
4. Analyze column fractions, 10 µl, by SDS-PAGE in 17% gels followed by Coomassie Blue staining.
5. Pool fractions containing mtSSB and spin-concentrate sample in a Centricon-30 concentrator (pre-treated with 5% Tween 20, *see* **Note 9**) to desalt. Use the dilution buffer to adjust the salt concentration of the sample to 100 mM NaCl.
6. Prepare two (10 ml) 12–30% glycerol gradients and chill on ice for at least 1 h.
7. Layer concentrated mtSSB pool onto each gradient, spin, and collect fractions as described in Steps 7 and 8 of **Section 3.6.3**. Analyze 10 µl per fraction by SDS-PAGE in 17% gels followed by staining with Coomassie Blue.
8. Pool peak fractions of tetrameric mtSSB, which typically sediments in fractions 20–31 (*see* **Note 10**).
9. Aliquot pooled mtSSB, freeze in liquid nitrogen, and store at –80°C (*see* **Notes 8 and 13**).

4. Notes

1. An aliquot of the homogenized cells can be analyzed under the microscope to check for cell lysis. If more than 10% of the cells are not lysed, the pellet may be resuspended, rehomogenized, and centrifuged again as in Steps 4–6.

2. All chromatographic and velocity sedimentation operations are performed at 0–4°C.
3. In addition to the DNA polymerase assay, the fractions can be analyzed by immunoblotting and by Bradford determination of protein concentration. These procedures may help to determine the fractions to be pooled.
4. Add 1/10 of the final amount of $(NH_4)_2SO_4$ and wait until the salt goes into the solution. Repeat until the total amount has been added. The sample must be stirred during incubation.
5. For the purification of the human pol γ–α, prepare the master mix such that each reaction contains 60 mM KCl.
6. The volume of *E. coli* cell culture may vary from 1 to 2 l. The materials used for cell harvest and lysis may be adjusted proportionally.
7. Each time it is noticed that the samples are getting warm during sonication, stop pulses and cool them for 1 min on ice.
8. Alternatively, samples may be stabilized by the addition of an equal volume of stabilization buffer (20 mM KPO_4, pH 7.6, 80% glycerol, 2 mM EDTA) and stored at –20°C.
9. Pre-treatment consists of filling up the Centricon-30 tubes with 5% Tween 20 and allowing them to sit overnight at room temperature.
10. Avoid fractions that sediment slowly, trailing toward the top of the gradient. These fractions are likely to contain dimers and monomers of mtSSB.
11. It is important to stain the SDS-polyacrylamide gels used for human mtSSB analysis throughout the purification with Coomassie Blue. We have observed that, especially for the gel analysis of the bacterial cell extract after induction with IPTG, a silver stained gel is not suitable for the detection of human mtSSB.
12. To obtain the soluble fraction of mtSSB-expressing *E. coli* cells, we use 1.5 mg/ml lysozyme in the 5X lysis solution (final concentration in cell suspension: 0.3 mg/ml). In the Blue Sepharose chromatography step of the human mtSSB purification, we have observed that the lysozyme has the same elution profile of human mtSSB, constituting the major contaminant of the Blue Sepharose pool. Despite the fact that human mtSSB and lysozyme have similar sizes (approx. 15 and 14 kDa, respectively), we are able to separate them in the glycerol gradient sedimentation step, because human mtSSB is a tetramer in solution and sediments as a 60-kDa protein. As an alternative cell lysis procedure, we have verified that a 5X buffer containing 1.25 M NaCl, 10 mM EDTA, and 10%

sodium cholate [prepared as a 20% stock, pH *7.4*, and stored at –20°C (cholic acid (Sigma) is dissolved in hot ethanol, filtered through Norit A (J.T. Baker Chemical Co.), and recrystallized twice before titration to pH 7.4 with sodium hydroxide)] is also very efficient (use final concentration of 1X buffer in the cell pellet suspension). Follow up the other steps as described in **Section 3.5.2**. Interestingly, because human and *Drosophila* mtSSBs elute differently from the Blue Sepharose column, most of the lysozymes used in the purification of *Drosophila* mtSSB elute during the wash of the column and does not constitute a major contaminant of the Blue Sepharose pool.

13. The purification procedure presented is suitable for most physical and biochemical experiments involving the human mtSSB, including analysis of quaternary structure, single stranded-DNA binding, pol γ stimulation *etc*. However, we note that on occasion a double-stranded DNA-unwinding contaminant is present in the preparation, which is detected in mtDNA helicase stimulation assays (data not shown). This contaminant can be eliminated by the addition of a phosphocellulose chromatographic step prior to the glycerol gradient sedimentation, which is performed similarly to that described in **Section 3.1.2.** with the following changes: the Blue Separose fraction is adjusted to an ionic equivalent of 60 mM potassium phosphate and loaded onto a phosphocellulose column equilibrated with 60 mM KPO_4, 10% glycerol, 2 mM EDTA, at a packing ratio of 0.5 mg protein/ packed ml; the column is washed with the same buffer and protein is eluted with a gradient from 60 to 150 mM KPO4, 10% glycerol, 2 mM EDTA, followed by a final elution step of 350 mM KPO4, 10% glycerol, 2 mM EDTA.

References

1. Gray, M. W., Burger, G., and Lang, B. F. (1999) Mitochondrial evolution. *Science* **283,** 1476–1481.
2. Kaguni, L.S. (2004) DNA polymerase gamma, the mitochondrial replicase. *Annu. Rev. Biochem.* **73,** 293–320.
3. Korhonen, J. A., Pham, X. H., Pellegrini, M., and Falkenberg, M. (2004) Reconstitution of a minimal mtDNA replisome *in vitro*. *EMBO J.* 23, 2423–2429.
4. Fan, L., Kim, S., Farr, C. L., Schaefer, K. T., Randolph, K. M., Tainer, J. A., and Kaguni, L. S. (2006) A novel processive mechanism for DNA synthesis revealed by structure, modeling and mutagenesis of the accessory subunit of human mitochondrial DNA polymerase. *J. Mol. Biol.* **358,** 1229–1243.
5. Fan, L., and Kaguni, L. S. (2001) Multiple regions of subunit interaction in *Drosophila* mitochondrial DNA polymerase: three functional domains in the accessory subunit. *Biochemistry* 40, 4780–4791.
6. Yakubovskaya, E., Chen, Z., Carrodeguas, J. A., Kisker, C., and Bogenhagen, D. F. (2006) Functional human mitochondrial DNA polymerase γ forms a heterotrimer. *J. Biol. Chem.* **281,** 374–382.
7. Longley, M. J., Graziewicz, M. A., Bienstock, R. J., and Copeland, W. C. (2005) Consequences of mutations in human DNA polymerase γ. *Gene* 354, 125–131.
8. Luoma, P. T., Luo, N., Löscher, W. N., Farr, C. L., Horvath, R., Wanschitz, J., Kiechl, S., Kaguni, L. S., and Suomalainen,

A. (2005) Functional defects due to spacer-region mutations of human mitochondrial DNA polymerase in a family with an ataxia-myopathy syndrome. *Hum. Mol. Genet.* **14,** 1907–1920.

9. Iyengar, B., Roote, J., and Campos, A. R. (1999) The *tamas* gene, identified as a mutation that disrupts larval behavior in *Drosophila melanogaster*, codes for the mitochondrial DNA polymerase catalytic subunit (*DNApol-γ125*). *Genetics* **153,** 1809–1824.
10. Luo, N., and Kaguni, L. S. (2005) Mutations in the spacer region of *Drosophila* mitochondrial DNA polymerase affect DNA binding, processivity, and the balance between pol and exo function. *J. Biol. Chem.* **280,** 2491–2497.
11. Iyengar, B., Luo, N., Farr, C. L., Kaguni, L. S., and Campos, A. R. (2002) The accessory subunit of DNA polymerase γ is essential for mitochondrial DNA maintenance and development in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **99,** 4483–4488.
12. Curth, U., Urbanke, C., Greipel, J., Gerberding, H., Tiranti, V., and Zeviani, M. (1994) Single-stranded DNA-binding proteins from human mitochondria and *Escherichia coli* have analogous physicochemical properties. *Eur. J. Biochem.* **221,** 435–443.
13. Thommes, P., Farr, C. L., Marton, R. F., Kaguni, L. S., and Cotterill, S. (1995) Mitochondrial single-stranded DNA-binding protein from *Drosophila* embryos. Physical and biochemical characterization. *J. Biol. Chem.* **270,** 21137–21143.
14. Farr, C. L., Wang, Y., and Kaguni, L. S. (1999) Functional interactions of mitochondrial DNA polymerase and single-stranded DNA-binding protein: template-primer DNA binding and initiation and elongation of DNA strand synthesis. *J. Biol. Chem.* **274,** 14779–14785.
15. Korhonen, J. A., Gaspari, M., and Falkenberg, M. (2003) TWINKLE has 5'→3' DNA helicase activity and is specifically stimulated by mitochondrial single-stranded DNA-binding protein. *J. Biol. Chem.* **278,** 48627–48632.
16. Maier, D., Farr, C. L., Poeck, B., Alahari, A., Vogel, M., Fischer, S., Kaguni, L. S., and Schneuwly, S. (2001) Mitochondrial single-stranded DNA-binding protein is required for mitochondrial DNA replication and development in *Drosophila melanogaster*. *Mol. Biol. Cell* **12,** 821–830.
17. Fernández-Moreno, M. A., Farr, C. L., Kaguni, L. S., and Garesse, R. (2006) *Drosophila melanogaster* as a model system to study mitochondrial function. *Methods Mol. Biol.* **372,** 33–49.
18. Wernette, C. M., and Kaguni, L. S. (1986) A mitochondrial DNA polymerase from embryos of *Drosophila melanogaster*. Purification, subunit structure, and partial characterization. *J. Biol. Chem.* **261,** 14764–14770.
19. Wang, Y., and Kaguni, L. S. (1999) Baculovirus expression reconstitutes *Drosophila* mitochondrial DNA polymerase. *J. Biol. Chem.* **274,** 28972–28977.
20. Farr, C. L., and Kaguni, L. S. (2002a) Purification strategies for *Drosophila* mtDNA replication proteins in native and recombinant form. DNA polymerase γ. *Methods Mol. Biol.* **197,** 273–283.
21. Farr, C. L., and Kaguni, L. S. (2002b) Purification strategies for *Drosophila* mtDNA replication proteins in native and recombinant form. Mitochondrial single-stranded DNA-binding protein. *Methods Mol. Biol.* **197,** 285–294.
22. Ziebarth, T. D., Farr, C. L., and Kaguni, L. S. (2007) Modular architecture of the hexameric human mitochondrial DNA helicase. *J. Mol. Biol.* **367,** 1382–1391.
23. Fansler, B. S., and Loeb, L. A. (1974) Sea urchin nuclear DNA polymerase. *Methods Enzymol.* **29,** 53–70.

Chapter 4

Functional Analysis of Mutant Mitochondrial DNA Polymerase Proteins Involved in Human Disease

Sherine S. L. Chan and William C. Copeland

Abstract

DNA polymerase γ (pol γ) is the only DNA polymerase within the mitochondrion and is thus essential for replication and repair of mtDNA. *POLG*, the gene encoding the catalytic subunit of pol γ, is a major locus for a wide spectrum of mitochondrial diseases with more than 100 known disease mutations. Thus, we need to understand how and why pol γ defects lead to disease. By using an extensive array of methods, we are developing a clearer understanding of how defects in pol γ contribute to disease. Furthermore, crucial knowledge concerning the role of pol γ in mtDNA replication and repair can be acquired. Here we present the protocols to characterize mutant DNA pol γ proteins, namely, assays for processive DNA synthesis, exonuclease activity, DNA binding, subunit interaction, and protein stability.

Key words: DNA polymerase γ, mitochondrial DNA polymerase, DNA replication, DNA repair, mitochondrial disease, enzyme assays, *POLG*, *POLG2*.

1. Introduction

Mitochondrial diseases affect 1/2,000 to 1/5,000 people in the general population *(1)*. A wide spectrum of mitochondrial disease affects both children and adults. Mutations in nuclear genes encoding mitochondrial DNA (mtDNA) replication components, such as *POLG* and *POLG2*, have been associated with disease *(2)*. DNA polymerase γ (pol γ) is a heterotrimeric enzyme consisting of a 140-kDa catalytic subunit (encoded by *POLG*) with high fidelity DNA polymerase and proofreading activities and a homodimeric 55-kDa accessory subunit (encoded by *POLG2*) for tight DNA binding and processive DNA synthesis *(3, 4)*. Pol γ is the only DNA polymerase within the mitochondrion, thus it is essential for

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_4 Springerprotocols.com

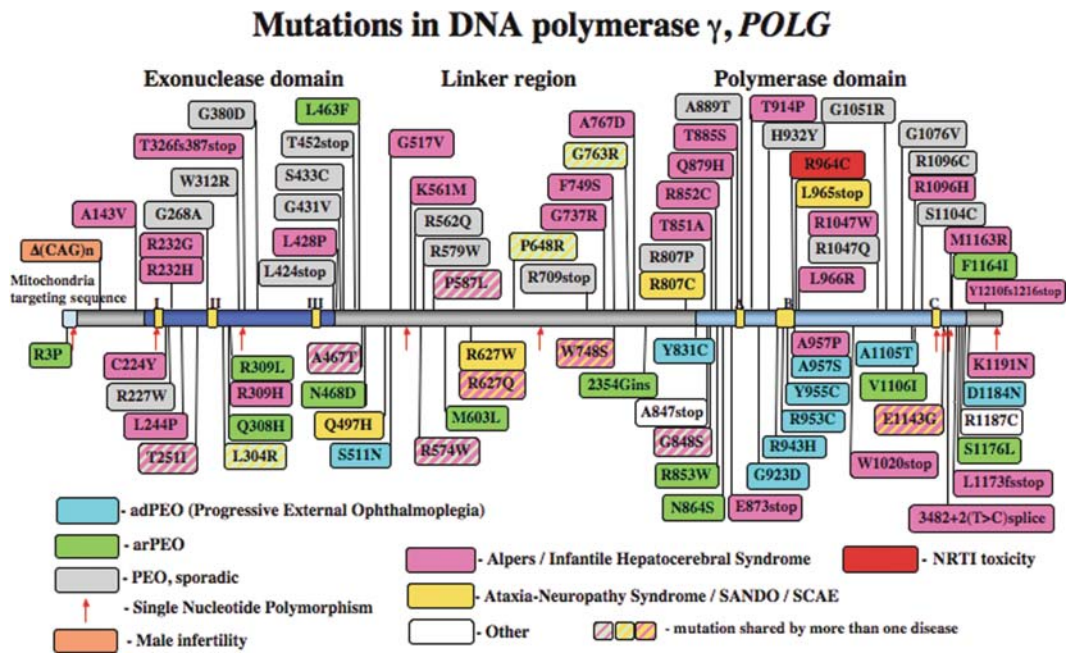


Fig. 4.1. Known disease mutations in *POLG*..

replication and repair of mtDNA. In particular, *POLG* is a major locus for mitochondrial disease (*see* **Fig. 4.1**), with more than 100 mutations associated with the fatal early childhood Alpers syndrome, midlife-onset ataxia neuropathy syndromes, late-onset progressive external ophthalmoplegia (PEO), male infertility, and susceptibility to nucleoside reverse transcriptase inhibitor drugs commonly used to treat HIV (*see* http://tools. niehs.nih.gov/polg/ for references). Furthermore, mutations within the accessory subunit gene (*POLG2*) are known to cause PEO *(5)*.

We and others have characterized a number of disease mutations, in order to determine the nature of the biochemical defects arising in these mutant proteins *(6–13)*. For example, the most common amino acid substitution in *POLG*, A467T, was shown to have a severe catalytic defect, as both polymerase and exonuclease activities were greatly reduced compared to wild-type (WT) enzyme *(11)*. Interestingly, this mutation also disrupts physical association between the catalytic and accessory subunits *(11)*. As more mutant pol γ variants are analyzed the structural basis for the biochemical defects in each enzyme is becoming clear *(12)*. Likewise, the involvement of single nucleotide polymorphisms in disease has been found to be important. In the case of pol γ with both W748S and E1143G in the same allele, the E1143G single-nucleotide polymorphism contributes both beneficial and detrimental effects to the protein *(12)*. Since more patients with mitochondrial disease are found with *POLG* mutations every year,

it is imperative that we understand the mechanisms by which these mutations cause mtDNA instability and ultimately mitochondrial disease, and the contributions of each mutation toward pathology.

By utilizing an extensive array of methods, we are developing a clearer understanding of how defects in pol γ contribute to disease. Furthermore, crucial knowledge concerning the role of pol γ in mtDNA replication and repair can be acquired. Here we present the protocols we use in the laboratory to characterize mutant DNA pol γ proteins, namely, assays to quantify polymerase activity, processivity, exonuclease activity, DNA binding, interaction between subunits, and protein stability.

2. Materials

2.1. Site-Directed Mutagenesis to Introduce DNA Polymerase γ Mutations

QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

2.2. Processivity and Primer Extension Assays

1. Enzyme dilution buffer: 50 mM Tris–HCl (pH 7.5), 10% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol, 50 μg/ml acetylated bovine serum albumin (BSA), 0.1% NP-40, 0.1 M NaCl.
2. T4 polynucleotide kinase.
3. Phosphor storage screen.
4. Typhoon 9400 phosphorimager (GE Healthcare, Piscataway, NJ).
5. NIH Image or ImageJ software (http://rsb.info.nih.gov/nih-image/).

2.3. Exonuclease Assay

1. Gel-purified 40mer oligonucleotide (5′-TCA CTG ATA TAC GTC CGA CTC TGA TGT ACA TGG TCA TGG T-3′).
2. Formamide stop solution: 95% deionized formamide, 0.01 M EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol.
3. 12% Polyacrylamide gel: 19:1 acrylamide, 6.7 M urea, 0.084% ammonium sulfate, 0.02% TEMED in 1X Tris–Borate–EDTA buffer.
4. Phosphor storage screen.
5. Typhoon 9400 phosphorimager (GE Healthcare, Piscataway, NJ).
6. NIH Image or ImageJ software (http://rsb.info.nih.gov/nih-image/).

2.4. Determination of DNA Binding by Electrophoretic Mobility Shift Assay

1. 38mer Oligonucleotide (5′-TTA TCG CAC CTA CGT TCA ATA TTA CAG GCG AAC ATA CT-3′).
2. Complementary 34mer oligonucleotide (5′-GTA TGT TCG CCT GTA ATA TTG AAC GTA GGT GCG A-3′).
3. Binding buffer: 10 mM HEPES-OH (pH 8.0), 0.2 mg/ml acetylated BSA, 2 mM dithiothreitol.
4. Gels: 6% native polyacrylamide (60:1 w/w acrylamide/bis-acrylamide), 20 mM HEPES-OH (pH 8.0), 0.1 mM EDTA, 0.06% ammonium persulfate, 0.1% TEMED.
5. Phosphor storage screen.
6. Typhoon 9400 phosphorimager (GE Healthcare, Piscataway, NJ).
7. NIH Image or ImageJ software (http://rsb.info.nih.gov/nih-image/).

2.5. Immunoprecipitation Assay to Determine Physical Association of both Subunits

1. Rabbit polyclonal antibodies (DPg) against recombinant pol γ *(6)*.
2. Protein G Sepharose 4 Fast Flow beads (GE Healthcare, Piscataway, NJ).
3. NuPAGE 4X LDS Sample Buffer (Invitrogen, Carlsbad, CA).
4. NuPAGE Novex 4–12% Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad, CA).
5. NuPAGE MOPS SDS Running Buffer (Invitrogen, Carlsbad, CA).
6. NuPAGE Antioxidant solution (Invitrogen, Carlsbad, CA).
7. Immobilon-P PVDF membrane (Millipore, Billerica, MA).
8. NuPAGE 20X transfer buffer (Invitrogen, Carlsbad, CA).
9. PBSN–BSA buffer: 0.05 M potassium phosphate buffer, pH 7.5, 0.15 M NaCl, 0.1% NP-40, and 0.1 mg/ml bovine serum albumin (BSA).
10. Buffer TNT: 0.1% Triton X-100, 50 mM Tris–HCl, pH 7.5, 0.5 M NaCl.
11. Buffer TN: 50 mM Tris–HCl, pH 7.5, 0.5 M NaCl.
12. Anti-penta-His monoclonal antibody (Qiagen, Valencia, CA).
13. PBSN buffer: 0.05 M KPO_4, pH 7.5, 0.15 M NaCl, 0.1% NP-40.
14. Goat anti-mouse IgG alkaline phosphatase conjugated secondary antibody (Bio-Rad, Hercules, CA).
15. Western Blue stabilized substrate for alkaline phosphatase reagent (Promega, Madison, WI).

16. 3% (w/v) Trichloroacetic acid (TCA): Make a 100% (w/v) stock solution by dissolving 500 g crystalline TCA in 227 ml water to make 500 ml total volume. From this stock solution, dilute to make a 3% solution.

2.6. Circular Dichroism Measurements to Determine Protein Stability

1. Jasco 810 Spectropolarimeter equipped with a Peltier thermal controller and 1-cm cell.
2. Dilution buffer: 10 mM potassium phosphate buffer (pH 7.5), 5% glycerol, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol, and 200 mM NaCl.
3. Graphing software with sigmoidal curve fitting (for example, KaleidaGraph from Synergy, Reading, PA).

3. Methods

3.1. Site-Directed Mutagenesis to Introduce DNA Polymerase γ Mutations

We use Stratagene's QuikChange site-directed mutagenesis kit to introduce the desired mutations into the pol γ construct.

1. The pQVSL11.4 baculoviral transfer vector encodes *POLG* cDNA without its mitochondrial targeting sequence *(14)*. This baculovirus transfer vector is the template used to introduce the mutation of interest, using site-directed mutagenesis. For example, codon 1143 was converted to the E1143G derivative with the QuikChange site-directed mutagenesis kit and the mutagenic primers 5′-CCT GGT GCG GG*G* GGA GGA CCG CTA CC-3′ and 5′-GGT AGC GGT CCT CC*C* CCC GCA CCA GG-3′.
2. After QuikChange, DNA is treated with the enzyme Dpn1 to digest and remove methylated template, and then XL1 Blue supercompetent cells are transformed according to the QuikChange protocol.
3. Introduced mutations are confirmed by sequencing the resultant baculovirus transfer vector.

3.2. Expression and Purification of the Catalytic Subunit of DNA Polymerase γ

1. Select recombinant baculovirus expressing the mutant pol γ catalytic subunit and amplify.
2. Transfect Sf9 insect cells in 30 confluent T_{175} tissue culture flasks.
3. Process and purify protein as described previously *(6, 8, 15)*.
4. MonoQ fractions containing each p140 derivative are flash-frozen in small aliquots in liquid nitrogen and stored at –80°C (*see* **Notes 1** and **2**; **Fig. 4.2**).

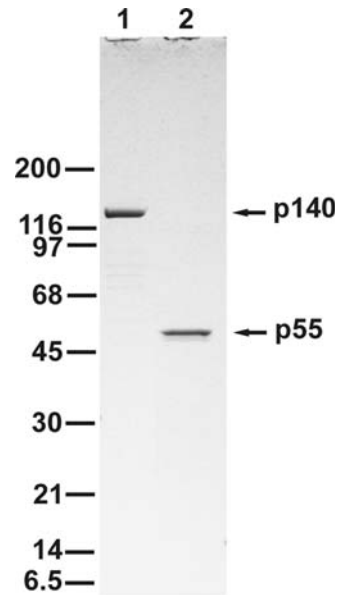


Fig. 4.2. DNA polymerase γ subunit composition. Lane 1: purified recombinant pol γ catalytic subunit (p140); lane 2: purified recombinant pol γ accessory subunit (p55).

3.3. Expression and Purification of the Accessory Subunit of DNA Polymerase γ

Express the His6 affinity-tagged p55 accessory subunit in *Escherichia coli* and purify to homogeneity as described previously *(16)* (*see* **Fig. 4.2**).

3.4. DNA Processivity Assay

The processivity of WT and mutant forms of pol γ is estimated by primer extension assay on an end-labeled, singly-primed M13 DNA substrate as described *(6)*, without the pre-incubation step.

3.4.1. Primer Extension Assay

3.4.1.1. Phosphorylation

1. In a chilled 1.5-ml polypropylene microfuge tube, add 10X T4 polynucleotide kinase buffer (to produce a final concentration of 1X), 20 pmol of the gel-purified 38mer, 25 pmol [γ-^{32}P]ATP, 12 U T4 polynucleotide kinase, and dH_20 to 25 µl.
2. Incubate reactions at 37°C for 60 min.
3. Heat at 80°C for 5 min to heat-kill the kinase.
4. Cool tube on ice.

3.4.1.2. Hybridization

1. Hybridize 5 pmol ^{32}P-labeled 35mer with 5.25 pmol M13mp18 DNA. Add TE buffer to 100 µl.
2. Vortex gently. Heat tube in a 400-ml beaker containing 95°C H_2O for 5 min.
3. Cool slowly to room temperature by leaving the beaker at room temperature.
4. Store at –20°C until needed.

3.4.1.3. Extension

1. Dilute polymerase in enzyme dilution buffer (*see* **Section 2**.2) to the desired concentration. Assemble reaction mixtures (10 µl) on ice with 0 or 100 mM NaCl, 25 µM of each dNTP, and 12 ng purified WT or mutant p140, with or without 9.6 ng of the p55 accessory subunit (gently mix p140 and p55 in a 1:2 molar ratio).
2. Reactions are incubated at 37°C for 30 min.
3. Quench reactions with 10 µl formamide stop solution.
4. Heat samples at >85°C for 3 min and analyze the products by denaturing polyacrylamide gel electrophoresis.
6. Dry the gel and expose to a phosphor storage screen.
7. Image the radioactivity on a Typhoon 9400 phosphor-imager.
8. Quantify bands using NIH Image software (*see* **Fig. 4.3**).

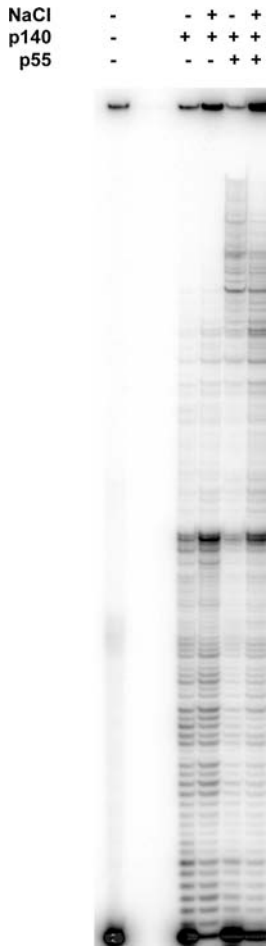


Fig. 4.3. Primer extension assay. Reactions were assembled with the indicated components.

3.4.2. Processivity Assay

The method is the same as above for the primer extension assay, except that heat-denatured calf thymus DNA harboring approximately 18 pmol of random 3′-ends is added to each of the reaction mixtures in **Section 3.4.1.3**. This acts as a polymerase trap, such that processivity can be determined for a single binding event.

3.5. Exonuclease Assay

The exonuclease assay we use in our laboratory has been described previously *(6, 11)*. Briefly, we assess exonuclease activity by monitoring degradation of a 5′-^{32}P-labeled 40mer in the absence of dNTPs.

1. Set up 1.5-ml polypropylene microfuge tubes on ice. Assemble reaction mixtures (10 µl) to contain 25 mM HEPES–KOH, pH 7.6, 1 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 0.05 mg/ml heat-treated BSA, 10 mM NaCl, 5 pmol of the labeled oligonucleotide, and 0.4–3.5 nM purified WT or mutant p140.
2. Incubate reactions at 37°C for 15 min.
3. Terminate reactions by placing tubes on ice and adding 10 µl formamide stop solution.
4. Heat samples at >85°C for 3 min and analyze 2.5 µl of the products by denaturing 12% polyacrylamide gel electrophoresis as described in **Section 3.4.1.3** (*see* **Fig. 4.4**).

3.6. Determination of DNA Binding by Electrophoretic Mobility Shift Assay

The $K_{d(DNA)}$ value for each form of p140 was determined by electrophoretic mobility shift assay *(17)*.

Fig. 4.4. Exonucleolytic degradation of DNA by WT and A467T pol γ. Lane 1: no enzyme control; lane 2: WT pol γ; lane 3: A467T pol γ.

3.6.1. Substrate Preparation – Phosphorylation

As described in **Section 3.4.1.1**.

3.6.2. Hybridization

1. To the chilled 1.5-ml polypropylene tube containing labeled 38mer, add 26 pmol unlabeled 34mer and water to a total volume of 40 µl. The labeled 38mer oligonucleotide primer is hybridized with a 1.3-fold molar excess of complementary 34mer to generate a primer–template substrate possessing a three base recessed 3′-end.
2. Mix gently, and hybridize by placing tube in a 400-ml beaker containing 95°C water. Let the water slowly cool to room temperature (about 1 h). Once the water is approximately 30°C the substrate is ready for use.
3. Assemble binding reactions (20 µl) on ice: binding buffer (*see* **Section 2.4**), 1 pmol of ^{32}P-primer–template, and 0, 0.5, 1, 1.5, 2, or 4 pmol of WT or mutant pol γ protein.
4. Bind at room temperature for 10 min.
5. Add 5 µl 50 mM HEPES-OH, pH 8.0, 50% glycerol to each reaction.
6. Resolve bound and unbound primer–template molecules by polyacrylamide gel electrophoresis through pre-chilled 6% native polyacrylamide gels at 4°C.
7. After 3 h at 120 V, dry the gel and expose to a phosphor storage screen.
8. Image the radioactivity on a Typhoon 9400 phosphorimager.
9. Quantify bands using NIH Image software (*see* **Fig. 4.5**).

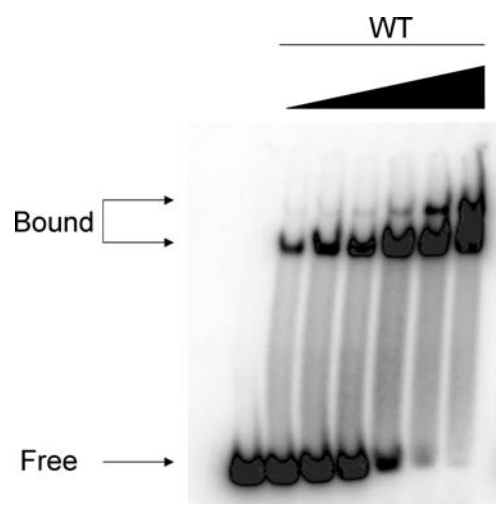


Fig. 4.5. DNA binding of WT pol γ catalytic subunit as assessed by electrophoretic mobility shift assay.

3.7. Immunoprecipitation Assay to Determine Physical Association of the Subunits

To determine subunit–subunit interaction, an immunoprecipitation experiment using anti-pol γ antibodies bound to Sepharose can be performed *(5, 11)*.

3.7.1. Construction of DPg-Sepharose

1. Resuspend 1.5 ml slurry of Protein G Sepharose 4 Fast Flow beads in 10 ml Buffer PBSN (*see* **Section 2.5**).
2. Gently spin down beads at 27 × g for 5 min, remove ethanol/supernatant.
3. Resuspend pellet in 15 ml Buffer PBSN.
4. Add 1.5 ml DPg anti-serum, mix end-over-end at 4°C for 6 h.
5. Recover beads by spinning down at 27 × g for 5 min.
6. Wash beads with 3 × 10 ml Buffer PBSN, store at 4°C, do not freeze.

3.7.2. Immunoprecipitation Experiment

1. Equilibrate the beads in PBSN–BSA (1 volume beads to 1 volume PBSN–BSA).
2. Aliquot 30 µl DPg-Sepharose beads per 1.5-ml polypropylene microfuge tube, place tube on ice.
3. Spin 2,700 × g at 4°C for 2 min.
4. Remove supernatant, add 400 µl PBSN–BSA to each tube and mix.
5. To separate tubes containing prepared DPg-Sepharose beads and PBSN–BSA add: 3 µg WT pol γ only; 3 µg WT p55 accessory subunit only; 3 µg mutant pol γ or p55 accessory subunit only; 3 µg WT pol γ and 3 µg WT p55; 3 µg mutant pol γ protein and 3 µg WT p55 (or 3 µg mutant p55 accessory subunit with 3 µg WT pol γ protein).
6. Rotate tubes end-over-end for 45 min at 4°C.
7. Collect beads by microcentrifugation at 2,700 × g for 2 min at 4°C.
8. Remove supernatant carefully by pipetting.
9. Wash beads twice with 1 ml cold PBSN–BSA, then once with PBSN.
10. Resuspend beads in 25 µl 2X LDS loading buffer (4X LDS loading buffer made 2X with PBSN).
11. Heat samples for 10 min at 70°C prior to analysis on a 4–12% polyacrylamide gel using NuPAGE MOPS running buffer and antioxidant solution (*see* **Note 3**).
12. After electrophoresis, electroblot the proteins onto an Immobilon-P PVDF membrane overnight at 80 mA using NuPAGE transfer buffer with 15% methanol and antioxidant solution.

13. Wash the membrane in Buffer TNT (*see* **Section 2.5**) for 15 min.
14. Block membrane with 5% dried milk in Buffer TN (*see* **Section 2.5**) at room temperature.
15. Incubate the blot with 0.2 μg/ml anti-penta-His monoclonal antibody in Buffer TN containing 0.1 mg/ml BSA for 2 h.
16. Wash the blot three times with Buffer TN for 10 min (*see* **Note 4**).
17. Incubate the blot in a 1/3,000 dilution of goat anti-mouse IgG alkaline phosphatase-conjugated secondary antibody for 1 h.
18. Wash blot thoroughly using three 10 min washes in Buffer TNT followed by three 10 min washes in Buffer TN.
19. Develop the blot with Western Blue alkaline phosphatase reagent.
20. Rinse in H_2O, then place the blot in 3% TCA for 15 min.
21. Wash in water for 2 min, air dry, and scan blot (*see* **Fig. 4.6**).

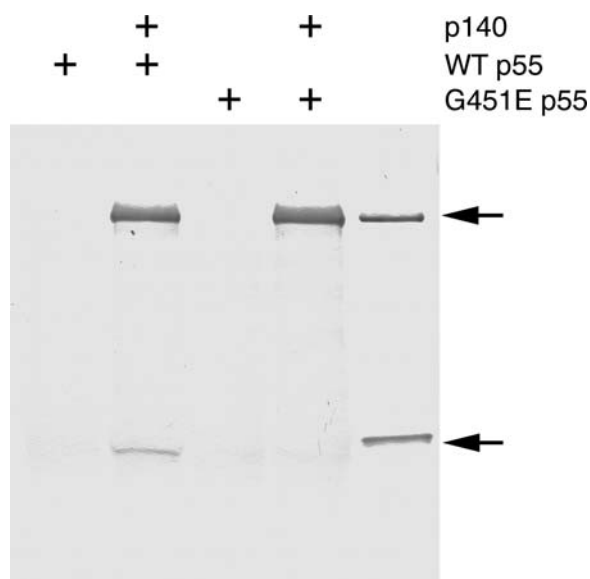


Fig. 4.6. Immunoprecipitation assay. The G451E mutant p55 subunit fails to co-immunoprecipitate with p140. Reproduced from Ref.*(5)*, University of Chicago Press.

3.8. Circular Dichroism Measurements to Determine Protein Stability

CD studies were performed according to the method of DeRose et al. *(18)* with the following modifications *(12)*. With circular dichroism spectroscopy, the relative amounts of α-helix, β-sheet, and random coil in a particular protein, as well as the intrinsic stability of the protein of interest can be determined.

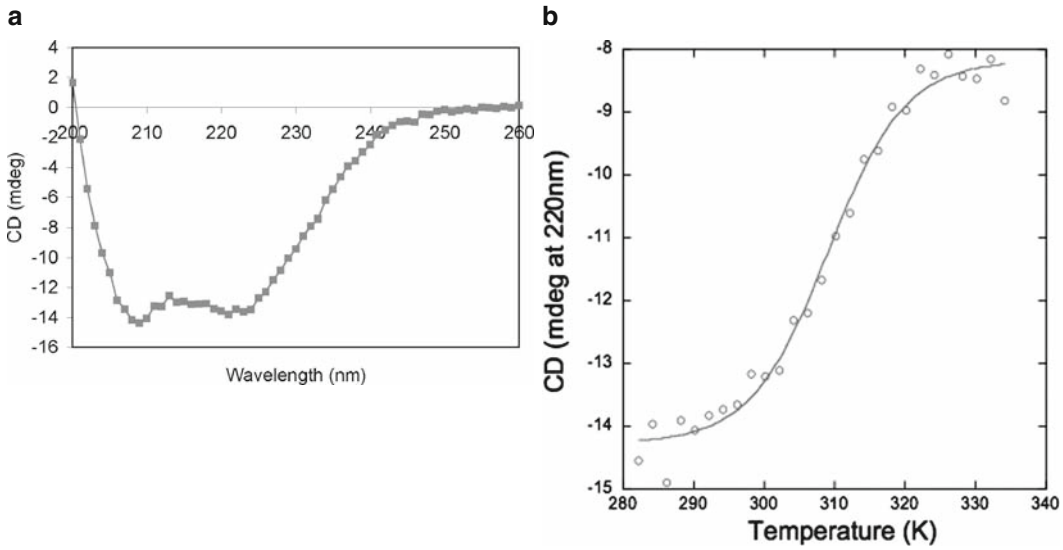


Fig. 4.7. Circular dichroism spectroscopy. (**A**) Circular dichroism spectrum of WT pol γ catalytic subunit; (**B**) thermal denaturation curve of WT pol γ at 220 nm.

1. Turn on spectropolarimeter and computer. Allow N_2 to equilibrate for 15 min. Allow water bath to cool down to 4°C.
2. Thaw pol γ protein aliquots on ice.
3. In a cooled 1-cm cell, add 1 ml pol γ protein at a concentration of 10 μg/ml (diluted with cold dilution buffer; *see* **Notes 5** and **6**) and a stir bar.
4. Measure in quadruplicate the CD spectra from 260 nm down to 190 nm at 4°C (**Fig. 4.7A**).
5. Determine the thermal stabilities of α-helices within the p140 proteins by measuring the ellipticity at 220 nm as the temperature is increased from 28°C to 60°C by 1°C/min, with stirring. Ellipticity should be sampled every 1°C.
6. Using a graphing program with sigmoidal curve fitting, determine the equation of each thermal denaturation curve. The melting point (T_m) will be the point of inflection of the thermal denaturation curve, while the value for the enthalpy change upon unfolding (ΔH_m) is the slope of the curve for each p140 protein (*see* **Fig. 4.7B**).

4. Notes

1. Make up all reactions in an ice bucket. Pol γ has a functional half-life at 42°C of less than 1 min without the accessory subunit or DNA *(11)*.

2. Refreeze as soon as possible in liquid nitrogen and store at –80°C. Enzyme preparations begin to lose DNA polymerase activity after approximately three freeze–thaw cycles. The enzyme buffer contains glycerol, salt, and β-mercaptoethanol, which are important for stability.
3. This combination of loading buffer and polyacrylamide gel helped to minimize background due to crossreactivity with rabbit immunoglobin heavy chains.
4. In the Western blot protocol, it is important to thoroughly wash the blot in order to further reduce crossreactivity and background.
5. Pol γ prefers to be stored in a high concentration of both glycerol and NaCl. However, these ingredients are in conflict with producing optimal CD spectra, as a higher background may result at shorter wavelengths (around 190 nm). Thus, a compromise is needed, as the conditions are not ideal for determining the amount of β-sheet. As pol γ appears to be highly α-helical, we have determined the thermal stability of the α-helices of pol γ at 220 nm.
6. Renaturation of pol γ is not possible, thus denatured protein samples must be discarded after melting curves are obtained.

Acknowledgments

The authors would like to thank Drs. Matt Longley and Kasia Bebenek for advice on the manuscript and Susanna Clark for technical assistance. This work was supported by an NIEHS intramural grant to W.C.C. and NIH Career Development Award (1K99-ES015555-01) to S.S.L.C.

References

1. Naviaux, R. K. (2004). Developing a systematic approach to the diagnosis and classification of mitochondrial disease. *Mitochondrion* **4**, 351–361.
2. Dimauro, S. and Davidzon, G. (2005). Mitochondrial DNA and disease. *Ann Med* **37**, 222–32.
3. Yakubovshaya, E., Chen, Z., Carrodeguas, J. A., Kisker, C. and Bogenhagen, D. F. (2006). Functional human mitochondrial DNA polymerase γ forms a heterotrimer. *J Biol Chem* **281**, 374–382.
4. Graziewicz, M. A., Longley, M. J. and Copeland, W. C. (2006). DNA polymerase gamma in Mitochondrial DNA Replication and Repair. *Chem Rev* **106**, 383–405.
5. Longley, M. J., Clark, S., Yu Wai Man, C., Hudson, G., Durham, S. E., Taylor, R. W., Nightingale, S., Turnbull, D. M., Copeland, W. C. and Chinnery, P. F. (2006). Mutant POLG2 Disrupts DNA Polymerase γ Subunits and Causes Progressive External Ophthalmoplegia. *Am J Hum Genet* **78**, 1026–34.
6. Longley, M. J., Ropp, P. A., Lim, S. E. and Copeland, W. C. (1998). Characterization of the native and recombinant catalytic subunit of human DNA polymerase γ:

identification of residues critical for exonuclease activity and dideoxynucleotide sensitivity. *Biochemistry* **37**, 10529–39.

7. Ponamarev, M. V., Longley, M. J., Nguyen, D., Kunkel, T. A. and Copeland, W. C. (2002). Active Site Mutation in DNA Polymerase γ Associated with Progressive External Ophthalmoplegia Causes Error-prone DNA Synthesis. *J Biol Chem* **277**, 15225–8.
8. Graziewicz, M. A., Longley, M. J., Bienstock, R. J., Zeviani, M. and Copeland, W. C. (2004). Structure-function defects of human mitochondrial DNA polymerase in autosomal dominant progressive external ophthalmoplegia. *Nat Struct Mol Biol* **11**, 770–6.
9. Chan, S. S. L., Longley, M. J., Naviaux, R. K. and Copeland, W. C. (2005). Mono-allelic POLG expression resulting from nonsense-mediated decay and alternative splicing in a patient with Alpers syndrome. *DNA Repair* **4**, 1381–9.
10. Luoma, P. T., Luo, N., Loscher, W. N., Farr, C. L., Horvath, R., Wanschitz, J., Kiechl, S., Kaguni, L. S. and Suomalainen, A. (2005). Functional defects due to spacer-region mutations of human mitochondrial DNA polymerase in a family with an ataxia-myopathy syndrome. *Hum Mol Genet* **14**, 1907–20.
11. Chan, S. S. L., Longley, M. J. and Copeland, W. C. (2005). The common A467T mutation in the human mitochondrial DNA polymerase (POLG) compromises catalytic efficiency and interaction with the accessory subunit. *J Biol Chem* **280**, 31341–6.
12. Chan, S. S. L., Longley, M. J. and Copeland, W. C. (2006). Modulation of the W748S mutation in DNA polymerase γ by the E1143G polymorphism in mitochondrial disorders. *Hum Mol Genet* **15**, 3473–83.
13. Yamanaka, H., Gatanaga, H., Kosalaraksa, P., Matsuoka-Aizawa, S., Takahashi, T., Kimura, S. and Oka, S. (2007). Novel Mutation of Human DNA Polymerase γ Associated with Mitochondrial Toxicity Induced by Anti-HIV Treatment. *J Infect Dis* **195**, 1419–25.
14. Lim, S. E. and Copeland, W. C. (2001). Differential incorporation and removal of antiviral deoxynucleotides by human DNA polymerase γ. *J Biol Chem* **276**, 23616–23.
15. Longley, M. J. and Copeland, W. C. (2002). Purification, separation and identification of the catalytic and accessory subunits of the human mitochondrial DNA polymerase. In *Mitochondrial DNA: Methods and Protocols* (Copeland, W. C., ed.), Vol. 197, pp. 245–258. Humana Press, Totowa, New Jersey.
16. Lim, S. E., Longley, M. J. and Copeland, W. C. (1999). The mitochondrial p55 accessory subunit of human DNA polymerase γ enhances DNA binding, promotes processive DNA synthesis, and confers N-ethylmaleimide resistance. *J Biol Chem* **274**, 38197–203.
17. Lim, S. E., Ponamarev, M. V., Longley, M. J. and Copeland, W. C. (2003). Structural Determinants in Human DNA Polymerase γ Account for Mitochondrial Toxicity from Nucleoside Analogs. *J Mol Biol* **329**, 45–57.
18. DeRose, E. F., Kirby, T. W., Mueller, G. A., Bebenek, K., Garcia-Diaz, M., Blanco, L., Kunkel, T. A. and London, R. E. (2003). Solution structure of the lyase domain of human DNA polymerase λ. *Biochemistry* **42**, 9564–74.

Chapter 5

Preparation of Human Mitochondrial Single-Stranded DNA-Binding Protein

Matthew J. Longley, Leslie A. Smith, and William C. Copeland

Abstract

Defects in mtDNA replication are the principle cause of severe, heritable metabolic disorders classified as mitochondrial diseases. In vitro analysis of the biochemical mechanisms of mtDNA replication has proven to be a powerful tool for understanding the origins of mitochondrial disease. Mitochondrial single-stranded DNA-binding protein (mtSSB) is an essential component of the mtDNA replication machinery. To facilitate ongoing biochemical studies, a recombinant source of mtSSB is needed to avoid the time and expense of human tissue culture. This chapter focuses on the subcloning, purification, and initial functional validation of the recombinant human mitochondrial single-stranded DNA-binding protein. The cDNA encoding the mature form of the human mtSSB protein was amplified from a HeLa cDNA library, and recombinant human mtSSB was overproduced in *Escherichia coli*. A procedure was developed to rapidly purify milligram quantities of homogenous, nuclease-free mtSSB that avoids DNA–cellulose chromatography. We show that, similar to *E. coli* SSB, human mtSSB assembles into a tetramer and binds single-stranded oligonucleotides in a 4-to-1 protein:oligonucleotide molar ratio.

Key words: Mitochondria, single-stranded DNA-binding protein, DNA replication, EMSA, tetramer, mtDNA, protein purification.

1. Introduction

Human mitochondria possess a circular, double-stranded DNA chromosome (16,569 bp) that is indispensible for the healthy growth of cells. Mitochondrial DNA (mtDNA) encodes 2 rRNAs, 22 tRNAs, and 13 mRNAs essential for electron transport and oxidative phosphorylation. Mutation of mtDNA causes a wide spectrum of neuromuscular degenerative diseases affecting many different tissues, and defects in mtDNA replication are the principle cause of metabolic disorders classified as "mitochondrial diseases." All of the factors required for replication and repair of the mitochondrial genome are

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_5 Springerprotocols.com

known to be encoded by the nucleus, imported into mitochondria as pre-proteins, and proteolytically processed into their mature forms. Efforts to define the genetics and the biochemical mechanisms by which cells maintain the integrity of their mtDNA are essential to understanding the origins of mitochondrial diseases.

Mitochondrial single-stranded DNA-binding protein (mtSSB) was discovered in an analysis of protein–mtDNA complexes derived from rat liver mitochondria that had been lysed with SDS, which revealed nucleoprotein fibrils within the single-stranded portions of both stable and expanding D-loops in replicative intermediates of rat liver mtDNA *(1)*. Current models of mtDNA replication predict replication intermediates that contain large regions of single-stranded DNA, and the abundant presence of 16-kDa mtSSB in these nucleoprotein fibrils strongly suggests that the mtSSB protein is an essential component of the mtDNA replication machinery. Also, native mtSSBs isolated from *Drosophila*, *Xenopus*, and humans (HeLa cells) have been reported to stimulate DNA polymerase γ from these organisms, dependent on the substrate utilized in vitro *(2–4)*. In addition, mtSSB has been shown in vitro to relieve replication stalling by *Xenopus laevis* pol γ within dT-rich template DNA sequences *(5)*. More recently, human mtSSB has been shown to stimulate the activity of the mtDNA helicase, and in vitro mtSSB is part of a minimal replisome complex along with the helicase, DNA polymerase γ, and its accessory protein *(6, 7)*. Mutations in the nuclear genes encoding mtDNA replication components, specifically the DNA polymerase γ (*POLG* and *POLG2*) and the helicase (*PEO1*), have been clearly linked with mitochondrial disease *(8, 9)*. The gene for human mtSSB has been cloned, and the protein has been shown to be homologous to *E. coli* SSB *(10)*. Although mtSSB has not yet been identified as a disease locus in humans, it is essential in yeast *(11)*, and participation in mtDNA replication makes mtSSB an obvious candidate locus for mitochondrial disease in humans.

Existing procedures for the purification of native mitochondrial or *E. coli* SSBs consistently utilize single-stranded DNA–cellulose chromatography as a primary purification step *(2, 12, 13)*. In our hands, recombinant mtSSB prepared from *E. coli* BL21(DE3) lysates by such protocols contained unacceptably high levels of nuclease activity that could not be resolved by additional chromatography on Affi-Gel Blue *(14)* or hydroxylapatite. Previous reports describing the purification of recombinant human mtSSB rely on animal cell culture as the source of protein and/or affinity tags to aid purification *(6, 15, 16)*. Here, we describe over-expression in *E. coli* of the mature form of human mtSSB without an affinity tag and without the mitochondrial targeting sequence, and a procedure for rapid purification of milligram quantities of homogenous, nuclease-free mtSSB that does not use DNA–cellulose chromatography is presented.

2. Materials

Utilizing oligonucleotide primers designed from the published nucleotide sequence of the human gene *(10)*, the cDNA for the human mtSSB was amplified from a HeLa cDNA library. Expression of the full-length human cDNA (aa 1–148, MW 17,249) in *E. coli* (JM105) from a pQE9 expression vector (Qiagen) generated recombinant protein that was almost entirely insoluble (data not shown). Similarly, Li and Williams demonstrated that alteration of the N-terminal amino acid sequence of murine mtSSB, such as by insertion of an affinity-tag to aid protein purification or even by the simple retention of the mitochondrial targeting presequence, adversely affected DNA binding and/or tetramerization of recombinant murine mtSSB *(15)*. Accordingly, we deleted the mitochondrial targeting sequence identified by Tiranti *(10)* and transferred the cDNA encoding the mature form (aa 17–148, MW 15,316) into the pET21a expression vector (Novagen). The resulting plasmid, pET21aHmtSSB, was used to transform *E. coli* JM105(DE3) to ampicillin resistance (*see* **Notes 1 and 2**). Ammonium sulfate was from Invitrogen, Carlsbad, CA. Ampicillin, IPTG, myo-inositol, and CHES were obtained from Sigma. *E. coli* single-stranded DNA-binding protein was purchased from United States Biochemical. Synthetic oligonucleotides were from Oligos Etc (Wilsonville, OR).

2.1. Growth and Induction

1. 2X YT bacterial growth media: 10 g yeast extract, 16 g Bacto-tryptone, 5 g NaCl, sterilized at 121°C in a steam autoclave.
2. 40% (w/v) Glucose: dissolved in water, sterilized by 0.45 μm filtration.
3. 100 mg/ml Ampicillin: dissolved in water, sterilized by 0.45 μm filtration.
4. 1 M IPTG: Dissolve isopropyl-β-D-thiogalactopyranoside in water at 0.238 g/ml, sterilized by 0.45 μm filtration.
5. Refrigerated centrifuge with a rotor capable of holding 1 l.

2.2. Cell Lysis

1. Buffer H: 30 mM HEPES–KOH (pH 7.6), 1 mM dithiothreitol, 0.25 mM ethylenediaminetetraacetic acid (EDTA), 0.25% (w/v) myo-inositol, 0.01% (v/v) NP-40, 0.1 mM phenylmethanesulfonyl fluoride (PMSF).
2. Branson Sonifier (model 450), equipped with a flat 0.5 inch disrupter tip.
3. Sorvall RC-5B refrigerated centrifuge with an SS34 rotor or the equivalent.

2.3. Affi-Gel Blue Chromatography

1. Affi-Gel Blue resin, 100–200 mesh (wet) was purchased from Bio-Rad.
2. Buffer H also containing 50 mM KCl.
3. Buffer H also containing 800 mM KCl.
4. Buffer H also containing 0.5 M KSCN.

2.4. Ammonium Sulfate Precipitation

1. Ammonium sulfate.
2. Ice bath, beaker with stir bar.
3. Sorvall RC-5B refrigerated centrifuge with an SS34 rotor or the equivalent.
4. Dialysis tubing with a 12,000 – 14,000 MW cutoff.
5. Dialysis buffer: 25 mM HEPES–KOH (pH 7.6), 2 mM 2-mercaptoethanol, 0.1 mM EDTA, 10% glycerol, 50 mM KCl.

2.5. MonoS Chromatography

1. MonoS (HR 5/5) cation exchange column.
2. Akta FPLC chromatography system.

2.6. MonoQ Chromatography

1. MonoQ (HR 5/5) anion exchange column.
2. *N*-Cyclohexyl-2-aminoethanesulfonic acid (CHES).
3. Buffer Q: 50 mM CHES–KOH (pH 9.5), 2 mM 2-mercaptoethanol, 0.1 mM EDTA, 10% glycerol.
4. Akta FPLC chromatography system.

2.7. Dialysis and Storage

1. Storage buffer: Buffer H also containing 0.25 M KCl and 25% glycerol.
2. Spectrophotometer and quartz cuvette, capable of measuring absorbance at 280 nm.

3. Methods

E. coli JM105(DE3) bearing a pET21a vector encoding the mature form of human mitochondrial SSB was treated with IPTG to induce gene expression and lysed by sonication. Cleared lysates prepared from 1-l cultures were applied to Affi-Gel Blue resin, extensively washed, and mtSSB was eluted with 0.5 M KSCN before fractional precipitation with 10–35% (saturation) ammonium sulfate. Following dialysis, the protein fraction that did not bind MonoS was adjusted to pH 9.3 and applied to a MonoQ FPLC column. Homogenous mtSSB was eluted from MonoQ with a linear salt gradient. Typical yield from a 1-l culture was 4–6 mg homogenous mtSSB (*see* **Table 5.1**). Detailed procedures are listed below.

Table 5.1
Purification of Recombinant Human Mitochondrial SSB

Fraction	Volume (ml)	Protein (mg/ml)	Total protein (mg)
I. Lysate	19	13	250
II. Affi-Gel Blue	52	0.40	21
III. Ammonium sulfate	2.1	6.7	14
IV. MonoS	3.0	1.5	4.4
V. MonoQ	5.8	0.74	4.3
VI. Dialysed	3.6	1.2	4.3

The cDNA for mature mtSSB was expressed in *E. coli*, and the protein was purified to homogeneity as described in **Sections 3.1–3.7**.

3.1. Growth and Induction

1. Grow an overnight culture (30 ml) of JM105(DE3) transformed with the pET21a HmtSSB plasmid at 37°C in 2X YT media supplemented with 0.1 mg/ml ampicillin.
2. Innoculate 1 l of 2X YT media supplemented with 0.1 mg/ml ampicillin and 0.1% (w/v) glucose with 5 ml of the overnight culture.
3. Incubate culture at 37°C on a rotary shaker. Periodically monitor growth by measuring light scattering at 600 nm.
4. When the culture density reaches A_{600} = 0.5, induce protein expression by adding 1 ml of 1 M ITPG to make a final concentration of 1 mM IPTG.
5. Continue growth at 37°C for 3 h. Harvest cells by centrifugation for 20 min at 4°C at 4,700 × g, such as in a Beckman J6-M centrifuge with a swinging bucket rotor at 4,000 rpm.
6. Resuspend the cell pellet (~5 g) in 5 ml of Buffer H. Freeze the suspension of induced cells with liquid nitrogen, and store the sample at –80°C.

3.2. Cell Lysis

1. Thaw suspension of JM105(DE3)pET21aHmtSSB on wet ice. Dilute the suspension with 10 ml of Buffer H also containing 50 mM KCl to hasten thawing (*see* **Note 3**).
2. Sonicate the sample for 45 s on a 50% duty cycle. Allow the sample to cool on ice for 2 min. Repeat the sonication and cooling steps two more times (*see* **Note 4**).
3. Clarify the whole cell lysate by centrifugation for 10 min at 17,000 × g, such as in a Sorvall RC-5B refrigerated centrifuge with an SS34 rotor at 12,000 rpm. Hold the supernatant on ice.

3.3. Affi-Gel Blue Chromatography

1. Pre-equilibrate the Affi-Gel Blue resin in Buffer H also containing 50 mM KCl by gravity settling in a beaker (*see* **Note 5**).
2. Transfer 14 ml of pre-equilibrated Affi-Gel Blue to a 1.5-cm I.D. chromatography column. Pack and wash the settled resin bed with 5 column volumes of Buffer H containing 50 mM KCl.
3. Apply the cleared lysate to the Affi-Gel Blue column by gravity flow at a rate not exceeding 0.5 ml/min.
4. Flush away unbound material in the lysate by washing the resin with 5 column volumes of Buffer H containing 50 mM KCl at a flow rate of 1.0–1.5 ml/min (*see* **Note 6**).
5. Elute weakly bound contaminating proteins by washing the resin with 5 column volumes of Buffer H containing 800 mM KCl at a flow rate of 1.0–1.5 ml/min.
6. Elute recombinant human mtSSB with 5 column volumes of Buffer H containing 0.5 M KSCN at a flow rate of 1.0–1.5 ml/min.

3.4. Ammonium Sulfate Precipitation

1. Transfer the Affi-Gel Blue eluate to a glass beaker on an ice bath.
2. Bring the solution to 10% (saturated) ammonium sulfate by adding solid ammonium sulfate at a ratio of 0.0549 g ammonium sulfate per ml of solution. Ammonium sulfate should be added slowly enough to prevent the accumulation of crystals in the beaker (*see* **Note 7**). After all the ammonium sulfate has dissolved, continue stirring on ice for an additional 15 min.
3. Transfer the slurry to a centrifuge tube, and clarify by centrifugation for 15 min at 17,000 $\times$ g, such as in a Sorvall RC-5B refrigerated centrifuge with an SS34 rotor at 12,000 rpm.
4. Transfer the supernatant to a fresh beaker and measure the new volume. Bring the supernatant to 35% (saturated) ammonium sulfate by slowly adding solid ammonium sulfate at a ratio of 0.1489 g ammonium sulfate per ml of solution. After all the ammonium sulfate has dissolved, continue stirring on ice for an additional 15 min.
5. Collect the precipitated protein by centrifugation for 15 min at 17,000 $\times$ g, as above.
6. Resuspend the well-drained protein precipitate in 2.0 ml of cold dialysis buffer, and dialyse for 1–2 h, until the conductivity of the sample approaches that of the dialysis buffer.
7. Clarify the dialysate by centrifugation for 10 min at 17,000 $\times$ g to remove any undissolved material. Hold the supernatant on ice.

3.5. MonoS Chromatography

1. Attach the MonoS (HR 5/5) column to an Akta FPLC chromatography system (*see* **Note 8**).
2. Equilibrate the MonoS column with at least 5 ml of dialysis buffer, to ensure complete equilibration of the column as judged by stable UV and conductivity baselines for the column effluent.
3. Inject the clarified dialysate onto the MonoS column, followed by 5 ml of dialysis buffer. Collect the unbound protein fraction that passes directly through the MonoS column (*see* **Note 9**).

3.6. MonoQ Chromatography

1. Measure the volume of the material passing through the MonoS column. Add 0.2 volumes of 0.5 M CHES–KOH (pH 9.5), which will raise the pH from 7.6 to approx. 9.3.
2. Attach the MonoQ (HR 5/5) column to an Akta FPLC chromatography system (*see* **Note 8**).
3. Equilibrate the MonoQ column with at least 5 ml of Buffer Q also containing 50 mM KCl. Ensure complete equilibration of the column as judged by stable UV and conductivity baselines for the column effluent.
4. Apply the near homogenous mtSSB (Fraction IV) to the MonoQ column. Flush unbound material from the column with 4 ml of equilibration buffer.
5. Elute the column with a 25 ml linear gradient of KCl (50–500 mM) in Buffer Q.
6. Identify fractions containing mtSSB as the major peak on the UV-profile. Homogenous mtSSB elutes at approx. 0.24 M KCl.

3.7. Dialysis and Storage

1. Dialyse the pooled mtSSB peak extensively against storage buffer at 4°C.
2. Determine the protein concentration of homogenous mtSSB with a *uv*-spectrophotometer, utilizing an extinction coefficient at 280 nm of 76,240 $M^{-1}cm^{-1}$ for tetramers (19,060 $M^{-1}cm^{-1}$ for monomers), as calculated from the primary amino acid sequence *(13, 17)*.
3. Store the dialyzed protein preparation in tightly sealed vials at –20°C.

3.8. Validation of Recombinant mtSSB

Validation experiments were performed both to verify the purity of recombinant, mature human mtSSB as prepared by this method, as well as to confirm the ability of the purified protein to form homotetramers and bind to single-stranded DNA.

3.8.1. Analysis of Purity

Established procedures for the purification of prokaryotic and mtSSB's often rely upon single-stranded DNA–cellulose chromatography as a terminal purification step. To avoid the possibility of DNA contaminating the mtSSB preparation, as well as to minimize the chances of enriching for other DNA metabolizing enzymes due to their intrinsic affinity to DNA, we sought to develop a rapid protocol for purifying recombinant mtSSB with a high yield that does not utilize DNA affinity chromatography. Samples taken throughout the purification were analyzed by SDS-PAGE (*see* **Fig. 5.1**). The high specificity of Affi-Gel Blue rendered the mtSSB >90% pure after the first chromatographic step. KSCN was removed by ammonium sulfate precipitation, and passage through a MonoS column removed many higher MW contaminants, including a contaminating nuclease activity. Although *E. coli* SSB and mtSSBs do not readily bind MonoS or MonoQ FPLC columns at neutral pH, MonoQ binds human mtSSB with high capacity at pH 9.5, permitting purification to homogeneity without resorting to DNA–cellulose chromatography.

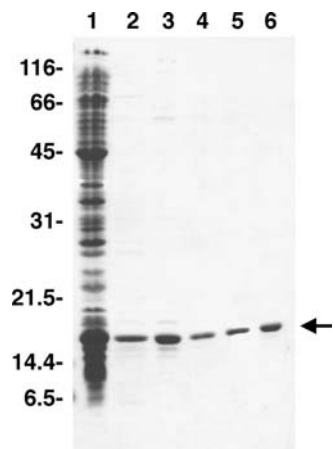


Fig. 5.1. Purification profile of recombinant, human, mitochondrial single-stranded DNA-binding protein. Proteins were resolved by 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1, cleared lysate; lane 2, Affi-Gel Blue; lane 3, ammonium sulfate; lane 4, MonoS; lane 5, MonoQ; lane 6, dialysis. The positions of molecular weight markers (kDa) are shown, and mtSSB is indicated by the arrow.

3.8.2. Tetramerization Measured by Analytical Ultracentrifugation

Equilibrium sedimentation analysis was performed to determine the native conformation of purified, recombinant, human mtSSB. In preparation for analytical ultracentrifugation, glycerol was removed from a sample of mtSSB (fraction V) by dialysis against a buffer containing 30 mM HEPES–KOH (pH 7.6), 2 mM 2-mercaptoethanol, 0.25 mM EDTA, 0.25 M KCl. Protein concentration was determined spectrophotometrically, as in **Section 3.7**. Samples (100 µl) were adjusted by dilution with dialysis buffer to protein concentrations of 32.1, 16.0, and 10.7 µM mtSSB

(monomers), and subjected to equilibrium sedimentation in an Optima XL-A analytical ultracentrifuge (Beckman Instruments) at 11,000 rpm at 4°C prior to obtaining absorbance profiles at 280 nm (*see* **Fig. 5.2**). Profiles were fit with Optima XL-A data analysis software, assuming a partial specific volume of 0.7261 cm^3/g and a solvent density of 1.0128 g/cm^3. The random distribution of residual absorbance values indicated that all three profiles fit very well to a model of a single species (*see* **Fig. 5.2**) with an average molecular weight of 62,600 ± 1,760 Da. Although attempts to model monomer–dimer or monomer–tetramer equilibria were confounded by the inability to detect SSB monomers at these protein concentrations, the subunit dissociation constant is estimated to be less than 10^{-8} M. Because the recombinant protein has a predicted molecular weight of 15,316 Da, the measured value indicates that recombinant human mtSSB exists as a tetramer in solution, as observed previously for native and recombinant forms of mtSSB from *X. laevis (3, 18)*, *Drosophila melanogaster (2)*, and humans *(13)*.

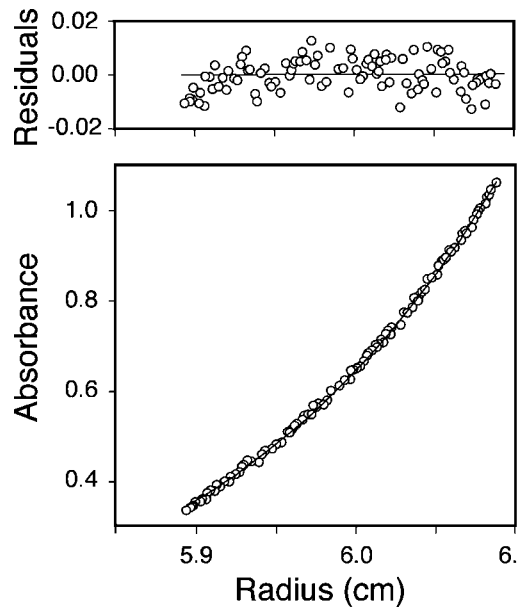


Fig. 5.2. Equilibrium analytical ultracentrifugation data. Recombinant human mtSSB (49 μg, fraction V) was subjected to equilibrium sedimentation, and a radial scan of the absorbance profile at 280 nm (*circles*) was fit to a model of a single ideal species (*solid line*). The random distribution of small residual values indicates only minor deviation from the ideal model.

3.8.3. DNA binding Confirmed by Electrophoretic Mobility Shift Assay

The ability of mtSSB tetramers to bind single-stranded DNA was confirmed by electrophoretic mobility shift assay, as described previously *(19)*. DNA binding by*E. coli* SSB, which also exists as a tetramer in solution *(20)*, was assessed as a positive control in side-by-side reactions. Various concentrations of human mtSSB or

E. coli SSB were combined in vitro with radiolabeled, single-stranded 34mer oligonucleotide, and mixtures were resolved by native PAGE (*see* **Fig. 5.3, upper panel**). This titration experiment indicates that 40 fmol of oligonucleotide is saturated by approximately four equivalents of either *E. coli* SSB monomers or mtSSB monomers, confirming the similar DNA-binding strength of each tetrameric species in vitro (*see* **Fig. 5.3**, **lower panel**).

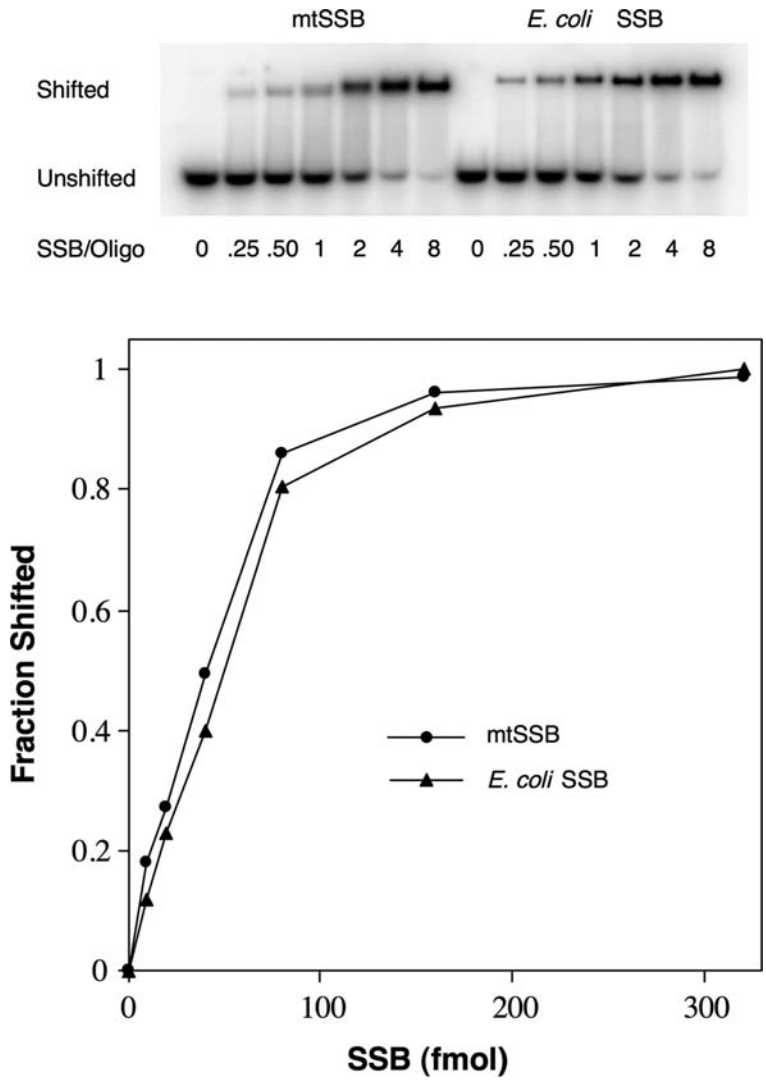


Fig. 5.3. DNA-binding properties of mtSSB and *E. coli* SSB were assessed by electrophoretic mobility shift assay. Binding mixtures (20 μl) contained 15 mM HEPES–KOH (pH 7.6), 0.5 mM EDTA, 1 mM dithiothreitol, 50 μg/ml acetylated BSA, 40 fmol of ^{32}P-labeled 34mer, and the indicated quantities of *E. coli* SSB or human mtSSB. Bound and unbound oligonucleotides were resolved on 10% native polyacrylamide gels and exposed to a phosphor storage screen. Radioactivity was imaged on a Typhoon 9400 phosphorimager and quantified with NIH Image software.

4. Notes

1. The *E. coli* strain JM105 harbors a mutated form of exonuclease I (*sbcB15*), which eliminates one source of minor exonuclease contamination in some preparations of SSB. Accordingly, JM105(DE3) is the host of choice for production of recombinant mtSSB.
2. JM105 was modified to contain the gene for T7 RNA polymerase with the Lambda DE3 Lysogenization kit, as described by the manufacturer (Novagen).
3. All steps in the purification should be performed on ice or at 4°C.
4. Good lysis is achieved with a Branson Sonifier (model 450) equipped with a flat, 0.5 inch disrupter tip. Operation at an output control setting of 5 delivers approx. 90–100 W of power to the sample. Care should be taken to fully immerse the probe, to avoid foaming that can occur due to the NP-40 in the lysis buffer.
5. New Affi-Gel Blue resin can leach unbound dye into your biological sample. Prior to first use, new resin should be washed in a solution consisting of 0.1 M acetic acid, 1.4 M NaCl, and 40% 2-propanol until no more dye leaches from the resin, followed by exhaustive washing in water. Then the washed resin can be transferred to the equilibration buffer.
6. Care should be taken to allow all of one buffer to enter the resin bed before changing to a new solution. This will reduce mixing of the two buffers and ensure a more abrupt transition between washing and elution conditions.
7. In advance, grind the ammonium sulfate into a powder with a mortar and pestle. Finely ground ammonium sulfate dissolves very readily, which prevents the accumulation of ammonium sulfate crystals in the bottom of the beaker.
8. All FPLC operations were performed at 4°C, with a flow rate of 1 ml/min and an upper pressure limit of 5 MPa.
9. Human mtSSB does not bind to MonoS under these buffer conditions. However, control experiments clearly indicate complete retention of contaminating 3'–5' exonuclease activity among the proteins that bind the MonoS column (data not shown).

Acknowledgments

The authors would like to thank Dr. Harvey Sage of the Department of Biochemistry at Duke University Medical Center for performing the analytical ultracentrifugation, as well as Drs. Leroy Worth (NIEHS) and Sherine Chan (NIEHS) for thoughtful comments on the manuscript. This work was supported by the Intramural Research Program of the National Institute of Environmental Health Sciences.

References

1. Van Tuyle, G. C. and Pavco, P. A. (1985). The rat liver mitochondrial DNA-protein complex: displaced single strands of replicative intermediates are protein coated. *J Cell Biol* **100**, 251–257.
2. Thommes, P., Farr, C. L., Marton, R. F., Kaguni, L. S. and Cotterill, S. (1995). Mitochondrial single-stranded DNA-binding protein from *Drosophila* embryos. Physical and biochemical characterization. *J Biol Chem* **270**, 21137–21143.
3. Mikhailov, V. S. and Bogenhagen, D. F. (1996). Effects of *Xenopus laevis* mitochondrial single-stranded DNA-binding protein on primer-template binding and 3'–>5' exonuclease activity of DNA polymerase γ. *J Biol Chem* **271**, 18939–18946.
4. Genuario, R. and Wong, T. W. (1993). Stimulation of DNA polymerase γ by a mitochondrial single-strand DNA binding protein. *Cell Mol Biol Res* **39**, 625–634.
5. Mikhailov, V. S. and Bogenhagen, D. F. (1996). Termination within oligo(dT) tracts in template DNA by DNA polymerase γ occurs with formation of a DNA triplex structure and is relieved by mitochondrial single-stranded DNA-binding protein. *J Biol Chem* **271**, 30774–30780.
6. Korhonen, J. A., Gaspari, M. and Falkenberg, M. (2003). TWINKLE has 5′ –> 3′ DNA helicase activity and is specifically stimulated by mitochondrial single-stranded DNA-binding protein. *J Biol Chem* **278**, 48627–48632.
7. Korhonen, J. A., Pham, X. H., Pellegrini, M. and Falkenberg, M. (2004). Reconstitution of a minimal mtDNA replisome in vitro. *EMBO J* **23**, 2423–2429.
8. Dimauro, S. and Davidzon, G. (2005). Mitochondrial DNA and disease. *Ann Med* **37**, 222–232.
9. Longley, M. J., Graziewicz, M. A., Bienstock, R. J. and Copeland, W. C. (2005). Consequences of mutations in human DNA polymerase γ. *Gene* **354**, 125–131.
10. Tiranti, V., Rocchi, M., DiDonato, S. and Zeviani, M. (1993). Cloning of human and rat cDNAs encoding the mitochondrial single-stranded DNA-binding protein (SSB). *Gene* **126**, 219–225.
11. Van Dyck, E., Foury, F., Stillman, B. and Brill, S. J. (1992). A single-stranded DNA binding protein required for mitochondrial DNA replication in *S. cerevisiae* is homologous to *E. coli* SSB. *EMBO J* **11**, 3421–3430.
12. Lohman, T. M., Green, J. M. and Beyer, R. S. (1986). Large-scale overproduction and rapid purification of the *Escherichia coli* ssb gene product. Expression of the ssb gene under lambda PL control. *Biochemistry* **25**, 21–25.
13. Curth, U., Urbanke, C., Greipel, J., Gerberding, H., Tiranti, V. and Zeviani, M. (1994). Single-stranded-DNA-binding proteins from human mitochondria and *Escherichia coli* have analogous physicochemical properties. *Eur J Biochem* **221**, 435–443.
14. Henricksen, L. A., Umbricht, C. B. and Wold, M. S. (1994). Recombinant replication protein A: expression, complex formation, and functional characterization. *J Biol Chem* **269**, 11121–11132.
15. Li, K. and Williams, R. S. (1997). Tetramerization and single-stranded DNA binding properties of native and mutated forms of murine mitochondrial single-stranded DNA-binding proteins. *J Biol Chem* **272**, 8686–8694.
16. Li, K. and Williams, R. S. (2002). Expression, purification, and *in vitro* assays of mitochondrial single-stranded DNA-binding protein. *Methods Mol Biol* **197**, 295–302.

17. Gill, S. C. and von Hippel, P. H. (1989). Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem* **182**, 319–326.
18. Mahoungou, C., Ghrir, R., Lecaer, J. P., Mignotte, B. and Barat-Gueride, M. (1988). The amino-terminal sequence of the *Xenopus laevis* mitochondrial SSB is homologous to that of the *Escherichia coli* protein. *FEBS Lett* **235**, 267–270.
19. Chan, S. S. L., Longley, M. J. and Copeland, W. C. (2006). Modulation of the W748S mutation in DNA polymerase γ by the E1143G polymorphism in mitochondrial disorders. *Hum Mol Genet* **15**, 3473–3483.
20. Lohman, T. M. and Ferrari, M. E. (1994). *Escherichia coli* single-stranded DNA-binding protein: multiple DNA- binding modes and cooperativities. *Annu Rev Biochem* **63**, 527–570.

Chapter 6

Methods for Assessing Binding of Mitochondrial Transcription Factor A (TFAM) to DNA

Atsushi Fukuoh and Dongchon Kang

Abstract

It is now recognized that mammalian mitochondrial DNA forms a higher structure called the nucleoid, corresponding to the nucleosome of nuclear DNA. Mitochondrial transcription factor A (TFAM), which was cloned as a transcription factor for mitochondrial DNA, is essential for the maintenance of mitochondrial DNA. In fact, TFAM markedly enhances the promoter-specific transcription of mitochondrial DNA. In addition, TFAM has an ability to bind to DNA in a sequence-independent manner and is abundant enough to cover an entire region of mitochondrial DNA. Over-expression of human TFAM in cells increases the amount of mitochondrial DNA almost in parallel with the TFAM. TFAM may stabilize mitochondrial DNA by packaging and regulate (or titrate) the amount of mitochondrial DNA. Thus, TFAM may play a crucial role in maintaining mitochondrial DNA as a main component of the nucleoid (or more appropriately mitochromosome).

Key words: Transcription, mtDNA, TFAM, transcription factor, nucleoid, mitochromosome.

1. Introduction

The circular 16.5 kbp of human mitochondrial genome codes for 13 proteins, 2 rRNAs, and 22 tRNAs. All the 13 proteins are essential subunits of a mitochondrial respiratory chain. The rRNAs and tRNAs are also essential for the synthesis of the proteins encoded by mitochondrial DNA (mtDNA). Given that the majority of ATP production depends on the respiratory chain, maintenance of the mitochondrial genome is unambiguously critical for individuals to live normally. mtDNA is considered more fragile than nuclear DNA. For example, mtDNA is easily attacked by reactive oxygen species produced by the respiratory chain

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_6 Springerprotocols.com

because mtDNA exists close to the respiratory chain *(1)*. Whereas nuclear DNA takes on a nucleosome structure winding around histone, human mtDNA had long been considered almost naked without such a higher structure. It is considered as one reason of the many for the fragility of mtDNA. Currently, it is being recognized that human mtDNA takes on a higher structure called a nucleoid structure and mitochondrial transcription factor A (TFAM) functions like histones in nuclear nucleosomes *(2, 3)*.

TFAM is essential for the maintenance of mtDNA *(4)*. TFAM is a first-characterized transcription factor of human mtDNA *(5)*. TFAM is composed of two high mobility group (HMG)-boxes and a basic carboxyl terminal region (C-tail). In vitro transcription assays reveal that TFAM indeed enhances mtDNA transcription by mitochondrial RNA polymerase in a promoter-specific fashion in the presence of mitochondrial transcription factor B (TFBM) *(6)*. The C-tail of TFAM is essential for the promoter-specific transcription because TFBM binds to the C-tail of TFAM *(7)* and TFAM lacking the C-tail does not initiate the transcription.

However, like many other HMG family proteins, TFAM has an ability to bind to DNA without sequence specificity. Recently, we have reported that the amount of human TFAM is sufficient to entirely cover mtDNA *(8)*, that most of TFAM molecules bind to mtDNA in human cells *(9)*, and TFAM indeed binds to entire regions of mtDNA in human cells *(10)*, suggesting that TFAM architecturally packages mtDNA to form a higher-order structure. Through formation of the higher-order structure, TFAM may stabilize mtDNA and regulate the amount of mtDNA because mtDNA amount is correlated with the amount of TFAM but not with the transcription level *(11)*. For the non-specific DNA binding or packaging of mtDNA, the C-tail of TFAM also plays an important role *(10)*. However, little remains known regarding how TFAM binds to mtDNA as a packaging factor. Hence, further investigation of the interaction between TFAM and mtDNA is required for a more complete understanding of mtDNA maintenance.

In this chapter, the methods for analyzing the TFAM–mtDNA interactions are described. As for TFAM in transcription reactions, see Ref. *(12)*.

2. Materials

2.1. Preparation of Recombinant TFAM

A DNA fragment encoding mature TFAM (42–246 amino acids residues) is inserted between *Bam*HI and *Eco*RI sites of the pPROEX-HTb (Invitrogen) to express N-terminally histidine-tagged TFAM (His-TFAM-full) *(8, 11)*.

2.2. MtChIP Assay

The MtChIP assay is used to examine the in vivo binding of TFAM to mtDNA *(10)*.

2.2.1. Preparation of Mitochondria: Buffer and Equipment

1. Phosphate-buffered saline with 20% glycerol.
2. Homogenization buffer: 10 mM HEPES–KOH (pH 7.4), 250 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA).
3. Percoll solutions: 20 mM Tris–HCl (pH 7.5), 250 mM sucrose, 1 mM EDTA, and 20% or 40% Percoll.
4. A power-driven Potter-Elvehjem glass-Teflon homogenizer.

2.2.2. Preparation of Antibody-Conjugated Magnetic Beads

1. Crosslinking buffer: 20 mM HEPES–KOH, pH 7.4, 250 mM sucrose, 2 mM EDTA, 1% formaldehyde, and 25 mM NaCl.
2. Immunoprecipitation (IP) buffer: 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% bovine serum albumin (BSA), and 0.5% NP-40.
3. Wash buffer: 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1% SDS.
4. Elution buffer: 0.1 M sodium bicarbonate, 1% SDS, and 10 mM dithiothreitol.
5. 5 M NaCl solution.
6. Digestion buffer: 25 mM EDTA, 10 mM Tris–HCl, pH 6.8, and 300 μg/ml proteinase K.
7. DNA buffer: 10 mM Tris–HCl, pH 8.5.
8. 1.0 M Glycine solution.

2.3. Supercoil-Inducing Activity of TFAM

2.3.1. Preparation of Closed Circular Plasmid

1. *Escherichia coli* DH5α.
2. pBR322 or other plasmid. Any plasmid is fine as long as it is supercoiled.
3. Topoisomerase I buffer: 10 mM Tris–HCl, pH 7.5, 1 mM EDTA.
4. 10% Sodium dodecyl sulfate (SDS) in MilliQ water.
5. Ethidium bromide solution (0.5 μg/ml).
6. Proteinase K solution: 2 mg/ml.

2.4. Kinetic Analysis of TFAM Binding to DNA (10)

1. TE buffer: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.
2. Annealing buffer (10X): 100 mM Tris–HCl, pH 8.0, 500 mM NaCl.
3. Running buffer: PBS with 0.005% Tween-20.
4. Regeneration buffer: PBS with 0.005% Tween-20 and 2 M NaCl.
5. BIAcore 1000 (GE Healthcare Bioscience).

6. Streptavidin-modified sensorchip SA (SA chip) (GE Healthcare Bioscience).
7. BIAevaluation version 4.1 (GE Healthcare Bioscience).

2.5. Electrophoretic Mobility Shift Assay (14)

1. 2X Annealing buffer: 100 mM Tris–HCl, pH 8.0, 20 mM $MgCl_2$, 10 mM dithiothreitol, 0.2 mM EDTA.
2. For preparation of the fluorescent isothiocyanate (FITC)-labeled JCT3, FITC-labeled JCT3R at its 5′-end is used.
3. 2X Binding buffer: 100 mM Tris–HCl, pH 8.0, 300 mM NaCl, 2 mM dithiothreitol, and 0.2% Triton X-100.
4. 6X Sample buffer: 10 mM Tris–HCl, pH 7.5, 50 mM EDTA, 30% glycerol, 0.06% bromophenol blue, and 0.12% Orange G.
5. Running buffer: 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA.

3. Methods

3.1. Preparation of Recombinant TFAM

Express the recombinant His-TFAM-full in *E. coli* BL21 cells. The recombinant His-TFAM-full (**Fig. 6.1**) is recovered from a soluble fraction after disruption of the cells by sonication *(8, 11)*. Purify the recombinant protein sequentially with four columns (GE Healthcare Bioscience): Ni^+-bound Chelating Sepharose, Heparin Sepharose, Sephacryl S-200 HR, and SP Sepharose. Dialyze His-TFAM-full in phosphate-buffered saline (PBS) containing 20% glycerol and store at –80°C (*see* **Note 1**).

3.2. MtChIP Assay

The MtChIP assay is used to examine the in vivo binding of TFAM to mtDNA *(10)*.

3.2.1. Preparation of Mitochondria

All steps are done at 4°C or on ice.

1. Harvest cells on a 10-cm dish (80% confluent grown) by scraping with a cell lifter into phosphate-buffered saline (PBS) (about 3 ml for one dish) and centrifuge at 600 × g for 10 min. Wash the cells by the centrifugation with 10 ml homogenization buffer.
2. Suspend the washed cells at 1×10^8 cells/ml in the homogenization buffer containing 1X protease inhibitor cocktail (Complete Mini; Rosche) and homogenize using a power-driven Potter-Elvehjem glass-Teflon homogenizer (2 ml size) with 20 strokes with tightly fitted pestle. After diluting two-fold with the homogenization buffer, centrifuge the homogenate at 900 × g for 10 min.

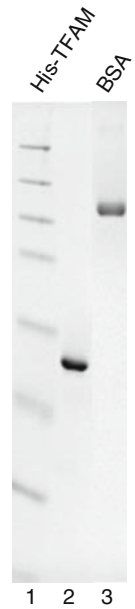


Fig. 6.1. **Purified recombinant TFAM.** Purified His-TFAM-full (655 ng) and bovine serum albumin (BSA fraction V, Sigma) (625 ng) are analyzed by SDS-polyacrylamide gel electrophoresis. Proteins are stained with Coomassie Brilliant Blue. Lane 1, molecular weight marker; lane 2, His-TFAM; lane 3, BSA.

3. Take the supernatant and centrifuge at 10,000 × g for 6 min. Homogenize the pellet in the homogenization buffer with the homogenizer with two or three strokes and centrifuge again. Collect the pellet, homogenize similarly, and use as a crude mitochondrial fraction.
4. Mix the crude mitochondrial fraction (adjusted to 2 ml with homogenization buffer) with 2 ml of 20% Percoll buffer (final 10%) and overlay onto a discontinuous Percoll density gradient (40% and 20% Percoll buffer, 4 ml each from bottom) in a 12-ml centrifugation tube.
5. After centrifugation at 70,000 × g for 1 h using an SW41Ti rotor (Beckman), take the mitochondrial fraction from the interface between 20% and 40% Percoll density gradients. Dilute the fraction with the homogenization buffer about fivefold and centrifuge at 10,000 × g for 6 min.
6. Wash the pellet once similarly and use as a purified mitochondrial fraction.

3.2.2. Preparation of Antibody-Conjugated Magnetic Beads

1. Conjugate affinity-purified anti-TFAM anti-rabbit IgG onto tosylactivated Dynabeads M-280 (Dynal, Oslo, Norway) (about 0.3 mg IgG for 1×10^9 beads) according to the manufacturer's instructions. Suspend the beads at 2×10^9 beads/ml in PBS/0.1% BSA.

Each of the following steps is done at 4°C or on ice unless otherwise stated.

2. *Crosslinking* – Suspend the pelleted mitochondria (about 2.5 mg protein isolated from 10 dishes) in 500 µl crosslinking buffer and incubate for 30 min. Stop the crosslinking reaction by the addition of 73.4 µl of 1.0 M glycine (final 125 mM). After centrifugation at 10,000 × g, wash the pellets with the crosslinking buffer containing glycine, suspend the pellets in 250 µl of PBS containing 1X Complete Mini Protease inhibitor, and move on to the next step (or centrifuge aliquots (50 µl each), discard the supernatants, and store at –80°C until moving on to the next step) (*see* **Note 2**).
3. *DNA fragmentation* – Sonicate 100 µl of the 250 µl sample and fragment DNA to about 600 bp. The sonication conditions are 24 cycles of 5-s sonication at output 20% and a 30-s interval on ice (Ultrasonic Processor Model GE50T).
4. *Immunoprecipitation* – After centrifugation at 10,000 × g, take 80 µl of the supernatant into two tubes (40 µl each). Add 2 ml of IP buffer to each tube for dilution.
5. Add the magnetic beads conjugated with affinity-purified anti-TFAM IgG or control rabbit IgG (5 µl) to each tube. After 1-h rotation, wash the beads three times with 1 ml IP buffer without BSA and two times with wash buffer by using magnet.
6. Then, suspend the beads in 10 µl of elution buffer at room temperature for 15 min. Collect the supernatant (the first elution) by separation with magnet, incubate the beads in another 10 µl of elution buffer at room temperature for 15 min again, and collect the second elution sample.
7. *DNA extraction* – Combine the first and second elution samples (total volume 20 µl). Add 0.8 µl of 5 M NaCl and incubate at 65°C for 6 h to reverse the crosslinking.
8. Add 1.5 µl of digestion buffer to 15 µl of the sample and incubate at 45°C for 60 min. Extract DNA from the samples with a PCR purification kit. Finally, resuspend the DNA in 50 µl of DNA buffer.
9. *Quantitative PCR* – The DNA is analyzed by quantitative PCR with a LightCycler (Rosche). The reaction mixture for PCR contains 4 µl of the DNA solution, 10 pmol each of primers, and 10 µl of SYBR Premix Ex-Taq in 20 µl. The cycle conditions are as follows: 95°C for 10 followed by 45 cycles of 95°C for 5 s, and 60°C for 20 s.
10. As quantification standards, amplify 1 ng, 10 pg, and 100 fg of total DNA of the cultured cells under the same reaction conditions (**Fig. 6.2**, lower panel).

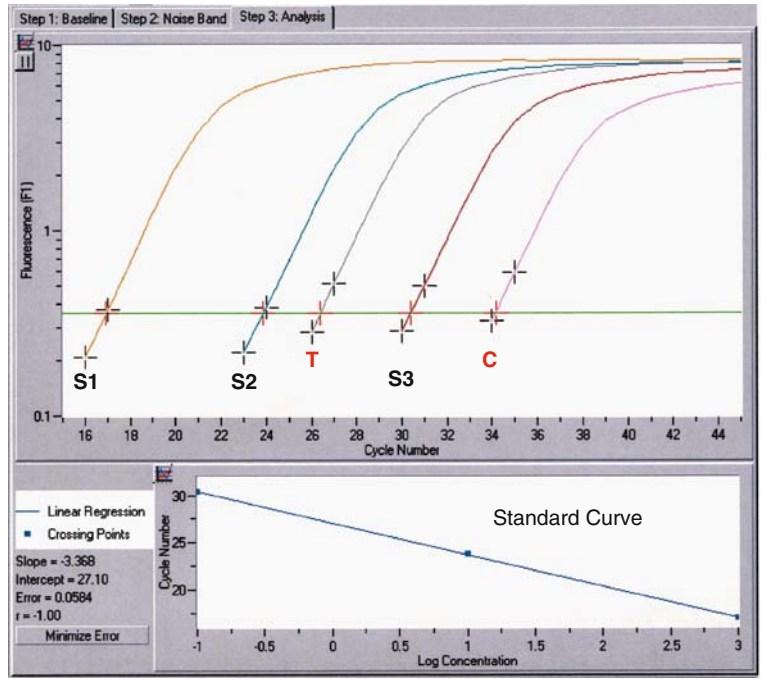


Fig. 6.2. **Real-time PCR for mtChIP**. The computer display of Light Cycler for PCR using Primer set 16. The upper panel shows amplification patterns of DNAs. S1, S2, and S3 stand for 1 ng, 10 pg, and 100 fg of standard DNA, respectively. T and C stand for immunoprecipitated DNAs with anti-TFAM and control IgG, respectively. The lower panel shows the standard curve drawn with S1–S3.

11. Determine the amount of mtDNA immunoprecipitated with anti-TFAM antibody for each region based on the standard DNA. Similarly, determine the mtDNA amount immunoprecipitated with control IgG. The amounts of mtDNA immunoprecipitated with control IgG should be below 3% of those with anti-TFAM antibody. Subtract the latter from the former to estimate the TFAM-specific immunoprecipitation (**Fig. 6.2**).
12. Refer to 32 pairs of specific primers at about 500-bp regular intervals over mtDNA (**Table 6.1**).

Table 6.1
Primer sets for the mtChIP assay, 32 pairs of specific primers, for the mtChIP assay, designed at about 500-bp regular intervals over mtDNA. nps, nucleotide position

Primer set	Forward primer reverse primer	(nps)(nps)
1	5′-TAGAGGCGACAAACCTACCG-3′ 5′-TCCTAGTGTCCAAAGAGCTG -3′	(1,983–2,002) (2,130–2,111)
2	5′-ATCACCTCTAGCATCACCAG-3′	(2,509–2,528)

(continued)

Table 6.1 (continued)

Primer set	Forward primer reverse primer	(nps)(nps)
	5′-AAGAGACAGCTGAACCCTCG-3′	(2,661–2,652)
3	5′-CGATGTTGGATCAGGACTATC-3′ 5′-AAGGCGCTTTGTGAAGTAGG-3′	(2,988–3,007) (3,167–3,148)
4	5′-TCTCACCATCGCTCTTCTAC-3′ 5′-AGTTTGATGCTCACCCTGATC-3′	(3,540–3,559) (3,679–3,659)
5	5′-TCGCCCTATTCTTCATAGCC-3′ 5′-AGAAGTAGGGTCTTGGTGAC-3′	(3,965–3,984) (4,103–4,084)
6	5′-ACACTCATCACAGCGCTAAG-3′ 5′-CGTGAGGAAATACTTGATGGC-3′	(4,518–4,537) (4,655–4,635)
7	5′-AGCAGTTCTACCGTACAACC-3′ 5′-GTTAGCTTGTTTCAGGTGCG-3′	(5,042–5,061) (5,182–5,163)
8	5′-ACTCTGCATCAACTGAACGC-3′ 5′-AAGCCAGTTGATTAGGGTGC-3′	(5,605–5,624) (5,722–5,703)
9	5′-AGGCTTTGGCAACTGACTAG-3′ 5′-ACTGTTCAACCTGTTCCTGC-3′	(6,131–6,150) (6,286–6,267)
10	5′-AGGCTTCGGAATAATCTCCC-3′ 5′-TGATTATGGTAGCGGAGGTG-3′	(6,650–6,669) (6,837–6,818)
11	5′-AACACTTTCTCGGCCTATCC-3′ 5′-AGGACTTTTCGCTTCGAAGC-3′	(7,186–7,205) (7,351–7,332)
12	5′-TCTGCTTCCTAGTCCTGTATG-3′ 5′-ATGAGGACTAGGATGATGGC-3′	(7,686–7,706) (7,812–7,793)
13	5′-ACAGTTTCATGCCCATCGTC-3′ 5′-CGGTAGTATTTAGTTGGGGC-3′	(8,196–8,215) (8,387–8,368)
14	5′-TTGCCACAACTAACCTCCTC-3′ 5′-TGTGGTAAGAAGTGGGCTAG-3′	(8,762–8,781) (8,918–8,899)
15	5′-TAACGCTCCTCATACTAGGC-3′ 5′-GGATTATCCCGTATCGAAGG-3′	(9,325–9,344) (9,459–9,440)
16	5′-TCACTATCTGCTTCATCCGC-3′ 5′-GACCCTCATCAATAGATGAG-3′	(9,850–9,869) (9,988–9,968)
17	5′-TCTGGCCTATGAGTGACTAC-3′ 5′-GAGGTGTGAGCGATATACTAG-3′	(10,361–10,380) (10,547–10,527)
18	5′-CAACCTATTTAGCTGTTCCCC-3′ 5′-TCGTGATAGTGGTTCACTGG-3′	(10,900–10,920) (11,032–11,013)
19	5′-GTCAATAGTACTTGCCGCAG-3′ 5′ ′-TAGGTCTGTTTGTCGTAGGC-3′	(11,440–11,459) (11,605–11,586)
20	5′-TAGTCACAGCCCTATACTCC-3′ 5′-AAACCCGGTAATGATGTCGG-3′	(11,961–11,980) (12,130–12,111)

(continued)

Table 6.1 (continued)

Primer set	Forward primer reverse primer	(nps)(nps)
21	5′-TCATGTGCCTAGACCAAGAAG-3′ 5′-ACGAACAATGCTACAGGGATG-3′	(12,497–12,517) (12,623–12,603)
22	5′-GGCAAATCAGCCCAATTAGG-3-′ 5′-GATAGCGCCTAAGCATAGTG-3′	(13,000–13,019) (13,185–13,166)
23	5′-AGACCACATCATCGAAACCG-3′ 5′-AAGCGAGGTTGACCTGTTAG-3′	(13,512–13,531) (13,646–13,627)
24	5′-TCCTAGACCTAACCTGACTAG-3′ 5′-GTAATTGAGATTGCTCGGGG-3′	(13,991–14,011) (14,175–14,156)
25	5′-AATAACACACCCGACCACAC-3′ 5′-GTAGTCCGTGCGAGAATAATG-3′	(14,548–14,567) (14,691–14,671)
26	5′-CTCCTGCTTGCAACTATAGC-3′ 5′-TGAGTAGCCTCCTCAGATTC-3′	(15,101–15,120) (15,253–15,234)
27	5′-CTATCCATCCTCATCCTAGC-3′ 5′-TGGTTGTCCTCCGATTCAGG-3′	(15,632–15,651) (15,772–15,753)
28	5′-ACCCATCAACAACCGCTATG-3′ 5′-TGTTGGTATCCTAGTGGGTG-3′	(16,070–16,089) (16,283–16,264)
29	5′-TATTAACCACTCACGGGAGC-3′ 5′-ACAGATACTGCGACATAGGG-3′	(20–39) (131–112)
30	5′-CACCAGCCTAACCAGATTTC-3′ 5′-AGAAAGGCTAGGACCAAACC-3′	(375–394) (671–652)
31	5′-ATAGAAGCCGGCGTAAAGAG-3′ 5′-ATCCCAGTTTGGGTCTTAGC-3′	(924–943) (1,076–1,057)
32	5′-GTCGAAGGTGGATTTAGCAG-3′ 5′-TTCGTCCAAGTGCACTTTCC-3′	(1,412–1,431) (1,600–1,581)

3.3. Supercoil-Inducing Activity of TFAM

TFAM induces the negative supercoiling by binding to DNA (**Fig. 6.3**A) *(10, 13)*.

1. Transform *E. coli* DH5α with pBR322 and extract the plasmid from the exponentially growing bacteria (OD 660 is below 1.0) by a conventional alkaline/SDS method. Alternatively, purchase the plasmid (e.g., TOYOBO, Osaka, Japan). Any kinds of plasmids are fine as long as they are supercoiled (*see* **Note 3**).
2. Treat supercoiled pBR322 plasmids (0.25 μg) with 2 U of topoisomerase I (Takara, Japan) in 20 μl topoisomerase I buffer for 30 min at 37°C (Step I in **Fig. 6.3A**).
3. Add 10 μl of TFAM solution (final 0.125, 0.25, 0.5, 1.0, 2.0 μM) at 37°C for another 30 min (Step II in **Fig. 6.3A**). Ethidium bromide (0.5 μg/ml) is used as a positive control at this step.

4. Add 3.3 μl of 10% SDS (final 1%) and 1 μl of 2 mg/ml proteinase K (final 60 μg/ml) to the reaction mixture and incubate for another 20 min at 56°C. Mix the mixture with an equal volume of chloroform:isoamylalcohol (24/1, v/v) and vortex vigorously to remove proteins.
5. Use an aliquot of the resultant aqueous phase for agarose gel electrophoresis (1% agarose gel) in buffer consisting of 45 mM Tris-phosphate, pH 7.5, 1 mM EDTA. During electrophoresis, a constant voltage of 2.5 V/cm is applied for 16 h at room temperature with continuous circulation of the buffer between the anode and cathode (1–2 ml/min). Stain the gel with 0.5 μg/ml ethidium bromide for 1 h and destain in water for 30 min before photodocumentation (**Fig. 6.3B**).

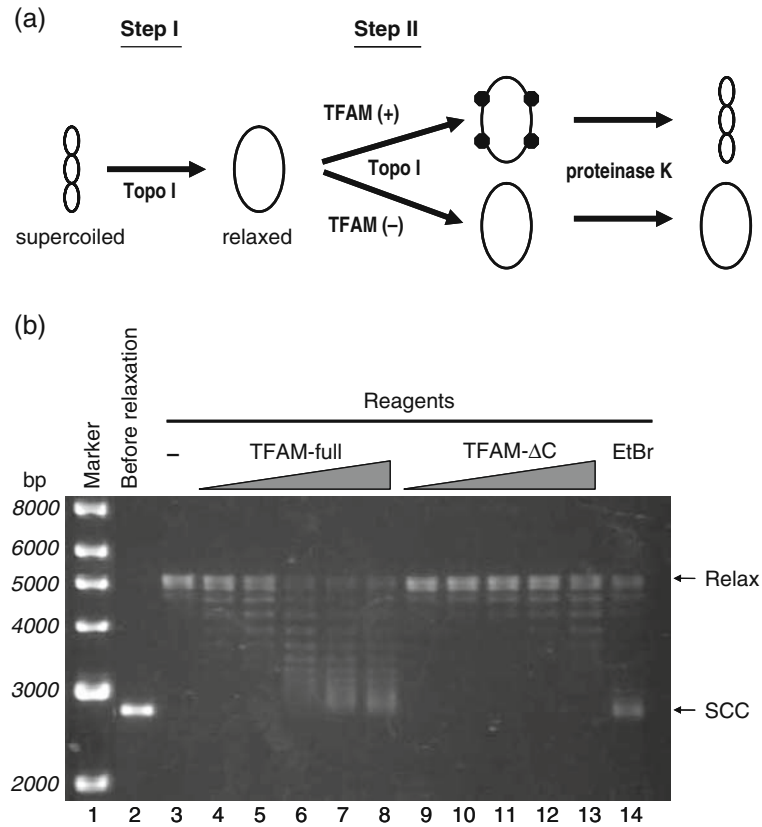


Fig. 6.3. **DNA supercoiling induced by TFAM-full and TFAM-ΔC.** The DNA unwinding activity of TFAM-full and TFAM-ΔC was analyzed. Plasmids, pBR322, were relaxed with topoisomerase I (lanes 3–14). The relaxed plasmids were incubated with nothing (lane 3), increasing concentrations (0.125, 0.25, 0.5, 1.0, 2.0 μM) of recombinant TFAM-full (lanes 4–8) or TFAM-ΔC (lanes 9–13), or 0.5 μg/mL ethidium bromide (lane 14) in the presence of topoisomerase I. Then the plasmids were electrophoresed on a 1% agarose gel. Lane 1, molecular weight marker; lane 2, plasmids before relaxation. Relax, relaxed form of pBR322; SCC, supercoiled circular pBR322.

3.4. Kinetic Analysis of TFAM Binding to DNA

Kinetics of TFAM binding to short DNA is analyzed by a surface plasmon resonance method *(10)*.

3.4.1. Preparation of Double-Stranded Oligonucleotides

1. Prepare a 30mer of biotinylated ssDNA and its complementary unbiotinylated ssDNA in TE buffer at 100 pmol/μl, respectively.
2. Mix 2.5 μl of biotinylated ssDNA and 5 μl of unbiotinylated ssDNA, 50 μl of annealing buffer (10X), and 442.5 μl water. Here, unbiotinylated ssDNA should be more than biotinylated ssDNA.
3. Denature the DNA mixture for 5 min at 95°C and cool down in a phased manner for annealing (60°C for 15 min, 37°C for 15 min, and 25°C for 15 min) by using a block incubator and then store at 4°C.

3.4.2. Preparation of TFAM Sample

1. Dialyze 100 μl of recombinant TFAM (0.35 mg/ml) against 1 l of the running buffer for 16 h. Repeat dialysis against the same buffer for 3 h, measure the final concentration of TFAM, and keep at 4°C.
2. Dilute TFAM with the running buffer to working concentrations just before loading.
3. Do all steps at a constant flow rate of 30 μl/min.
4. The 30 bp of biotinylated double-stranded DNA is immobilized onto an SA chip surface, resulting in the capture of approx. 450 RU of the DNA (*see* **Note 4**).
5. Inject various concentrations of TFAM protein (5–nM) over the reference and DNA-immobilized surfaces with a KINJECT program.
7. Monitor the association for 120 s (60 μl) and the dissociation for 180 s (90 μl) (**Fig. 6.4**).
8. After each measurement, regenerate the SA chip surface by injecting 60 μl of the regeneration buffer.
9. The association rate constant (K_a) and the dissociation rate constant (K_d) can be calculated according to the BIA evaluation version 4.1 by global fitting using a program named 1:1 (Langmuir) binding model. The association and dissociation on the empty sensor chip is subtracted as a background for the calculation of kinetic parameters.

3.5. Electrophoretic Mobility Shift Assay (14)

3.5.1. Preparation of Four-Way DNA Junction

1. Mix 40 μl of 2X annealing buffer and 10 μl each of four oligonucleotides.
2. The annealing is done with a PCR machine. The conditions are as follows: 85°C for 5 min, 65°C for 5 min, 55°C for 5 min, 45°C for 5 min, 35°C for 5 min, 25°C for 5 min, and 4°C for ever. Take 90 min for decreasing the temperature between each step. A designated JCT3 four-way (34 nucleotides

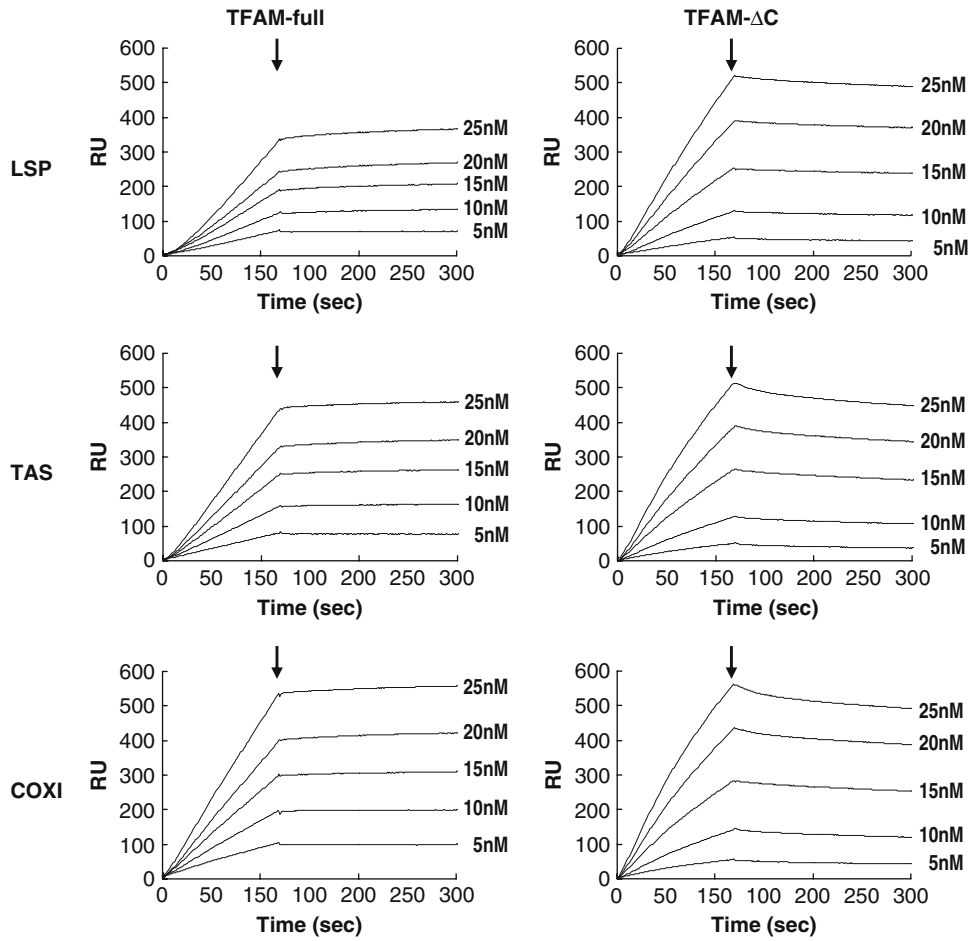


Fig. 6.4. **Binding of TFAM to oligonucleotides.** The binding of TFAM to immobilized 30mer oligonucleotides was started by flowing the buffer containing the indicated recombinant proteins (5, 10, 15, 20, and 25 nM) in PBS with 0.005% Tween-20. After about 2 min, the dissociation reaction was started by flowing the buffer without the recombinant protein. An arrow indicates the buffer change. LSP, light strand promoter (nps 420–449); TAS, termination-associated sequence (nps 16,151–16,180); COXI, cytochrome *c* oxidase I (nps 6,991–7,020).

for each strand) is shown in **Fig. 6.5**. For preparation of the fluorescent isothiocyanate (FITC)-labeled JCT3, FITC-labeled JCT3R at its 5′-end is used.

3. Prepare10 µl of the standard reaction mixture containing 20 nM FITC-labeled four-way DNA junction and the indicated concentration of TFAM-full (5 µl of 2X binding buffer, 0.2 µl of 5 µM four-way, and 4.8 µl of TFAM plus water) and incubate at 25°C for 20 min.
4. Add 2 µl of 6X sample buffer and run the DNA–protein complexes on a 5% LongRanger® polyacrylamide gel (14 × 12 cm, height × width) in running buffer consisting of 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA at a constant 25 mA (*see* **Note 5**).

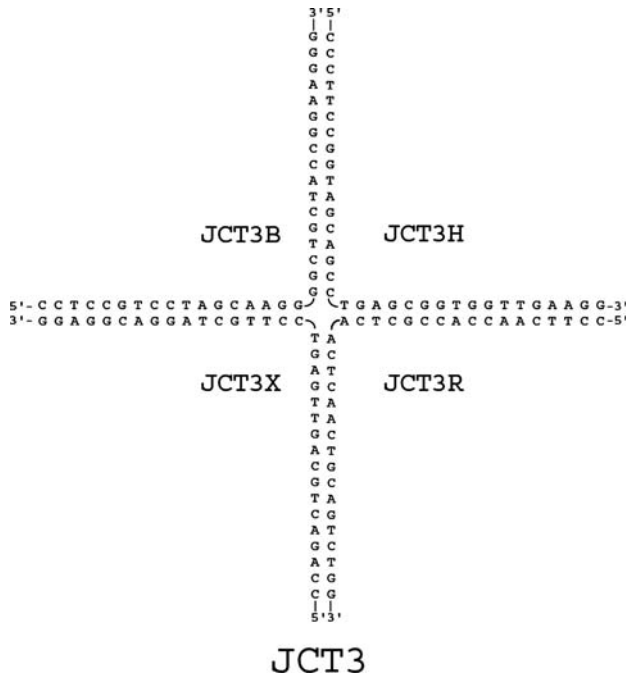


Fig. 6.5. **Structure of JCT3 four-way junction.** The four-way is constructed by annealing the four 34mer oligonucleotides.

5. Scan the gel and quantify the DNA–protein complexes with an image scanner Fluoroimager 595 (Amersham Pharmacia Biotech) (**Fig. 6.6A**).

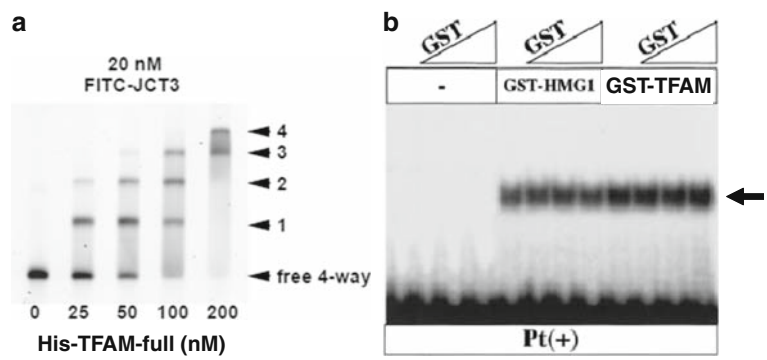


Fig. 6.6. **Electrophoretic gel mobility shift assay. A.** The binding of His-TFAM to FITC-labeled JCT3 is shown. A stepwise increase in TFAM binding is seen. **B.** Binding of purified GST fusion proteins to ^{32}P-labeled double-stranded oligonucleotides (22mer, 0.4 ng/μl) which are cisplatin-treated. Increasing amounts of GST (10, 30, and 90 ng) were incubated with GST-HMG1 or GST-TFAM (30 ng). The arrow indicates DNA–protein complexes. TFAM binds to the damaged DNA as strongly as HMG1 does.

4. Notes

1. Typically, 0.4 mg of TFAM (>95% purity) is obtained from 1 l bacterial culture (**Fig. 6.1**). Truncated TFAM molecules that may be cleaved between the two HMG boxes in the cells are major contaminating proteins.
2. To examine the whole regions of mtDNA, mitochondria prepared from ten dishes may be needed. The crosslinking conditions (incubation time and concentration of formaldehyde) are important and so may be adjusted according to the results.
3. One critical point is that the plasmid should be well supercoiled and be minimally nicked. The plasmid is better prepared from bacteria in the same growing conditions. The pHs around the anode and cathode are changing during electrophoresis. To get a clear electrophoretic pattern, it is important to exchange the running buffer between the anode and cathode. It is OK that you take the running buffer at one side and add to the other side with a pipette periodically (for example, every 1 h).
4. The length of oligonucleotide is important. If long DNA is used, more than one TFAM molecule may bind to the DNA, which may make the binding curve fitting difficult. Another possible problem is that TFAM might bind to DNA as a multimer. The caution may be required for analyzing and explaining the results.
5. It is recommended to do electrophoresis in a cold room for keeping the low temperature. However, TFAM binding to a four-way DNA junction is considerably stable and so electrophoresis at room temperature is usually safe. TFAM binds to damaged DNA more strongly than to intact DNA. **Fig. 6.6B** shows GST-tagged TFAM binding to ^{32}P-labeled linear double-stranded oligonucleotide which is treated with cisplatin *(15)*.

Acknowledgments

This work was supported in part by the Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Technology, Sports, and Culture of Japan.

References

1. Kang, D., Hamasaki, N. (2003) Mitochondrial oxidative stress and mitochondrial DNA. *Clin. Chem. Lab. Med.* **41**, 1281–1288.
2. Kang, D., Hamasaki, N. (2005) Mitochondrial transcription factor A in maintenance of mitochondrial DNA: overview of its

multiple roles. *Ann. N.Y. Acad. Sci* **1042**, 101–108.

3. Kang, D., Kim, S.H., Hamasaki, N. (2007) Mitochondrial transcription factor A (TFAM): roles in maintenance of mtDNA and cellular functions. *Mitochondrion* **7**, 39–44.
4. Larsson, N.G., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., et al. (1998) Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nat. Genet.* **18**, 231–236.
5. Parisi, M.A., Clayton, D.A. (1991) Similarity of human mitochondrial transcription factor 1 to high mobility group proteins. *Science* **252**, 965–9.
6. Ekstrand, M.I., Falkenberg, M., Rantanen, A., Park, C.B., Gaspari, M., Hultenby, K., et al. (2004) Mitochondrial transcription factor A regulates mtDNA copy number in mammals. *Hum Mol Genet* **13**, 935–44.
7. McCulloch, V., Shadel, G.S. (2003) Human mitochondrial transcription factor B1 interacts with the C-terminal activation region of h-mtTFA and stimulates transcription independently of its RNA methyltransferase activity. *Mol Cell Biol* **23**, 5816–24.
8. Takamatsu, C., Umeda, S., Ohsato, T., Ohno, T., Abe, Y., Fukuoh, A., et al. (2002) Regulation of mitochondrial D-loops by transcription factor A and single-stranded DNA-binding protein. *EMBO Rep.* **3**, 451–456.
9. Alam, T.I., Kanki, T., Muta, T., Ukaji, K., Abe, Y., Nakayama, H., et al. (2003) Human mitochondrial DNA is packaged with TFAM. *Nucleic Acids Res* **31**, 1640–5.
10. Ohgaki, K., Kanki, T., Fukuoh, A., Kurisaki, H., Aoki, Y., Ikeuchi, M., et al. (2007) The C-terminal tail of mitochondrial transcription factor a markedly strengthens its general binding to DNA. *J Biochem (Tokyo)* **141**, 201–11.
11. Kanki, T., Ohgaki, K., Gaspari, M., Gustafsson, C.M., Fukuoh, A., Sasaki, N., et al. (2004) Architectural role of TFAM in maintenance of human mitochondrial DNA. *Mol. Cell. Biol.* **24**, 9823–9834.
12. Falkenberg, M., Gaspari, M., Rantanen, A., Trifunovic, A., Larsson, N.G., Gustafsson, C.M. (2002) Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA. *Nat Genet* **31**, 289–94.
13. Iwaasa, M., Umeda, S., Ohsato, T., Takamatsu, C., Fukuoh, A., Iwasaki, H., et al. (2002) 1-Methyl-4-phenylpyridinium ion (MPP+), a toxin that can cause Parkinsonism, alter branched structures of DNA. *J. Neurochem.* **82**, 30–37.
14. Ohno, T., Umeda, S., Hamasaki, N., Kang, D. (2000) Binding of human mitochondrial transcription factor A, an HMG box protein, to a four-way DNA junction. *Biochem. Biophys. Res. Commun.* **271**, 492–498.
15. Yoshida, Y., Izumi, H., Torigoe, T., Ishiguchi, H., Itoh, H., Kang, D., Kohno, K. (2003) p53 Physically interacts with mitochondrial transcription factor a and differentially regulates binding to damaged DNA. *Cancer Res.* **63**, 3729–3734.

Chapter 7

Inducible Expression in Human Cells, Purification, and In Vitro Assays for the Mitochondrial DNA Helicase Twinkle

Steffi Goffart and Hans Spelbrink

Abstract

Mitochondrial DNA (mtDNA) maintenance can be and has been studied in a wide variety of organisms, some more tractable than others. We use human and mouse cell culture models to study proteins and mechanisms of mtDNA replication. Recent advances in cell culture systems allow us to streamline the analysis of replication proteins both in vivo in cell culture and in vitro following protein purification. One such system, the inducible 293 Flp-In™ TREx™ system, will be described here in detail with the emphasis on the mitochondrial DNA helicase Twinkle, in particular its mitochondrial purification following over-expression, and basic activity and multimerization assays.

Key words: Twinkle, helicase, mtDNA, replication, mitochondria.

1. Introduction

It is now generally accepted that mitochondrial DNA (mtDNA) is organized in multi-protein DNA complexes termed nucleoids (*see (1)* and references therein). Recent studies have shown that not only in yeast but also in various mammals the number and variety of potential nucleoid proteins are surprising (*see (2)* and references therein). Amongst the nucleoid proteins are also those proteins that are directly implicated in replication and repair of mtDNA, such as Twinkle.

The mitochondrial DNA helicase Twinkle was first identified as a partial human cDNA sequence on the basis of its homology with phage and bacterial helicases *(3)*. We subsequently derived its full-length cDNA sequence and showed that mutations in the

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_7 Springerprotocols.com

gene for Twinkle were associated with autosomal dominant Progressive External Ophthalmoplegia (adPEO) with multiple mtDNA deletions *(4)*. It has subsequently been shown that Twinkle is indeed a DNA helicase *(5)*, as was predicted on the basis of its similarity with the phage T7 primase/helicase gp4 and its involvement in an mtDNA maintenance disorder. In addition, the demonstration that Twinkle together with mitochondrial single-stranded DNA-binding protein (mtSSB) and polymerase gamma (POLG) is capable of highly processive DNA synthesis *(6)* firmly established Twinkle as at least one of the likely replicative mtDNA helicases. This was confirmed by us in a recent paper, demonstrating that inducible expression of dominant negative mutations affecting the helicase activity of Twinkle causes mtDNA depletion by replication stalling *(7)*. There does not appear to be a homolog of Twinkle in *Saccharomyces cerevisiae* and to date it is still unclear what the replicative mitochondrial helicase in this organism is.

Helicases couple the hydrolysis of a nucleotide to the unwinding of dsDNA, dsRNA, or RNA–DNA hybrids. They are defined by various parameters including oligomeric state, substrate specificities, directionality, and processivity (*see* Ref. *(8)* for a recent, detailed review). Defining these parameters generally forms part of the initial characterization of a helicase. For Twinkle it was shown that it likely forms multimers as does T7 gp4. In vitro helicase activity prefers a fork-like substrate consisting of a short dsDNA segment and a single-stranded 5′-tail *(5)*. The assay that we use and is presented here is based on these conditions. In our hands Twinkle prefers UTP, GTP, and dGTP for hydrolysis, largely in agreement with Korhonen 2003. ATP can also be hydrolyzed efficiently. As the phage T7 gp4 protein Twinkle has 5′–3′ directionality *(5)*. Characterizing helicases by means of the various biochemical parameters might sometimes seem superfluous because of similarity with already characterized homologous proteins, it nevertheless can give surprising and interesting results. For example, the yeast mitochondrial helicase Suv3p is essentially an RNA helicase as part of the so-called mitochondrial degradosome *(9)*. The human homolog, however, has been shown to preferentially unwind dsDNA in contrast to the yeast enzyme that preferentially unwinds dsRNA *(10)*, suggesting a different biological function of the human protein.

In order to study the effects of mtDNA maintenance protein expression directly in human cell culture and to purify the studied proteins for in vitro functional assays, we have adopted an inducible system, the so-called Flp-In^{TM}TREx^{TM} system. In combination with the commercially available human embryonal kidney (HEK) 293 Flp-In^{TM}TREx^{TM} cells which are well suited for high-level protein expression we can rapidly analyze both in vivo effects of expression of, e.g., Twinkle mutants and isolate sufficient quantities of protein for in vitro biochemical assays *(7)*. This approach is obviously applicable to other mitochondrial (maintenance) proteins

(*see* e.g. *(11)*). Here we describe the establishment of stable inducible 293 Flp-In™ TREx™ cells, mitochondrial isolation, and purification of recombinant Twinkle helicase using His-affinity purification, the basic Twinkle helicase assay and a multimerization assay using glutaraldehyde crosslinking.

2. Materials

2.1. Cultivation of Flp-In™ TREx™ -293 Cells

1. Flp-In™ TREx™-293 core kit (Invitrogen).
2. Enzymes and material for cloning the specific transgene into pcDNA5/FRT/TO.
3. Dulbecco's Modified Eagle Medium, high glucose (DMEM) with 10% v/v fetal bovine serum, FBS (*see* **Note 1**), and 2 mM L-glutamine.
4. Selective antibiotics (InvivoGen): Zeocin 100 mg/ml stock, Blasticidin 10 mg/ml stock, Hygromycin 100 mg/ml stock.
5. Uridine 25 mg/ml in H_2O, filter-sterilize, and store in 1 ml aliquots at –20°C (*see* **Note 2**).
6. Phosphate-buffered saline, PBS.
7. Doxycycline or Tetracycline, 10 μg/ml in ethanol, store at –20°C (*see* **Note 3**).
8. DMSO, cell-culture tested.
9. Transfection reagent, e.g., Fugene (Roche) or similar.
10. Cell culture material, e.g., sterile plastic dishes, pipettes, and cryovials.

2.2. Isolation of Mitochondria and Subsequent Protein Purification from Flp-In™ TREx™ -293 Cells

1. 10X Homogenization buffer: 400 mM Tris–HCl, pH 7.8, 250 mM NaCl, 50 mM $MgCl_2$. Autoclave and store at +4°C.
2. 1X and 0.1X homogenization buffer. Diluted from 10X stock.
3. Protease inhibitor mix, e.g., complete EDTA-free protease inhibitor cocktail tablets (Roche) (*see* **Note 4**).
4. Tight Potter (ca. 5 ml) (Teflon/glass) and rotator (*see* **Note 5**).

2.2.1. Mitochondrial Isolation

Optional:

5. Ultracentrifuge with swingout rotor and appropriate tubes of 2–10 ml.
6. 1 and 1.5 M Sucrose solutions in 1X homogenization buffer.
7. Cytochalasin B (Sigma), 10 mg/ml in DMSO, store at –20°C and protect from light, *very toxic!*

2.2.2. Purification of His-Tagged Proteins from Isolated Mitochondria

1. TALON metal affinity resin (Clontech) (*see* **Note 6**).
2. Protease inhibitor mix (*see* **Note 4**).

3. Lysis buffer (*see* **Note 7**): 50 mM KH_2PO_4, pH 7.5, 1 M NaCl, 0.5% Triton X-100.
4. Wash buffer (*see* **Note 8**): 25 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10% glycerol, 20 mM imidazole.
5. Elution buffer (*see* **Notes 8 and 9**): 25 mm Tris–HCl, pH 7.5, 100 mM NaCl, 20% glycerol, 250 mM imidazole, 100 mM L-arginine–HCl, pH 7.5.
6. Sonicator.

2.3. Assays with the Purified Twinkle Helicase

2.3.1. Helicase Substrate Labeling

1. M13mp18(+) single-stranded DNA (Amersham Pharmacia).
2. Complementary oligonucleotide; the 20 nucleotides that will anneal to the M13 vector are in *italics* (5′-ACA TGA TAA GAT ACA TGG ATG AGT TTG GAC AAA CCA CAA C*GT AAA ACG ACG GCC AGT GCC*-3′).
3. T4 Polynucleotide kinase with reaction buffer.
4. [^{32}P]γ-ATP, 5,000–6,000 Ci/mmol, 10 mCi/ml.
5. 1X DNA hybridization buffer (400 mM Tris–HCl, pH 7.5, 1 M NaCl).
6. Centricon-100 concentration tubes (Amicon) (*see* **Note 10**).
7. Heatblock or waterbath at 37°C and 75°C.

2.3.2. In Vitro Helicase Assay

1. Mitochondrial protein extract or purified protein.
2. Radioactively labeled helicase substrate.
3. Nucleotide solutions at 3 mM, store at –20°C (*see* **Note 11**).
4. Helicase buffer: 25 mM Tris–HCl, pH 7.6, 4.5 mM $MgCl_2$, 50 mM NaCl, 100 μg/ml BSA, 1 mM DTT, 10% glycerol, 100 mM L-arginine–HCl, pH 7.6.
5. Loading buffer: 90 mM EDTA, 6% SDS, 30% glycerol, 0.25% bromophenol blue.
6. Vertical slab gel system (10–15 cm length) with comb teeth width = 5 mm.
7. Acrylamide/bisacrylamide 10% gel (29:1) in 1X TBE buffer.
8. TBE (1X) running buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA).
9. Gel dryer.
10. Autoradiography equipment (phosphorimager and/or X-ray film).

2.3.3. Oligomerization Assay by Glutaraldehyde Crosslinking

1. Purified protein.
2. Oligomerization buffer: 25 mM Tris–HCl, pH 7.6, 4.5 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, 10% glycerol, 100 mM L-arginine–HCl, pH 7.6.

3. Glutaraldehyde solution, store at +4°C, handle under a fume hood, very toxic!
4. SDS sample buffer, 2X: 50 mM Tris, pH 6.8, 0.01% Serva Blue G, 4% SDS, 12% glycerol, 100 mM DTT.
5. NuPAGE Novex 4–7% Tris–acetate gradient gels (Invitrogen) and running buffer.
6. HiMark pre-stained protein standard (Invitrogen).
7. Gel running apparatus for 10 × 10 cm vertical gels.
8. Blotting apparatus.
9. Nitrocellulose membrane.
10. Blocking reagent.
11. Primary and secondary antibody.
12. Chemiluminescence detection for regular Western blotting.

3. Methods

3.1. Cultivation of Flp-In™ TREx™ -293 Cell Lines

Various inducible mammalian expression systems are available. Perhaps the most commonly used are those that use a tetracycline responsive (cis) element (TRE) in combination with a protein that binds to this element and activates transcription in the absence of tetracycline (e.g., doxycycline (DOX)) (Tet-off) or binds to the element and activates transcription in the presence of DOX (Tet-on). In our experience these basic so-called Tet systems are very tedious since they require isolation and characterization of many single-cell colonies and are prone to so-called position effects, meaning that depending on where the transgenic construct integrates in the host-cell genome expression levels vary and can suffer from epigenetic silencing. The result is that, even when great care is taken to isolate true single-cell colonies and maintain selection for the antibiotic selection present on the expression plasmid, a mosaic transgene expression is usually observed by immunofluorescence and the expression deteriorates over time even when the protein of interest is not induced. Most of these typical problems of the basic Tet systems are overcome in the Flp-In™ TREx™ system by the use of a site-specific recombinase, the so-called Flip-recombinase (for details *see* www.invitrogen.com). The commercially available Flp-In™TREx™-293 cell line is engineered to have a single Flip recombination site at a stable and transcriptionally active locus. By co-transfection of a Flip-recombination proficient plasmid containing the gene of interest following a TRE and a plasmid expressing the Flip-recombinase itself, the DOX responsive gene of interest gets integrated at a predetermined genomic location. The result is that all transfected and "Flip-recombined" cells have the gene of interest at the same active locus and thus that all

derived transgenic lines should in fact be isogenic. One immediate advantage is that single-cell colony picking is not necessary. Using the appropriate antibiotic selection and careful handling of the cells we typically obtain ready-to-use transgenic lines within 2 months starting from the initial transfection and with very little additional work. In addition, the far majority of lines that we have obtained so far show that >99% of cells express the recombinant protein with little variation in expression levels at a given DC concentration, as based on immunofluorescence examination. The one drawback with the system, as with all Tet systems, is that the expression regulation is not absolutely tight: in non-induced cells a very low level of protein expression can be observed even under tetracycline-free conditions. This is acceptable under most circumstances, but can impair the cells if an especially deleterious protein is expressed. In this case also, maintaining a "Tet-free" stock in culture for a prolonged period of time can lead to counterselection of the transgene or outgrowth of the few (<1%) of cells that do not appear to express the transgene.

3.1.1. Maintenance of Flp-In™ TREx™-293 Cells

1. Flp-In™ TREx™-293 cells are grown in DMEM containing 10% FBS, 2 mM L-glutamine at 37°C under 100% humidity, and 5–8.5% CO_2. As selective antibiotics, 100 μg/ml Zeocin and 15 μg/ml Blasticidin are added to the parental cell line containing the tet-repressor only. To maintain this selective pressure medium with selective antibiotics is replaced every 2–3 days. The medium should be pre-warmed before adding to the cells (*see* **Note 12**).
2. For best growth rates cells should be around 40–80% confluent and split when they reach 90% confluency, usually after 3–4 days. To passage the cells they are resuspended in either PBS or fresh medium by pipetting and the appropriate portion is transferred to a new cell culture dish containing fresh medium with the selective antibiotics. A dilution ratio of 1:4–1:6 is recommendable in most cases.
3. To keep cell stocks for longer time they should be frozen in liquid nitrogen. Harvest cells in logarithmic growth phase (ca. 60–80% confluency) into a 15-ml tube, centrifuge at 400 × g for 5 min, and resuspend the cell pellet in DMEM containing 20% FBS, 2 mM glutamine, and 10% DMSO. Transfer the cell suspension to several cryovials and slowly cool down to –80°C (*see* **Note 13**).

   Transfer the vials after 12–48 h into a liquid nitrogen container. Cultured cells remain viable for several years in liquid nitrogen (*see* **Note 14**) .Thaw cells by removing the cryovial from liquid nitrogen and placing it in a 37°C waterbath. As soon as the suspension is thawed, transfer it to a cell culture dish and dilute with growth medium. Replace the

medium after 3–16 h by fresh medium to remove all traces of DMSO. Selective antibiotics should be added 2 days after thawing the cells.

3.1.2. Establishing Stable Flp-In™TREx™-293 Cell Lines Containing a Transgene

1. To obtain stable Flp-In™TREx™ expression lines, the parental cell line is co-transfected with a Tet-repressor-regulated expression vector (such as pcDNA5/FRT/TO) containing the transgene and pOGG44 containing the Flip-recombinase gene. For transfection of the parental Flp-In™TREx™-293 cells, cells are grown without antibiotics to 40–60% confluency and then transfected using any established transfection method and a ratio of 1:9 of pcDNA5/FRT/TO to pOGG44 (*see* **Note15**).
2. Forty-eight hours after transfection the cells are split to ca. 25% confluency. Two hours later 150 μg Hygromycin and 15 μg/ml Blasticidin are added to select transgenic cells. The cell culture medium is replaced every 3–4 days with fresh medium containing these antibiotics. After 1–2 weeks small clonal colonies can be observed under microscope. When these colonies become overconfluent and contain several hundred cells all the colonies on a dish are resuspended and the cells spread more evenly. This is repeated until the culture plate is full.
3. As soon as sufficient amounts of cells are obtained the cell line should be checked for transgene expression with and without induction by doxycycline. A portion of the cells are seeded into two wells of a 6-well plate and 10 ng/ml doxycycline is added to one well. After 48 h the cells are harvested and analyzed for expression of the desired protein using Western blot analysis.
4. If the transgene is expressed, the cell line is expanded and aliquots are frozen to keep an early passage stock (*see* **Note 16**).
5. Hygromycin and Blasticidin should be added also to established cell lines (*see* **Note 17**).

3.2. Isolation of Mitochondria and Subsequent Protein Purification from Flp-In ™ TREx ™ -293 Cells

Once a cell line is established to express a recombinant protein cells can be cultivated to large quantities for protein purification. We typically express recombinant proteins with a His tag for further purification. Since most proteins we study are exclusively mitochondrial, an initial but highly beneficial purification step (since it removes many nuclear contaminants that might have similar activities as the proteins we study) isolates mitochondria from the rest of the cell. We here describe two crude mitochondrial isolation methods and further purification by means of a sucrose step gradient. His-tagged protein purification in the case of Twinkle

typically uses TALON, which is a Cobalt-based affinity-matrix, but similar methods using Nickel matrices are equally applicable. It is beyond the scope of this chapter to discuss various alternative protocols for His-protein purification.

3.2.1. Purification of Mitochondria from Flp-In ™TREx™-293 Cells

(Method based on Gaines and Attardi 1984 (12).)

1. Harvest the Flp-In™ TREx™-293 cells expressing the desired His-tagged protein by washing them off in 1X PBS and centrifuging at 400 × g for 5 min at 4°C.
2. Remove the supernatant and resuspend the cell pellet in 5 vol 0.1 × homogenization buffer and centrifuge again for 5 min at 400 × g and at 4°C.
3. Remove the supernatant and resuspend cells in 5 vol 0.1 × homogenization buffer + 1X protease inhibitors.
4. Incubate the cell suspension for 5 min on ice.
5. Transfer the cell suspension to a pre-cooled Potter and homogenize the cells with ca. 15 strokes at 1,500 rpm.
6. Check the efficiency by diluting a small amount of cell suspension in 1X PBS + 0.01% Trypan Blue and streaking it onto a miscroscopy slide (*see* **Note 18**).
7. Immediately add 1/10 vol of 10X homogenization buffer and transfer the suspension to a fresh centrifuge tube.
8. Wash potter and pestle with 2 ml 1X homogenization buffer and combine this with the homogenized cell suspension.
9. Centrifuge for 5 min at 800 × g and at 4°C (*see* **Note 19**).
10. Transfer the supernatant to a fresh centrifuge tube and repeat the centrifugation step.
11. Transfer the supernatant to a fresh centrifuge tube and centrifuge for 10 min at 12,000 × g and at 4°C (*see* **Note 20**).
12. Remove the supernatant and resuspend the mitochondrial pellet in 1 × homogenization buffer + 1X proteinase inhibitors.
13. Centrifuge again for 10 min at 12,000 × g and at 4°C.
14. The pellet containing a crude mitochondrial fraction is now ready for use or can be purified further using a sucrose gradient (*see* **Note 21**). The mitochondrial pellet can be snap-frozen in liquid nitrogen and stored at –80°C.

Optional sucrose gradient purification step

15. Resuspend the mitochondrial pellet in ca. 4 vol 1X homogenization buffer and overlay it onto a two-step sucrose gradient containing 1.5 M sucrose and 1 M sucrose in 1X homogenization buffer.
16. Centrifuge in an ultracentrifuge with swingout-rotor for 1 h at 45,000 × g.

17. Remove the upper phase leaving a small layer of solution above the mitochondrial band (*see* **Note 22**).
18. Pipette the mitochondrial layer off using a cutoff pipette tip with wide opening.
19. Dilute the mitochondrial layer fraction with 3 vol 1X homogenization buffer and pellet the mitochondria for 5 min at 12,000 × g and at 4°C.
20. The pellet now contains purified mitochondria free of cytosolic and nuclear contaminations.

Alternative cell breakage with cytochalasin B

(Method essentially as described in Yasukawa et al., 2005(13)).

21. Harvest the cells as described above.
22. Resuspend the cell pellet in 10 ml DMEM containing 10 μg/ml cytochalasin B (*see* **Note 23**).
23. Transfer the cell suspension into a cell culture dish and incubate it for 30 min in the cell culture incubator.
24. Carefully transfer the cell suspension to a 50-ml centrifuge tube and fill up with cold DMEM (*see* **Note 24**).
25. Pellet the cells by centrifuging for 5 min at 400 × g and at 4°C.
26. Remove the supernatant and resuspend the cells in 5 ml 1X homogenization buffer containing protease inhibitors.
27. Continue with Step 5 of the protocol above, omitting Step 7 (addition of 10X homogenization buffer).

3.2.2. Purification of His-Tagged Proteins from Isolated Mitochondria

1. Dissolve a fresh or frozen mitochondrial pellet in >20 vol of lysis buffer containing 1X protease inhibitors.
2. Sonicate thoroughly while keeping the sample on ice (*see* **Note 25**).
3. Add 100–300 μl of TALON resin and rotate the tube at 4°C for 1–2 h.
4. Pellet the resin by centrifuging for 5 min at 1,000 × g and at 4°C in a swingout-rotor.
5. Remove the supernatant and resuspend the resin in the same volume of lysis buffer containing 1X protease inhibitors.
6. Pellet the resin again by centrifugation for 5 min at 1,000 × g and at 4°C.
7. Resuspend the resin in 1 ml wash buffer and centrifuge for 5 min (1,000 × g 4°C) (*see* **Note 26**).
8. Remove the supernatant and repeat this wash step twice.
9. Remove all liquid and resuspend the resin in 1 vol elution buffer.
10. Incubate the suspension on ice for 5 min with occasional mixing.

11. Centrifuge again for 5 min at 1,000 × g and at 4°C to pellet the resin.
12. Transfer the supernatant containing the eluted protein to a fresh tube, carefully avoiding the resin.
13. Aliquot the protein extract and snap-freeze in liquid nitrogen. Store at –80°C.

3.3. Assays with the Purified Twinkle Helicase

Both T7 gp4 and Twinkle use similar helicase assays requiring a fork-like substrate *(5)*. It should, however, be emphasized here that different helicases can have very different substrate requirements and this may require extensive testing of a wide variety of substrates. The assay we use is very similar to the assay originally published *(5)*. It requires the annealing of a ~60-bp oligonucleotide to, for example, single-stranded M13 DNA. The oligo has at its 3'-end a 20-bp complementary sequence to the M13 vector, while the 5'-end 40-bp should not anneal to the M13 vector. Similar substrates can also be made using two partially complementary oligos but this requires a slightly different purification method of the labeled substrate.

The helicase assay itself measures the release of the annealed oligo from the M13 vector DNA in the presence of a nucleotide triphosphate that is hydrolyzed by the helicase to provide the necessary energy for the strand separation. The annealed substrate and released oligo are easily separated on a non-denaturing acrylamide gel due to the large difference in size. An example result of a helicase assay is depicted in **Fig. 7.1**.

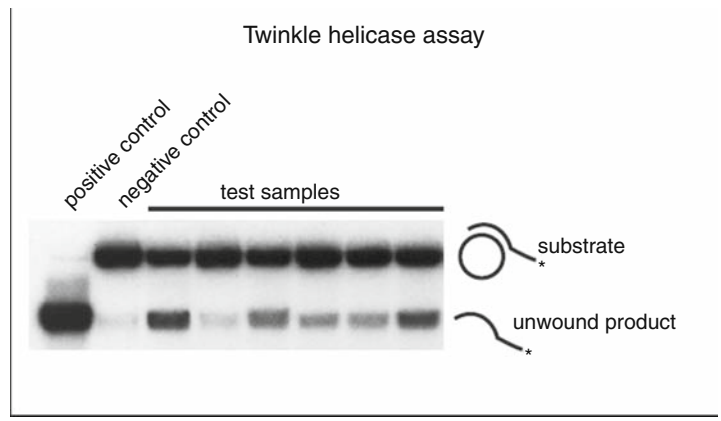


Fig. 7.1. **Example result of a helicase assay using purified Twinkle.** Wild-type Twinkle and variants were purified from Flp-In™TREx™-293 cells and used in a helicase assay using the methods described in this chapter. Positive control illustrates the heat-denatured substrate sample while the negative control in this case is substrate without added protein. Some degree of unwinding is observed with most samples, most strongly in the case of wild-type Twinkle which is the sample next to the negative control.

The second assay we apply in the analysis of mutations of the Twinkle protein is an oligomerization assay by means of glutaraldehyde crosslinking. Although the protocol given here is perhaps not the most sensitive to changes in the oligomeric state of Twinkle, it does have the benefit that it can be applied to relatively small quantities of protein. It is crucial in this assay to have controls in the form of a sample that is not glutaraldehyde crosslinked, which is required to show the monomeric form of Twinkle on Western blots. A second control that we include in the assay is a splice variant of Twinkle, called Twinky that does not show abundant higher order multimers on native PAGE *(4)* and also in this crosslinking assay does not appear to form multimers (*see (7)* Supplementary Figures). Using this assay and also using native page analysis, Twinkle shows multiple multimeric forms including a form that is the size of a hexamer, which is believed to be the active form of the T7 gp4 protein *(14)*.

3.3.1. Helicase Substrate Labeling and Annealing

1. Add 20 pmol of oligonucleotide to 25 µl 1X kinase reaction buffer containing 5 µCi [^{32}P]γ-ATP and 10 U T4 polynucleotide kinase (*see* **Note 27**).
2. Incubate for 45 min at 37°C to end-label the DNA.
3. Inactivate the polynucleotide kinase by incubating for 10 min at 75°C.
4. Mix 2.5 µl of the reaction mix (containing 2 pmol oligonucleotide) with 3 µg M13mp18(+) DNA.
5. Adjust the volume to 100 µl with 1X DNA hybridization buffer.
6. Incubate for 5 min at 75°C and let slowly cool down to =30°C to allow annealing of the complementary strands.
7. Add 1 ml 1X DNA hybridization buffer and transfer the solution to a Centricon-100 tube.
8. Centrifuge for 10 min at 1,000 × g and discard the flowthrough (*see* **Note 28**).
9. Add 1 ml DNA hybridization buffer, repeat centrifugation, and discard the flowthrough.
10. Repeat the wash as in Step 8 once more.
11. Invert the column into a fresh collection tube and collect the remaining liquid by centrifuging for 1 min at 1,000 × g.
12. Measure the volume of the eluate to determine the approximate concentration of the substrate.
13. Store the substrate at +4°C for up to 2 weeks (*see* **Note 29**).

3.3.2. Helicase Activity Assay

1. Thaw the frozen protein extracts on ice.
2. Mix 1–10 ng of protein with 400 pmol substrate in 25 µl helicase assay buffer.

3. Include two control samples containing only substrate, but no protein, and one sample containing a mock extract prepared from cells that do not express the studied transgene (*see* **Note 30**).
4. Start the reaction by adding 3 mM nucleotide, and incubate for 30 min at 37°C.
5. Stop the reaction by adding 15 µl loading buffer.
6. Heat-denature one of the two control samples for 3 min at 95°C (*see* **Note 31**).
7. Load 20 µl of each sample on a 10% native TBE gel.
8. Run the gel in 1X TBE at 10 V/cm for 1–2 h until the bromophenol blue front has moved ca. 6 cm into the gel.
9. Disassemble the gel system, transfer the gel to Whatman paper, and dry on a vacuum gel dryer.
10. Expose to X-ray film or phosphorimager.
11. To compare relative unwinding activities we quantify the amount of unwound product as well as the still double-stranded substrate. For each sample the activity is then expressed as radioactivity in the product band per total radioactivity (substrate plus product band).

3.3.3. Oligomerization Assay by Glutaraldehyde Crosslinking

1. Thaw the frozen protein extracts on ice.
2. Dilute 1–10 ng of protein in 23 µl oligomerization buffer. Include one control sample that will not be crosslinked.
3. Incubate for 5 min at room temperature to allow the formation of oligomers.
4. Dilute glutaraldehyde to 0.25% in oligomerization buffer (*see* **Note 32**).
5. Add 2 µl glutaraldehyde dilution to all protein dilutions except for the control sample and start the crosslinking reaction by vortexing.
6. Incubate for 10 min at room temperature.
7. Stop the reaction by adding 15 µl loading buffer.
8. Heat-denture the samples immediately for 10 min at 95°C.
9. Load the samples onto a denaturing 4–7% Tris–acetate gel, include one lane with the non-crosslinked control sample and one with the protein standard.
10. Separate at 120 V until the loading dye has reached the bottom of the gel.
11. Blot the gel onto nitrocellulose membrane and detect the protein using a conventional Western blotting protocol.

4. Notes

1. The fetal bovine serum used should be tetracycline-free. Guaranteed tetracycline-free medium can be obtained from various suppliers, but also many batches of regular serum are essentially tetracycline-free. The serum can be tested for absence of tetracycline by monitoring transgene expression levels by Western blot analysis of TREx or other tetracycline-regulated cells grown in medium with the regular serum compared to commercial tet-free serum. To establish a TREx cell line with unknown effects of the transgene it is recommended to use guaranteed tet-free medium.
2. Uridine is added to the growth medium to support survival and growth of cells with impaired mitochondrial function. Uridine auxotrophy has been suggested to be the result of a severe drop in the activity of dihydroorotate dehydrogenase *(15)*. Cells with fully functional mitochondria grow better without uridine addition.
3. Both tetracycline and doxycycline can be used to control the Tet-repressor system. Doxycycline has the advantage of higher stability in solutions and better solubility, but it is still advisable to make fresh solutions every 10 days to ensure the concentration is accurate.
4. Protease inhibitors are recommended to avoid protein degradation during the mitochondrial preparation. Homemade or commercial mixes of protease inhibitors can be used, but if a His-affinity purification is planned, EDTA should be avoided, as it strips the metal ions of the affinity resin.
5. Also other homogenizer types can be used, but the number of strokes has to be tested for each homogenizer, as the shearing force differs substantially. If a Dounce homogenizer is used, perform manual strokes without rotating.
6. Various commercial resins are available for His-affinity purification, the most common ones using Ni^{2+} as the capture ion (e.g., Ni^{2+}-NTA agarose). TALON resin uses Co^{2+} ions and has a slightly higher specificity and thus can reduce the number of contaminating proteins. The advantage of one resin type over the other can vary for each protein, so a comparison might be useful.

7. A lysis buffer containing high concentrations of salt facilitates the dissociation of DNA-binding proteins from the mitochondrial DNA. If needed the salt concentration can be decreased down to 100 mM NaCl, but the protein yield might be reduced. TritonX-100 can be substituted by other detergents compatible with the resin. Anionic detergents like SDS or sodium deoxycholate should be avoided since they interfere with the His-affinity binding.
8. Low salt wash and elution buffer can be adjusted to the conditions of the downstream assay. As a general guideline a near-neutral buffer pH, 50–100 mM NaCl and 10–20% glycerol are recommended components to ensure protein stability. Imidazole binds competitively to the metal ions of the resin and elutes bound proteins. Up to 40 mM imidazole in the wash buffer can be used to reduce non-specific binding of contaminating proteins to the resin, while higher concentrations may lead to dissociation of His-tagged proteins.
9. L-Arginine improves the solubility of many proteins and prevents aggregation.
10. Centricon tubes facilitate the separation of annealed substrate from unincorporated oligonucleotides if the substrate has a molecular weight much higher than the oligonucleotide alone. If two oligonucleotides are annealed to form a small molecular weight molecule, a separation by native acrylamide gel electrophoresis is recommended.
11. The unwinding activity of helicases requires the hydrolysis of nucleotides as energy source. ATP is the most commonly used nucleotide, but does not necessarily give the highest activity. One of the initial steps to characterize a newly identified helicase is therefore the determination of its substrate specificity. Thus, the enzyme activity using various nucleotides at various concentrations might be compared to analyze the nucleotide specificity. As nucleotide solutions are degraded easily, aliquot the nucleotide solution and avoid repetitive freezing and thawing.
12. Two hundred and ninety-three cells are only loosely attached to the growth surface, so care should be taken to avoid rough movements and direct pipetting onto the cell layer, as cells might be washed off.
13. A cooling speed of approximately 1°C/min is required to avoid the formation of ice-crystals in the cytosol that would puncture the cell membranes. The easiest way to achieve an appropriate cooling rate is commercial freezing pots that contain isopropanol and which are placed directly into

a –80°C freezer. A cheaper method is to wrap the cryovials thickly in tissue paper or pack them into a small styrofoam box before placing them at –80°C.

14. To check the quality of a batch of frozen cells thaw one vial several days after freezing. About 30–80% of the cells should be viable and attach to the dish surface within a few hours.
15. Calcium phosphate transfection or various liposomic transfection methods can be applied. In our hands Fugene-6 (Roche Biosciences) has shown least cell toxicity and gives high transfection rates. It is recommendable to transfect 2–3 10 cm plates for each construct. An average transfection will give 3–15 founder colonies per plate. Since all cells integrate the transgene into the same genomic locus, clone picking is not required and all cells on a plate can be pooled.
16. In the case of very deleterious proteins the very low leakiness of the system can impair cell growth even when no protein expression can be detected. In this case the cells might loose the inducibility after cultivation for longer times. For this reason and to avoid any long-term selection of adapted cells, thawing of an early passage stock is recommended every 6–8 weeks. For this reason it is also recommendable to prepare a large quantity of vials for storage in N_2^{liq} as soon as possible after establishing a line.
17. Depending on the transgene the cells can be kept without selective pressure for up to 2 weeks, e.g., for large-scale experiments requiring large amounts of culture medium and thus antibiotics. In any case it is recommended to keep at least one cell "stock" plate under constant selection.
18. The membranes of broken cells are only faintly visible under the microscope, while intact cells are round, clearly defined and are not stained by Trypan Blue. Intact nuclei of 293 cells appear oval and blue, while extensive breakage of nuclei results in a blue, grainy sediment. About 70% broken cells with little nuclei breakage are ideal. If required add more strokes to reach sufficient homogenization. If extensive nuclei rupture is observed reduce the amount of strokes.
19. This step pellets unbroken cells, nuclei, and membrane fragments. The mitochondria stay in the supernatant, which should appear cloudy.
20. This centrifugation step pellets the mitochondria, the supernatant should be clear.
21. This mitochondrial pellet contains low amounts of various cellular organelles. If a further extraction of specific proteins from the mitochondrial lysate is planned, no additional purification of the intact mitochondria is needed. If a purer

mitochondrial preparation is required further separation of mitochondria from the cytosolic and nuclear contaminants by sucrose gradient purification is recommended. Other types of gradients can also be applied for this purification step.

22. The buffer phase above the 1 M sucrose layer contains nuclear and cytosolic proteins and should be removed before pipetting off the mitochondrial layer to avoid contamination. Intact mitochondria form a sharp dense layer between the two sucrose densities. If an additional diffuse band is visible, mitochondria were damaged, e.g., by too rough homogenization or pipetting.
23. Cytochalasin B degrades the cytoskeleton of cells, increasing the fragility of the cell membrane without swelling.
24. Handle cytochalasin B-treated cells gently and avoid rough pipetting, as this can already disrupt the cell membrane.
25. Sonication facilitates the dissociation of DNA-binding proteins from the mitochondrial DNA and at the same time breaks the mitochondrial DNA into easily soluble fragments. With a Vibra cell sonicator (Sonic) ca. 1 min sonication in 1 s interval with 2 s break at 70% amplitude is recommended. If no sonication is applied, the yield of protein might be lower and insoluble DNA might cause clogging of the resin in later steps. To avoid the latter a high-speed centrifugation step to pellet the DNA (20 min >12,000 × g at 4°C) is recommended, in this case continue with the supernatant.
26. A convenient way of handling the wash and elution steps is to transfer the resin in this step to small spin columns (e.g., from Qiagen). The columns allow fast and complete removal of the wash solutions and higher recovery of the elution fraction.
27. Only one of the DNA strands in the substrate is radioactively labeled at the 5′-end, usually the smallest.
28. The flowthrough contains non-annealed oligonucleotide and free ATP from the labeling reaction.
29. After longer storage the substrate degrades and increasing amount of single-stranded oligonucleotide makes the determination of enzymatic separation in a helicase assay difficult.
30. A mock protein preparation is recommended to ensure that the observed enzyme activity is due to the protein of interest and not due to a contamination.
31. The heated control sample can be used as positive control with 100% free product, while the non-heated control gives the substrate to product ratio in the absence of enzyme activity.
32. Glutaraldehyde is toxic and volatile, so handle all solutions and samples containing glutaraldehyde under a fume hood.

Acknowledgments

S.G. and H.S. are supported by the Academy of Finland, the Medical Research Fund of Tampere University Hospital and the European Community's Sixth Framework Programme for Research, Priority 1 "Life sciences, genomics and biotechnology for health," contract number "LSHM-CT-2004-503116."

References

1. Chen, X. J., and Butow, R. A. (2005) The organization and inheritance of the mitochondrial genome. *Nat. Rev. Genet.***6,** 815–25.
2. Holt, I. J., He, J., Mao, C. C., Kirk-Up, J. D., Martinsson, P., Sembongi, H., Reyes, A., and Spelbrink, J. N. (2007) Mammalian mitochondrial nucleoids: Organizing an independently minded genome. *Mitochondrion.***7**, 311–321.
3. Leipe, D. D., Aravind, L., Grishin, N. V., and Koonin, E. V. (2000) The bacterial replicative helicase DnaB evolved from a RecA duplication. *Genome Res.***10,** 5–16.
4. Spelbrink, J. N., Li, F. Y., Tiranti, V., Nikali, K., Yuan, Q. P., Tariq, M., Wanrooij, S., Garrido, N., Comi, G., Morandi, L., Santoro, L., Toscano, A., Fabrizi, G. M., Somer, H., Croxen, R., Beeson, D., Poulton, J., Suomalainen, A., Jacobs, H. T., Zeviani, M., and Larsson, C. (2001) Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria. *Nat. Genet.***28,** 223–31.
5. Korhonen, J. A., Gaspari, M., and Falkenberg, M. (2003) TWINKLE Has 5' -> 3' DNA helicase activity and is specifically stimulated by mitochondrial single-stranded DNA-binding protein. *J. Biol. Chem.***278,** 48627–32.
6. Korhonen, J. A., Pham, X. H., Pellegrini, M., and Falkenberg, M. (2004) Reconstitution of a minimal mtDNA replisome in vitro. *Embo J.***23,** 2423–9.
7. Wanrooij, S., Goffart, S., Pohjoismaki, J. L., Yasukawa, T., and Spelbrink, J. N. (2007) Expression of catalytic mutants of the mtDNA helicase Twinkle and polymerase POLG causes distinct replication stalling phenotypes. *Nucleic Acids Res.***35,** 3238–51.
8. Singleton, M. R., Dillingham, M. S., and Wigley, D. B. (2007) Structure and mechanism of helicases and nucleic acid translocases. *Annu. Rev. Biochem.***76,** 23–50.
9. Dziembowski, A., Piwowarski, J., Hoser, R., Minczuk, M., Dmochowska, A., Siep, M., van der Spek, H., Grivell, L., and Stepien, P. P. (2003) The yeast mitochondrial degradosome. Its composition, interplay between RNA helicase and RNase activities and the role in mitochondrial RNA metabolism. *J. Biol. Chem.***278,** 1603–11.
10. Minczuk, M., Piwowarski, J., Papworth, M. A., Awiszus, K., Schalinski, S., Dziembowski, A., Dmochowska, A., Bartnik, E., Tokatlidis, K., Stepien, P. P., and Borowski, P. (2002) Localisation of the human hSuv3p helicase in the mitochondrial matrix and its preferential unwinding of dsDNA. *Nucleic Acids Res.***30,** 5074–86.
11. Pohjoismaki, J. L., Wanrooij, S., Hyvarinen, A. K., Goffart, S., Holt, I. J., Spelbrink, J. N., and Jacobs, H. T. (2006) Alterations to the expression level of mitochondrial transcription factor A, TFAM, modify the mode of mitochondrial DNA replication in cultured human cells. *Nucleic Acids Res.***34,** 5815–28.
12. Gaines, G., and Attardi, G. (1984) Highly efficient RNA-synthesizing system that uses isolated human mitochondria: new initiation events and in vivo-like processing patterns. *Mol. Cell. Biol.***4,** 1605–17.
13. Yasukawa, T., Yang, M. Y., Jacobs, H. T., and Holt, I. J. (2005) A bidirectional origin of replication maps to the major noncoding region of human mitochondrial DNA. *Mol. Cell***18,** 651–62.
14. Crampton, D. J., Ohi, M., Qimron, U., Walz, T., and Richardson, C. C. (2006) Oligomeric states of bacteriophage T7 gene 4 primase/helicase. *J. Mol. Biol.***360,** 667–77.
15. Morais, R., Desjardins, P., Turmel, C., and Zinkewich-Peotti, K. (1988) Development and characterization of continuous avian cell lines depleted of mitochondrial DNA. *In Vitro Cell Dev. Biol.***24,** 649–58.

Chapter 8

Purification Strategy for Recombinant Forms of the Human Mitochondrial DNA Helicase

Tawn D. Ziebarth and Laurie S. Kaguni

Abstract

In this chapter, we present a streamlined purification for the production of near-homogeneous and high yield recombinant forms of the human mitochondrial DNA helicase. Minimizing the number of steps and the time elapsed for purification of this enzyme facilitates studies of its structure and mechanism and allows elucidation of native features of both wild-type- and human disease-related forms.

Key words: mitochondrial DNA replication, DNA helicase, human, DNA unwinding.

1. Introduction

The human mtDNA helicase is a member of the *Escherichia coli* DnaB-like family of replicative helicases, also known as Superfamily 4 *(1)*. These family members contain five conserved sequence motifs, including the classic Walker A and Walker B motifs that participate directly in the ATPase binding and hydrolysis activity required for DNA unwinding (*2, 3)*. The human enzyme transduces the hydrolysis energy for replication fork advancement and translocates along ssDNA in a 5′–3′ direction *(4)*. Its native conformation is that of a hexamer, with an overall modular architecture comprising distinct N- and C-terminal domains *(1)*. A unique feature of the enzyme is a regulatory role in ATP hydrolysis for its extreme N- and C-termini *(1)*. In addition, amino acid residue R609 has been identified as the arginine finger that serves to ligate the γ phosphate of the incoming ATP molecule during the catalytic cycle *(1)*.

To date, biochemical and physical data are limited for this novel enzyme, and further analysis is mandated by its direct link to a human disease associated with multiple mtDNA deletions *(5)*. This finding,

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_8 Springerprotocols.com

in conjunction with studies revealing the role for the mtDNA helicase in mtDNA maintenance and regulation of mtDNA copy number, reveals its importance for proper mitochondrial physiology and in turn, overall cellular processes *(6)*. In this chapter, we present a streamlined purification for recombinant forms.

2. Materials

2.1. Recombinant Human Mitochondrial DNA Helicase Purification from Spodoptera frugiperda Cells

1. 1*M* Tris–HCl, pH 7.5, pH 8.0, stored at 24°C.
2. 0.5*M* Ethylenediaminetetraacetic acid (EDTA), pH 8.0.
3. 5*M* Sodium chloride (NaCl).
4. 2*M* Tris–HCl, pH 6.8, pH 8.8.
5. 0.2*M* Phenylmethylsulfonyl fluoride (PMSF) in isopropanol. Store aliquots at –20°C.
6. 1*M* Sodium metabisulfite, prepared as a 1.0*M* stock solution at pH 7.5 and stored at –20°C.
7. Leupeptin is prepared as a 1 mg/ml stock solution in 50 m*M* Tris–HCl, pH 7.5, 2 m*M*EDTA, and stored at –20°C.
8. 1*M* Dithiothreitol (DTT). Store aliquots at –20°C.
9. 14*M* 2-Mercaptoethanol (β-Me). Store at 4°C.
10. 30% Polyacrylamide (29:1; acrylamide:bisacrylamide). Store at 4°C.
11. 10% Sodium dodecyl sulfate (SDS).
12. 4X Resolving gel buffer: 1.5*M* Tris–HCl, pH 8.8, 0.4% SDS.
13. 4X Stacking gel buffer: 0.5*M* Tris–HCl, pH 6.8, 0.4% SDS.
14. 5X SDS-PAGE running buffer: 0.125*M* Tris base, 0.95*M* glycine, 0.5% SDS.
15. 5X SDS-PAGE loading buffer: 50% glycerol, 2*M* Tris base, 0.25*M* DTT, 5% SDS, 0.1% bromophenol blue. Aliquots are stored at –20°C.
16. 10% Ammonium persulfate (APS).
17. TC-100 Insect cell culture medium and fetal bovine serum (Life Technologies).
18. Amphotericin, penicillin-G, and streptomyocin (Sigma).
19. Insect cell transfection buffer and Grace's medium (PharMingen).
20. *S. frugiperda* (Sf9) cells.
21. Baculoviruses encoding N- and C-terminally His-tagged human mitochondrial helicase, obtained from Dr. Maria Falkenberg (Karolinska Institute).

22. Phosphate-buffered saline, stored at 4°C.
23. Buffer A: 50 m*M* Tris–HCl, pH 8.0, 0.6*M*NaCl, 10% glycerol, 10 m*M*2-mercaptoethanol.
24. Nickel-nitrilotriacetic acid agarose resin (Qiagen).
25. 1*M* Imidazole, stored at 24°C.
26. Heparin Sepharose agarose resin (Amersham Pharmacia Biotech).
27. Polyallomer tubes (14 × 89 mm, Beckman)

2.2. Human Mitochondrial DNA ATPase Assay

1. Recombinant human mitochondrial DNA helicase at approx. 200 μg/ml.
2. 1*M* Tris–HCl, pH 7.5, stored at 24°C.
3. 1*M* $MgCl_2$, stored at 24°C.
4. 0.29 mg/ml Bovine serum albumin (BSA, Sigma).
5. 82 m*M*ATP, store aliquots at 4°C.
6. 1*M* Dithiothreitol (DTT). Store aliquots at –20°C.
7. [γ-^{32}P]ATP.
8. 2.0 mg/ml DNase I-activated calf thymus DNA.
9. 0.5*M* Ethylenediaminetetraacetic acid (EDTA), pH 8.0.
10. Polygram polyethyleneimine cellulose paper (Brinkmann Instruments, Inc.)
11. 50 m*M* ADP/ATP, store aliquots at 4°C.
12. 1*M* Formic acid.
13. 0.5*M* Lithium chloride (LiCl).

3. Methods

3.1. Recombinant Human Mitochondrial DNA Helicase Purification from S. frugiperda Cells

3.1.1. sf9 Cell Growth and Protein Over-expression

1. Grow *S. frugiperda* cells (2.0 l) in TC-100 insect cell culture media containing 10% (v/v) fetal bovine serum at 27°C to a cell density of 2×10^6, dilute to a cell density of 1×10^6 with TC100 containing 10% fetal bovine serum.
2. Infect with baculovirus encoding N- or C-terminally His-tagged helicase (complete cDNA sequence lacking the mitochondrial presequence) at a multiplicity of infection of 5 at 27°C.
3. Harvest cells at 72 h postinfection by centrifugation and wash with an equal volume of cold phosphate-buffered saline twice.
4. Recentrifuge, discard supernatant, and freeze pellet in liquid nitrogen. Store at –80°C.

3.1.2. Soluble Cytoplasmic Fraction Preparation

All buffers contain 1 m*M* PMSF, 10 m*M* sodium metabisulfite, 2 μg/ml leupeptin, and 10 m*M* 2-mercaptoethanol. Perform all steps at 0–4°C.

1. Thaw cells and resuspend in 1/45 volume of original cell culture in 25 m*M* Tris–HCl, pH 8.0. Allow cells to sit for 20 min.
2. Homogenize cells in a Dounce homogenizer using 20 strokes with a tight pestle.
3. Adjust homogenate to 1 *M* NaCl followed by centrifugation at 45,000 rpm for 27 min in a 60 Ti rotor. Use Buffer A to balance.
4. Extract supernatant (fraction I)

3.1.3. Nickel-Nitrilotriacetic Acid Chromatography

1. Dilute fraction I with an equal volume of Buffer A and load onto a nickel-nitrilotriacetic acid column (2.5 ml resin/l of cells) equilibrated with Buffer A containing 10 m*M* imidazole at a flow rate of 12 ml/h.
2. Wash the column with equilibration buffer at a flow rate of 20 ml/h.
3. Elute the column with Buffer A containing steps of 25 m*M*, 250 m*M*, and 500 m*M* imidazole. Protein will elute with buffer containing 250 m*M* imidazole.
4. Analyze fractions by SDS-PAGE on 10% minigels followed by silver staining, and pool fractions accordingly (fraction II)

3.1.4. Heparin Sepharose Chromatography

1. Dilute fraction II to an ionic equivalent of 150 m*M* NaCl and load onto a Heparin Sepharose column (2.7 mg/ml resin) equilibrated with buffer containing 20 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, 10% glycerol, 0.5 m*M* EDTA at a flow rate of 3–5 ml/h (*see* **Note 1**).
2. Wash the column with equilibration buffer containing 200 m*M* NaCl at a flow rate of 9 ml/h.
3. Elute the column with equilibration buffer containing salt steps of 0.6 and 1 *M* NaCl. Protein will elute at 350–700 m*M* NaCl (*see* **Note 2**).
4. Adjust eluted fractions to 1.0 *M* NaCl.
5. Analyze fractions by SDS-PAGE on 10% minigels followed by silver staining, and pool fractions accordingly (fraction III).

3.1.5. Glycerol Gradient Sedimentation

1. Layer fraction III onto pre-formed 12–30% glycerol gradients containing 35 m*M* Tris–HCl, pH 7.5, 330 m*M* NaCl, 1 m*M* EDTA, prepared in polyallomer tubes for use in a Beckman SW 41 rotor.
2. Centrifuge at 140,000 × g for 37 h at 3°C, then fractionate by collecting four-drop (200 μl) fractions.

3. Analyze fractions by SDS-PAGE on 10% minigels followed by silver staining, and pool fractions accordingly (fraction IV).
4. Freeze fraction IV in liquid nitrogen and store at –80°C. Samples are analyzed by SDS-PAGE on 10% gels to evaluate purity and yield (*see* **Note 3**).

3.2. ATPase Assay

This assay measures the amount of ATP hydrolyzed to ADP + inorganic phosphate by the human mtDNA helicase in a DNA-independent and dependent manner.

1. Each reaction (0.02 ml) contains 20 m*M* Tris–HCl, pH 7.5, 4 m*M* $MgCl_2$, 0.1 mg/ml bovine serum albumin, 10% glycerol, 0.5 m*M* ATP, 10 mM dithiothreitol, 4 μCi of [γ-^{32}P]ATP, 100 μ*M* DNase I-activated calf thymus DNA, and 0.05 μg N- and C- terminally His-tagged human mtDNA helicase. Prepare the reaction mix with and without DNA in a microcentrifuge tube on ice. Add the radioactivity last. Vortex and centrifuge briefly in the microcentrifuge.
2. Dispense the mix, 20 μl, to pre-chilled and numbered microcentrifuge tubes on ice.
3. Add the enzyme, 0.25 μl, to each tube avoiding bubbles and mix gently by flicking the tube three times.
4. Incubate the tubes for 15 min at 37°C.
5. Stop the reactions with addition of EDTA to 20 m*M* and transfer to ice.
6. Divide Polygram polyethyleneimine cellulose paper into 1 × 1 cm lanes.
7. Spot 0.5 μl ADP/ATP marker in each 1-cm lane. Allow paper to dry.
8. Spot 2.0 μl reaction sample slowly while drying.
9. Develop Polygram CEL 300 PEI/UV paper in 1*M* formic acid and 0.5*M* lithium chloride for 30 min.
10. Allow paper to dry.
11. Visualize ADP and ATP spots under UV light and cut out each band.
12. Count radioactivity in each band in scintillation fluid.

4. Notes

1. Precipitate will form as ionic strength decreases to 150 m*M* NaCl. As the sample loads onto the column, this precipitate will accumulate on the resin bed.

2. As the ionic strength increases during the elution, the precipitate will go back into solution.
3. The purity and yield of the final preparations are determined by SDS-PAGE followed by silver staining. **Figure 8.1** shows a typical purification of the recombinant form of mtDNA helicase.

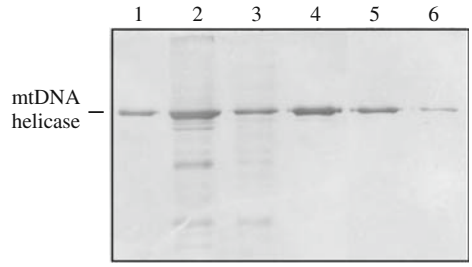


Fig. 8.1. Baculovirus over-expression and purification of the human mtDNA helicase. Protein fractions were denatured and electrophoresed in a 10% SDS-polyacrylamide gel. Proteins were detected by silver staining. Lane 1:*Hs* mtDNA helicase (0.3 μg); lane 2: whole cell fraction; lane 3: soluble extract (fraction I, 0.3 μg); lane 4: Nickel-NTA pool (fraction II, 0.8 μg); lane 5: Heparin Sepharose pool (fraction III, 0.4 μg); lane 6: glycerol gradient pool (fraction IV, 0.1 μg).

References

1. Ziebarth TD, Farr CL, Kaguni LS. Modular architecture of the hexameric human mitochondrial DNA helicase. J Mol Biol 2007;367(5):1382–91.
2. Caruthers JM, McKay DB. Helicase structure and mechanism. Curr Opin Struct Biol 2002;12(1):123–33.
3. Walker JE, Saraste M, Runswick MJ, Gay NJ. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. Embo J 1982;1(8):945–51.
4. Korhonen JA, Gaspari M, Falkenberg M. TWINKLE Has 5′ -> 3′ DNA helicase activity and is specifically stimulated by mitochondrial single-stranded DNA-binding protein. J Biol Chem 2003;278(49):48627–32.
5. Spelbrink JN, Li FY, Tiranti V, et al. Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria. Nat Genet 2001;28(3):223–31.
6. Tyynismaa H, Sembongi H, Bokori-Brown M, et al. Twinkle helicase is essential for mtDNA maintenance and regulates mtDNA copy number. Hum Mol Genet 2004;13(24):3219–27.

Chapter 9

Methods for Studying Mitochondrial Transcription Termination with Isolated Components

Paola Loguercio Polosa, Stefania Deceglie, Marina Roberti, Maria Nicola Gadaleta, and Palmiro Cantatore

Abstract

Characterization of the basic transcription machinery of mammalian mitochondrial DNA has been greatly supported by the availability of pure recombinant mitochondrial RNA polymerase (mtRNAP) and accessory factors, which allowed to develop a reconstituted in vitro transcription system. This chapter outlines a general strategy that makes use of a minimal promoter-independent transcription assay to study mitochondrial transcription termination in animal systems. We used such a system to investigate the transcription termination properties of the sea urchin factor mtDBP, however, it is applicable to the study of transcription termination in a variety of organisms, provided that the pure mtRNAP and the transcription termination factor are available.

The assay here described contains the recombinant proteins mtRNAP and mtDBP, both expressed in insect cells, and a template consisting of a 3′-tailed DNA construct bearing the sequence bound by mtDBP. Transcription by the RNA polymerase produces run-off and terminated molecules, the size of the latter being consistent with RNA chain arrest in correspondence of the mtDBP–DNA complex. Transcription termination is protein-dependent as addition of increasing amounts of mtDBP to the assay causes a decrease in the intensity of the run-off and the gradual appearance of short-terminated molecules. Furthermore, we report a method, based on pulse-chase experiments, which allows us to distinguish between the true termination and the pausing events.

Key words: Transcription factors, transcription termination, sea urchin mtDBP, mtRNA polymerase, termination assay, pulse-chase experiment.

1. Introduction

Animal mitochondrial DNA (mtDNA) is a circular, closed, double-stranded molecule of 15–17 kb. It has a conserved gene content, lacks introns, and contains a main non-coding region (D-loop region) that is the most variable part of the molecule,

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_9 Springerprotocols.com

both in size and in sequence. This region hosts regulatory signals for the replication and expression of the organelle genome. While in vertebrates mtDNA has a conserved gene organization, in invertebrates there are remarkable differences both with respect to vertebrates and among invertebrates themselves. The different gene organization is associated with distinct RNA synthesis mechanisms: in vertebrates the transcription process is almost invariant, whereas peculiar modes of RNA synthesis for each organism take place in invertebrates. Vertebrate mtDNA is transcribed by one L- and two H-strand transcription units. Transcription of L-strand gives rise to one mRNA (ND6), eight tRNAs, and the H-strand replication primer. Of the two H-strand transcription units, one is responsible for the synthesis of the two 16S and 12S rRNAs, and of $tRNA^{Phe}$ and $tRNA^{Val}$, whereas the other directs the synthesis of the remaining tRNAs and of 12 mRNAs. Since mRNA genes are in most of the cases flanked by tRNA genes, it has been proposed that the tRNA structure represents a recognition signal for an RNAseP-like processing enzyme which would generate the mature transcripts (for review *see* Refs. *(1, 2)*). An example of such enzyme may be the recently described *Drosophila* RNase Z *(3)*.

Studies on mitochondrial transcription in invertebrates have been limited to a few experimental systems such as sea urchin, *Drosophila*, and *Artemia (4–7)*. In sea urchin the mtDNA is transcribed by multiple and overlapping transcription units, probably originating from AT-rich conserved sequences located in six different positions of the genome *(4)*. In *Drosophila* the peculiar gene organization, consisting of blocks of genes oriented in opposite direction, argues for a mechanism based on the existence of distinct transcription initiation sites located at the beginning of each block of genes *(6)*.

Despite differences in the mechanism of transcription between vertebrates and invertebrates, the mitochondrial transcription apparatus appears rather conserved in all animals. Transcription initiation depends on a single subunit, phage-like, mitochondrial RNA polymerase (mtRNAP) and on three protein factors, namely TFAM, TFB1M, and TFB2M. According to a recently proposed model, the HMG-like protein TFAM binds DNA and causes a conformational change, thus allowing TFB1M or TFB2M to bind DNA, as well as the recruitment of the mtRNAP *(2)*. It has been recently reported that a further protein factor, named TERF3, acts as a negative regulator of RNA initiation *(8)*.

mtDNA transcription is also regulated at the level of transcription termination. In particular it has been found that the factor mTERF, which binds downstream of the 16 rRNA gene, is involved in the termination of the ribosomal transcription unit *(9)*. It has been recently reported that mTERF, by interacting also with the non-coding region, could regulate the differential

expression of the two H-strand transcription units by means of a looping mechanism *(10)*. mTERF homologs have been described also in sea urchin and in *Drosophila*. In the sea urchin *Paracentrotus lividus* the DNA-binding protein mtDBP functions as transcription termination factor by binding two homologous sequences located in the non-coding region and at the boundary of oppositely transcribed ND5 and ND6 genes, respectively *(11)*. Furthermore, mtDBP has a contrahelicase activity that likely modulates mtDNA synthesis *(12)*. In *Drosophila* the protein DmTTF terminates transcription in vivo by binding two regions of *Drosophila* mtDNA containing the converging ends of block of genes coded by opposite strands *(13–15)*.

Identification of mitochondrial transcription initiation sites and characterization of the transcription apparatus were pursued by different in vivo and in vitro experimental approaches. Relevant insights into the mechanism of RNA synthesis in human were obtained initially from in organello studies and, later, from the reconstitution of in vitro transcription systems using partially or completely purified components. The latter approach, which permits the dissection of different steps in the transcription pathway, is described in this report. Here we will focus on the procedures that are used to study transcription termination in sea urchin mitochondria; the described methods allowed us to map a protein-dependent transcription termination site and to establish whether the protein factor mediates true transcription termination rather than RNA polymerase pausing.

2. Materials

2.1. Protein Preparation

1. mtDBP and mtRNAP cDNA cloning and protein purification are described in detail in Ref. *(16)*.

2.2. 3′-Tailed DNA Template Preparation

1. To produce the 98-bp 3′-tailed template, TermNCR(R), bearing the mtDBP binding site in opposite orientation with respect to the direction of transcription, two complementary oligonucleotides are used, that are 98 and 114 nt long; the latter contains a 3′-tail of 16 dC (*see* **Notes 1–3**).
2. Oligonucleotides are PAGE-purified; they are dissolved in double-distilled water (*see* **Note 4**) at 10 pmol/μl and stored at –20°C.
3. 2X Annealing buffer: 33.2 mM Tris–HCl, pH 7.5, 13.2 mM $MgCl_2$, 0.1 M NaCl.

2.2.1. Polyacrylamide Gel Electrophoresis of the DNA Template

1. Handcast 10% polyacrylamide mini-gel (8.3 cm width × 7.3 cm length × 0.75 mm thickness) in 1X TBE, run with Mini-Protean Electrophoresis Cell (Bio-Rad).
2. Thirty percent acrylamide/bis solution (29:1 with 3.3% C).
3. *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine, TEMED.
4. Ammonium persulfate (APS): prepare 10% solution in double-distilled water, store at +4°C for about 1 month.
5. DNA size marker: GeneRuler 50-bp DNA ladder (Fermentas).

2.3. Gel-Mobility Shift Assay

1. 10X Binding buffer: 0.1 M Tris–HCl, pH 8.0, 3 M NaCl, 0.1 M $MgCl_2$.
2. Bovine serum albumin (BSA) (1 mg/ml).
3. Poly [d(I-C)] (Roche).
4. 46mer double-stranded oligonucleotide containing mtDBP binding site, labeled at its 5′-termini with [γ-^{32}P]ATP (3,000 Ci/mmol, 10 μCi/μl).
5. Recombinant and purified mtDBP from baculovirus-infected cells (*see Ref. (16)*).
6. 1X Dilution buffer: 10 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 500 mM NaCl, 20% glycerol, 1 mM DTT added prior to use.
7. 6X Loading dye (Fermentas)
8. 50% Glycerol.
9. Typhoon 8600 Phosphor Imaging System (Molecular Dynamics).
10. Storage phosphor screen.
11. Gel dryer.
12. X-ray film.

2.4. In Vitro Transcription Assay

1. mtDBP and mtRNAP proteins (*see* **Section 2.1**).
2. mtDBP dilution buffer for transcription assay: 20% glycerol, 10 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, pH 8.0; add fresh DTT to 1 mM.
3. DEPC-treated double-distilled water.
4. 5X Transcription buffer: 200 mM Tris–HCl, pH 8.0, 125 mM NaCl, 40 mM $MgCl_2$, 10 mM spermidine $(HCl)_3$ (Sigma), 0.5 mg/ml BSA (Sigma). Make 5X transcription buffer in DEPC-treated double-distilled water; store at –20°C.
5. 25 mM DTT in DEPC-treated double-distilled water; store at –20°C.
6. DNA template (*see* **Section 2.2**).

7. NTPs final concentration in the reaction mixture is 1 mM ATP, 0.3 mM CTP, 0.3 mM GTP, 0.0125 mM UTP. Make a 25X NTP mixture in DEPC-treated double-distilled water, dispense in small aliquots and store at –20°C.
8. [α-^{32}P]UTP (800 Ci/mmol, 20 μCi/μl).
9. RNaseOUT (Invitrogen).
10. Stop buffer: 10 mM Tris–HCl, pH 7.4, 0.5% SDS, 0.2 M NaCl, 10 mM EDTA, pH 8.0.
11. 20 mg/ml Glycogen, RNA grade.
12. Cold ethanol.
13. Phenol extraction: mixture containing phenol (saturated with Tris, pH 8.0, and stabilized with 0.1% hydroxyquinoline), chloroform, and isoamyl alcohol 25:24:1, v/v.
14. DNase buffer: 40 mM Tris–HCl, pH 7.5, 6.0 mM $MgCl_2$ in DEPC-treated double-distilled water.
15. DNase I.
16. Urea dye: 3.5 M urea, 0.5X TBE, 0.01% bromophenol blue, 0.01% xylene cyanol. Store the dye at –20°C.

2.5. Transcription Termination Assay

1. Materials as above (**Section 2.4**).

2.6. Gel Electrophoresis of the Transcription Products

1. Heat block at 95°C to denature the samples.
2. Handcast 12% polyacrylamide/7 M urea mini-gel (8.3 cm width × 7.3 cm length × 0.75 mm thickness) in 1X TBE, prepared and run with Mini-Protean Electrophoresis System.
3. 10X TBE: 0.9 M Tris, 0.87 M boric acid, 25 mM EDTA, pH 8.3 with boric acid.
4. Ammonium persulfate, APS: *see* Step 4, **Section 2.3**.
5. Thirty percent acrylamide/bis solution (29:1 with 3.3% C).
6. *N*,*N*,*N*,*N*′-Tetramethyl-ethylenediamine, TEMED.
7. Labeled RNA size marker for small RNAs (Ambion Decade Marker System). Prepare the labeled RNAs following Ambion protocol, store it at –80°C. It is stable for up to 6 months.
8. Storage phosphor screen.
9. Phosphorimager.
10. Gel dryer.
11. X-ray film.

2.7. Pulse-Chase Transcription Termination Experiment

1. Materials as in **Section 2.4**.

2.8. Gel Electrophoresis Analysis of Transcription Products from the Pulse-Chase Experiment

1. Materials as in **Section 2.6**.
2. X-ray film.
3. Laser densitometer (LKB Ultroscan XL).

3. Methods

The detailed understanding of the various steps of the transcription process is based on the use of a pure in vitro system containing a DNA template, the mtRNAP, and the individual factors involved in the transcription machinery. A highly purified human transcription system was developed by Falkenberg et al., suitable to study transcription initiation *(17)*. The system contains the recombinant proteins mtRNAP, TFAM, TFB1M or TFB2M, and short mtDNA fragments bearing the specific promoters HSP or LSP. Studies of the transcription elongation properties of nuclear or mitochondrial RNA polymerases, as well as their transcription arrest, can be performed independently of initiation factors and promoter recognition. This analysis is carried out by using nonselective templates consisting of a double-stranded oligonucleotide containing a short single-stranded sequence, needed for polymerase loading. Protein-dependent arrest of RNA polymerase can be derived from true termination of transcription or from polymerase pausing. In the first case, both the enzyme and the RNA dissociate from the template and the transcription event is definitively interrupted; in the case of pausing, the termination factor slows down the polymerase, which remains bound to the template and is able to resume transcription afterward.

Below we describe the use of a minimal sea urchin mitochondrial transcription system that contains the recombinant proteins mtRNAP and mtDBP, both expressed in insect cells and a nonselective template bearing the mtDBP binding site. This system allows one to detect a protein-mediated transcription termination event and, furthermore, to distinguish between true transcription termination and RNA polymerase pausing. The same procedure can be used to study transcription termination by heterologous RNA polymerases (*see* **Note 5**).

3.1. Protein Preparation

1. mtRNAP and mtDBP are recombinant proteins lacking the mitochondrial presequence; mtRNAP contains seven histidines at the N-terminus. The cDNAs for mtRNAP and mtDBP were cloned in the BacPAK6 baculovirus vector (Clontech). Each protein was purified from 400 ml of baculovirus-infected Sf9 insect cells. Chromatography fractions were immediately snap-frozen in liquid nitrogen and stored at –80°C. Positive fractions, as detected by immunoblotting, gel-shift or transcription assay, were thawed on ice, gently mixed by tipping the tube, dispensed into 50 µl aliquots, snap-frozen in liquid nitrogen, and stored at –80°C. Details of protein purification are reported in Ref. *(16)*.

3.2. DNA Template Preparation

1. In an Eppendorf tube combine 200 pmol of each oligonucleotide (that is, 20 µl each of a 10 pmol/µl solution), 50 µl of 2X annealing buffer, and 10 µl of double-distilled water. Final concentration of oligonucleotide should be 2 pmol/µl. Two hundred picomoles of double-stranded DNA is sufficient for gel electrophoresis analysis and for several transcription reactions.
2. Perform denaturation and annealing steps in a PCR thermocycler as follows: denaturation for 10 min at 98°C, sequential annealing steps for 1 min at temperatures that are 2 degrees below the preceding, ranging from 98 to 20°C, final incubation for 5 min at 20°C. The annealed template is stored at –20°C; it is stable for several weeks.
3. Check that no single-stranded DNA is left in the mixture by comparing the migration on a 10% polyacrylamide mini-gel of about 5 pmol of annealed and single-stranded oligonucleotides.

3.3. Mobility-Shift Titration of the Amounts of mtDBP for the Transcription Termination Assay

1. The probe used in the gel-shift assay is a double-stranded 46mer oligonucleotide, containing the 25-bp mtDBP binding site.
2. The probe is labeled at its 5′-termini with [γ-^{32}P]ATP according to standard procedure (*see* Ref. *(18)*).
3. To set up the DNA-binding reaction, for each sample to be tested, place an Eppendorf tube on ice, add 50 fmol of probe labeled to a specific activity of about 3,400 cpm/fmol, 0.5 µg of poly [d(I-C)], 2 µl of 10X binding buffer, 1 µg of BSA.
4. Make serial dilutions of mtDBP with ice-cold dilution buffer; we usually test 0.05, 0.5, 1.0, and 2.0 pmol of mtDBP by adding to each sample not more than 5 µl of each dilution.
5. Make up the final volume (20 µl) with double-distilled water.
6. Add the specific amount of protein, mix gently, incubate for 20 min at 25°C.

7. Make a blank containing 2 µl of 50% glycerol and all the reagents except the protein; incubate as before.
8. Pre-run the gel (16 cm width × 20 cm length × 1.5 mm thickness, 6% polyacrylamide gel in 0.5X TBE) for 40 min at 200 V (constant current) in the cold room.
9. Add 2 µl of 6X loading dye only to the blank, load onto the gel.
10. Run the gel at 300 V, for about 1 h, until the front dye, xylene cyanol, reaches about 5 cm from the gel bottom.
11. Vacuum dry the gel and analyze by phosphorimaging. **Figure 9.1** shows a typical mobility-shift titration assay carried out by incubating a constant amount of labeled probe (50 fmol per reaction) with different amounts of mtDBP (0.05, 0.25, 0.35, and 0.75 pmol) corresponding to a DNA/protein ratio of 1:1, 1:5, 1:7, and 1:15. At ratio 1:15 the DNA is almost completely bound as very little free DNA is present; therefore, for the termination assays, we chose a DNA/protein ratio of up to 1:10.

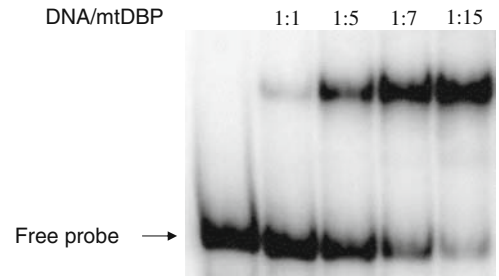


Fig. 9.1. **Mobility-shift titration of recombinant mtDBP purified from baculovirus-infected insect cells.** The assay was performed with 50 fmol of the end-labeled double-stranded 46mer oligonucleotide, bearing the mtDBP binding sequence, and 0.05, 0.25, 0.35, and 0.75 pmol of mtDBP (DNA/protein ratio is 1:1, 1:5, 1:7, and 1:15). Samples were analyzed on a 6% polyacrylamide gel, followed by phosphorimaging analysis. At 1:15 the DNA is almost completely bound as very little free DNA is present.

3.4. In Vitro Transcription Assay with mtRNAP: Preparation of Samples and Reaction

1. A standard transcription reaction is carried out in a 25-µl volume. All the components listed in the Materials section (*see* **Section 2.5**) are made ready and placed on ice, except for the protein fraction that is thawed just prior to addition.
2. In an Eppendorf tube, placed on ice, combine 2 pmol (1 µl) of DNA template, 5 µl of 5X transcription buffer, 1 µl of 25 mM DTT, 1 µl of 25X NTP mixture, 0.5 µl of [α-^{32}P]UTP (corresponding to 10 µCi), 28 U of RNaseOUT (*see* **Note 6**).
3. The mixture is vortexed and briefly spun in an Eppendorf microfuge; add the required volume of DEPC-treated water and, finally, 5 µl of mtRNAP, corresponding to 0.15 pmol of

the recombinant protein. The sample is gently mixed by tipping the tube and incubated at 30°C in a water bath for 30 min.

4. The reaction is stopped with 150 µl of Stop buffer, followed by the addition of 0.12 mg/ml glycogen and one phenol extraction.
5. Nucleic acids are precipitated by adding 2.5 volumes of ethanol pre-chilled at –20 °C and keeping the samples at –80°C for 2 h. Then, nucleic acids are pelleted by centrifuging at full speed in a microfuge at 4°C for 20 min.
6. Each pellet (no need to dry it) is dissolved in 150 µl of DNase buffer by pipetting up and down with a Gilson micropipette and incubated with 0.7 U of DNaseI at 37°C for 10 min. After adding 0.2 M NaCl, nucleic acids are ethanol precipitated for 12 h (overnight) at –20°C. Alternatively, you may keep at –80°C for 2 h (*see* **Note 7**).

3.5. Transcription Termination Assay

1. Samples are set up as above (*see* Steps 1, 2, **Section 3.4**).
2. Dilutions of mtDBP are made to have 6, 8, 16, and 20 pmol of protein, possibly in a volume not greater than 5 µl. In the control sample, which lacks mtDBP, dilution buffer is added that corresponds to the highest volume of protein added to the assay.
3. Add to the sample the required volume of double-distilled water and the proper amount of mtDBP or an equivalent volume of mtDBP-containing buffer. Incubation of the DNA template with the termination factor is then performed for 20 min at 23°C.
4. Add 5 µl of mtRNAP (it does not need to be diluted), mix gently.
5. Follow Steps 3–6 of **Section 3.4**.

3.6. Gel Electrophoresis Analysis of Transcription Products

1. Prepare a 0.75-mm thick, 12% polyacrylamide/7 M urea mini-gel in 1X TBE, using the Mini-Protean Electrophoresis Cell (Bio-Rad). The gel should polymerize in about 1 h.
2. Carefully remove the comb, assemble the gel following the Bio-Rad manual instructions, and thoroughly wash the wells with running buffer using a syringe with a proper needle.
3. Prepare the sample of labeled Decade RNA marker: take 2 µl of the labeled RNA and dilute to 200 µl with DEPC-treated water, heat at 80°C for 10 min and transfer 5 µl to an Eppendorf tube placed on ice (*see* below). Store the remaining at –80°C.
4. The final pellets from the last centrifugation (Step 6, **Section 3.4**) are air-dried by keeping the tubes on ice, cap opened, under the fume hood for a few minutes. Then pellets

are dissolved in 12 µl of urea dye by brief vortexing, heated at 80°C for 10 min, and loaded onto the gel, alongside with the Decade RNA marker.

5. Run the gel at 125 V (constant current) for about 2 h until the front dye, xylene cyanol, reaches 2 cm from the gel bottom.
6. After the run, wash the gel twice with distilled water for 10 min, vacuum-dry for 1 h and analyze by phosphorimaging. Alternatively vacuum-dry the gel using a gel dryer (for 60 min at 80°C) and expose with an X-ray film for 12 h at –80°C in the presence of an intensifying screen.
7. The size of the reaction products is determined by using the labeled Decade RNA marker (*see* also **Note 8**).
8. Quantification of data can be done by densitometry scanning of the digital signal or by laser densitometry of the X-ray film (*see* below). An example of results produced by the transcription termination experiment is shown in **Fig. 9.2A,B**. While in the absence of mtDBP (lane 1) a run-off product of about 100 nt is obtained, the addition of increasing amounts of the termination factor (lanes 2–5) causes the decrease in the intensity of the run-off band and the gradual appearance of shorter transcripts. The size of these transcripts (between 50 and 55 nt) corresponds to that of molecules arrested at the protein binding site.

3.7. Pulse-Chase Transcription Termination Experiment

1. The reaction (*see* **Section 3.4**) is performed in a 100-µl volume: the reaction mixture contains 8 pmol (4 µl) of template, 20 µl of 5X transcription buffer, 4 µl of 25X NTPs mixture, and 3.5 µl of [α-^{32}P]UTP corresponding to 70 µCi.
2. After pre-incubation with 48 pmol of mtDBP for 15 min at 23°C, 20 µl of mtRNAP-containing fraction is added and incubation is continued for 30 min at 30°C.
3. Cold UTP is added to a final concentration of 2 mM, a 20-µl volume of each reaction is taken at 0, 30, 100, 200 min and immediately processed following Steps 4–6 of **Section 3.4**.

3.8. Gel Electrophoresis Analysis of Transcription Products from the Pulse-Chase Experiment

1. Perform Steps 1–6 of **Section 3.6**.
2. To measure the ratio between the terminated and the run-off transcripts, we performed quantitative analysis of the digital signal using phosphorimaging or we scanned the X-ray film with a laser densitometer equipped with gel evaluation software. An example of the results of the pulse-chase transcription experiment is shown in **Fig. 9.3**. If pausing of the mtRNAP occurred, then the ratio between the terminated and the run-off transcripts would decrease progressively with

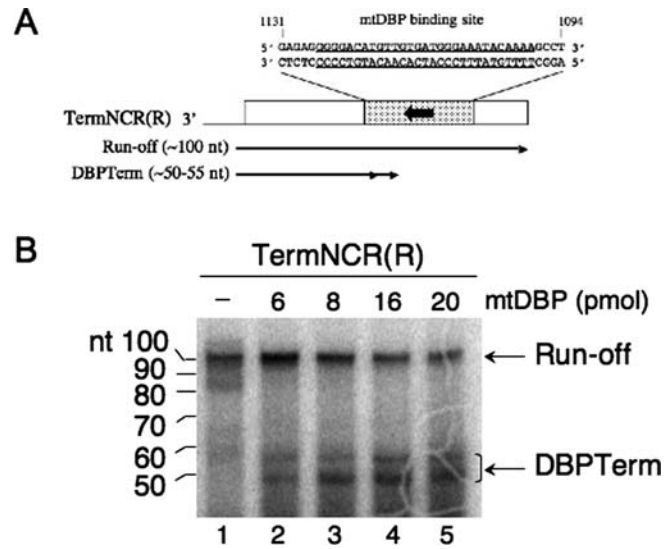


Fig. 9.2. **Transcription termination assays with recombinant mtRNAP and mtDBP.** (**A**) Scheme of the 98-bp 3′-tailed DNA TermNCR(R) construct, used in the transcription assays. The filled boxes indicate the 38 bp of the non-coding region of *P. lividus* mtDNA (the sequence and nucleotide position are shown); the mtDBP binding site, as from DNaseI footprinting analysis *(11)*, is underlined. The arrow enclosed in the boxes marks the orientation of mtDBP target site with respect to transcription direction. The open boxes represent the flanking sequences (41 and 19 bp, respectively), which are unrelated to sea urchin mtDNA; the thin line refers to the 3′-tail. Run-off and terminated transcripts are indicated by arrowed lines. (**B**) Transcription termination assay. Transcription reactions were performed in the presence of about 0.15 pmol of recombinant mtRNAP, 2 pmol of the template, and the indicated amounts of mtDBP, corresponding to a DNA/protein ratio of 1:3, 1:4, 1:8, and 1:10. DBPTerm refers to the terminated products generated by mtDBP. Positions of the RNA markers corresponding to the ^{32}P-5′ end labeled RNA ladder, Decade Markers (Ambion), are shown on the left. Samples were analyzed on a 12% polyacrylamide/7 M urea mini-gel, followed by phosphorimaging analysis. The figure shows that, while in the absence of mtDBP a run-off product of about 100 nt is obtained (lane 1), the addition of increasing amounts of the protein factor (lanes 2–5) causes the progressive decrease in the intensity of the run-off band and the gradual appearance of shorter transcripts. The size of these transcripts (between 50 and 55 nt) corresponds to that of molecules arrested at the protein binding site (modified from Ref. *(16)* with permission).

time, due to resumption of elongation by the stalled enzyme. In the case of transcription termination, which involves dissociation of the enzyme and RNA from the template, the above ratio should not change with time. Quantification of the run-off and the terminated bands indicates that the transcript ratio remains constant, with the shorter transcript persisting for up to 200 min of incubation, a time longer than the half-life of the mtDBP–DNA complex (150 min, Ref. *(11)*). Therefore we conclude that mtDBP promotes true transcription termination rather than polymerase pausing.

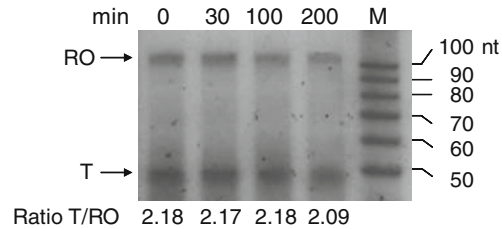


Fig. 9.3. **Pulse-chase of transcription elongation by mtRNAP in the presence of mtDBP.** After a pulse-label of 15 min with [α-^{32}P]UTP, the reaction was chased with an excess of unlabeled UTP. Samples (20 μl) were taken at the indicated time-points and analyzed on a 12% polyacrylamide/7 M urea mini-gel, followed by phosphorimaging analysis. Values below the picture of the gel indicate the ratio of the labeling of the terminated (T) to run-off (RO) transcripts for each time-point. The ratio does not change appreciably, with the shorter transcript persisting for up to 200 min of incubation. This implies that mtDBP promotes real transcription termination rather than pausing (modified from Ref. *(16)* with permission).

4. Notes

1. When you construct the template for transcription termination assay, make sure that the predicted relative size of terminated and run-off transcripts is such that they do not migrate too closely in the gel.
2. Usually, dC is preferred to other nucleotides to make the template tail, as it increases the transcription activity of the RNA polymerase II *(19)*, and, presumably, of the mitochondrial enzyme. Concerning the length of the 3′-overhang, 15–16 nt (preferably not less, for an efficient initiation) are as good as 100 nt; however, if you employ oligonucleotides, 15 or 16 nt long tails are obviously more convenient than longer tails, given the difficulties and costs of long oligonucleotide synthesis.

   3′-tailed templates may also be prepared by adding the poly(dC) tail to a double-stranded DNA fragment obtained by PCR. Tailing can be performed by a C-tailing reaction in the presence of Terminal deoxynucleotidyl Transferase (TdT). Methods for the dC-tailing reaction with TdT are reported in many manuals (*see* Ref. *(18)*). Since it is known that the TdT reaction produces tails of variable length that could affect the transcription efficiency, it is important to optimize the reaction conditions in order that the size of the dC tails varies between about 20 and 500 nt, with the average tail length being around 100 nt. The length and heterogeneity of poly(dC) extensions are checked by comparing on a 10% polyacrylamide gel the migration of tailed and untailed DNA:

the untailed product should run as a tight band whereas the tailed fragment should migrate more slowly and form a slight smear. Heterogeneity of the extensions does not affect the size of the transcription products since transcription on tailed templates has been shown to initiate at sites that are three to six residues outside the tail-duplex junction, independently of the length of the tail *(19)*.

3. To allow complete annealing of the oligonucleotides, design, if possible, sequences that do not form strong secondary structures.
4. All solutions are prepared in water that has a resistivity of 18.2 MΩ cm. This is referred as "double-distilled water" in the text.
5. Termination experiments could be also performed with heterologous polymerases (e.g., human mtRNAP or bacteriophage T3 or T7 RNAP), if you wish to investigate the ability of a termination factor to arrest the elongation by heterologous polymerases. Such an assay can be performed either with non-selective templates or with templates containing the specific promoter, provided that they all contain the target site of the termination factor. An experiment of this type was carried out to demonstrate the termination role of the *Drosophila* factor DmTTF *(15)*.
6. In vitro transcription reactions with purified mitochondrial RNA polymerases are performed in excess of DNA template. Therefore the optimal ratio of enzyme to template in the transcription reaction should be determined. This is performed by preliminary experiments in the presence of a fixed amount of enzyme and variable amounts of template. RNA polymerase should not be saturating to prevent the possibility that large amounts of enzyme are stalling at the end of the linear template and blocking subsequent enzyme molecules.
7. Nucleic acids precipitation is effective at –20°C, even at very low concentrations. In the case of RNA transcripts formed during a transcription reaction, the use of co-precipitant (purified RNase-free glycogen) is essential for effective RNA precipitation. In this case the 2-h incubation at –80°C gives at least 90% recovery.
8. Usually, the pattern of the transcription products obtained with a termination assay is quite simple as it shows only two prominent bands (*see* **Fig. 9.2B**): the higher molecular weight band, corresponding to the run-off, and the shorter product whose size corresponds to that of a molecule ending at the protein–DNA complex. A protein-dependent termination event is characterized by the fact that the intensity of the shorter band varies proportionally with the amount of the

added termination factor, whereas that of the run-off bands decreases (*see* **Fig. 9.2B**). In some cases, however, it can be desirable to map exactly the terminated molecules. This is done by S1 protection analysis of the labeled transcripts in the presence of a complementary riboprobe. S1-resistant products are then separated on a 5% polyacrylamide/7 M urea gel. The procedure to perform this assay is reported in Ref. *(20)*.

Acknowledgments

This work was supported by grants from M.I.U.R.-COFIN PRIN 2005 and "Università di Bari": Progetto di Ricerca di Ateneo 2006, and by a Short-Term Fellowship from Federation of European Biochemical Societies (FEBS) to P.L.P.

References

1. Montoya J, Lopez Perez MJ, Ruiz-Pesini E (2006) Mitochondrial DNA transcription and disease: past present and future. *Biochim. Biophys. Acta* **1757**, 11179–1189.
2. Falkenberg M, Larsson NG, Gustafsson CM (2007) DNA replication and transcription in mammalian mitochondria. *Annu. Rev. Biochem.* **76**, 679–699.
3. Dubrovsky EB, Dubrovskaya VA, Levinger L, Schiffer S, Marchfelder A (2004) Drosophila RNase Z processes mitochondrial and nuclear pre-tRNA 3′ ends in vivo. *Nucleic Acids Res.* **32**, 255–262.
4. Cantatore P, Roberti M, Loguercio Polosa P, Mustich A, Gadaleta MN (1990) Mapping and characterization of Paracentrotus lividus mitochondrial transcripts: multiple and overlapping transcription units. *Curr. Genet.* **17**, 235–245.
5. Berthier F, Reanaud M, Alziari S, Durand R (1986) RNA mapping on *Drosophila* mitochondrial DNA: precursors and template strands. *Nucleic Acids Res.* **14**, 4519–4533
6. Roberti M, Loguercio Polosa P, Bruni F, Musicco C, Gadaleta MN, Cantatore P (2003) DmTTF, a novel mitochondrial transcription termination factor that recognizes two sequences of *Drosophila melanogaster*mitochondrial DNA, *Nucleic Acids Res.* **31**, 1597–1604.
7. Carrodeguas JA, Vallejo CG (1997) Mitochondrial transcription initiation in the crustacean Artemia franciscana. *Eur. J. Biochem.* **250**, 514–523.
8. Park CB, Asin-Cayuela J, Camara Y, Shi Y, Pellegrini M, Gaspari M, Wibom R, Hultenby K, Erdjument-Bromage H, Tempst P, Falkenberg M, Gustafsson CM, Larsson NG (2007) MTERF3 is a negative regulator of mammalian mtDNA transcription. *Cell.* **130**, 273–285.
9. Kruse B, Narashiman N, Attardi G (1989) Termination of transcription in human mitochondria: identification and purification of a DNA binding protein that promotes termination. *Cell.* **58**, 391–397.
10. Martin M, Cho J, Cesare AJ, Griffith JD, Attardi G (2005) Termination factor-mediated DNA loop between termination and initiation sites drives mitochondrial rRNA synthesis. *Cell.* **123**, 1227–1240
11. Loguercio Polosa P, Roberti M, Musicco C, Gadaleta, MN, Quagliariello E, Cantatore P (1999) Cloning and characterisation of mtDBP, a DNA-binding protein which binds two distinct regions of sea urchin mitochondrial DNA. *Nucleic Acids Res.,* **27**, 1890–1899.
12. Loguercio Polosa P, Deceglie S, Roberti M, Gadaleta MN, Cantatore P (2005) Contrahelicase activity of the mitochondrial transcription termination factor mtDBP. *Nucleic Acids Res.* **33**, 3812–3820.
13. Roberti M, Bruni F, Loguercio Polosa P, Gadaleta MN, Cantatore P (2006) The

Drosophila termination factor DmTTF regulates in vivo mitochondrial transcription. *Nucleic Acids Res.* **34**, 2109–2116.

14. Roberti M, Fernandez-Silva P, Loguercio Polosa P, Fernandez Vizarra E, Bruni F, Deceglie S, Montoya J, Gadaleta MN, Cantatore P (2005) In vitro transcription termination activity of the Drosophila mitochondrial DNA-binding protein DmTTF, *Biochem. Biophys. Res. Commun.* **331**, 357–362.
15. Roberti M, Bruni F, Loguercio Polosa P, Manzari C, Gadaleta MN, Cantatore P (2006). The Drosophila termination factor DmTTF regulates in vivo mitochondrial transcription. *Nucleic Acids Res.* **34**, 2109–2116.
16. Loguercio Polosa P, Deceglie S, Falkenberg M, Roberti M, Di Ponzio B, Gadaleta MN, Cantatore P (2007) Cloning of the sea urchin mitochondrial RNA polymerase and reconstitution of the transcription termination system. *Nucleic Acids Res.* **35**, 2413–2427.
17. Falkenberg M, Gaspari M, Rantanen A, Trifunovic A, Larsson NG, Gustafsson CM (2002) Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA. *Nat. Genet.* **31,** 289–294.
18. Sambrook J, Russel DW (2001) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor New York.
19. Dedrick RL, Chamberlin MJ (1985) Studies on transcription of 3'-extended templates by mammalian RNA polymerase II. Parameters that affect the initiation and elongation reactions. *Biochemistry,* **24,** 2245–2253.
20. Micol V, Fernandez-Silva P, Attardi G (1996) Methods Enzymol. Academic Press 264, 158–173.

Chapter 10

Oxidative Phosphorylation: Synthesis of Mitochondrially Encoded Proteins and Assembly of Individual Structural Subunits into Functional Holoenzyme Complexes

Scot C. Leary and Florin Sasarman

Abstract

The bulk of ATP consumed by various cellular processes in higher eukaryotes is normally produced by five multimeric protein complexes (I–V) embedded within the inner mitochondrial membrane, in a process known as oxidative phosphorylation (OXPHOS). Maintenance of energy homeostasis under most physiological conditions is therefore contingent upon the ability of OXPHOS to meet cellular changes in bioenergetic demand, with a chronic failure to do so being a frequent cause of human disease. With the exception of Complex II, the structural subunits of OXPHOS complexes are encoded by both the nuclear and the mitochondrial genomes. The physical separation of the two genomes necessitates that the expression of the 13 mitochondrially encoded polypeptides be co-ordinated with that of relevant nuclear-encoded partners in order to assemble functional holoenzyme complexes. Complex biogenesis is a highly ordered process, and several nuclear-encoded factors that function at distinct stages in the assembly of individual OXPHOS complexes have been identified.

Key words: Mitochondria, oxidative phosphorylation mtDNA, pulse-chase labeling, mitochondrial translation, holoenzyme assembly, blue native PAGE.

1. Introduction

The mitochondrial content of most eukaryotic cells is largely a reflection of their bioenergetic requirements. Transient increases in cellular ATP demand can generally be met by the existing population of mitochondria, because the abundance of the five multimeric protein complexes (I–V) that catalyze oxidative phosphorylation (OXPHOS) is in slight excess of that required to maintain energy homeostasis under normal conditions *(1)*; however, chronic

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_10 Springerprotocols.com

bioenergetic shortfalls resulting from either genetic perturbations or environmental and physiological stimuli trigger an adaptive response, in which the mitochondrial content of the cell is increased in an attempt to restore energy homeostasis *(2, 3)*.

Both the maintenance of existing levels of OXPHOS complexes and the adaptive increases in their abundance that accompany mitochondrial biogenesis require the de novo synthesis of individual structural subunits. Thirteen proteins critical to the biogenesis of Complexes I, III, IV, and V are encoded by mitochondrial DNA (mtDNA), and a large number of nuclear-encoded accessory factors regulate their expression at either the transcriptional or the translational level *(4)*. The stability of newly synthesized mitochondrial proteins is dependent upon their insertion into the inner mitochondrial membrane and subsequent assembly with relevant nuclear partners to form functional holoenzyme complexes, a process that is frequently facilitated by one or more nuclear-encoded assembly factors. The apparent interdependence of the biogenesis of some OXPHOS complexes may be explained by their eventual organization into higher order structures termed supercomplexes *(5)*.

Changes in both the synthesis and the stability of mitochondrially encoded proteins can be readily quantified in cultured cells by labeling with radioactive (^{35}S) methionine in the presence of the appropriate inhibitors of cytoplasmic translation. Differences in the absolute levels of the holoenzymes themselves can be assessed by blue-native polyacrylamide gel electrophoresis (BN-PAGE), followed by conventional immunoblotting with commercially available antibodies. Both of these techniques are straightforward and may provide considerable mechanistic insight into the molecular genetic basis of not only human disease but also physiological adaptation to a range of intrinsic and extrinsic stimuli.

2. Materials

2.1. Pulse-Chase Labeling of the Mitochondrial Translation Products

2.1.1. Labeling of Mitochondrially Synthesized Proteins with Radioactive (^{35}S) Methionine and Cysteine

1. Labeling medium: Dulbecco's Modified Eagle's Medium (DMEM) without methionine and cysteine (Gibco – Invitrogen Corp., Carlsbad, CA), supplemented with 10% dialyzed fetal bovine serum (FBS), 1X glutamax and 110 mg/l sodium pyruvate (*see* **Note 1**). Store at 4°C.
2. Regular DMEM supplemented with 10% FBS. Store at 4°C.
3. Phosphate-buffered saline (PBS) reconstituted from tablets and sterilized by autoclaving. Store at room temperature.
4. Inhibitors of cytoplasmic translation: emetine (Sigma–Aldrich, St. Louis, MO) for pulse-labeling or anisomycin (Sigma–Aldrich)

for chase-labeling. In each case, prepare a 2 mg/ml solution in PBS, and sterilize by passing through a 0.2-μm syringe filter (Sarstedt, Newton, NC). Make fresh as required.

5. Pro-Mix L-(^{35}S) in vitro cell-labeling mix, >1,000 Ci/mmol (EasyTag, PerkinElmer Life and Analytical Sciences, Woodbridge, ON) (*see* **Note 2**). Store at 4°C. Observe handling and storage conditions required for this particular radioactive isotope.
6. Chloramphenicol (CAP, Sigma–Aldrich) for chase-labeling. Prepare a 1 mg/ml solution in regular DMEM without serum (*see* **Note 3**), then sterilize by passing through a 0.2-μm syringe filter. Stable at 4°C for up to 1 week.
7. Cell lifters (Corning, Inc. Life Sciences, Lowell, MA).

2.1.2. Sample Preparation

1. Gel loading buffer (2X): 186 mM Tris–HCl, pH 6.7 – 6.8, 15% glycerol, 2% sodium dodecyl sulfate (SDS), 0.5 mg/ml bromophenol blue, 6% β-mercaptoethanol (β-ME). Store at room temperature. Add β-ME just before use.
2. Micro-BCA^{TM} Protein Assay Kit (Pierce Biotechnology, Rockford, IL).
3. High Intensity Ultrasonic Processor (Sonics & Materials, Inc., Danbury, CT).

2.1.3. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Separating buffer (4X): 1.5 M Tris–HCl (pH 8.8), 8 mM EDTA-Na_2, 0.4% SDS. Store at room temperature.
2. Stacking buffer (4X): 0.5 M Tris–HCl (pH 6.8), 8 mM EDTA-Na_2, 0.4% SDS. Store at room temperature.
3. Thirty percent acrylamide/bisacrylamide solution (37.5:1) (Bioshop Canada, Burlington, ON, Canada). Avoid exposure to unpolymerized solution, as it is a neurotoxin. Store at 4°C.
4. *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED, Bioshop, Canada). Store at 4°C.
5. Ammonium persulfate (APS): prepare a 10% solution in double-distilled water. Make fresh as required.
6. Running buffer (1X): To 3 l double-distilled water (total volume required for one run), add 9.08 g Tris base, 43.25 g glycine, and 3.0 g SDS. Do not pH. Store at room temperature.
7. Molecular weight markers: Page Ruler^{TM} Pre-stained Protein Ladder (Fermentas, Glenburnie, MD).
8. WIZ Peristaltic Pump (Teledyne Isco, Lincoln, NE).

2.1.4. Generation and Analysis of the Data

1. SGD2000 Digital Slab Gel Dryer (Thermo Fisher Scientific, Waltham, MA).

2. Storm 840 Gel and Blot Imaging System (GE Healthcare).

2.2. Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE)

2.2.1. Cell Culture and Sample Preparation

1. PBS and DMEM supplemented with 10% FBS, prepared and stored as described in **Section 2.1.1.**
2. Trypsin solution (0.05% final in PBS) (Gibco – Invitrogen Corp.). Store at 4°C.
3. Bovine serum albumin (BSA) diluted to 1 mg/ml in double-distilled water and Bradford reagent (Bio-Rad, Hercules, CA).
4. Digitonin (EM Biosciences, San Diego, CA) resuspended at 4 mg/ml in PBS (*see* **Note 4**).
5. Blue native (BN) sample buffer: 0.5 ml 3X gel buffer (1.5 M aminocaproic acid [Sigma–Aldrich], 150 mM Bis-tris [Bioshop, Canada], pH 7.0), 0.5 ml 2 M aminocaproic acid, and 4 µl 500 mM EDTA (*see* **Note 5**). Stable for 6–12 months when stored at 4°C.
6. Lauryl maltoside (Roche) as a 10% solution in double-distilled water (*see* **Note 6**).
7. Coomassie Brilliant Blue G-250 (SBG) (Bio-Rad) as a 5% solution in 0.75 mM aminocaproic acid (*see* **Note 7**). Store indefinitely at 4°C.

2.2.2. BN-PAGE

1. Acrylamide/bisacrylamide (AB) mix: 48% acrylamide, 1.5% bisacrylamide [99.5%T, 3%C] (Bioshop, Canada) (*see* **Note 8**). Store for 6–12 months at 4°C.
2. 3X Gel buffer (*see* **Section 2.2.1**).
3. 87% Glycerol (EM Biosciences) stock solution (in water). Store indefinitely at room temperature.
4. Colorless cathode buffer: 15 mM Bis-tris, 50 mM Tricine (EM Biosciences), pH 7.0.
5. Blue cathode buffer: colorless cathode buffer containing 0.02% SBG.
6. Anode buffer: 50 mM Bis-tris, pH 7.0 (*see* **Note 9**).
7. APS and TEMED, prepared and stored as described in **Section 2.1.3**.
8. Protein standards: high molecular weight native marker kit (Pharmacia) (*see* **Note 10**).

2.2.3. Western Blotting and Immunodetection

1. Trans-blot SD semi-dry transfer cell (Bio-Rad).
2. Transfer buffer: To 1 l double-distilled water, add 5.8 g Tris base, 2.93 g glycine, 0.75 g SDS, and 200 ml methanol. Do not pH. Store at room temperature.

3. Nitrocellulose (Pall Corporation, Mississauga, ON, Canada) and Whatman 3 M paper (Schleicher & Schuell, New Jersey, NJ).
4. Tris-buffered saline (TBS) (10X): To 2 l of double-distilled water, add 48.4 g Tris base, 160 g NaCl. Do not pH (*see* **Note 11**). Store indefinitely at room temperature.
5. Barnstead Lab Line Maxi Rotator (VWR scientific, Mississauga, ON, Canada).
6. Blocking solution: 5% BSA dissolved in 1X TBS supplemented with 0.1% Tween-20 (TBS-T).
7. Primary and secondary antibody solution: TBS-T supplemented with 2% BSA.
8. Primary antibodies (Mitosciences, Eugene, OR) for Complexes I (anti-39 kDa), II (anti-SDHA), III (anti-core 1), IV (anti-COX I or anti-COX IV), and V (anti-ATPase α).
9. Secondary antibody: anti-mouse IgG conjugated to horseradish peroxidase (Cedarlane Laboratories, Burlington, ON, Canada). Enhanced chemiluminescent (ECL) reagents (Cell Signaling Technology, Danvers, MA) and Hyclone CL film (Denville Scientific, Inc., Metuchen, NJ).
10. Stripping solution: To 0.5 l of double-distilled water, add 31.5 ml Tris–HCl (pH 7.5), 10 g SDS, and 3.9 ml β-ME (*see* **Note 12**).
11. Sciera shaking water bath (Bellco Biotechnology, Vineland, NJ).

3. Methods

Pulse-labeling of mitochondrial translation products allows for an assessment of both the expression of individual proteins and the global rate of mitochondrial protein synthesis. Chase-labeling further permits for an evaluation of the stability of mitochondrially encoded proteins, and by extension, their assembly into the multimeric holoenzyme complexes of OXPHOS. In both cases, cells are exposed to a mixture of radiolabeled methionine and cysteine in the presence of an inhibitor of cytoplasmic translation, which allows for the specific radiolabeling of mitochondrially encoded proteins. There are, however, three main differences between pulse- and chase-labeling: first, the length of the chase, defined as the incubation time in regular, "cold" medium following removal of the radiolabel; second, the type of inhibition of cytoplasmic translation (i.e., irreversible versus reversible); and third,

exposure to chloramphenicol, a reversible inhibitor of mitochondrial translation that is only used for chase-labeling. With pulse-labeling, the short duration of the chase (i.e., 10 min) allows for the use of an irreversible inhibitor of cytoplasmic translation such as emetine. In contrast, a reversible inhibitor of cytoplasmic translation is required when chase-labeling, because cells are maintained in culture for a period of time that is sufficient (up to 17 h) to quantify the rate of degradation of radiolabeled proteins. For this purpose, we use anisomycin, although several other groups prefer cycloheximide *(6, 7)*. Finally, in chase-labeling, cells are exposed to chloramphenicol prior to incubation with the radioisotope. This results in the accumulation of a pool of nuclear-encoded structural subunits within the mitochondria, an event which facilitates the assembly of nascent OXPHOS complexes subsequent to the radiolabeling of mitochondrially encoded subunits *(8)*.

As outlined in this chapter, the pulse-chase labeling procedure can be applied to all types of adherent cells, independent of either the species of origin or their proliferative state (i.e., dividing versus terminally differentiated) and regardless of whether they are transformed, primary, or immortalized. It is important to recognize, however, that the characteristic pattern of mitochondrial translation is unique to each individual species, even when the identical cell type is being considered. Variation across both the individuals and the tissues within a single species is also possible. This variation can be qualitative, with differences in the electrophoretic mobility of a specific protein *(6, 7)* or quantitative, with differences in the overall abundance of mitochondrial translation products (also *see* **Fig. 10.1**). Such qualitative differences can be due to neutral polymorphisms, while quantitative differences likely reflect different energetic requirements across cell types. It is therefore essential that all of the appropriate controls be included in each experiment.

Pioneered in the early 1990s by Schagger and colleagues *(9, 10)*, BN-PAGE has emerged as the technique of choice to examine the assembly and abundance of OXPHOS complexes within the inner mitochondrial membrane. In its original form, Coomassie dyes were used to impart the charge shift necessary for detergent solubilized proteins to be fractionated by size in a non-ionic gel and buffer system. The technique has since been refined to permit studies of the organization of OXPHOS complexes into higher order structures known as supercomplexes *(11)*; however, it is described here in its simplest form, which results in the release of OXPHOS complexes from the inner mitochondrial membrane in either their monomeric (Complexes I, II, IV, and V) or dimeric forms (Complex III). Detailed instructions are provided for using digitonin to prepare an enriched mitoplast fraction starting from whole cells *(12)*, an approach we favor because much smaller amounts of starting material are required for downstream analyses.

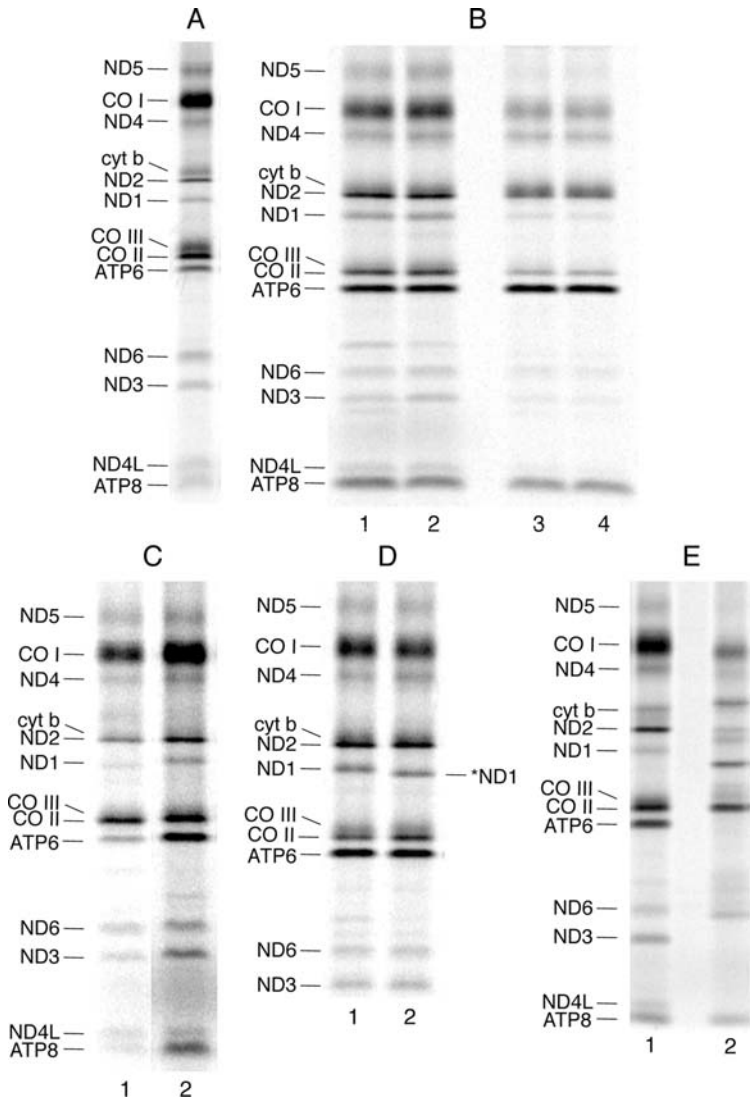


Fig. 10.1. *In vivo analysis of mitochondrial translation by pulse-chase labeling. Panel A*: Typical pattern of pulse-labeled mitochondrial translation products in human cultured cells, shown here for immortalized myoblasts. The 13 mitochondrially synthesized proteins are indicated at the left of the panel: ND, subunits of Complex I; CO, subunits of Complex IV; ATP, subunits of Complex V; and cyt b, subunit of Complex III. *Panel B*: Pulse- (1,2) and chase- (3,4) labeling of two lines of immortalized human fibroblasts. Note that in chase-labeling the two Complex V subunits are preferentially stabilized, a characteristic event resulting from the addition of CAP. *Panel C*: Increased levels of mitochondrial translation products in the transformed cell line HEK293 (2), when compared to a line of immortalized myotubes (1). The two lanes are part of the same gel and have been placed side-by-side to facilitate comparison. *Panel D*: Different migration of the ND1 subunit (most likely due to a neutral polymorphism) in two different lines of immortalized human fibroblasts analyzed by pulse-labeling. *Panel E*: Difference between human and mouse cultured cells in the overall pattern of pulse-labeled mitochondrial translation products, shown here for the human osteosarcoma line 143B (1) and the mouse myeloma line A9 (2). In all panels, each lane contains 50 μg of total cellular protein.

If the starting material is either abundant or prevents the use of such an approach (e.g., autopsy/biopsy material), mitochondria may be isolated by differential centrifugation prior to solubilization in lauryl maltoside. It is also important to note that although we focus on the analysis of cultured human cell lines, BN-PAGE can also be used to study OXPHOS complexes in a range of tissues and cell types derived from both model organisms and non-traditional species. The primary limitation when extending this methodology to investigate non-traditional paradigms, however, is the availability of primary antibodies that will recognize structural subunits of the various OXPHOS complexes. While some commercially available antisera against human proteins do crossreact with their homologues in other species *(13)*, these must be tested empirically.

3.1. Pulse-Chase Labeling of the Mitochondrial Translation Products

3.1.1. Labeling of Mitochondrially Synthesized Proteins with Radioactive (^{35}S) Methionine and Cysteine

1. One 60-mm tissue culture plate is required for each cell line to be labeled. Split dividing cells such that on the day of the experiment, they are between 75 and 90% confluent (*see* **Note 13**).
2. If cells will be chase-labeled, prepare the CAP solution.
3. For chase-labeling only, aspirate growth medium from each plate 22–24 h prior to the start of the labeling procedure, and add 4.8 ml fresh growth medium and 200 µl CAP solution (total volume of 5 ml/plate, final CAP concentration of 40 µg/ml).
4. At least 30 min before the start of the labeling procedure, pipette the total volumes of labeling medium (2 ml/plate) and of DMEM+10% FBS (5 ml/plate) that are required for the entire experiment into two separate tissue culture plates, and place them in the incubator. This step will allow the media to equilibrate to 5% CO_2 and 37°C (*see* **Note 14**).
5. For each plate to be labeled, aspirate the growth medium and wash twice with 3 ml PBS.
6. Add 2 ml equilibrated labeling medium/plate, and incubate for 30 min (*see* **Note 15**). During this time, prepare and sterilize a 2 mg/ml solution of either emetine (pulse-labeling) or anisomycin (chase-labeling).
7. Add 100 µl of the appropriate inhibitor of cytoplasmic translation (final concentration of 100 µg/ml) to each plate and incubate for 5 min.
8. Add 400 µCi of EasyTag labeling mixture to each plate (final concentration of 200 µCi/ml) and incubate for 60 min.
9. Remove labeling mixture from cells and dispose off it according to University guidelines for the handling of radioisotopes. For pulse-labeling, add 5 ml of equilibrated DMEM+10% FBS/plate and return to the incubator for 10 min. For chase-

labeling, wash cells once with either DMEM+10% FBS or with PBS, and chase in DMEM+10% FBS (5 ml/plate) for up to 17.5 h (*see* **Note 16**).

10. Wash cells three times with PBS (*see* **Note 17**).
11. Using the cell lifter, scrape cells in 0.7–0.8 ml ice-cold PBS and then use a pipette to transfer the entire volume to an Eppendorf tube. Repeat with an additional 0.7–0.8 ml ice-cold PBS to collect cells remaining on the plate, and transfer to the same Eppendorf tube (total volume of ~1.5 ml) (*see* **Note 18**).
12. Collect cells by centrifugation at 1,500 × g for 10 min at 4°C.
13. Aspirate PBS and resuspend the pellet in 200 µl ice-cold PBS. From this point onward, keep cells on ice until they are resuspended in gel loading buffer. Samples may now be stored at –80°C for later use or the procedure may be continued (*see* **Note 19**).

3.1.2. Sample Preparation

1. Use the Micro-BCA™ Protein Assay Kit to determine the protein concentration of each sample. Duplicates of 5 and 10 µl are used, and the protein concentration for each of the duplicates must be within 10–15% of each other, otherwise the measurement should be repeated.
2. For each sample, spin down the desired amount of protein (usually 50 µg) by centrifugation at >10,000 × g for 20 min at 4°C (*see* **Note 20**).
3. Resuspend the pellet in 10 µl of 2X gel loading buffer (room temperature) and 10 µl of double-distilled water.
4. Sonicate samples for 3–8 s at an output control of 60.
5. Spin samples at room temperature for 10–15 min at >10,000 × g or until the bubbles resulting from sonication have disappeared.

3.1.3. SDS-PAGE

1. These instructions assume the use of a PROTEAN II xi gel system from Bio-Rad Laboratories. Rinse the glass plates, spacers, combs, and casting stand gaskets several times with deionized, then with double-distilled water, followed by a final rinse in 70–95% ethanol. Air-dry.
2. Prepare 12 ml of a 15% gel mixture by combining 6 ml acrylamide/bisacrylamide solution, 3 ml of 4X separating buffer, 2.9 ml of double-distilled water, 60 µl of 10% APS, and 6 µl of TEMED. Prepare 12 ml of a 20% gel mixture by combining 8 ml acrylamide/bisacrylamide solution, 3 ml of 4X separating buffer, 0.9 ml of double-distilled water, 60 µl of 10% APS, and 6 µl of TEMED.

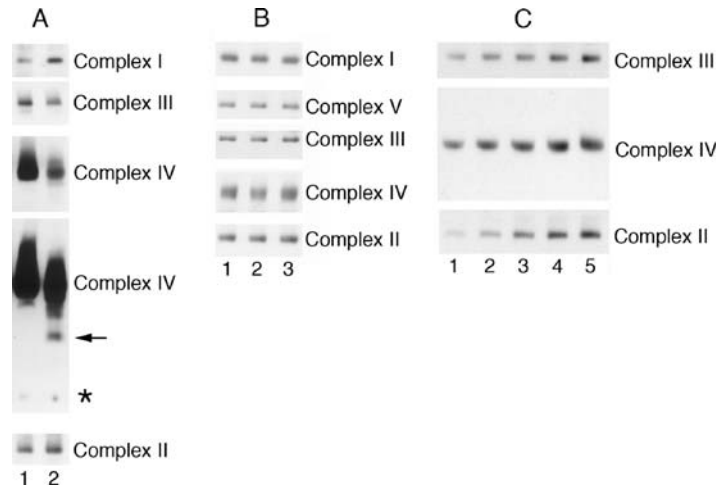


Fig. 10.2. *Representative BN-PAGE gels of samples prepared by differential digitonin permeabilization. Panel A*: Control human fibroblasts either without (1) or with (2) stable expression of a short-hairpin RNA that results in the knockdown of COX11, an assembly factor critical to Complex IV biogenesis. While the assembly of Complexes I, II, and III is unaffected, there is a significant accumulation of both monomeric COXIV (asterisk) and the S2 assembly intermediate (*arrow*). *Panel B*: The abundance of OXPHOS complexes in mouse spinal cord extracts (1–3). *Panel C*: The levels of Complexes II, III, and IV in HEK293 cells exposed to an increasing digitonin to protein ratio (1–5:0.2–1.6 mg digitonin:mg protein). In all panels, a total of 10 μg of protein was loaded per lane.

3. Using the WIZ Peristaltic Pump at its maximum flow rate, pour a 1.0-mm thick, 15–20% gradient gel by using the entire volume (24 ml) of the 15% and 20% gel solutions (*see* **Fig. 10.3**, for detailed instructions). Overlay the gradient gel with double-distilled water.
4. Once the gradient gel has polymerized, pour off the water overlay and dry the area above the gel with Whatman paper. Prepare the stacking gel by mixing 1.04 ml of acrylamide/bisacrylamide solution, 2.5 ml of 4X stacking buffer, 6.5 ml of double-distilled water, 50 μl of 10% APS, and 10 μl TEMED, and pour it on top of the separating gel until it begins to overflow. Insert the comb and allow the stacking gel to polymerize (*see* **Note 21**).
5. Once the stacking gel has set, remove the comb by pulling it straight up slowly and gently. Rinse the wells three times with double-distilled water.
6. Assemble the electrophoresis unit and add the running buffer to the inner and outer chambers of the unit. Load the whole 20 μl of each sample in an individual well (*see* **Note 22**). Reserve at least one well for the pre-stained molecular weight markers (15–20 μl/well).

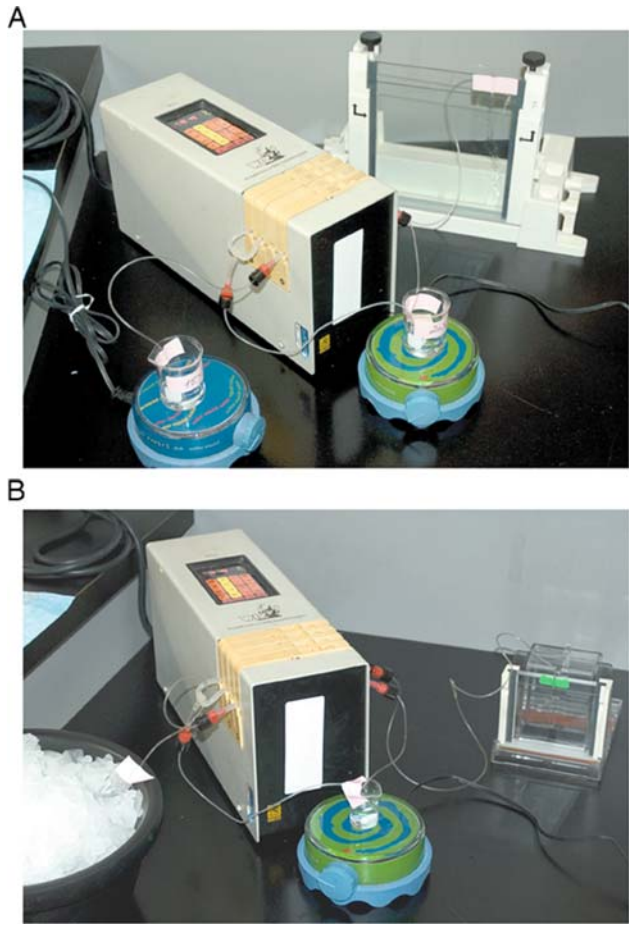


Fig. 10.3. *Organization, assembly, and casting of translation and BN-PAGE gels.* Clean and assemble the components required to cast either gel type (see relevant Methods sections for detailed instructions). Run double-distilled water through the WIZ Peristaltic Pump for roughly 5 min at a flow rate of 99 to ensure that the tubing is clean. Completely empty the tubing of all double-distilled water, and secure the relevant lines of tubing to either the beakers or the gel casting apparatus using tape, as shown in panels A (translation gel setup) and B (BN-PAGE gel setup). While the organization of the tubing does not differ between the two setups, note that the low percentage solution for the BN-PAGE gel is kept on ice and is not stirred while the gel is poured. Once the tubing is secured to both beakers, add the lowest percentage acrylamide solution to the left most beaker. Turn on the pump set to a flow rate of maximum for translation gels and 75 for BN-PAGE gels, and let the low percentage solution flow through the tubing until it almost reaches the beaker that will contain the highest percentage solution. Immediately add the high percentage solution to the right most beaker, and exhaust the entire volume of both solutions prior to stopping the pump. Overlay gently with water and allow at least 1 h for polymerization.

7. Complete the assembly of the electrophoresis unit and connect to a power supply. Run the gel at 10 mA for 16–16.5 h or until the lowermost (usually 11 kDa) molecular weight marker is at 1 cm from the bottom of the glass plates.

3.1.4. Generation and Analysis of the Data

1. At the end of the run, disconnect the electrophoresis unit from the power supply and disassemble it. Separate the glass plates sandwiching the gel by vigorously twisting one of the spacers. Remove and discard the stacking gel, and cut one corner of the separating gel to follow its orientation.
2. Rinse the gel by submerging it in a vessel containing double-distilled water, and transfer it by hand to a piece of thick filter paper cut to the dimensions of the gel. Cover the gel with Saran Wrap.
3. Dry gel under vacuum at 60°C for 1 h by using the SGD2000 Digital Slab Gel Dryer or equivalent.
4. Expose to a phosphorimager cassette for at least 3 days, then scan with the Storm 840 Gel and Blot Imaging System. Analyze the resultant image with the help of the ImageQuant TL Software. For characteristic patterns of mitochondrial translation analyzed by pulse-chase labeling and visualized by this method, *see* **Fig. 10.1**.

3.2. BN-PAGE

3.2.1. Cell Culture and Sample Preparation

1. Harvest a confluent 100-mm plate of cells by washing once in PBS, incubating in 4 ml of trypsin for 10 min, and neutralizing with an equal volume of medium (*see* **Note 23**).
2. Following neutralization, pellet the cells in a 15-ml Falcon tube, resuspend the pellet in 1 ml ice-cold PBS, transfer to an Eppendorf tube, and spin at 14,000 × g for 2 min at 4°C.
3. Resuspend the pellet in an arbitrary volume of ice-cold PBS and quantify the protein concentration using the Bradford assay.
4. Re-pellet the cells and resuspend in ice-cold PBS to a final concentration of 5 mg/ml for human fibroblasts (*see* **Note 24**).
5. Add an equal volume of digitonin at 4 mg/ml, mix the tube twice by inversion, and incubate on ice for 10 min.
6. Dilute with ice-cold PBS to a final volume of 1.5 ml, and spin for 10 min at 10,000 × g at 4°C.
7. Remove supernatant without disturbing the pellet, and wash it gently with 1 ml ice-cold PBS to completely remove residual digitonin.
8. Add BN sample buffer at half the volume that was initially required to resuspend the cell pellet at 5 mg/ml, and add lauryl maltoside at 1/10th of the BN buffer volume (*see* **Note 25**).

9. Resuspend pellet carefully by pipetting up and down 10–20 times, taking care not to foam the detergent (*see* **Note 26**).
10. Following a 20 min extraction on ice, spin at 20,000 × g for 20 min at 4°C.
11. Carefully remove the supernatant, transfer to a new Eppendorf tube, and quantify the protein concentration using the Bradford assay (*see* **Note 27**).
12. Add a volume of SBG that corresponds to half the volume of lauryl maltoside used in Step 8, and store at –20°C (*see* **Note 28**).

3.2.2. BN-PAGE

1. Prepare the glass plates, spacers, combs, and casting stand gaskets for either the Bio-Rad Mini-Protean II or Mini-Protean 3 gel system as described in **Section 3.1.3.**
2. Prepare 10 ml of a 6% gel mixture in a 15 ml-Falcon tube by combining 3.3 ml of 3X gel buffer, 1.2 ml of AB mix, and 5.44 ml of double-distilled water. Prepare the same volume of a 15% gel mixture in another 15-ml Falcon tube by combining 3.3 ml of 3X gel buffer, 3.0 ml of AB mix, 1.68 ml of double-distilled water, and 2 ml of 87% glycerol. Chill both solutions on ice for at least half an hour.
3. Add 60 µl of 10% APS and 4 µl of TEMED to the 6% gel mixture, and 10 µl of 10% APS and 2 µl of TEMED to the 15% gel mixture. Mix both solutions by inversion several times, and place on ice.
4. Set the WIZ Peristaltic Pump to a flow rate of 75, and pour a 1.0-mm thick, 6–15% gradient gel using 2.8 and 2.3 ml of the 6 and 15% gel stock solutions, respectively (*see* **Fig. 10.3**, for detailed instructions). Gently overlay the gradient gel with double-distilled water by gravity flow from 1-ml syringes with 24 gauge needles, and allow 1 h for polymerization (*see* **Note 29**).
5. While the gradient gel is polymerizing, prepare and chill 5 ml of stacking gel solution by combining 1.64 ml of 3X gel buffer, 0.4 ml of AB mix, and 2.87 ml of double-distilled water in a 15-ml Falcon tube.
6. Once the gradient gel has polymerized, pour off the overlay and use a Kimwipe to ensure complete removal of all residual water. Add 60 µl of 10% APS and 6 µl of TEMED to the stacking gel solution, mix by inversion several times, and pour it on top of the separating gel until it begins to overflow. Insert a 15-well comb.
7. Upon polymerization of the stacking gel, gently remove the comb by slowly pulling it straight up. Rinse the wells three times with colorless cathode buffer.

8. Add blue cathode buffer to the wells, and assemble the portion of the electrophoresis unit that will form the cathode (i.e., inner) chamber. Load wells with equal amounts of protein, while reserving at least one well for the molecular weight markers (*see* **Note 30**).
9. Insert the cathode chamber into the gel tank, and fill it with blue cathode buffer. Completely fill the gel tank (i.e., outer chamber) with anode buffer (*see* **Note 31**), fully assemble the electrophoresis unit, and connect to a power supply. Run the gel for 30–45 min at 35 V. Increase to 75 V for another half an hour and to 90–100 V for the remainder of the run.
10. Once the dye front is one third of the way through the gradient gel, stop the run and replace the blue cathode buffer with colorless cathode buffer (*see* **Note 32**). Continue electrophoresis until the dye front reaches the bottom of the gradient gel.

3.2.3. Western Blotting and Immunodetection

1. While the gel is running, cut six pieces of Whatman paper and nitrocellulose membrane to the exact dimensions of the gel(s) to be transferred.
2. Equilibrate the membrane in transfer buffer shortly before the end of the run. At the end of the run, disconnect the electrophoresis unit from the power supply and disassemble it. Separate the glass plates sandwiching the gel by twisting one of the spacers. Remove and discard the stacking gel, and cut the bottom right-hand corner of the gradient gel to mark its orientation.
3. Leaving the gel on the glass plate to which it has adhered, fully immerse it in a vessel containing transfer buffer, and gently rock it back and forth until it physically separates from the plate.
4. Prepare the apparatus for transfer by wetting a piece of Whatman paper and placing it on the cathode plate of the transfer apparatus. Carefully remove all bubbles by rolling a borosilicate tube over the Whatman paper. Dab away excess transfer buffer with Kimwipes as required (*see* **Note 33**).
5. Repeat Step 4 with two more pieces of Whatman paper and finally with the nitrocellulose membrane.
6. Handling the gel by its bottom end, carefully place it on the membrane. Remove any bubbles between it and the membrane by very gently rolling the borosilicate tube over the gel as many times as necessary.
7. Repeat Step 4 three more times.

8. Wet the anode plate with double-distilled water, fully assemble the apparatus, and transfer for 1 h at a constant milliamperage of 0.8 mA/cm^2 of nitrocellulose membrane.
9. At the end of the transfer, disassemble the apparatus, discard the gel(s), and place the nitrocellulose membrane in a vessel containing an ample volume of TBST.
10. Shake the membrane for 5 min, replace TBST with blocking solution, and continue shaking at room temperature for at least 1 h.
11. Replace the blocking solution with that containing the primary antibody solution and rock overnight at 4°C (*see* **Note 34**).
12. The following day, remove the primary antibody and wash the membrane six times for 5 min per wash with shaking at room temperature using ample volumes of TBST.
13. Incubate the membrane for 1 h in the secondary antibody solution, and repeat washes as outlined in Step 12.
14. Combine both ECL reagents with double-distilled water such that they each represent 1/20th of the final volume (10–20 ml total). Remove TBST from the vessel as completely as possible and replace with the ECL solution. Rock for 1 min. Using a pair of forceps, dab the membrane against the side of the vessel to remove excess ECL solution, and place it in a transparent acetate leaflet. Remove all bubbles with the help of a borosilicate tube.
15. Once in the darkroom, take multiple exposures of the membrane and develop the film.
16. Return the membrane to a vessel containing TBST. If stripping is not required, repeat Steps 10–15 with a different primary antibody. Should stripping be necessary, incubate the membrane in stripping buffer for 30 min while gently rocking in a water bath set at 50°C. Promptly remove stripping solution and wash the membrane extensively with TBST (4X 15 min) while shaking at room temperature. Repeat Steps 10–15 (*see* **Note 35**).

4. Notes

1. Certain formulations of DMEM without methionine and cysteine contain sodium pyruvate, while others do not. Check before adding!

2. Pure (^{35}S) methionine results in the strongest signal and the best signal/noise ratio. However, EasyTag mixture of (^{35}S) methionine and cysteine is considerably less expensive and gives a comparable result (approximately 75% of the signal intensity compared with pure (^{35}S) methionine).
3. Add warm medium to the CAP powder and incubate in a water bath at 37°C with occasional vortexing to help dissolve the powder.
4. Commercially available sources of digitonin contain impurities that affect its solubility in aqueous solutions. Full dissolution of digitonin in PBS requires boiling for 5 min. The solution is then cooled on ice and is stable for approx. 4 h. It should not be reused for preparation of samples on subsequent days.
5. The addition of protease inhibitors is not required when preparing native extracts from cultured cells.
6. Lauryl maltoside requires minimal heating or vortexing to go into solution. While it is stable indefinitely at 4°C, its efficacy declines with time. As a result, it should be made fresh the day that samples are prepared to ensure the most consistent results.
7. The use of SBG from other commercial sources results in a considerable increase in the non-specific background during Western blotting and should therefore be avoided.
8. In the event that the AB mix precipitates during storage, heat at 42°C and vortex periodically until it goes back into solution.
9. Cathode and anode buffers can be stored indefinitely at room temperature once they have been prepared.
10. High molecular weight markers are resuspended in 100 µl BN sample buffer, followed by the addition of 10 µl of 5% SBG. Loading 5–10 µl per lane allows for visualization of all five markers by Coomassie staining; however, only a subset of these are visible by Ponceau staining if they are transferred onto a nitrocellulose membrane.
11. We have found that this unbuffered form of TBS helps with consistent immunodetection of OXPHOS complexes. This may be attributable to the exposure by high pH of epitopes that are otherwise either partially or fully masked in this native gel system.
12. The stripping solution is supplemented with β-mercaptoethanol immediately prior to its use.
13. Starting with less than a 75% confluent plate might result in insufficient protein for SDS-PAGE analysis. However, cells must also be less than 100% confluent, as they should still be able to divide during radiolabeling.

14. Label a maximum of six plates at a time, otherwise it will be difficult to respect the required timing for several steps of the procedure.
15. Throughout the labeling procedure it is important that individual plates be placed directly on the shelf of the incubator, rather than stacked on top of each other. This ensures that all the plates have an equal opportunity to equilibrate in terms of temperature and CO_2 concentration.
16. The purpose of the short chase of 10 min in pulse-labeling is to allow the ribosomes to finish translating any radiolabeled proteins; otherwise, any "hot" protein shorter than the full-length species will run at a different size. While the short chases can be universally done in DMEM+10%FBS, longer chases should be done in cell-specific medium (e.g., chase myoblasts in myoblast-specific medium).
17. Be gentle when washing cells loosely attached to the plate, such as large myotubes or certain transformed cell lines.
18. Alternatively, in the case of myotubes, an enriched population of fused cells can be obtained by selective trypsinization: trypsinize cells for about 2 min or until fused cells start lifting (unfused myoblasts will take at least 5 min to trypsinize). Dilute trypsin by adding 5 ml PBS to the plate and transfer trypsinized cells to a 15-ml Falcon tube. Rinse plate with another 5 ml PBS and add to the same 15-ml tube. Collect cells by centrifugation at 1,500 × g for 5 min. Aspirate PBS, then resuspend pellet in ~1.5 ml cold PBS and transfer to an Eppendorf tube.
19. Remember to dispose appropriately of all materials that come in contact with the radioisotope: pipettes, cell plates, Eppendorf tubes, pipette tips, cell lifters, etc.
20. Especially when labeling a transformed cell line for the first time, run 25 and 50 μg of total cellular protein to verify the linearity of the resulting signal; 50 μg of total cellular protein should be within the linear range for most un-transformed cells.
21. This gel system allows the use of 15- and 20-well combs. While the 20-well comb has the obvious advantage of a higher number of samples per run, the 15-well comb will result in better definition of the bands and a higher resolution between lanes, both of which improve the quantification of the signal.
22. To save time, prepare the running buffer while the separating gel polymerizes. Likewise, start preparing the samples after pouring the stacking gel.
23. One confluent 100-mm plate of human fibroblasts or myoblasts will yield sufficient material to prepare and analyze a sample at least twice, assuming that 10–20 μg of total protein are loaded per lane.

24. If a different cell type or the same cell type from another species is being prepared for BN-PAGE, it is important to ensure that the digitonin to protein ratio that is used achieves maximal enrichment for OXPHOS complexes without promoting either their dissociation or degradation. In our experience, a digitonin to protein ratio of 0.8 and 1.2 is appropriate for generating enriched mitoplasts from human fibroblasts and myoblasts, respectively.
25. In our experience, roughly half of the total cellular protein is depleted upon treatment of human fibroblasts (and myoblasts) with digitonin. The purpose of reducing the volume of BN buffer used in the solubilization of the enriched mitoplast pellet reflects a desire to maintain a protein concentration of roughly 2–4 mg/ml at this stage of the isolation. If a different digitonin to protein ratio is used in the preparation of enriched mitoplasts from other cell lines, the volume of BN buffer to be used should also be adjusted accordingly.
26. It is impossible to generate a homogeneous solution when resuspending the pellet. Once the solution has been repeatedly pipetted up and down as described in Step 9, small, tight fragments of "insoluble" material should be visible. Do not use more vigorous means of solubilizing the pellet (e.g., homogenizing) since this may promote the dissociation of OXPHOS complexes.
27. The final protein concentration should be between 1 and 3 mg/ml. Lower protein concentrations may result in the dissociation of OXPHOS complexes, while higher protein concentrations may lead to their anomalous or inconsistent migration due to partial release from either the inner mitochondrial membrane or higher order complexes (i.e., supercomplexes).
28. Freezing at –20°C only preserves the integrity of OXPHOS complexes for 1–2 months. For long-term storage, samples may be kept indefinitely at –80°C.
29. The volumes specified for pouring the gradient and stacking gels are sufficient to cast up to three gels. These may be scaled either up or down based on individual needs; however, no more than four gradient gels should be prepared from the same solutions as there is an increased risk of polymerization within the pump tubing.
30. Loading 10–20 μg of total protein per lane will permit for the immunodetection of all five OXPHOS complexes. Samples are loaded prior to filling the cathode chamber with blue cathode buffer due to the extreme difficulty one otherwise has visualizing the wells.

31. It is very difficult to generate a perfectly sealed cathode chamber. Completely filling the gel tank with anode buffer therefore serves to minimize the potential leaking of cathode buffer into the anode chamber.
32. The cathode buffer is changed from blue to colorless at this stage of electrophoresis to minimize the amount of Coomassie that remains in the gel at the end of the run. This will not affect the electrophoretic mobility of the proteins and greatly enhances the signal to noise ratio upon Western blotting.
33. The presence of excess transfer buffer will affect the quality of protein transfer to the membrane. Transferring more than two gels at a time using this particular system will also adversely affect the quality of transfer.
34. The monoclonal antisera raised against structural subunits of OXPHOS can be repeatedly frozen and thawed, thus extending their lifespan, if their stock solution is diluted with TBST supplemented with BSA as opposed to milk. The only exception is anti-ATPase α, which, even in BSA, retains its immunoreactivity for a maximum of 2–3 freeze/thaw cycles.
35. Stripping is only necessary if the complexes to be detected are of similar sizes (e.g., Complexes III and V), and repeated stripping should be avoided since it will result in an unwanted loss of protein from the membrane. Sequential blotting for Complexes IV, V, and II, followed by stripping of the membrane and subsequent blotting for Complexes I then III is therefore preferable.

References

1. Villani, G. and Attardi, G. (1997) *In vivo* control of respiration by cytochrome *c* oxidase in wild-type and mitochondrial DNA mutation-carrying human cells. *Proc. Natl. Acad. Sci. USA* **94**, 1166–1171.
2. Moyes, C.D., Battersby, B.J. and Leary, S.C. (1998) Regulation of muscle mitochondrial design. *J. Exp. Biol.* **201**, 299–307.
3. Nisoli, E., Clementi, E., Moncada, S. and Carruba, M.O. (2004) Mitochondrial biogenesis as a cellular signaling framework. *Biochem. Pharmacol.* **67**, 1–15.
4. Shoubridge, E.A. and Sasarman, F. Mitochondrial translation and human disease, in Translational control in Biology and Medicine (Mathews, M.B., Sonenberg, N. and Hershey, J.W.B., Ed.), CSHL Press, Cold Spring Harbor, NY, pp. 775–801.
5. Acín-Pérez, R., Bayona-Bafaluy, M.P., Fernández-Silva, P., Moreno-Loshuertos, R., Pérez-Martos, A., Bruno, C., Moraes, C.T. and Enríquez, J.A. (2004) Respiratory complex III is required to maintain complex I in mammalian mitochondria. *Mol. Cell.* **13**, 805–815.
6. Chomyn, A. (1996) *In vivo* labeling and analysis of human mitochondrial translation products. *Methods Enzymol.* **264**, 197–211.
7. Fernández-Silva, P., Acín-Pérez, R., Fernández-Vizarra, A., Pérez-Martos, A. and Enríquez, J.A. (2007) *In vivo* and *in organello* analyses of mitochondrial translation. *Methods Cell Biol.* **80**, 571–588.
8. Costantino, P. and Attardi, G. (1977) Metabolic properties of the products of mitochondrial protein synthesis in HeLa cells. *J. Biol. Chem.* **252**, 1702–1711.
9. Schagger, H. and von Jagow G. (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem.* **199**, 223–231.

10. Schagger, H., Bentlage, H., Ruitenbeek, W., Pfeiffer, K., Rotter, S., Rother, C., Bottcher-Purkl, A. and Lodemann, E. (1996) Electrophoretic separation of multiprotein complexes from blood platelets and cell lines: technique for the analysis of diseases with defects in oxidative phosphorylation. *Electrophoresis* **17**, 709–714.
11. Pfeiffer, K., Gohil, V., Stuart, R.A., Hunte, C., Brandt, U., Greenberg, M.L. and Schagger, H. (2003) Cardiolipin stabilizes respiratory chain supercomplexes. *J. Biol. Chem.* **278**, 52873–52880.
12. Klement, P., Nijtmans, L.G., Van den Bogert, C. and Houstek, J. (1995) Analysis of oxidative phosphorylation complexes in cultured human fibroblasts and amniocytes by blue-native-electrophoresis using mitoplasts isolated with the help of digitonin. *Anal. Biochem.* **231**, 218–224.
13. Leary, S.C., Lyons, C.N., Rosenberger, A.G., Ballantyne, J.S., Stillman, J. and Moyes, C.D. (2003) Fiber-type differences in muscle mitochondrial profiles. *Am. J. Physiol. (Regul. Integr. Comp. Physiol.)* **285**, R817–R826.

Chapter 11

Reactive Oxygen Species Production by Mitochondria

Adrian J. Lambert and Martin D. Brand

Abstract

Oxidative damage to cellular macromolecules is believed to underlie the development of many pathological states and aging. The agents responsible for this damage are generally thought to be reactive oxygen species, such as superoxide, hydrogen peroxide, and hydroxyl radical. The main source of reactive species production within most cells is the mitochondria. Within the mitochondria the primary reactive oxygen species produced is superoxide, most of which is converted to hydrogen peroxide by the action of superoxide dismutase. The production of superoxide by mitochondria has been localized to several enzymes of the electron transport chain, including Complexes I and III and glycerol-3-phosphate dehydrogenase. In this chapter the current consensus view of sites, rates, mechanisms, and topology of superoxide production by mitochondria is described. A brief overview of the methods for measuring reactive oxygen species production in isolated mitochondria and cells is also presented.

Key words: Superoxide, hydrogen peroxide, Complex I, Complex III, succinate, rotenone, antimycin, myxothiazol.

1. Introduction

The term "free radical" is used to denote any atom or molecule with an unpaired electron in its outermost shell. In biology, the term tends to have negative connotations, since free radicals are associated with high, indiscriminate reactivity that can lead to molecular damage. The free radical theory of aging is based on this premise *(1)*. The term reactive oxygen species (ROS) is usually used to signify any oxygen-containing molecule (radical or non-radical) capable of initiating some kind of deleterious reaction. These include superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), peroxyl radical ($RO_2^{\bullet}$), alkoxyl radical ($RO^{\bullet}$), hydroperoxyl radical ($HO_2^{\bullet}$), hypochlorous acid (HOCl),

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_11 Springerprotocols.com

and singlet oxygen ($^1O_2$). Research has tended to focus on the negative aspects of ROS, however, they do participate in essential functions, such as redox signaling and the killing of bacteria by neutrophils and macrophages *(2)*.

Most work on ROS has centered on superoxide, hydrogen peroxide, and the hydroxyl radical, which are formed as follows. The one-electron reduction of oxygen results in the formation of superoxide (reaction 11.1), dismutation of two superoxide molecules yields hydrogen peroxide and oxygen (reaction 11.2), and the oxidation of ferric iron by hydrogen peroxide yields the hydroxyl radical and the hydroxide anion (reaction 11.3):

$$O_2 + e^- \rightarrow O_2^{\bullet -} \quad (11.1)$$

$$2O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2 \quad (11.2)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + OH^- \quad (11.3)$$

The discovery of superoxide dismutase (SOD), a naturally occurring enzyme that catalyzes reaction (11.2), was a key event in demonstrating the importance of ROS in vivo *(3)*. This importance was confirmed more recently by the demonstration that mice null for the mitochondrial form of SOD (MnSOD or SOD2) exhibit severe pathologies and have a markedly curtailed lifespan *(4, 5)*. In addition to SOD, cells contain many other systems dedicated to the control of ROS levels within the cell. These include small metabolites (e.g., ascorbic acid, α-tocopherol) that scavenge ROS and enzymes such as catalase and glutathione peroxidase that convert ROS to less reactive products. Despite the elaborate defense systems, it appears that control over ROS levels in the cell is not perfect. Overproduction or imperfect removal of ROS results in oxidative stress, which is associated with oxidative damage to DNA, proteins, and lipids. Excessive (or limited) ROS may also perturb redox signaling pathways, which may lead to aberrant gene expression and cellular dysfunction. There is now a wealth of evidence indicating that ROS are intimately involved in the development of major diseases, including cancer, diabetes, Parkinson's disease, and Alzheimer's disease *(6)*.

Mitochondria are responsible for the majority of ATP production in the cell. The energy for ATP synthesis comes from the electrochemical proton gradient across the mitochondrial inner membrane. This gradient (called proton motive force) is composed of a pH component (ΔpH) and an electrical component ($\Delta\psi$), and is formed by the enzymes of the electron transport chain as they pump protons from the mitochondrial matrix to the intermembrane space. The energy required for the proton pumping is obtained as the electrons are passed from carriers in Complex I with high potential energy through Complex III to carriers in Complex IV with lower potential energy. Pairs of electrons enter the chain from reduced substrates at Complex I, Complex II, and

other dehydrogenases, and reduce oxygen to water at Complex IV. However, at certain sites within the chain, electrons are passed singly to carriers such as FeS centers whose chemistry demands that they take electrons one at a time or to flavins or quinones that have stable one-electron reduced intermediates. It is these single electrons that may occasionally react directly with oxygen instead of passing on down the respiratory chain to Complex IV, resulting in superoxide formation (reaction 11.1). Understanding the sites, mechanisms, topology, and regulation of superoxide production by mitochondria is of great importance to understanding the damage ROS cause and the removal and repair systems that need to be in place, and the majority of this review is focused on these areas. First, we provide an overview of the methods commonly deployed to detect and measure ROS.

2. Detection of Reactive Oxygen Species in Mitochondria and Cells

2.1. Measurement of Superoxide in Isolated Mitochondria

Most of our understanding of the mechanics of ROS production has been obtained using isolated mitochondria, because of their experimental accessibility. Most superoxide detection systems rely on the reaction of some compound with superoxide that results in a change in the characteristics of that compound, such as a change in absorbance, fluorescence, luminescence, or paramagnetism. Detector compounds include acetylated cytochrome *c*, epinephrine, dihydroethidium, lucigenin, coelenterazine, and DMPO *(7)*. However, in intact mitochondria, direct detection of superoxide that is produced in the matrix using these probes is problematic for various reasons. Firstly, since superoxide does not cross the mitochondrial inner membrane, it cannot diffuse out of the mitochondria, thus the probe must be delivered into the matrix where it may undergo a variety of non-specific reactions. Of particular concern is the ability of some probes to undergo redox cycling which results in the production of superoxide by the probe itself. Secondly, if the probe is charged or becomes charged after reacting with superoxide, then it may distribute across the mitochondrial inner membrane according to the membrane potential, which may change its physico-chemical properties. Direct quantification of the rate of superoxide production is difficult as "standard" superoxide cannot be added to the matrix to generate a standard curve.

There are two alternative methods to measure mitochondrial superoxide production, both of them are indirect. The first is by the inactivation of aconitase, which is a mitochondrial matrix enzyme containing a labile iron in its iron–sulfur cluster that is superoxide sensitive. At any given moment, mitochondrial aconitase activity

depends on its rate of inactivation by superoxide (and possibly other ROS) and its rate of reactivation by reduction and replacement of iron into the cluster. The aconitase assay is based on the following scheme where (11.4) is catalyzed by aconitase, (11.5) is catalyzed by isocitrate dehydrogenase, and the reaction is monitored by following the rate of increase in NADPH absorbance or fluorescence:

$$\text{Citrate} \rightarrow \text{Isocitrate} \tag{11.4}$$

$$\text{Isocitrate} + \text{NADP}^+ \rightarrow \alpha - \text{Ketoglutarate} + \text{NADPH} + \text{H}^+ \tag{11.5}$$

Typically, mitochondria are incubated under the conditions of interest for a specified amount of time. The mitochondria are then lysed and the aconitase activity determined. A relative drop in activity compared to controls is an index of superoxide produced during the incubation period *(8–10)*. One drawback with this method is that quantification of absolute rates of superoxide production is not possible.

The second (and most commonly used) way to assess superoxide production indirectly is by measuring hydrogen peroxide, which readily diffuses out of the mitochondria and is amenable to simple detection protocols. The method relies on the fact that most of the superoxide produced by mitochondria is converted to hydrogen peroxide by endogenous (and if desired, externally added) superoxide dismutase. The method assumes that any removal processes of hydrogen peroxide in the matrix do not change under different experimental conditions. Common assays used to assess mitochondrial hydrogen peroxide production are based on the oxidation of a reduced detector compound coupled to the enzymatic reduction of hydrogen peroxide by horseradish peroxidase (HRP) (11.6 to 11.8)

$$\text{HRP} + \text{H}_2\text{O}_2 \rightarrow \text{HRP} - \text{H}_2\text{O}_2 \tag{11.6}$$

$$\text{HRP} - \text{H}_2\text{O}_2 + \text{AH}_2 \rightarrow \text{HRP} + 2\text{H}_2\text{O} + \text{A} \tag{11.7}$$

or

$$\text{HRP} - \text{H}_2\text{O}_2 + 2\text{AH} \rightarrow \text{HRP} + 2\text{H}_2\text{O} + \text{A}_2 \tag{11.8}$$

where AH_2 is typically amplex red or dihydrochlorofluorescein, and AH is *p*-hydroxyphenylacetic acid or homovanillic acid *(7)*. The increase in A or A_2 is followed fluorometrically. The advantage of this method is that quantification is easily achievable by means of a standard curve obtained by the addition of known amounts of hydrogen peroxide to the system. This allows for direct comparison of rates from different laboratories, a selection of which are presented in **Table 11.1**. However, as with all methods, consideration should be given to appropriate controls. For example,

Table 11.1
Rates of hydrogen peroxide production in mitochondria isolated from rat

Tissue	Substrates	Rate	Reference
Skeletal muscle	Succinate	2.54	*(30, 47)*
	Succinate + rotenone	0.05	
	Pyruvate and malate	0.02	
	Pyruvate and malate + rotenone	0.47	
Heart	Succinate	0.63	*(28)*
	Succinate + rotenone	0.06	
	Glutamate and malate	0.004	
	Glutamate and malate + rotenone	0.22	
Heart	Succinate	2.8	*(37)*
	Succinate + rotenone	0.1	
	Pyruvate and malate	0.05	
	Pyruvate and malate + rotenone	0.25	
Heart	Succinate	1.03	*(49)*
	Succinate + rotenone	0.52	
	Pyruvate and malate	1.83	
	Pyruvate and malate + rotenone	3.62	
Brain	Succinate	1.39	*(32)*
	Succinate + rotenone	0.17	
	Glutamate and malate	0	
	Glutamate and malate + rotenone	0.43	
Brain	Succinate	0.39	*(31)*
	Succinate + rotenone	0	
	Glutamate and malate	0	
	Glutamate and malate + rotenone	0.25	

Rates are in nmol H_2O_2/min/mg mitochondrial protein.

considerable background rates of fluorescence increase were obtained with homovanillic acid as a probe, even in the absence of mitochondria *(11)*.

2.2. Measurement of Reactive Oxygen Species in Intact Cells

To determine the physiological or pathological importance of ROS production by mitochondria, measurements need to be made in intact cells, but such measurements are much less reliable than those on isolated mitochondria. Most methods for the detection of ROS in cells rely on changes in fluorescence of a detector compound. Typically, the cells are incubated with the probe for a given time, then the cells are examined by either confocal microscopy, flow cytometry, or conventional fluorescence. In general the techniques are fraught with potential hazards, such as autooxidation of the probes when illuminated, non-selectivity for particular kinds of ROS, redox cycling, toxicity, and loss of the fluorophore from the

cell. Nonetheless, many studies have employed various probes as indicators of ROS production in cells *(7)*. The common probes in use are briefly described below.

Dihydroethidium (DHE, also called hydroethidine), undergoes a two-electron oxidation to ethidium, the fluorescence of which increases when it binds to DNA. The oxidation appears to be relatively specific for superoxide *(12)*. However, since ethidium is a cation it will tend to accumulate inside the mitochondria according to the magnitude of the membrane potential, $\Delta\psi$. This will alter its availability to bind nuclear and mitochondrial DNA, thus the fluorescent yield obtained does not necessarily reflect the rates of superoxide formation *(13)*. Furthermore, ethidium is not the only fluorescent product of DHE oxidation, as 2-hydroxyethidium is also formed, and there is evidence that DHE can catalyze the dismutation of superoxide *(12, 14)*. A recent study showed that more reliable results are obtainable by HPLC with electrochemical detection, rather than detection by fluorescence *(14)*. The attachment of DHE to the lipophilic cation triphenylphosphonium (TPP^+) via a hexyl carbon chain results in a compound termed MitoSOX red, which is targeted to mitochondria by the TPP^+ moiety. There is evidence to suggest that this probe offers improved selectivity for mitochondrial superoxide produced in the matrix *(15)*.

Dihydroflourescein (H_2F) and related compounds such as dihydrochlorofluorescein (H_2DCF), carboxydihydrochlorofluorescein (carboxy-H_2DCF), and dihydrochlorofluorescein diacetate (H_2DCF-DA) are commonly used indicators of cytoplasmic hydrogen peroxide production *(16)*. These probes are oxidized from the poorly fluorescent dihydro-compounds to the highly fluorescent parent molecules. However, they appear to have poor and undefined specificity, as they can apparently be oxidized by reactive nitrogen species and cellular peroxidases even in the absence of hydrogen peroxide *(7, 17, 18)*. Dihydrorhodamine 123 is another compound that is oxidized to a fluorescent parent compound, rhodamine 123. Like ethidium, this hydrophilic cation will accumulate into the mitochondrial matrix depending on the membrane potential, and like H_2DCF and related compounds, its specificity is poorly defined.

With all currently available probes for detecting cellular ROS, there is uncertainty as to the precise nature of the pathways involved in generating the fluorescent signals. This indicates that extreme care must be taken when interpreting the results obtained with these probes. At best they may be used as qualitative indicators of general oxidative stress, as opposed to the precise quantification of specific ROS. Ideally, experiments designed to compare levels of oxidative stress between one physiological condition and another should employ a variety of probes in conjunction with several positive and negative controls.

3. Superoxide Production by Isolated Mitochondria

3.1. Introduction

The interest in ROS production by mitochondria began over 35 years ago *(19–21)* and there are several reviews on the subject *(22–26)*. Typical experiments involve isolation of mitochondria from tissues or cells, followed by incubation under a defined set of conditions with the chosen detection system. **Table 11.1** shows a small selection of the absolute rates of hydrogen peroxide production obtained by various laboratories. Clearly the rates obtained from specific tissues are somewhat variable. There are many potential sources of this variability, such as the method employed for isolating mitochondria, which can impact on the overall purity and quality of the mitochondrial preparation. Another explanation is the differences in incubation conditions and application of appropriate controls, which vary considerably from laboratory to laboratory. Despite this variability in absolute rates, there are several consistent findings on mitochondrial superoxide production that have emerged, and it is on these findings that we focus in the following sections. We stress the sites of superoxide production and the topology of ROS production from these sites, since ROS produced at the matrix surface of the inner membrane, into the inner membrane itself, or on the outer surface of the membrane are likely to have very different physiological and pathological effects. This topology is likely to be important, given the location of mitochondrial DNA in the matrix and attached to the matrix surface of the inner membrane, and the presence in the inner membrane of polyunsaturated phospholipids that are susceptible to attack by ROS to produce lipid peroxides that can go on to generate further lipooxidative damage and produce other well-known markers of oxidative stress, such as hydroxynonenal, TBARS, and protein carbonyls.

3.2. Complex I

NADH–ubiquinone oxidoreductase (Complex I) is the first enzyme of the electron transport chain. It oxidizes NADH generated by the tricarboxylic acid cycle and reduces ubiquinone to ubiquinol with the concurrent pumping of four protons across the mitochondrial inner membrane. Relatively, the eukaryotic enzyme is huge, comprising some 46 subunits with a combined mass of almost 1 MDa. The enzyme's redox centers comprise a flavin mononucleotide (FMN), numerous iron–sulfur (Fe–S) clusters, and a ubiquinone (Q) binding site, with the accepted sequence of electron transfers being NADH → FMN → Fe–S → Q. Overall, Complex I has an L-shaped structure, with the hydrophobic arm (containing the Q-binding site) embedded in the membrane and the hydrophilic arm (containing the FMN and the iron–sulfur clusters) protruding into the matrix. The mechanism of Complex I (how the electron transfer is coupled to proton pumping) is unknown *(27)*.

The rate of superoxide production by Complex I can vary considerably. One of the main factors influencing the rate is which respiratory substrates are present. The highest rates are generally seen in the presence of succinate *(28–34)*. During succinate oxidation, electrons are transported down the electron transport chain, which results in proton pumping by Complexes III and IV (**Fig. 11.1A**). The resulting proton motive force and reduced Q pool drive electrons thermodynamically uphill into Complex I, reducing NAD^+ to NADH. When rotenone, a specific Complex I Q-site inhibitor, is added under these conditions, the rate of superoxide production is markedly diminished (*see* **Table 11.1** and **Fig. 11.1B**) *(28–34)*. The standard interpretation of this result is that superoxide is generated by Complex I during reverse electron transport from QH_2 to NAD^+. Relatively high rates of superoxide production are also obtained with glycerol-3-phosphate as substrate. In a similar fashion to succinate, these high rates are diminished by rotenone, indicating again that Complex I produces superoxide during reverse electron transport *(35)*.

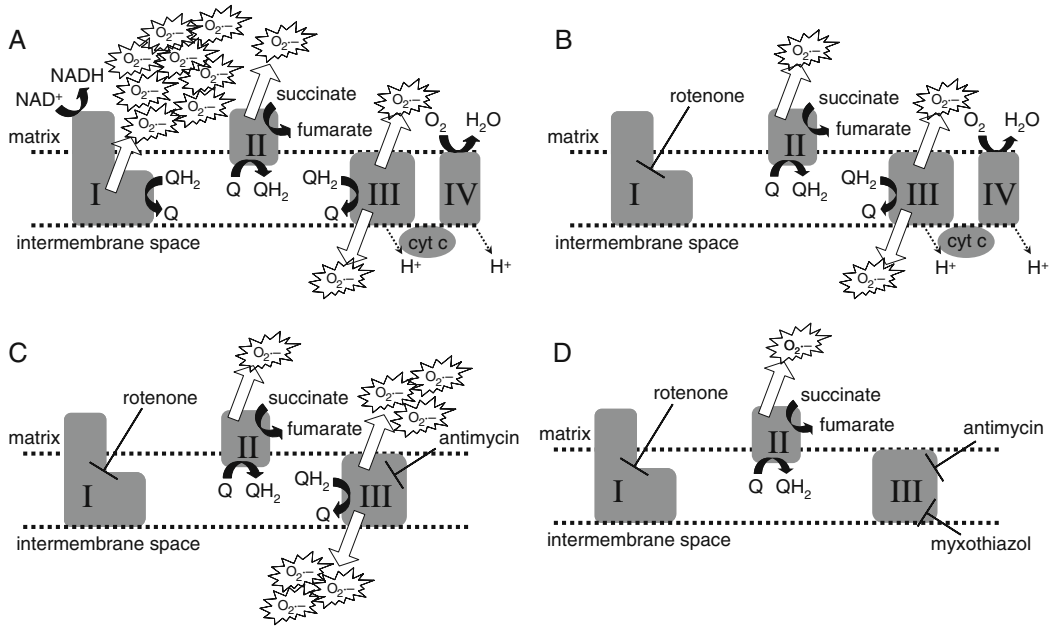


Fig. 11.1 Modes of superoxide production by the mitochondrial electron transport chain with succinate as substrate. Mitochondria oxidizing succinate produce a high proton motive force by proton pumping at Complexes III and IV. The superoxide production rate is high, mostly directed toward the matrix and almost exclusively from Complex I during reverse electron transport (**A**). Addition of the Complex I inhibitor rotenone inhibits this high rate; superoxide production at other sites is relatively low (**B**). In the presence of the Complex III center *i* inhibitor antimycin, there is no proton pumping and proton motive force is zero. Superoxide production rates are high, directed both to the matrix and intermembrane space in equal proportions, and located primarily at center *o* of Complex III (**C**). Addition of the Complex III center *o* inhibitor myxothiazol blocks center *i* and diminishes the high rate seen with antimycin (**D**).

High rates of superoxide production during reverse electron transport are apparent in mitochondria from several different tissues (brain, heart, skeletal muscle, and kidney) *(28–32, 36)*. Most of the work has been performed on rat mitochondria, but the observation also applies to mitochondria from a variety of vertebrate species and *Drosophila (35, 37)*. This strongly suggests that high superoxide production rates from Complex I during reverse electron transport are a universal property of isolated mitochondria, however, it is unknown if this mode of superoxide production occurs in whole cells. Some reports indicate that in intact cells, rotenone decreases ROS formation *(38–41)*, suggesting that the superoxide-producing site during reverse electron transport in Complex I is active in cells. Other studies report an increase in ROS production in cells treated with rotenone *(42–45)*, suggesting that the forward mode of superoxide production is in operation (see below).

Providing mitochondria with substrates that result in forward electron transport, such as pyruvate plus malate to generate NADH, results in relatively low rates of superoxide production (**Table 11.1** and **Fig. 11.2A**) *(28, 29, 31, 32, 34, 36, 46, 47)*. Under these conditions, it is not known where in the mitochondria the superoxide originates. However, in the presence of Complex I inhibitors, this low rate can be increased several fold, suggesting that Complex I is capable of significant superoxide production during forward electron transport (**Table 11.1** and **Fig. 11.2B**). This mode of superoxide production is apparent in mitochondria from various tissues from a variety of species *(28, 29, 31, 32, 34, 36, 37, 46–50)*.

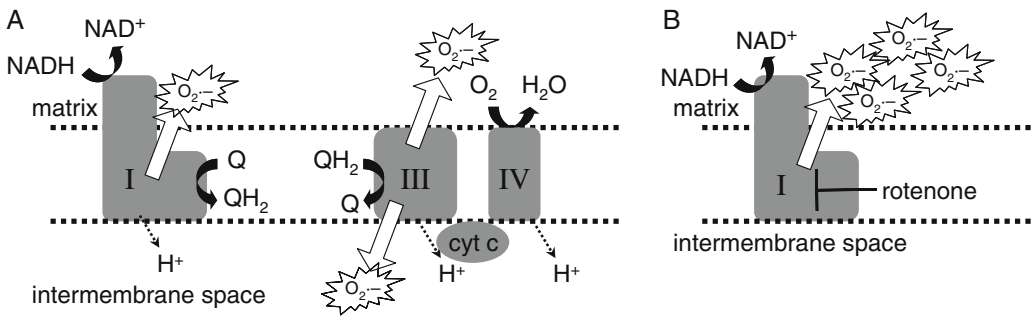


Fig. 11.2. Modes of superoxide production by the mitochondrial electron transport chain with pyruvate and malate/glutamate as substrates. The substrates generate NADH, and a high proton motive force results from proton pumping by Complexes I, III, and IV. Superoxide production rates are relatively low (**A**). Addition of the Complex I inhibitor rotenone to the system results in elevated superoxide production rates in the matrix from Complex I (**B**). Proton pumping is inhibited and proton motive force is zero, however, imposition of a proton motive force under these conditions doubles superoxide production *(47)*.

In addition to which substrates are present, there are several other factors that influence the rates of superoxide production by Complex I in isolated mitochondria. In particular, the magnitude

of the components of proton motive force (ΔpH and $\Delta\psi$) across the mitochondrial inner membrane has a strong influence during both forward and reverse electron transport *(28, 30–32, 34, 47, 51, 52)*. This phenomenon has led to the concept of "uncoupling to survive" that suggests that lowering proton motive force by mild uncoupling will lower superoxide production while still allowing for sufficient ATP production. One of the effects of this will be lowered oxidative damage and increased lifespan *(53, 54)*.

The actual sites of superoxide production within Complex I are unresolved. Any of the electron-carrying centers (FMN, iron–sulfur clusters, Q) is in theory capable of donating an electron to oxygen to yield superoxide. In the isolated enzyme, there is a strong evidence that superoxide is produced at the flavin site, by the reaction of the reduced form of the flavin ($FMNH_2$) with oxygen by a bi-molecular ping–pong mechanism *(55)*. Other authors have also concluded that the FMN site is where superoxide originates in Complex I *(31, 56)*. However, there are arguments that it is the iron–sulfur clusters that produce superoxide, in particular, clusters N1-a and N2 have been proposed *(29, 57)*, as well as iron–sulfur clusters in general *(58)*. The Q site has also been suggested as the main site of superoxide production *(33, 47)*. This lack of agreement may reflect the differences in methodology: as discussed above, the rate (and the site) of superoxide production by Complex I will vary depending on the conditions employed. In addition, some of the inhibitors employed to dissect out the sites of production are poorly characterized and non-specific. The current working model of Complex I superoxide production in this laboratory is that in energized mitochondria (i.e., in the presence of proton motive force), the majority of superoxide originates from the Q site. The other sites (particularly FMN) produce superoxide at modest rates that only become apparent in de-energized mitochondria when the flavin is kept reduced *(47)*.

Regarding topology, there is no detectable superoxide production by Complex I to the intermembrane space, because addition of SOD to mitochondria producing superoxide at Complex I does not increase the rate of hydrogen peroxide formation *(11, 59)*. Therefore, Complex I probably delivers superoxide exclusively to the mitochondrial matrix. From the structure of the complex, this is inevitable when the superoxide is produced at the flavin site. If superoxide is produced at the Q site of Complex I, this site must deliver the superoxide to the matrix and not to the intermembrane space.

3.3. Complex III

Ubiquinol (QH_2) is generated by Complex I and by other oxidoreductases that do not pump protons, in animals chiefly Complex II, glycerol-3-phosphate dehydrogenase, and the electron transferring flavoprotein ubiquinone oxidoreductase (ETF-QOR).

Ubiquinone–cytochrome *c* oxidoreductase (Complex III) transfers electrons from this ubiquinol (which is present in the lipid bilayer of the inner membrane) to cytochrome *c* in the intermembrane space. Two protons are pumped (and two more protons are dumped non-electrogenically into the intermembrane space) for every pair of electrons transferred; the mechanism of the coupling of electron transfer to proton pumping is described by the relatively well-understood Q-cycle mechanism *(60)*.

The native rates of superoxide production by Complex III are relatively low compared to the rates from Complex I during reverse electron transport. For example, during succinate oxidation in the presence of rotenone (where Complex I is functionally removed) the rates of hydrogen peroxide production are low (**Table 11.1** and **Fig. 11.1B**) *(28–34)*. Since it is diminished by myxothiazol, an inhibitor of Complex III, much of this low rate is from Complex III, and little (if any) is from other producers, such as ubiquinone or Complex II *(11, 35)*. The main evidence that Complex III can produce superoxide at high rates comes from the marked increase in the rate of production observed in the presence of antimycin *(11, 31, 34, 36, 61–63)*. This inhibitor is specific for center *i* of Complex III, where it blocks electron transfer from the *b* hemes to the quinone. This results in an increase in the formation of a semiquinone at center *o* in the complex, which can donate an electron to oxygen yielding superoxide at considerable rates (**Fig. 11.1C**) *(64)*. Center *o* inhibitors of Complex III (such as myxothiazol or stigmatellin), prevent electrons from accessing center *o*, and lower the high rates of superoxide production observed in the presence of antimycin (**Fig. 11.1D**) *(11, 28, 34, 61, 62, 65)*. This is taken as evidence that the main site of superoxide production within Complex III is the semiquinone at center *o*. Like Complex I, superoxide production by Complex III apparently exhibits dependence on the membrane potential *(30, 51, 66)*, but this effect and relative importance of ΔpH and $\Delta\psi$ have not been investigated in detail.

In terms of topology, addition of SOD to mitochondria that are producing superoxide at Complex III results in an increase in the rate of hydrogen peroxide production *(10, 11, 59)*. This indicates that Complex III is capable of producing superoxide toward the intermembrane space. Studies in mitoplasts (mitochondria with the outer membrane removed) also indicate that there is superoxide production directed outwards from the mitochondria *(67)*. The current consensus view is that about 50% of the superoxide from center *o* of Complex III is matrix directed, and the other 50% is released into the intermembrane space. Superoxide from center *o*, which is near the intermembrane surface of the inner membrane, is presumably produced in the protonated form as the hydroperoxyl radical into the membrane bilayer, and subsequently deprotonates and escapes as superoxide to both sides of the membrane *(59)*.

3.4. Succinate Dehydrogenase

Succinate dehydrogenase (SDH, succinate–ubiquinone oxidoreductase, Complex II) faces the inner surface of the mitochondrial inner membrane and reduces Q to QH_2while converting succinate to fumarate during the operation of the tricarboxylic acid cycle. Mutations in this enzyme are associated with a variety of diseases in humans, and although the mechanistic details of the pathways are still being elucidated, overproduction of ROS has been implicated *(68)*. In *Caenorhabditis elegans*, defects in the enzyme result in elevated superoxide production rates and abnormal energy metabolism *(69)*. SDH appears to produce only superoxide, as no hydrogen peroxide generation was detected, at least in the *Escherichia coli* enzyme *(70)*. Compared to the high rates seen from Complex I during reverse electron transport, or Complex III in the presence of antimycin, the rates of superoxide production by SDH in mammalian mitochondria are very low. For example, in the presence of succinate, rotenone, and stigmatellin or myxothiazol, where electrons cannot enter either Complex I or Complex III, most of the superoxide presumably originates at SDH. Under these conditions, only very low rates of hydrogen peroxide generation are observed *(28, 34, 61, 62, 65)*. SDH contains an FAD site, three iron–sulfur clusters, a heme group, and a Q-binding site. It appears that it has evolved to produce very little ROS under normal conditions, by suppressing the formation of a flavin radical in the active site; the rates during succinate oxidation by fumarate reductase, which normally catalyzes the same reaction in reverse in *E. coli* and lacks the suppression mechanism, are very much greater *(70)*.

3.5. Glycerol-3-Phosphate Dehydrogenase

The glycerol-3-phosphate (G3P) shuttle provides a mechanism for the transport of electrons from cytosolic NADH to the mitochondrial electron transport chain. Two isoenzymes of glycerol-3-phosphate dehydrogenase (GPDH) participate in this shuttle. The cytosolic form catalyzes the oxidation of NADH by dihydroxyacetone phosphate and the mitochondrial form (whose active site faces the outer side of the mitochondrial inner membrane) is a G3P–ubiquinone oxidoreductase that oxidizes G3P and reduces Q to QH_2, thereby feeding electrons into the electron transport chain. G3P-dependent hydrogen peroxide production has been reported in mitochondria from various sources, including heart, brain, and kidney from mouse; brown adipose tissue from hamsters and rats; and flight muscle from *Drosophila (34–36, 71–73)*. This implies that G3PDH is capable of superoxide production. The rate of superoxide production by G3PDH in isolated *Drosophila* mitochondria was estimated to be about 70% of the total rate, the other 30% coming from Complex I *(35)*. Unlike Complexes I and III, superoxide production by GPDH is not very sensitive to membrane potential *(35)*. Like Complex III, the production is sensitive to externally added SOD, leading to the

conclusion that about 70% of the superoxide production is directed to the intermembrane space and 30% to the mitochondrial matrix *(10)*. The site of superoxide production within the enzyme in unknown, but it is likely to be the flavin.

3.6. Other ROS-Producing Enzymes in Mitochondria

During β-oxidation of fatty acids, the electron transferring flavoprotein ubiquinone oxidoreductase (ETF-QOR) catalyzes the oxidation of electron transferring flavoprotein with the concomitant reduction of Q to QH_2. The enzyme is located in the mitochondrial inner membrane and possesses an FAD group and an iron–sulfur cluster. The ETF-QOR appears to be capable of producing superoxide, as measurable rates of hydrogen peroxide production are obtained in the presence of palmitoyl carnitine. This rate is unaffected by externally added SOD, indicating that production occurs in, or is directed to, the matrix *(11)*.

A number of other sites within mitochondria have emerged as capable of ROS generation. These include dihydrolipoyl dehydrogenase-containing enzymes such as α-ketoglutarate dehydrogenase and pyruvate dehydrogenase *(74, 75)*, and NADH–ubiquinone oxidoreductases other than Complex I *(76)*. The production of ROS by these sites is not as well characterized and understood as production from the enzymes discussed above.

4. Summary

In isolated mitochondria a reasonably clear picture of the substrate and inhibitor dependence of superoxide production has emerged. High rates are seen with succinate, and the superoxide is most likely to originate from Complex I during reverse electron transport. During forward electron transport into Complex I, superoxide production rates are low, but they can be increased by Complex I inhibitors. Relatively low rates are seen from Complex III; these can be increased by antimycin and subsequently lowered by myxothiazol. It is unclear which sites of mitochondrial superoxide production are active in vivo, whether or not they are the same in all tissues under all conditions, and how they are regulated, and more work is required in this area.

Acknowledgments

AJL is supported by a Research into Aging Fellowship, MDB is supported by the Medical Research Council and the Wellcome Trust (066750/B/01/Z).

References

1. Harman, D. (1992) Free radical theory of aging. *Mutat. Res.* **275,** 257–266.
2. Droge, W. (2002) Free radicals in the physiological control of cell function. *Physiol. Rev.* **82,** 47–95.
3. McCord, J. M. and Fridovich, I. (1969) Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **244,** 6049–6055.
4. Lebovitz, R. M., Zhang, H., Vogel, H., Cartwright, J., Jr., Dionne, L., Lu, N., Huang, S. and Matzuk, M. M. (1996) Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. *Proc. Natl. Acad. Sci. USA* **93,** 9782–9787.
5. Li, Y., Huang, T. T., Carlson, E. J., Melov, S., Ursell, P. C., Olson, J. L., Noble, L. J., Yoshimura, M. P., Berger, C., Chan, P. H., Wallace, D. C. and Epstein, C. J. (1995) Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat. Genet.* **11,** 376–381.
6. Halliwell, B. and Gutteridge, J. M. C. (1999) *Free radicals in biology and medicine*, Oxford University Press Inc, NY
7. Tarpey, M. M., Wink, D. A. and Grisham, M. B. (2004) Methods for detection of reactive metabolites of oxygen and nitrogen: *in vitro* and *in vivo* considerations. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **286,** R431–444.
8. Gardner, P. R. (2002) Aconitase: sensitive target and measure of superoxide. *Meth. Enzymol.* **349,** 9–23.
9. Talbot, D. A. and Brand, M. D. (2005) Uncoupling protein 3 protects aconitase against inactivation in isolated skeletal muscle mitochondria. *Biochim. Biophys. Acta* **1709,** 150–156.
10. Miwa, S. and Brand, M. D. (2005) The topology of superoxide production by complex III and glycerol 3-phosphate dehydrogenase in *Drosophila* mitochondria. *Biochim. Biophys. Acta* **1709,** 214–219.
11. St-Pierre, J., Buckingham, J. A., Roebuck, S. J. and Brand, M. D. (2002) Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J. Biol. Chem.* **277,** 44784–44790.
12. Benov, L., Sztejnberg, L. and Fridovich, I. (1998) Critical evaluation of the use of hydroethidine as a measure of superoxide anion radical. *Free Radic. Biol. Med.* **25,** 826–831.
13. Budd, S. L., Castilho, R. F. and Nicholls, D. G. (1997) Mitochondrial membrane potential and hydroethidine-monitored superoxide generation in cultured cerebellar granule cells. *FEBS Lett.* **415,** 21–24.
14. Zhao, H., Joseph, J., Fales, H. M., Sokoloski, E. A., Levine, R. L., Vasquez-Vivar, J. and Kalyanaraman, B. (2005) Detection and characterization of the product of hydroethidine and intracellular superoxide by HPLC and limitations of fluorescence. *Proc. Natl. Acad. Sci. USA* **102,** 5727–5732.
15. Robinson, K. M., Janes, M. S., Pehar, M., Monette, J. S., Ross, M. F., Hagen, T. M., Murphy, M. P. and Beckman, J. S. (2006) Selective fluorescent imaging of superoxide *in vivo* using ethidium-based probes. *Proc. Natl. Acad. Sci. USA* **103,** 15038–15043.
16. Negre-Salvayre, A., Auge, N., Duval, C., Robbesyn, F., Thiers, J. C., Nazzal, D., Benoist, H. and Salvayre, R (2002) Detection of intracellular reactive oxygen species in cultured cells using fluorescent probes. *Meth. Enzymol.* **352,** 62–71.
17. O'Malley, Y. Q., Reszka, K. J. and Britigan, B. E. (2004) Direct oxidation of 2',7'-dichlorodihydrofluorescein by pyocyanin and other redox-active compounds independent of reactive oxygen species production. *Free Radic. Biol. Med.* **36,** 90–100.
18. Rota, C., Chignell, C. F. and Mason, R. P. (1999) Evidence for free radical formation during the oxidation of 2'-7'- dichlorofluorescin to the fluorescent dye 2'-7'-dichlorofluorescein by horseradish peroxidase: possible implications for oxidative stress measurements. *Free Radic. Biol. Med.* **27,** 873–881.
19. Hinkle, P. C., Butow, R. A., Racker, E. and Chance, B. (1967) Partial resolution of the enzymes catalyzing oxidative phosphorylation. XV. Reverse electron transfer in the flavin-cytochrome beta region of the respiratory chain of beef heart submitochondrial particles. *J. Biol. Chem.* **242,** 5169–5173.
20. Jensen, P. K. (1966) Antimycin-insensitive oxidation of succinate and reduced nicotinamide-adenine dinucleotide in electron-transport particles. I. pH dependency and hydrogen peroxide formation. *Biochim. Biophys. Acta* **122,** 157–166.
21. Loschen, G., Flohe, L. and Chance, B. (1971) Respiratory chain linked H_2O_2 production in pigeon heart mitochondria. *FEBS Lett.* **18,** 261–264.

22. Andreyev, A. Y., Kushnareva, Y. E. and Starkov, A. A. (2005) Mitochondrial metabolism of reactive oxygen species. *Biochemistry (Mosc)* **70,** 200–214.
23. Brand, M. D., Affourtit, C., Esteves, T. C., Green, K., Lambert, A. J., Miwa, S., Pakay, J. L. and Parker, N. (2004) Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. *Free Radic. Biol. Med.* **37,** 755–767.
24. Jezek, P. and Hlavata, L. (2005) Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism. *Int. J. Biochem. Cell Biol.* **37,** 2478–2503.
25. Raha, S. and Robinson, B. H. (2000) Mitochondria, oxygen free radicals, disease and ageing. *Trends Biochem. Sci.* **25,** 502–508.
26. Turrens, J. F. (2003) Mitochondrial formation of reactive oxygen species. *J. Physiol.* **552,** 335–344.
27. Brandt, U. (2006) Energy converting NADH:quinone oxidoreductase (complex I). *Annu. Rev. Biochem.* **75,** 69–92.
28. Hansford, R. G., Hogue, B. A. and Mildaziene, V. (1997) Dependence of H_2O_2 formation by rat heart mitochondria on substrate availability and donor age. *J. Bioenerg. Biomembr.* **29,** 89–95.
29. Kushnareva, Y., Murphy, A. N. and Andreyev, A. (2002) Complex I-mediated reactive oxygen species generation: modulation by cytochrome *c* and $NAD(P)^+$ oxidation-reduction state. *Biochem. J.* **368,** 545–553.
30. Lambert, A. J. and Brand, M. D. (2004) Superoxide production by NADH:ubiquinone oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner membrane. *Biochem. J.* **382,** 511–517.
31. Liu, Y., Fiskum, G. and Schubert, D. (2002) Generation of reactive oxygen species by the mitochondrial electron transport chain. *J. Neurochem.* **80,** 780–787.
32. Votyakova, T. V. and Reynolds, I. J. (2001) $\Delta\Psi_m$-Dependent and -independent production of reactive oxygen species by rat brain mitochondria. *J. Neurochem.* **79,** 266–277.
33. Ohnishi, S. T., Ohnishi, T., Muranaka, S., Fujita, H., Kimura, H., Uemura, K., Yoshida, K. and Utsumi, K. (2005) A possible site of superoxide generation in the complex I segment of rat heart mitochondria. *J. Bioenerg. Biomembr.* **37,** 1–15.
34. Gyulkhandanyan, A. V. and Pennefather, P. S. (2004) Shift in the localization of sites of hydrogen peroxide production in brain mitochondria by mitochondrial stress. *J. Neurochem.* **90,** 405–421.
35. Miwa, S., St-Pierre, J., Partridge, L. and Brand, M. D. (2003) Superoxide and hydrogen peroxide production by *Drosophila* mitochondria. *Free Radic. Biol. Med.* **35,** 938–948.
36. Kwong, L. K. and Sohal, R. S. (1998) Substrate and site specificity of hydrogen peroxide generation in mouse mitochondria. *Arch. Biochem. Biophys.* **350,** 118–126.
37. Lambert, A. J., Boysen, H. M., Buckingham, J. A., Yang, T., Podlutsky, A., Austad, S. N., Kunz, T. H., Buffenstein, R. and Brand, M. D. (2007) Low rates of hydrogen peroxide production by isolated heart mitochondria associate with long maximum lifespan in vertebrate homeotherms. *Aging Cell* **6**, 607–618.
38. Li, Y. and Trush, M. A. (1998) Diphenyleneiodonium, an NAD(P)H oxidase inhibitor, also potently inhibits mitochondrial reactive oxygen species production. *Biochem. Biophys. Res. Commun.* **253,** 295–299.
39. Parthasarathi, K., Ichimura, H., Quadri, S., Issekutz, A. and Bhattacharya, J. (2002) Mitochondrial reactive oxygen species regulate spatial profile of proinflammatory responses in lung venular capillaries. *J. Immunol.* **169,** 7078–7086.
40. Schuchmann, S. and Heinemann, U. (2000) Increased mitochondrial superoxide generation in neurons from trisomy 16 mice: a model of Down's syndrome. *Free Radic. Biol. Med.* **28,** 235–250.
41. Vrablic, A. S., Albright, C. D., Craciunescu, C. N., Salganik, R. I. and Zeisel, S. H. (2001) Altered mitochondrial function and overgeneration of reactive oxygen species precede the induction of apoptosis by 1-O-octadecyl-2-methyl-*rac*-glycero-3-phosphocholine in p53-defective hepatocytes. *FASEB J.* **15,** 1739–1744.
42. Barrientos, A. and Moraes, C. T. (1999) Titrating the effects of mitochondrial complex I impairment in the cell physiology. *J. Biol. Chem.* **274,** 16188–16197.
43. Li, N., Ragheb, K., Lawler, G., Sturgis, J., Rajwa, B., Melendez, J. A. and Robinson, J. P. (2003) Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. *J. Biol. Chem.* **278,** 8516–8525.
44. Nakamura, K., Bindokas, V. P., Kowlessur, D., Elas, M., Milstien, S., Marks, J. D., Halpern, H. J. and Kang, U. J. (2001) Tetrahydrobiopterin scavenges superoxide in

dopaminergic neurons. *J. Biol. Chem.* **276,** 34402–34407.

45. Siraki, A. G., Pourahmad, J., Chan, T. S., Khan, S. and O'Brien, P. J. (2002) Endogenous and endobiotic induced reactive oxygen species formation by isolated hepatocytes. *Free Radic. Biol. Med.* **32,** 2–10.
46. Kudin, A. P., Bimpong-Buta, N. Y., Vielhaber, S., Elger, C. E. and Kunz, W. S. (2004) Characterization of superoxide-producing sites in isolated brain mitochondria. *J. Biol. Chem.* **279,** 4127–4135.
47. Lambert, A. J. and Brand, M. D. (2004) Inhibitors of the quinone-binding site allow rapid superoxide production from mitochondrial NADH:ubiquinone oxidoreductase (Complex I). *J. Biol. Chem.* **279,** 39414–39420.
48. Barja, G. and Herrero, A. (1998) Localization at complex I and mechanism of the higher free radical production of brain nonsynaptic mitochondria in the short-lived rat than in the longevous pigeon. *J. Bioenerg. Biomembr.* **30,** 235–243.
49. Herrero, A. and Barja, G. (1997) Sites and mechanisms responsible for the low rate of free radical production of heart mitochondria in the long-lived pigeon. *Mech. Ageing Dev.* **98,** 95–111.
50. Herrero, A. and Barja, G. (1998) H_2O_2 production of heart mitochondria and aging rate are slower in canaries and parakeets than in mice: sites of free radical generation and mechanisms involved. *Mech. Ageing Dev.* **103,** 133–146.
51. Korshunov, S. S., Skulachev, V. P. and Starkov, A. A. (1997) High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS Lett.* **416,** 15–18.
52. Liu, S. S. (1997) Generating, partitioning, targeting and functioning of superoxide in mitochondria. *Biosci. Rep.* **17,** 259–272.
53. Brand, M. D. (2000) Uncoupling to survive? The role of mitochondrial inefficiency in ageing. *Exp. Gerontol.* **35,** 811–820.
54. Speakman, J. R., Talbot, D. A., Selman, C., Snart, S., McLaren, J. S., Redman, P., Krol, E., Jackson, D. M., Johnson, M. S. and Brand, M. D. (2004) Uncoupled and surviving: individual mice with high metabolism have greater mitochondrial uncoupling and live longer. *Aging Cell* **3,** 87–95.
55. Kussmaul, L. and Hirst, J. (2006) The mechanism of superoxide production by NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria. *Proc. Natl. Acad. Sci. USA* **103,** 7607–7612.
56. Johnson, J. E., Jr., Choksi, K. and Widger, W. R. (2003) NADH-Ubiquinone oxidoreductase: substrate-dependent oxygen turnover to superoxide anion as a function of flavin mononucleotide. *Mitochondrion* **3,** 97–110.
57. Genova, M. L., Ventura, B., Giuliano, G., Bovina, C., Formiggini, G., Parenti Castelli, G. and Lenaz, G. (2001) The site of production of superoxide radical in mitochondrial complex I is not a bound ubisemiquinone but presumably iron-sulfur cluster N2. *FEBS Lett.* **505,** 364–368.
58. Herrero, A. and Barja, G. (2000) Localization of the site of oxygen radical generation inside the complex I of heart and nonsynaptic brain mammalian mitochondria. *J. Bioenerg. Biomembr.* **32,** 609–615.
59. Muller, F. L., Liu, Y. and Van Remmen, H. (2004) Complex III releases superoxide to both sides of the inner mitochondrial membrane. *J. Biol. Chem.* **279,** 49064–49073.
60. Trumpower, B. L. (1990) The protonmotive Q cycle. Energy transduction by coupling of proton translocation to electron transfer by the cytochrome bc_1 complex. *J. Biol. Chem.* **265,** 11409–11412.
61. Chen, Q., Vazquez, E. J., Moghaddas, S., Hoppel, C. L. and Lesnefsky, E. J. (2003) Production of reactive oxygen species by mitochondria: central role of complex III. *J. Biol. Chem.* **278,** 36027–36031.
62. McLennan, H. R. and Degli Esposti, M. (2000) The contribution of mitochondrial respiratory complexes to the production of reactive oxygen species. *J. Bioenerg. Biomembr.* **32,** 153–162.
63. Raha, S., McEachern, G. E., Myint, A. T. and Robinson, B. H. (2000) Superoxides from mitochondrial complex III: the role of manganese superoxide dismutase. *Free Radic. Biol. Med.* **29,** 170–180.
64. Cape, J. L., Bowman, M. K. and Kramer, D. M. (2007) A semiquinone intermediate generated at the Qo site of the cytochrome bc1 complex: Importance for the Q-cycle and superoxide production. *Proc. Natl. Acad. Sci. USA* **104,** 7887–7892.
65. Turrens, J. F., Alexandre, A. and Lehninger, A. L. (1985) Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Arch. Biochem. Biophys.* **237,** 408–414.
66. Talbot, D. A., Lambert, A. J. and Brand, M. D. (2004) Production of endogenous

matrix superoxide from mitochondrial complex I leads to activation of uncoupling protein 3. *FEBS Lett.* **556,** 111–115.

67. Han, D., Williams, E. and Cadenas, E. (2001) Mitochondrial respiratory chain-dependent generation of superoxide anion and its release into the intermembrane space. *Biochem. J.* **353,** 411–416.
68. Rustin, P., Munnich, A. and Rotig, A. (2002) Succinate dehydrogenase and human diseases: new insights into a well-known enzyme. *Eur. J. Hum. Genet.* **10,** 289–291.
69. Senoo-Matsuda, N., Yasuda, K., Tsuda, M., Ohkubo, T., Yoshimura, S., Nakazawa, H., Hartman, P. S. and Ishii, N. (2001) A defect in the cytochrome b large subunit in complex II causes both superoxide anion overproduction and abnormal energy metabolism in *Caenorhabditis elegans. J. Biol. Chem.* **276,** 41553–41558.
70. Messner, K. R. and Imlay, J. A. (2002) Mechanism of superoxide and hydrogen peroxide formation by fumarate reductase, succinate dehydrogenase, and aspartate oxidase. *J. Biol. Chem.* **277,** 42563–42571.
71. Tretter, L., Takacs, K., Hegedus, V. and Adam-Vizi, V. (2007) Characteristics of alpha-glycerophosphate-evoked H_2O_2 generation in brain mitochondria. *J. Neurochem.* **100,** 650–663.
72. Drahota, Z., Chowdhury, S. K., Floryk, D., Mracek, T., Wilhelm, J., Rauchova, H., Lenaz, G. and Houstek, J. (2002) Glycerophosphate-dependent hydrogen peroxide production by brown adipose tissue mitochondria and its activation by ferricyanide. *J. Bioenerg. Biomembr.* **34,** 105–113.
73. Sekhar, B. S., Kurup, C. K. and Ramasarma, T. (1987) Generation of hydrogen peroxide by brown adipose tissue mitochondria. *J. Bioenerg. Biomembr.* **19,** 397–407.
74. Gazaryan, I. G., Krasnikov, B. F., Ashby, G. A., Thorneley, R. N., Kristal, B. S. and Brown, A. M. (2002) Zinc is a potent inhibitor of thiol oxidoreductase activity and stimulates reactive oxygen species production by lipoamide dehydrogenase. *J. Biol. Chem.* **277,** 10064–10072.
75. Starkov, A. A., Fiskum, G., Chinopoulos, C., Lorenzo, B. J., Browne, S. E., Patel, M. S. and Beal, M. F. (2004) Mitochondrial alpha-ketoglutarate dehydrogenase complex generates reactive oxygen species. *J. Neurosci.* **24,** 7779–7788.
76. Fang, J. and Beattie, D.S. (2003) External alternative NADH dehydrogenase of *Saccharomyces cerevisiae*: a potential source of superoxide. *Free Radic. Biol. Med.* **34,** 478–488.

Chapter 12

Measuring mtDNA Damage Using a Supercoiling-Sensitive qPCR Approach

Sam W. Chan and Junjian Z. Chen

Abstract

Compromised mitochondrial DNA structural integrity can have functional consequences for mitochondrial gene expression and replication leading to metabolic and degenerative diseases, aging, and cancer. Gel electrophoresis coupled with Southern blot and probe hybridization and long PCR are established methods for detecting mtDNA damage. But each has its respective shortcomings: gel electrophoresis is at best semi-quantitative and long PCR does not offer information on the structure. To overcome these limitations, we developed a new method with real-time PCR to accurately quantify the mtDNA structural damage/repair and copy number change. We previously showed that the different mtDNA structures (supercoiled, relaxed circular, and linear) have profound influences on the outcome of the real-time PCR amplification. The supercoiled structure is inhibitory to the PCR amplification, while relaxed structures are readily amplified. We will illustrate the use of this new method by quantifying the kinetics of mtDNA damage and repair in LNCaP prostate cancer cells induced by exogenous H_2O_2 treatments. The use of this new method on clinical samples for spontaneous mtDNA damage level will also be highlighted.

Key words: mtDNA supercoiling, oxidative damage, DNA repair, copy number, real-time PCR.

1. Introduction

Recent studies have shown that alterations in mitochondrial DNA (mtDNA) can induce functional changes that play important roles in metabolic and degenerative diseases, aging, and cancer *(1)*. The mitochondrion is responsible for energy production during cellular respiration, and the electron transport chain (ETC) lies at the center of this metabolic function. Situated in the inner membrane, the ETC of the mitochondria supplies energy to the cell through oxidative phosphorylation of ADP to ATP. However, the ETC is

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_12 Springerprotocols.com

also a major source of reactive oxygen species (ROS) *(2, 3)*. Due to its close proximity and lack of protecting histones, the mtDNA is susceptible to oxidative damage, which can potentially lead to changes in mitochondrial gene expression and somatic mutations in many human cancers *(4–8)*. In the cells, mtDNA is composed of a mixture of supercoiled, relaxed circular, and linear DNA. The mature mtDNA has a supercoiled structure with an average of 100 negative super-helical turns *(9)*. This supercoiled conformation is susceptible to strand breaks induced by oxidative damage, a single-strand break can lead to the disruption and relaxation of the supercoiled structure. Maintaining the integrity of the mtDNA structure is crucial to normal mitochondria function, since the supercoiled conformation is required for the initiation of mtDNA replication and transcription *(10–12)*.

Several techniques have been developed to study the mtDNA damage. Gel electrophoresis is frequently used to detect mtDNA structural damage and conformational changes, but this assay is not quantitative and it involves a very tedious process requiring the coupling of Southern blot and probe hybridization. On the other hand, long PCR allows the quantification of mtDNA damage, however, it does not offer information on the mtDNA structure. To overcome these shortcomings, a new approach was developed with real-time PCR to quantify structural damage and repair of mtDNA and copy number change. This method is based on several key findings *(13)*: (a) the PCR amplification efficiencies of supercoiled and relaxed DNA are different, the supercoiled structure of the DNA inhibits PCR amplification while relaxed DNA are amplified uninhibited (*see* **Note 1**); (b) a heat treatment prior to real-time PCR can be used to artificially introduce strand breaks and relax the mtDNA, allowing quantification of the total amount of mtDNA copies. Thus, this new method is useful for quantifying not only the structural damage and copy number change of mtDNA in stressed cells in culture, but also the level of spontaneous damage of mtDNA from clinical samples. In this chapter, this new approach will be illustrated by studying the mtDNA damage responses to acute oxidative stress in treated LNCaP prostate cancer cells.

2. Materials

2.1. Cell Collection for LNCaP (see Note 2)

1. PBS-CMF, phosphate-buffered saline without $CaCl_2$, $MgCl_2$ (GIBCO, Invitrogen, cat. no. 20012-027).
2. PBS-CMF/0.5 mM EDTA, to obtain 0.5 mM EDTA, add 500 µl of 0.5 M EDTA to 500 ml of PBS-CMF; 0.5 M EDTA, pH 8.0 (GIBCO, Invitrogen, cat. no. 15575-020).

3. Trypsin/EDTA solution: 0.05% trypsin + 0.02% EDTA.
4. RPMI media 1640, 1 × 0.1 μm filtered, with L-glutamine.
5. Fetal bovine serum (FBS).
6. Penicillin–streptomycin.
7. Complete RPMI medium: make by removing 50 ml RPMI from 500 ml bottle, adding 50 ml FBS (to get 10% FBS) and adding 5 ml penicillin–streptomycin.
8. Poly-L-lysine (PLL) coated-dish for LNCaP cells: 5 ml PLL + 495 ml distilled water, DNAse and RNAse free (*see* **Note 3**).

2.2. DNA Extraction with Genomic-Tip

2.2.1. Cell Culture

1. QIAGEN Blood & Cell Culture DNA Kit Mini. The kit contains the following: C1, G2, QBT, QC, QF buffers, protease, 25 genomic tips.
2. RNase A 2.5 ml (100 mg/ml).
3. Distilled water, DNAse and RNAse free.
4. 1X Tris/EDTA buffer solution (TE buffer): 10 mM Tris–KOH, pH 8.0 ± 0.1, and 1 mM EDTA.
5. Isopropanol (2-propanol) for molecular biology, minimum 99%.
6. 70% Ethanol: Mix 35 ml of 100% ethanol into 15 ml of RNase and DNase free water.

2.2.2. Snap-Frozen Tissue Samples

1. Refer to material in **Section 2.2.1** for cell culture with the following additional material.
2. Proteinase K.
3. Glass tissue grinder, ground glass Potter-Elvehjem type (Kontes).
4. Sterile surgical scalpel blades and handle.

2.3. DNA Quantification

2.3.1. Fluorometric Quantification of dsDNA Using PicoGreen ®

1. 96-Well plate, black opaque, fluorometry compatible (Perkin–Elmer, OptiPlate-96F).
2. Quant-iT™ PicoGreen ® dsDNA Assay Kit (Invitrogen, cat. no. P7589). The kit contains PicoGreen dye, 20X TE buffer, and lambda DNA standard (100 μg/ml).
3. Multi-well plate reader with fluorometric capabilities at excitation/emission wavelengths of 480 nm/520 nm, respectively (Perkin–Elmer 1420. Multilabel Counter Victor^{3}V).

2.3.2. Quantification by Nanodrop

1. Nanodrop spectrophotometer instrument.
2. Distilled water, DNAse and RNAse free.
3. 1X Tris/EDTA buffer solution (TE buffer): pH 8.0 ± 0.1, 10 mM Tris, and 1 mM EDTA.

2.4. Template DNA Preparation and Heat Treatment

1. PCR platform: GeneAmp PCR system 9700 (Applied Biosystems).
2. 1X Tris/EDTA buffer solution, pH 8.0 ± 0.1, 10 mM Tris, and 1 mM EDTA.

2.5. mtDNA Structural Damage and Repair Analysis Using Real-Time PCR Bio-Rad System

1. 2X IQ™ SYBR® Green Supermix 500 × 50 µl reactions (Bio-Rad, cat. no. 170-8882).
2. Primers at ~10 µM to achieve final 300 nM concentration for each reaction (primer sequences are listed in **Table 12.1**).
3. Distilled water, DNAse and RNAse free (GIBCO, Invitrogen, cat. no. 10977-015).

Table 12.1
Primer sequences

Gene	Forward primer 5′–3′	Reverse primer 5′–3′
CO2 (mtDNA)	CCCCACATTAGGCTTAAAAACAGAT	TATACCCCCGGTCGTGTAGCGGT
D-loop (mtDNA)	TATCTTTTGGCGGTATGCACTT TTAACAGT	TGATGAGATTAGTAGTATGGGA GTGG
β-Actin	TCACCCACACTGTGCCCATCTACGA	CAGCGGAACCGCTCATCAGCG GAACCGCTCAT
β-Globin	GTGCACCTGACTCCTGAGGAGA	CCTTGATACCAACCTGCCCAG

4. $MgCl_2$ solution, 50 mM (Bio-Rad, cat. no. 170-8872).
5. PCR plates, 96-well, DNAse and RNase free (Bio-Rad, cat. no. 2239441).
6. Optical tape for plates (Bio-Rad, cat. no. 2239444).
7. Real-time PCR detection system: MyIQ™ Single Color Real-Time PCR Detection System with iCycler™ thermocycler base (Bio-Rad).

2.6. Applied Biosystems (ABI) System

1. Power SYBR® Fast Green PCR MASTER MIX.
2. Primers at ~10 µM to achieve final 300 nM concentration for reaction (primer sequences are listed in **Table 12.1**).
3. Distilled water, DNAse and RNAse free.
4. PCR plates, 96-well, MicroAmp™ Fast Optical 96-well Reaction Plate with Barcode (0.1 ml).
5. Optical tape for plates (ABI).
6. Real-time PCR detection system: 7500 Fast Real-Time PCR System (ABI).

3. Methods

The supercoiled structure of mtDNA is very sensitive to artifacts and can be easily disrupted when not carefully handled. So it is important to always handle the cells and DNA samples with care. The following protocols are designed to minimize potential artificial DNA damage. This section will go through the logical workflow of the experiment from (**Section 3.1**) cell collection ➔ (**Section 3.2**) DNA extraction of (**Section 3.2.1**) cells or (**Section 3.2.2**) frozen tissues ➔ (**Section 3.3**) DNA quantification ➔ (**Section 3.4**) template DNA preparation and dilution ➔ (**Section 3.5**) real-time PCR analysis.

LNCaP cells are used here as an example for the cell collection protocol. During cell collection, it is important to keep in mind to not introduce artificial damage to the cells by mishandling them. A QIAGEN Blood & Cell Culture DNA Kit will be used for DNA extraction. This kit is recommended since it is an ion-exchange system, which does not oxidize purines during isolation, unlike phenol-based methods *(14)* (*see* **Note 4**). The QIAGEN recommended protocol has been modified (*see* **Note 5**) in order to recover the mtDNA as well as the nuclear DNA. The protocol will also cover working with frozen tissue samples (*see* **Note 6**).

Following the DNA extraction, the concentrations of the DNA samples need to be quantified in order to prepare a 10 ng/μl DNA working solution and the 1 ng/μl and 5 ng/μl DNA templates. It is recommended (*see* **Note 7**) to perform a two-step quantification process where the first round will determine the stock concentrations; the second round will determine and confirm the concentrations of the 10 ng/μl dilutions prepared from the first round. There are two recommended methods that can be used: (**Section 3.3.1**) fluorometric quantification of dsDNA using PicoGreen® and (**Section 3.3.2**) quantification by Nanodrop ® spectrophotometer (*see* **Note 8**).

Prior to the real-time PCR analysis, half of the 1 ng/μl DNA templates will be heat-treated. The original and heated 1 ng/μl DNA templates will be used for detecting the mitochondrial markers (CO2 and/or D-loop). The 5 ng/μl templates will be used for nuclear gene markers (β-actin and β-globin). The method used for real-time PCR analysis is based on the relative quantification model suggested by Pfaffl *(15)*.

3.1. Cell Collection (LNCaP)

Protocol for LNCaP cells (*see* **Note 1**)

1. Remove RPMI medium from the dish by suction.
2. Add gently by using the side of the dish, 5 ml PBS-CMF/EDTA, incubate for 1 min at room temperature

3. Remove the PBS-CMF/EDTA and add 2.5 ml of trypsin, and incubate for 5 min at 37°C.
4. Resuspend cells by adding 6 ml of RPMI medium to inactivate trypsin; gently aspirate up and down with pipette to get rid of clumps.
5. Transfer cell suspension to a 15-ml tube, and put on ice.
6. Take a small aliquot of cell suspension aside for cell counting (should be between 2 and 5 million cells).
7. Centrifuge at ~180 × g for 6 min.
8. Remove medium and resuspend cells in 1 ml PBS-CMF (if the cell counting is more than 5 million cells, use 2 ml of PBS then in Step 9 split into two 1.5-ml tubes).
9. Transfer to 1.5-ml tube, centrifuge at a high speed for 2 min.
10. Remove supernatant but not completely, leave just enough to cover the cell pellet. Put on ice.
11. Store at –80°C.

3.2. DNA Extraction with Genomic-Tip for Cell Culture and Snap-Frozen Tissue

This is a modified protocol based on the QIAGEN protocol. Do not use the C1 buffer (*see* **Note 5**). Pre-warm the QF buffer to 50°C in a water bath. Cool the 70% ethanol to –20°C.

3.2.1. For Cell Cultures

Prepare protease solution by adding 1.4 ml of distilled water into powdered protease. The recommended amount of cells is 2–5 million.

1. Thaw cells on ice; resuspend cells by flicking the tube with fingers.
2. Add 1 ml G2 digest buffer to tube. Before adding G2, re-flick until cells are resuspended. After adding G2, immediately vortex at a maximum speed for 25 s precisely (be consistent for all samples). Do one sample at a time and let the sample rest at room temperature while moving onto the other samples. Centrifuge briefly afterward.
3. Add 3 μl RNase A to each sample. Invert to mix and centrifuge for several seconds.
4. Add 25 μl of reconstituted protease. Invert to mix, but do not centrifuge afterward. Incubate at 50°C for 1 h in water bath. Continue to Step 5.

3.2.2. For Snap-Frozen Tissue Samples

Work with one sample at a time, it is important to carry out the following steps as fast as possible since the tissue sample can degrade rapidly after thawing. Do not thaw the samples until needed for cutting or grinding (*see* **Note 9**). Homogenize gently with hands, any mechanized assistance will disrupt the mtDNA structure. It is recommended to carry out Steps 1–3 on ice.

1. Prepare a mix of 1.8 ml G2 buffer + 4 µl RNAse A per sample, mix well.
2. Weigh tissue sample (around 10–50 mg) and cut to smaller pieces if needed (*see* **Note 10**).
3. Add the tissue pieces and ~300 µl of the G2 buffer/RNAse A mix into the glass tissue grinder to homogenize. Grind by hand and take care of not over-grinding as this will disrupt the mtDNA structure. Collect homogenate into a 2-ml tube. Wash grinder with remaining G2 buffer/RNAse A mix and transfer to 2-ml tube (*see* **Note 11**).
4. Add 100 µl proteinase K (*see* **Note 12**). Invert to mix, but do not spin. Incubate at 50°C for 2 h. Do the next sample by repeating Steps 1–4. Then continue to Step 5.

DNA Extraction with Genomic-Tips

5. About 15 min before the incubation at Step 4, equilibrate each genomic-tip with 2 ml of QBT buffer and let the buffer drip down.
6. Take the samples out of water bath, and vortex digested samples for 10 s at a maximum speed. Then transfer each sample to a genomic-tip.
7. After the samples have drained through the tip, the DNA will be trapped in the resin. Wash the tips with 3 × 1 ml of QC buffer.
8. Elute the DNA by adding 910 µl of QF buffer twice (pre-warmed to 50°C) into a 15-ml tube.
9. Mix the eluted DNA by inverting the tubes and centrifuge at ~188 × g. Aliquot solution into two 2-ml microcentrifuge tubes equally (~850 µl).
10. Precipitate the DNA by adding 700–800 µl of isopropanol to each tube bringing the volume up to ~1.65 ml. Mix by inverting and incubate for 10 min at room temperature (*see* **Note 13**). Centrifuge at 15,000–18,000 × g for 20 min at 4°C (*see* **Note 14**).
11. Remove and discard supernatant by taking care of not losing the DNA pellet at the bottom of the tube. This pellet contains nuclear and mitochondrial DNA.
12. Wash the DNA pellet with 500 µl of 70% EtOH (–20°). Centrifuge at 15,000–18,000 × g for 10 min at 4°C.
13. Remove and discard the supernatant. Repeat the washing step from Step 12.
14. Remove the supernatant, three samples at a time, and let the DNA pellet air-dry a bit (*see* **Note 15**).
15. Dissolve DNA pellet with 80–200 µl of TE buffer. Aim for a final concentration of ~ 60 ng/µl up to ~200 ng/µl.

16. Let the DNA samples sit at 4°C overnight to ensure that the DNA is completely dissolved.

3.3. DNA Quantification (see Note 7)

Should perform duplicate readings at least.

3.3.1. Fluorometric Quantification of dsDNA Using PicoGreen ®

3.3.1.1. First Round of Quantification of the Stock DNA Samples

1. Thaw the PicoGreen® dye at room temperature in the dark.
2. Mix samples by gently flicking the tube. Centrifuge briefly.
3. Prepare the lambda DNA standard. Recommended dilution series of 2X from 20 to 0.625 ng/μl (seven standards).
4. Aliquot 99 μl of TE buffer and 1 μl of DNA sample into a single well. Repeat for duplicate well and go to next sample. It is recommended to first prepare a mix of 198 μl TE buffer and 2 μl DNA in a microcentrifuge tube and then aliquot the mix into two wells, 100 μl each.
5. Aliquot 90 μl TE buffer and 10 μl of the lambda standard into a well. Repeat for duplicate well and go to next lambda standard.
6. Prepare PicoGreen® solution as much as needed: 5 μl of stock PicoGreen® into 1 ml TE buffer; 100 μl of this mixture is needed per well (keep in dark).
7. Aliquot 100 μl of PicoGreen® mix into each well, then mix by pipetting up and down.
8. Incubate at room temperature and in the dark for 10 min.
9. Perform fluorometric reading with a multiplate reader with excitation/emission wavelengths of 480 nm/520 nm.
10. Calculate the concentration of the stock DNA samples with the standards.
11. Prepare a 10 ng/μl dilution of the DNA samples (volume: around 200 μl).

3.3.1.2. Second Round of Quantification of the 10 ng/μl DNA Samples

Used to confirm the concentration and to get a more precise reading.

1. Repeat the steps from "First Round of Quantification"
2. But add 90 μl of TE buffer and 10 μl of 10 ng/μl of DNA sample into each well instead of 1 μl, since the samples have been diluted.
3. Calculate and confirm the concentrations of 10 ng/μl DNA samples. All subsequent dilutions from the 10 ng/μl DNA samples will be based on these results.

3.3.2. Quantification by Nanodrop ® (for Model ND-1000) (see Note 8)

1. Choose the nucleic acid reading option from the Nanodrop software.
2. Initialize the machine with distilled water. Pipette 1 μl of water onto reading pedestal. Click OK.

3. Wipe off water with clean tissue; make a blank reading with 1 μl TE buffer.
4. Put 1 μl of DNA sample and click measure.
5. Take triplicate readings of each sample and use the average of the results.
6. From stock, prepare the 10 ng/μl dilutions. Re-quantify the 10 ng/μl to get more precise measurements.

3.4. Template DNA Preparation and Heat Treatment

1. Prepare 5 ng/μl dilutions in TE buffer from the 10 ng/μl samples (volume: 200 μl).
2. Prepare 1 ng/μl dilutions in TE buffer from the 5 ng/μl samples (volume: 100 μl).
3. Aliquot 50 μl of 1 ng/μl DNA into a PCR tube.
4. Perform heat treatment on 50 μl of 1 ng/μl DNA by following this PCR protocol: 95°C for 6 min and 10°C for cool down.
5. 5 ng/μl DNA will be used for quantifying nuclear markers, and 1 ng/μl original and heated DNA will be used for mitochondrial markers.
6. Prepare a series of five standards by using the stock DNA of the control sample. Recommended range: five times dilution series starting from 40 ng/μl (*see* **Note 16**).

3.5. mtDNA Structural Damage and Repair Analysis Using Real-Time PCR

To ensure optimal reproducibility of the replicates, always prepare a master mix containing the SYBR Green Supermix and primers, and use aliquots of this mixture with individual template DNA samples. For mitochondrial markers: use 1 ng/μl original and heated templates. For nuclear markers: 5 ng/μl templates. Generally, the reaction can be run from 20 to 50 μl/well depending on the kit. This protocol will use 30 μl/well in triplicates on the Bio-Rad system with LNCaP cells that have been treated with H_2O_2 to induce oxidative damage at varying time-points.

1. Mix samples and standards gently by flicking the tube with fingers. Centrifuge briefly.
2. Prepare and label 0.5-ml tubes for each sample and standard.
3. Prepare the total amount of master mix needed by adding the reagents in this specific order, based on six samples (i.e., six original and six heated 1 ng/μl DNA) with five standards (**Table 12.2**).
4. Aliquot 5.4 μl of DNA template into a 0.5-ml tube, then add 84.6 μl of the master mix. Mix well by pipetting up and down. Put tube on ice until ready to load into wells.
5. Repeat Step 4 for each sample and standard.

Table 12.2
(*see* Note 17)

Template points (triplicate, 30 µl/well)	Reactions				
5 DNA standards	15X			1X	54X
6 Original 1 ng/µl	18X	⟶	Distilled H_2O	10.8 µl	583.2
DNA					µl
6 Heated 1 ng/µl	18X		50 mM $MgCl_2$	0.6 µl	32.4 µl
DNA			10 µM Forward	0.9 µl	48.6 µl
1 Blank	3X		primer		
	54X		10 µM Reverse	0.9 µl	48.6 µl
			primer		
			2X SYBR Mix	15 µl	810 µl
			(Bio-Rad)		
				28.2 µl	1,522.8
					µl

6. Aliquot each template mix into three wells (30 µl per well). Take extra care to deposit the mix at the bottom of well without creating bubbles. Avoid crosscontamination of the wells. Do not forget to aliquot blanks as well.
7. Seal the plate with optical tape. Centrifuge the plate briefly if necessary to get rid of excessive bubbles.
8. Follow this PCR protocol for mtDNA markers on the Bio-Rad system (for ABI system, *see* **Note 18**): cycle 1 (1X), 95.0°C for 1.5 min; cycle 2 (30X), Step 1 at 95.0°C for 20 s, Step 2 at 61.0°C for 30 s; cycle 3 (1X), 95.0°C for 1 min; cycle 4 (1X), 55.0°C for 1 min; cycle 5 (40X), 55.0°C for 10 s with an increase of 1.0°C after each repeat for collecting melt curve data. Enable real-time data collection at cycle 2 Step 2.
9. Run at least one nuclear DNA marker to use as the reference gene for the analysis (*see* **Fig. 12.1**). To run nuclear gene, calculate the amount of each reagents based on five standards, six 5 ng/µl DNA samples, and one blank (36X). PCR protocol (for ABI system, *see* **Note 19**): use the same protocol from Step 8, but replace 30X with 40X at cycle 2.
10. The data can be analyzed with any relative expression software tool (REST) which is based on the Pfaffl formula:

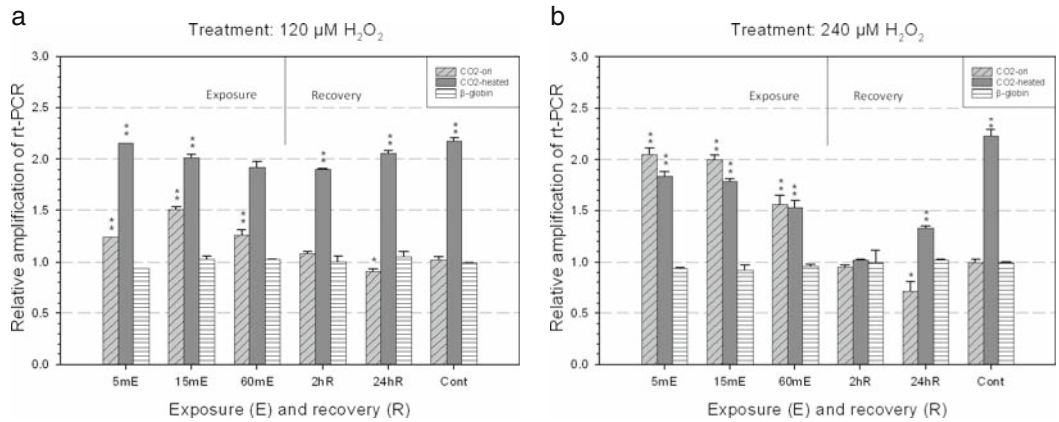


Fig. 12.1. **MtDNA damage responses to oxidative damage in LNCaP prostate cancer cells induced by exogenous H_2O_2 treatment.** Two concentrations (120 μM and 240 μM) of H_2O_2 were used to induce oxidative stress in LNCaP cells. For exposure, LNCaP cells were treated for 5, 15, and 60 min to induce DNA damage. For recovery, LNCaP cells were first treated for 60 min, and then allowed to recover in fresh medium for 2 and 24 h. The results have been normalized with the nuclear marker β-actin; a second nuclear marker β-globin is shown to indicate the lack of structural change in the target nuclear genes. CO2 is the mitochondrial marker: the diagonally stripped gray bars represent the original mtDNA fraction and the solid gray bars represent the heated mtDNA fraction. The heated fraction represents the total amount of relaxed mtDNA, any decrease or increase in the signal of the heated fractions can infer changes of the total amount of mtDNA. An increase in signal of the original fraction represents an increase in relaxed mtDNA, inferring structural damage, while a decrease may represent repair given that the total amount of mtDNA remains unchanged. (**A**) At low concentration of H_2O_2 (120 μM), LNCaP cells showed early increase of mtDNA structural damage from 5 minE to 60 minE when compared to control, but the damage was repaired 24 h after the treatment. (**B**) While at a higher concentration of H_2O_2 (240 μM), LNCaP showed acute early mtDNA structural damage, reflecting complete disruption of mtDNA structure in the original fraction. The damage was not repaired 24 h after the treatment but resulting in a loss of at least half amount of the mtDNA copy number. Statistics were performed by one-way ANOVA, compared to original fraction of control (* = $p<0.05$, ** = $p<0.01$) (*see* **Note 21**).

$$R = \frac{(Efficiency_{\text{target gene}})^{\Delta CPtarget(Mean\ Control - Mean\ sample)}}{(Efficiency_{\text{reference gene}})^{\Delta CPref(Mean\ Control - Mean\ sample)}} \qquad (12.1)$$

Assign a nuclear gene as the reference gene and use the original 1 ng/μl from the control sample as the calibrator for analysis (**Fig. 12.2**) (*see* **Note 20**).

4. Notes

1. The supercoiled structure inhibits the binding of oligonucleotide primers, preventing further amplification. In contrast, the relaxed forms (open circular and linear) allow effective primer binding and subsequent DNA polymerization. Thus, the relaxed forms of mtDNA are good substrates for PCR but not the supercoiled mtDNA.

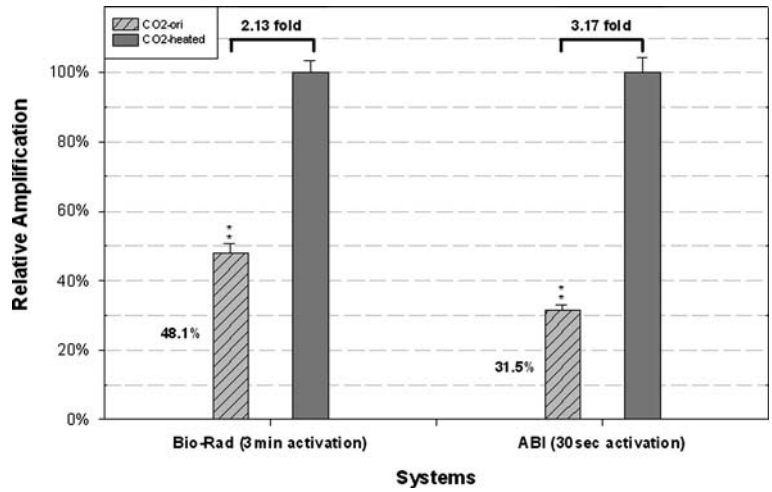


Fig. 12.2. **Baseline levels of mtDNA damage in non-treated prostate cancer cells analyzed by two systems: Bio-Rad vs ABI.** The baseline level of damage and the spontaneous damage are two distinct features. The baseline level of damage is the amount of mtDNA damage that is being detected by the systems. Artifacts introduced during the sample preparation and the initial heat-activation of real-time PCR can increase the baseline level of damage. On the other hand, spontaneous damage represents the actual amount of endogenous mtDNA damage of non-treated samples. The mtDNA structure is heat sensitive, and consequently the initial enzyme activation step at 95°C of the real-time PCR protocol can potentially introduce artificial damage to the mtDNA structure. So by shortening this step, it is possible to reduce this artificial damage. This figure compares the relative amplification of non-treated C4-2 cells, an isogenic variance of LNCaP, DNA between Bio-Rad and ABI systems (3 min and 30 s of initial 95°C activation, respectively). With the Bio-Rad system, 48.1% of the total mtDNA was found to be damaged, while the ABI system detected 31.5% of damage, which was a reduction of 34.5% between systems. Substantial amount of artificial damage has been removed with the ABI system. As such the baseline level of damage detected with the ABI system is more representative of the spontaneous level of mtDNA damage. The differences observed between the two systems can be attributed to the shorter initial heat-activation time as well as the different enzyme chemistry used. Statistics were performed with *t*-test, the two groups were found significantly different [Bio-Rad *(3* min), $n=4$; ABI (30 s), $n=3$; ** = $p<0.01$] (*see* **Note 22**).

2. Listed material and method for prostate cancer LNCaP cells. Substitute with equivalent reagents and buffers to cell line used.
3. LNCaP cells detach easily on non-PLL-treated surfaces. After preparing the PLL solution, it is recommended to filter it with a 0.2-μm filter. Add 3 ml PLL solution to each 100-mm dish and incubate for 5 min, remove all solution. Dry in fume hood overnight.
4. There are other methods such as DNA extraction by Trizol, but it has been shown that the Trizol procedure is too harsh and will disrupt all structural features from the mtDNA.
5. The steps involving C1 buffer has been removed. The C1 step is used for isolating the nucleus by lysing the cell membrane and discarding all remaining organelles, in consequence the mitochondria and the mtDNA will be lost.
6. Fresh snap-frozen tissue samples are suitable, but paraffin-embedded fixed tissues are not, due to crosslinking.

7. The two-step DNA quantification is optional but recommended. Since this will limit the dynamic range of the samples during real-time PCR amplification, the results tend to be more precise.
8. A conventional spectrophotometer can also be used for the two-step DNA quantification. We recommend using the Nanodrop spectrophotometer over a conventional spectrophotometer. The former method does not require the use of a cuvette for holding and reading the samples, and requires very little amount of the sample (1 μl per reading) for precise measurement.
9. It is not recommended to repeatedly freeze and thaw the same samples as this can degrade the quality of the DNA. If the tissue sample is planned to be used on separate occasions, cut the tissue sample to smaller pieces and thaw only what is needed.
10. Work fast, it is not recommended to thaw the tissue sample until it is ready to be homogenized in the glass homogenizer. If the tissue sample is too hard to cut, thaw on wet ice.
11. After the homogenization, there will still be residue thin pieces of tissue that are not completely homogenated. Do not continue grinding because this will disrupt the DNA structure, but transfer everything to a 2-ml tube. The digestion with proteinase K will digest the remaining pieces.
12. Proteinase K from QIAGEN is used instead of the protease included in the kit because it is more efficient and will digest more readily the remaining bits of tissue in the homogenate.
13. Unless the sample has been exposed to harsh treatments during experiments, the precipitated DNA should look like long intertwined DNA fibers. Generally, the presence of intertwined DNA fibers is an indication of well-preserved DNA.
14. The intertwined DNA is mostly nuclear DNA; mtDNA is actually quite small and hard to *see* when in suspension. The high-speed centrifugation is needed to bring down the mtDNA and to form a pellet of nuclear and mtDNA.
15. Do not over-dry as this will make it harder to dissolve back in TE buffer. By the time the supernatant from the third tube is carefully removed, the pellet from the first tube should be dry enough.
16. A standard is needed because the real-time PCR analysis is based on Pfaffl's relative quantification model. The standard is not needed for the analysis if the calculations are based on $\Delta\Delta$Ct method, but the efficiencies of different markers have to be optimized and validated.

17. It is recommended to add an additional 3–4 μl of distilled water to the final mix because inevitably some of the mix will be lost due to pipetting.
18. Protocol for mtDNA markers on the ABI system: cycle 1 (1X), 95.0°C for 30 s; cycle 2 (30X), Step 1 at 95.0°C for 3 s, Step 2 at 61.0°C for 30 s; cycle 3 (1X), add melt curve. Enable real-time data collection at cycle 2 Step 2.
19. Protocol for nuclear DNA on the ABI system: cycle 1 (1X), 95.0°C for 30 s; cycle 2 (40X), Step 1 at 95.0°C for 3 s, Step 2 at 61.0°C for 30 s; cycle 3 (1X), add melt curve. Enable real-time data collection at cycle 2 Step 2.
20. There are various REST tools that are freely available on the Internet: REST-XL, REST-MCS, and others.
21. Similar results were obtained by analyzing with the D-loop mitochondrial marker.
22. Similar results were observed with other cell lines, frozen kidney tissues, and blood leukocyte samples. The Power SYBR® Fast Green PCR MASTER MIX from ABI and its 7500 Fast Real-Time PCR System platform made it possible to cut down the initial 95°C activation time to 30 s (*see* **Note 18**). This should be taken into consideration when comparing clinical samples where little differences in spontaneous damage levels must be determined. But it is unnecessary when performing relative comparisons between samples in experiments where the samples are subjected to high levels of damage caused by treatments because the artificial damage caused by artifacts is evenly distributed.

References

1. Wallace, D.C. (2005) A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu. Rev. Genet.*, **39**, 359 407.
2. Turrens, J.F. and Boveris, A. (1980) Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem. J.*, **191**, 421–427
3. Richter, C. (1995) Oxidative damage to mitochondrial DNA and its relationship to ageing. *Int. J. Biochem. Cell Biol.*, **27**, 647–653
4. Toyokuni, S., Okamoto, K., Yodoi, J. and Hiai H. (1995) Persistent oxidative stress in cancer. *FEBS Lett.* **358**, 1–3.
5. Herrmann, P.C., Gillespie, J.W., Charboneau, L., Bichsel, V.E., Paweletz, C.P., Calvert, V.S., Kohn, E.C., Emmert-Buck, M.R., Liotta, L.A., et al. (2003) Mitochondrial proteome: altered cytochrome c oxidase subunit levels in prostate cancer. *Proteomics.* **3**, 1801–1810.
6. Fliss, M.S., Usadel, H., Caballero, O.L., Wu, L., Buta, M.R., Eleff, S.M., Jen, J. and Sidransky, D. (2000) Facile detection of mitochondrial DNA mutations in tumors and bodily fluids. *Science.* **287,** 2017–2019.
7. Parrella, P., Xiao, Y., Fliss, M., Sanchez-Cespedes, M., Mazzarelli, P., Rinaldi, M., Nicol, T., Gabrielson, E., Cuomo, C., et al. (2001) Detection of mitochondrial DNA mutations in primary breast cancer and fine-needle aspirates. *Cancer Res.* **61,** 7623–7626.
8. Chen, J.Z., Gokden, N., Greene, G.F., Mukunyadzi, P. and Kadlubar, F.F. (2002) Extensive somatic mitochondrial mutations

in primary prostate cancer using laser capture microdissection. *Cancer Res.* **62**, 6470–6474.

9. Radloff, R., Bauer, W. and Vinograd, J. (1967) A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. *Proc. Natl. Acad. Sci. USA*, **57**, 1514–1521
10. Robberson, D.L. and Clayton, D.A. (1972) Replication of mitochondrial DNA in mouse L cells and their thymidine kinase-derivatives: displacement replication on a covalently-closed circular template. *Proc. Natl. Acad. Sci USA*, **69**, 3810–3814
11. Bogenhagen, D. and Clayton, D.A. (1978) Mechanism of mitochondrial DNA replication in mouse L-cells: introduction of superhelical turns into newly replicated molecules. *J. Mol. Biol.*, **119**, 69–81
12. Clayton, D.A. (1982) Replication of animal mitochondrial DNA. *Cell*, **28**, 693–705
13. Chen, J., Kadlubar F.F. and Chen J.Z. (2007) DNA supercoiling suppresses real-time PCR: a new approach to the quantification of mitochondrial DNA damage and repair. Nucleic Acids Res. **35**(4), 1377–1388
14. Ayala-Torres, S., Chen, Y., Svoboda, T., Rosenblatt, J. and Van Houten, B. (2000) Analysis of gene-specific DNA damage and repair using quantitative polymerase chain reaction. *Methods*, **22**, 135–147.
15. Pfaffl, M. W. (2001) A new mathematical model for relative quantification for real-time RT-PCR. *Nucleic Acid Res.* **29**, 9 00

Chapter 13

Quantitative Analysis of Oxidized Guanine, 8-Oxoguanine, in Mitochondrial DNA by Immunofluorescence Method

Mizuki Ohno, Sugako Oka, and Yusaku Nakabeppu

Abstract

8-Oxoguanine (8-oxoG), an oxidized form of guanine, is one of the major mutagenic lesions generated under oxidative stress. Oxidative damage in mitochondrial DNA has been implicated as a causative factor for a wide variety of degenerative diseases as well as for cancer during aging. We established a quantitative method for in situ detection of 8-oxoG in mitochondrial DNA in a single-cell level using a monoclonal antibody. Specific detection of 8-oxoG in mitochondrial DNA was confirmed by pre-treatment of samples with DNase I or MutM, the latter excising 8-oxoG opposite C in DNA. We then analyzed 8-oxoG dynamics in mitochondrial DNA of the wild-type and 8-oxoG DNA glycosylase (OGG1)-deficient mouse cells after exposure to hydrogen peroxide. Intensities for the 8-oxoG immunoreactivity in mitochondrial DNA were increased immediately after the exposure to hydrogen peroxide in both types of cells. The increased intensities returned to basal levels within a few hours only in wild-type cells, but not in OGG1-deficient cells which exhibited the increased intensities even 24 h after the exposure. These results indicate that OGG1 is a major enzyme for excision repair of 8-oxoG in mitochondrial DNA in mouse cells, and that our method described here is appropriate to study 8-oxoG dynamics in mitochondrial DNA.

Key words: 8-oxoguanine, OGG1, immunodetection, immunofluorescence, mitochondrial DNA, oxidative stress, hydrogen peroxide, base excision repair.

1. Introduction

Reactive oxygen species (ROS), generated as byproducts of mitochondrial respiration or as a consequence of exposure to environmental agents, are known to oxidize DNA. Oxidative damage to cellular DNA often causes mutagenesis as well as programmed cell death. The former may result in carcinogenesis, while the latter has been linked to degenerative disorders *(1–3)*. Among many classes

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_13 Springerprotocols.com

of DNA damage caused by ROS, an oxidized form of guanine base, 8-oxoguanine (8-oxoG) is considered to play a major role in mutagenesis and carcinogenesis, because 8-oxoG can pair with adenine as well as cytosine.

8-OxoG accumulates in both nuclear and mitochondrial DNA (mtDNA) during aging, and the level of accumulation is known to increase in patients with various neurodegenerative diseases, such as Parkinson's disease (PD) or Alzheimer's disease (AD) *(4, 5)*, suggesting that an accumulation of 8-oxoG in cellular DNA contributes to cell death. We recently demonstrated that accumulation of 8-oxoG in mtDNA causes mitochondrial dysfunction and Ca^{2+} release followed by the activation of calpain, resulting in cell death. It is thus important to quantitatively evaluate the levels of 8-oxoG in mtDNA in order to understand the cellular response to the oxidative stress in mitochondria. To this end, we have developed several methods to detect and quantify levels of 8-oxoG in cellular DNA, based on a separation of 8-oxo-2′ -deoxyguanosine in enzyme-digested DNA preparation from cells or tissues on a liquid chromatography with electrochemical detection or MS/MS analysis or in situ immunological detection of 8-oxoG in cultured cells or various tissues derived from mouse or human *(6–9)*.

We herein describe an in situ detection of 8-oxoG in mtDNA in cultured cells or mouse brain sections, using a monoclonal antibody against 8-oxoG (N45.1). In order to ensure the specific and accurate detection of 8-oxoG with the monoclonal antibody, we developed specific pre-treatments. Because the antibody used here rather selectively reacts with 8-oxoG in single-stranded DNA, and it also efficiently reacts with 8-oxoG in RNA, it is required to remove RNA from samples and denature DNA prior to reaction with the primary antibody against 8-oxoG (**Table 13.1**). However, different conditions are required for the denaturation of nuclear and mitochondrial DNA probably because of differences in DNA packaging. We therefore have carefully examined the effects of various combinations of pre-treatment with RNase A or DNase I and denaturation with HCl or NaOH, on in situ detection of 8-oxoG in mitochondrial DNA, and the results are summarized in **Table 13.1** and **Fig. 13.1**. We found that the pre-treatment with RNase A and denaturation with NaOH give clear and specific cytoplasmic immunofluorescent signals for 8-oxoG which are co-localized with signals from MitoTracker (**Fig. 13.1A**) or with immunofluorescent signals from mitochondrial transcription factor A (TFAM) which binds to mitochondrial DNA (**Fig. 13.1B**)*(10)*. When cells are exposed to hydrogen peroxide, cytoplasmic immunofluorescent signals for 8-oxoG detected after RNase A pre-treatment and NaOH-denaturation are dramatically increased in comparison to control cells, and those signals are diminished by either pre-treatment with MutM (an enzyme excising 8-oxoG opposite cytosine in DNA, *see* **Note 1**)

Table 13.1
Combination of nucleases and denaturation methods on the detection of 8-oxoG using the monoclonal anti-8-oxoG antibody, N45.1

		Pretreatment with nuclease			
		None	RNase A	DNase I	RNase A + DNase I
Denaturation	None	rRNA+ Cytoplasmic RNA	weak cytoplasmic signal	rRNA+ Cytoplasmic RNA	No signal
	HCl	rRNA+ Cytoplasmic RNA	Nuclear DNA	rRNA+ Cytoplasmic RNA	No signal
	NaOH	N.D	Mitchondrial DNA	poor morphology	poor morphology

The signal patterns of the immunofluorescence for intracellular 8-oxoG detected by the combination of each pretreatment with nuclease and denaturation method are summarized.

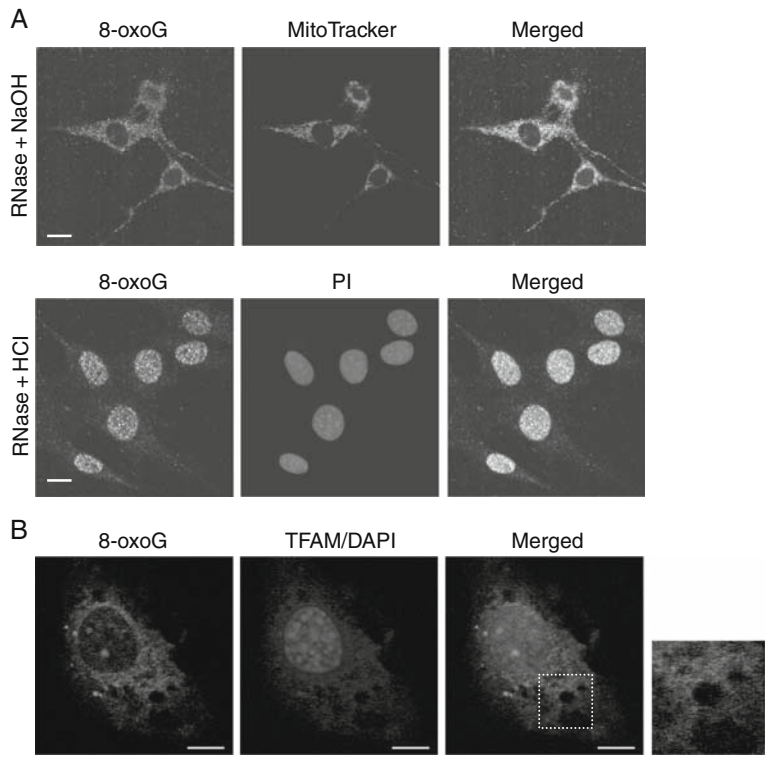


Fig. 13.1. **Immunodetection of 8-oxoG in mitochondrial DNA.** A. Immunofluorescence detection of intracellular 8-oxoG in NIH3T3 cells. *Top panels*: cytoplasmic 8-oxoG immunoreactivity is detected in a sample pre-treated with RNase A and denatured with NaOH (RNase + NaOH). Mitochondria are stained with MitoTracker. *Bottom panels*: nuclear 8-oxoG immunoreactivity is detected in a sample pre-treated with RNase A and

or with DNase I (**Fig. 13.2**). Cytoplasmic immunofluorescent signals for 8-oxoG increase after the exposure to oxidative stress, then return to the basal level within a few hours in wild-type MEFs, however, those in OGG1-null MEFs remain high even 24 h after the exposure (**Fig. 13.3**). From these experiments, we conclude that the denaturation with NaOH after RNase A treatment is the

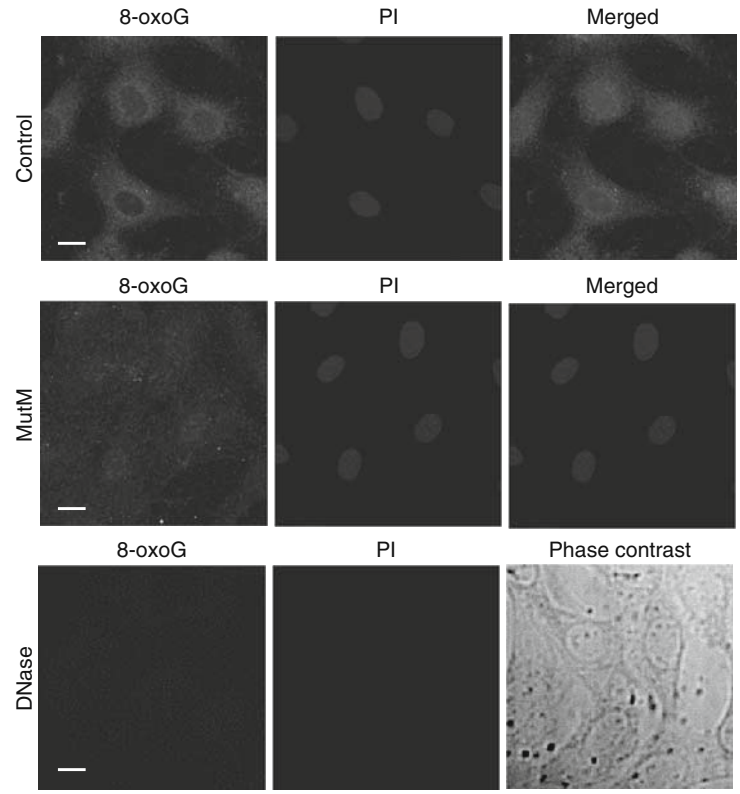


Fig. 13.2 **Cytoplasmic 8-oxoG immunoreactivity detected in a sample pre-treated with RNase A and denatured with NaOH is diminished by MutM or DNAse I treatment.** NIH3T3 cells are cultured on a slide glass. Cells are exposed to hydrogen peroxide for 30 min prior to fixation. Increased 8-oxoG immunoreactivity in cytoplasm after exposure to H_2O_2. Control sample is pre-treated with RNase and denatured with NaOH. Cytoplasmic signals of 8-oxoG immunoreactivity are largely diminished by the additional pre-treatment with MutM (MutM). Cytoplasmic signals of 8-oxoG immunoreactivity are completely diminished by the additional pre-treatment with DNase I (DNase). Although there is no PI signal, cells remained intact (phase contrast).

Fig. 13.1 (continued) denatured with HCl (RNase + HCl). Nuclear DNA is stained with propidium iodide (PI). Bar: 10 μm. B. Cytoplasmic 8-oxoG immunoreactivities were co-localized with mitochondrial transcription factor A (TFAM) in Nu-hOGG1 MEFs which were exposed to 50 μM menadione for 1 h. A part of the merged image was magnified. Nuclei were counterstained with DAPI. Bar: 10 μm. (Adapted from **Ref. *(12).***)

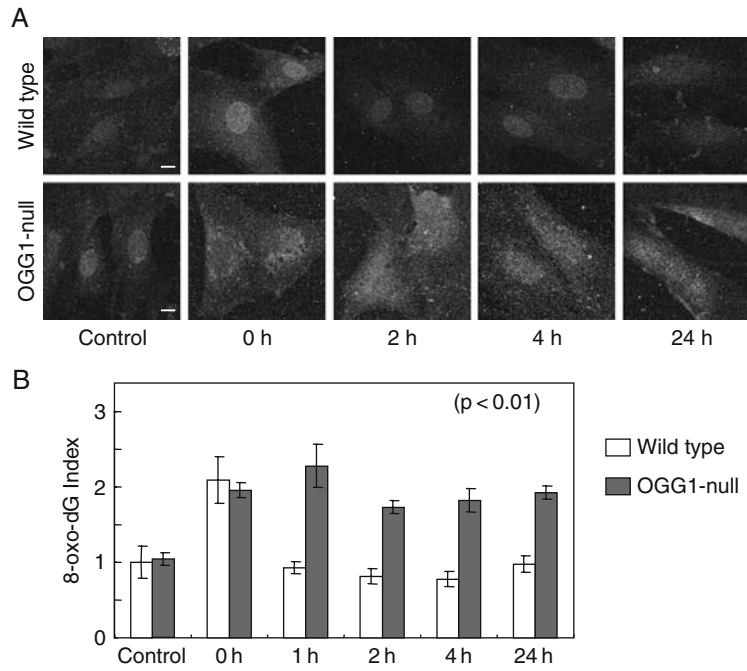


Fig. 13.3. **Dynamics of 8-oxoG immunoreactivity in mitochondrial DNA after exposure of wild-type and OGG1-null MEFs to hydrogen peroxide.** MEFs are exposed to H_2O_2 for 30 min, and at given time cells are subjected to 8-oxoG immunofluorescence microscopy with RNase pre-treatment and NaOH denaturation. Control: no exposure to H_2O_2. Nuclei were counterstained with propidium iodide. Bar: 20 μm. 8-OxoG immunoreactivity in mtDNA increased immediately after exposure to H_2O_2 (0 h), and the levels in wild-type cells were greatly reduced within 2 h after the exposure and returned to the basal level thereafter (*top panels*), while those in OGG1-null cells were not reduced even 24 h after the exposure and remained high (*middle panels*). B. 8-OxoG index in mtDNA was determined and plotted as a bar graph. $p<0.01$, repeated measurement ANOVA.

most suitable pre-treatment to detect 8-oxoG in mitochondrial DNA of cultured cells. To detect mitochondrial 8-oxoG in mouse brain sections, the denaturation step with NaOH should be avoided because the treatment causes disruption of tissue structure resulting in unreproducible results *(9)*.

2. Materials

2.1. Cell Lines

1. NIH3T3 cells *(11)*.
2. Wild-type and *Ogg1*$^{-/-}$ mouse embryo fibroblasts (MEFs). The generation of *Ogg1*$^{-/-}$ mice has been described previously *(12)*. *Ogg1*$^{-/-}$ and wild-type MEFs are isolated from embryos that were obtained by mating *Ogg1*$^{+/-}$ female and male mice.

3. Nu-hOGG1 MEFs: *Ogg1*$^{-/-}$ MEFs immortalized as above were transfected with a vector pcDNA3.1Hyg(−):hOGG1-1a encoding the nuclear form of hOGG1 *(13)*.

2.2. Cell Culture

1. Growth medium A: Dulbecco's Modified Eagle's Medium (DMEM, low glucose, GIBCO 11885) supplemented with 10% calf serum (CS, GIBCO 200-6170A), penicillin–streptomycin (GIBCO 15140).
2. Growth medium B: Dulbecco's Modified Eagle's Medium (DMEM, high glucose, GIBCO 11995-065) supplemented with 10% fetal bovine serum (FBS, GIBCO), penicillin–streptomycin (GIBCO 15140).
3. Solution of trypsin (0.25%) and ethylenediamine tetraacetic acid (EDTA, 1 mM, IBL 23315).
4. Phosphate-buffered saline: Dissolve five tablets of PBS (Dulbecco's 28-103-05 FH) in distilled water, make up to 1,000 ml, autoclave.
5. Poly-L-lysine coated glass slides (thickness 0.9–1.2 mm, pre-cleaned, 76 × 26 mm): Before starting the culture, prepare culture glasses as follows. Set pre-cleaned slide glasses to the slide holder, wrap with aluminum foil, and autoclave. Put sterile slide glass into the culture dish. Culture cells on slide glass for immunological staining.
6. MitoTracker Red (Molecular Probes M-7512).
7. Medium containing hydrogen peroxide: 0.5 mM hydrogen peroxide (H_2O_2) in serum -free DMEM. Prepare medium containing H_2O_2 freshly for each experiment. Use within 30 min of preparation.
8. 2-Methyl-1.4-naphthoquinone (menadione) is dissolved in PBS at 500 mM, stored in single-use aliquots at –30°C. Dilute with low glucose DMEM without FBS and penicillin–streptomycin and filter through 0.2-μm filter (Millipore, Corp., Bedford, MA, USA) for use.
9. Fixative: cold methanol (100%) stored at –20°C.
10. Paraformaldehyde (PFA, Nacalai tesque, for electron microscopic grade): 4% (w/v) in PBS, use fresh solution for each experiment. PFA should be handled under the hood because of toxicity. Transfer PFA powder to sterile bottle, add PBS to make 4% solution, put magnetic stir bar in the bottle, cap, heat bottle with stirring at 60°C for 2–h until completely dissolved (use water bath with magnetic stirrer).

2.3. Immunological Detection of Cellular 8-OxoG

1. Anti-8-oxoG monoclonal antibody (clone N45.1, Nikken SEIL Co., Ltd).
2. Goat polyclonal anti-mitochondrial transcription factor A (TFAM) antibody (SC-23588 Santa Cruz).

3. RNase solution: Stock solution; 10 mg/ml RNase A (Sigma) in 10 mM Tris–HCl (pH 7.5), 15 mM NaCl. To inactivate contaminating DNase, incubate RNase solution at 98°C for 15 min, then it leave at room temperature until cool. Store at –20°C until use. Working solution: 1/2 dilution of stock solution with same buffer.
4. DNase solution: 1 U/μl of DNase I (Sigma) in 50 mM Tris–HCl (pH 7.5), 0.1 mM $MgCl_2$.
5. MutM solution: 10 μg/ml of MutM protein (Fapy DNA glycosylase, Sigma) in nicking buffer [10 mM Tris–HCl (pH 7.5), 5 μM $ZnCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 1.5% glycerol, 100 μg/ml BSA] (*see* **Note 1**).
6. HCl solution: 2 N HCl in distilled water. Prepare freshly.
7. NaOH solution: 50 mM NaOH in 50% ethanol. Prepare freshly.
8. Tris-base neutralization solution: 50 mM Tris-base in distilled water.
9. Blocking solution: 1X BlockAce (Dainippon Pharmaceutical, Japan).
10. Dilution solution for antibodies: 1/10 dilution of BlockAce with distilled water.
11. Wash solution: PBD:PBS + 0.1% Triton-X 100.
12. Secondary antibody: anti-mouse IgG Alexa-488 conjugate (Molecular Probes).
13. Counter staining solution (Propidium Iodide solution, PI, Sigma): Stock solution: 1.5 mM (1 mg/ml) PI in water, store at 4°C. Working solution: diluting the stock solution 1:3,000 in PBS (500 nM). DNA and RNA are both stained with PI.
14. Anti-fade mounting media for fluorescence microscopy: Vectashield (Vector Lab) is used to prevent rapid fluorescence quenching during microscopic examination.

2.4. Immunodetection of Mitochondrial 8-OxoG in Mouse Brain Section

1. Sucrose solutions: Prepare 20% and 30% sucrose (w/v) in PBS.
2. Disposable plastic dish for tissue freezing (Cryomold, Sakura).
3. Tissue freezing medium (O.C.T compound, Sakura).
4. 6-Well culture dish (Nunc).
5. Painting brush: A small and soft painting brush is useful to pick up free-floating tissue section from solution without damage.

6. DAPI solution for nuclear DNA staining: Prepare stock solution (0.5 mg/ml), keep at 4°C, avoiding light.
7. Rabbit anti-VDAC polyclonal antibodies were described previously *(10)*.
8. Other solutions required are as above (**Section 2.3**).

2.5. Microscope and Software

1. A fluorescence microscope Axioskop2 equipped with a CCD camera AxioCam (Carl Zeiss).
2. Digital image processing and camera control: AxioVision software (Carl Zeiss).
3. Images processing: Adobe Photoshop 7.0 Software (Adobe systems, Inc).

3. Methods

3.1. Cell Culture

1. NIH3T3 cells are maintained in growth medium A, with 5% CO_2 at 37°C. The cells on culture dishes are treated with trypsin–EDTA solution and passaged once per 3 days (1:3).
2. MEF culture: Primary MEFs are maintained in growth medium B, with 5% CO_2 at 37°C.
3. When primary culture of MEFs reaches 90% confluence, cells on culture dishes are treated with trypsin–EDTA solution and passaged (1:3).
4. For immunofluorescent microscopy, cells are directly cultured on poly-L-lysine coated glass slides. Put a sterile slide glass on a 100-mm culture dish, add 5 ml of pre-warmed growth medium to cover the surface of slide glass with medium, and add 2–5 $\times 10^5$ cells in 5 ml of growth medium to the culture dish with slide glass, gently mix, and incubate in the CO_2 incubator.
5. When multiple cell slides are necessary, a sterile square dish (sterile no. 2 square scale, Eiken Chemical Co., Ltd) can be used with five slides, add 5 ml of pre-warmed growth medium, then 0.8–1.2 $\times 10^6$ cells in 20 ml of growth medium are added.
6. The cells are cultured for 16 h on slide glasses and then are used for the experiments.
7. For mitochondrial staining, medium is changed with freshly prepared medium containing 400 nM MitoTracker Red, and cells are incubated 45–60 min in the CO_2 incubator. Remove the medium from the culture by aspiration, and add gently 6 ml of pre-warmed complete growth medium per 100-mm

dish. Incubate the culture at 37°C for 5 min to allow unbound MitoTracker to diffuse into the medium. Repeat this procedure once before fixation.

8. Fixation: Remove the medium by aspiration, and rinse cells with cold PBS (4°C), transfer slides to cold methanol, fix for at least 10 min at –20°C. Slides can be kept in methanol for a week at –20°C.

3.2. Hydrogen Peroxide Exposure

1. The cells cultured on a slide glass are gently rinsed with a pre-warmed serum-free medium, and then are incubated with 0.5 mM of H_2O_2 in a serum-free medium for 30 min at 37°C (*see* **Note 2**).
2. After 30 min, the medium is removed by aspiration, and then cells are incubated in pre-warmed complete medium containing serum. After the 30-min exposure to H_2O_2, cells are fixed at 0, 2, 4, and 24 h later.
3. The slides are washed three times with cold PBS at given time.
4. Fix slides with cold methanol for at least 10 min. Slides are kept in methanol at –20°C until use.

3.3. Pre-treatment for Immunodetection of 8-OxoG in Mitochondrial DNA

1. Fixed slides with methanol are rinsed with PBS twice at room temperature (RT).
2. To eliminate RNA, the slides are treated with RNase solution for 1 h at 37°C (*see* **Note 3**).
3. Rinse the slides with PBD three times, 3 min for each, at RT. Transfer slides into PBS.
4. To obtain the negative control or confirm specificity of the immunoreactivity, pre-treatment with MutM or DNase I is performed. Incubate slides with MutM solution or DNase solution for 1 h at 37°C. Then slides are rinsed with PBD three times, 3 min for each, at RT (refer **Fig. 13.2**).
5. Slides are incubated with 4% PFA for 10 min at RT to re-fix cells, then rinsed with PBD three times, 3 min for each, at RT (*see* **Note 4**).
6. Denaturation of mitochondrial DNA: Dip slides into NaOH solution for 7 min at RT, then rinse with PBS three times, 3 min for each, at RT.

3.4. Pre-treatment for Immunodetection of 8-OxoG in Nuclear DNA

1. Fixed slides with methanol are rinsed with PBS twice at RT.
2. To eliminate RNA, slides are treated with RNase solution for 1 h at 37°C.
3. Rinse slides with PBD three times, 3 min for each, at RT. Transfer slides to PBS.
4. To obtain the negative control or confirm specificity of the immunoreactivity, pre-treatment with MutM or DNase I is

performed. Incubate slides with MutM solution or DNase solution for 1 h at 37°C. Then slides are rinsed with PBD three times, 3 min for each, at RT.

5. Denaturation of nuclear DNA: Rinse slides with distilled water, then dip the slides into 2 N HCl for 10 min.
6. Dip slides into Tris-base solution for 10 min.
7. Rinse slides with PBS for three times, 3 min for each, at RT.

3.5. Immunofluorescence Staining

1. Pre-treated slide is placed in blocking solution prepared in a Coplin Jar for 10 min at RT to reduce non-specific signals.
2. Diluted mouse anti-8-oxoG monoclonal antibody (1:10 in dilution solution), with appropriately diluted other antibodies if necessary, is applied to the slide, which is then covered with a plastic cover slip and incubated for 2 h at RT in a dark, humidified chamber.
3. Rinse the slide with PBD three times, 3 min each, at RT with gentle shaking.
4. Place the slide in blocking solution for 10 min at RT.
5. Diluted secondary antibody (1:300 in dilution solution) is applied to the slide, which is then covered with a plastic cover slip and incubated for 40 min at RT in a dark, humidified chamber.
6. Rinse the slide with PBD three times, 3 min each, at RT with gentle shake.
7. Dip the slide into PI solution for 5 min to stain nuclear DNA at RT, and then rinse the slide with PBD.
8. Mount the slide with anti-fade mounting media. Store the slide at 4°C until observation under the microscope.

3.6. Fluorescent Microscopy and Image Analysis

1. Fluorescent images are observed using an Axioskop2 equipped with an AxioCam. A digital image of each fluorescent signal is captured separately as a gray-scale image. Merging of the images with different fluorescences is performed using the AxioVision software. Captured images are processed using Adobe Photoshop 7.0 software.
2. To obtain an 8-oxoG index, each image of 8-oxoG immunofluorescence is converted to 256-level gray-scale, whereas each image of nuclear PI staining is converted to a binary monochrome using the Adobe Photoshop.
3. From the gray-scale image of 8-oxoG immunofluorescence, the signals present in areas corresponding to nuclei represented by the monochrome PI image are deleted, and then the image with only cytoplasmic signals for 8-oxoG immunofluorescence is saved as a tiff file.

4. The tiff file of image with the cytoplasmic 8-oxoG immunofluorescence is opened using NIH Image 1.61 software.
5. An area of a single cell is manually defined, and an integrated pixel density for each cell is determined with a uniform threshold.
6. At least 30 cells are analyzed, and an average value of the integrated pixel density per cell is calculated as the 8-oxoG index for each sample.

3.7. Immunodetection of Mitochondrial 8-OxoG in Mouse Brain Section

3.7.1. Sample Preparation

1. Animals deeply anesthetized with pentobarbital (30 mg/kg i.p.) are perfused intracardially with saline followed by cold 4% paraformaldehyde (PFA) in 0.1 M PBS.
2. Brains are removed and fixed in 4% PFA for 12 h at 4°C.
3. Cryoprotection: Rinse fixed brains with 20% sucrose, and transfer to 20% sucrose for 24 h at 4°C. Next, brains are transferred to 30% sucrose for 24 h at 4°C.
4. Rinse brains with PBS twice, remove extra liquid.
5. Cut in half and trim fixed brain, then set it onto the cryomold.
6. Add O.C.T compound and freeze brain immediately to set cryomold on metal block in liquid nitrogen. Keep brain at –80°C until use.
7. Serial sections (40 mm thick) are cut on a cryostat and each section is collected as a free-floating section in a well filled with PBS, and processed immediately for immunodetection.

3.7.2. Detection of 8-OxoG in Mitochondrial DNA in Mouse Brain

To detect 8-oxoG in mtDNA in tissue section, the alkali denaturation step is not recommended because the treatment causes disruption of tissue structure resulting in unreproducible results. Thus, avoid this step *(9)*.

1. Aspirate PBS carefully (do not aspirate floating tissue) from the well, add 500 μl of pre-warmed RNase solution. Incubate for 1 h at 37°C.
2. Aspirate RNase solution carefully, add PBD, wash for 10 min at RT with gentle shake. Repeat this step for three times.
3. Aspirate PBD carefully, add blocking solution, and incubate for 30 min at RT.
4. Aspirate blocking solution carefully, add 1:100 diluted anti-8-oxoG antibody and anti-VDAC antibodies, and incubate for overnight at 4°C.
5. Aspirate antibody solution carefully, add PBD, and wash for 10 min at RT with gentle shake. Repeat this step three times.
6. Aspirate PBD carefully, add 1 ml of blocking solution, and incubate for 30 min at RT.

7. Aspirate blocking solution carefully, add 500 ml of 1:300 diluted proper Alexa Fluor-labeled secondary antibodies, and incubate for 45 min at RT.
8. Aspirate secondary antibody solution carefully, add PBD, and wash for 10 min at RT with gentle shake. Repeat this step three times. Add DAPI solution for last PBD (1:5,000 dilution of stock solution with distilled water).
9. The free-floating sections are picked up using painting brush and set onto the slide glass, mount with Vectashield. Keep slide at 4°C until use.
10. Confocal images (**Fig. 13.4**) are acquired under Eclipse TE300 (Nikon, Japan) equipped with the Radiance 2100 laser scanning confocal microscope system (Bio-Rad Laboratories).

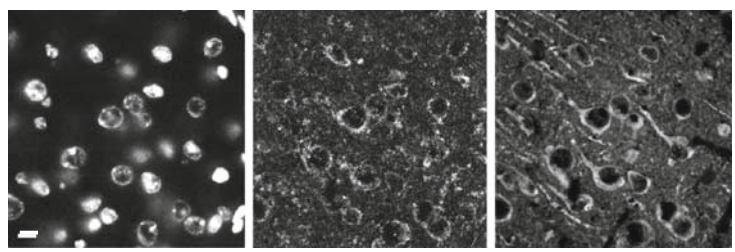


Fig. 13.4. **Immunodetection of mitochondrial 8-oxoG in mouse brain.** The sections were subjected to immunofluorescent staining with the anti-8-oxoG antibody after RNase treatment. Laser scanned confocal images from the cerebral cortex stained for nuclear DNA with DAPI, 8-oxoG, and VDAC are shown. Weak peri-nuclear immunoreactivities for 8-oxoG were observed in large neurons, and these immunoreactivities were mostly co-localized with the mitochondrial protein VDAC immunoreactivity. Scale bar: 10 μm. (Adapted from Ref. *(9)*.)

4. Notes

1. MutM is a bacterial enzyme, 8-oxoG DNA glycosylase that has an activity to excise 8-oxoG opposite cytosine in duplex DNA. This protein is also called FPG (formamidopyrimidine–DNA glycosylase). OGG1 is a functional homolog of MutM.
2. NIH3T3 and MEFs cells are not killed by exposure to H_2O_2 under these conditions (0.5 mM, 30 min). You will see shrinking of cells after 30-min exposure, and then recovery within a few hours.
3. Complete digestion of RNA in the fixed cells is required to obtain a clear immunofluorescent signal. The concentration of RNase A used here is much higher than that in usual

reagents for molecular biological experiments. During handling of this solution, careful attention should be paid to avoid contamination of equipment and other reagents with the RNase. Use humidified chamber, jars, and pipettes prepared only for this treatment. RNase activity on glassware or metalware can be inactivated by dry-oven at 200°C for 2 h.

4. Re-fixation by PFA is necessary only when cells are treated with NaOH.

Acknowledgments

We extend our special thanks to all other members of our laboratory for useful comments on the manuscript. This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (grant numbers: 18013038, 20013034), and the Japan Society for the Promotion of Science (grant numbers: 16390119, 18300124).

References

1. Ames, B. N., Shigenaga, M. K. and Hagen, T. M. (1993) Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. USA* **90,** 7915–7922.
2. Nakabeppu, Y., Tsuchimoto, D., Yamaguchi, H. and Sakumi, K. (2007) Oxidative Damage in Nucleic Acids and Parkinson's Disease. *J. Neurosci. Res.* **85,** 919–934.
3. Nakabeppu, Y., Sakumi, K., Sakamoto, K., Tsuchimoto, D., Tsuzuki, T. and Nakatsu, Y. (2006) Mutagenesis and carcinogenesis caused by the oxidation of nucleic acids. *Biol. Chem.* **387,** 373–379.
4. Shimura-Miura, H., Hattori, N., Kang, D., Miyako, K., Nakabeppu, Y. and Mizuno, Y. (1999) Increased 8-oxo-dGTPase in the mitochondria of substantia nigral neurons in Parkinson's disease. *Ann. Neurol.* **46,** 920–924.
5. Nunomura, A., Perry, G., Aliev, G., Hirai, K., Takeda, A., Balraj, E. K., et al. (2001) Oxidative damage is the earliest event in Alzheimer disease. *J. Neuropathol. Exp. Neurol.* **60,** 759–767.
6. Tsuruya, K., Furuichi, M., Tominaga, Y., Shinozaki, M., Tokumoto, M., Yoshimitsu, T., et al. (2003) Accumulation of 8-oxoguanine in the cellular DNA and the alteration of the OGG1 expression during ischemia-reperfusion injury in the rat kidney. *DNA Repair* **2,** 211–229.
7. Yoshimura, D., Sakumi, K., Ohno, M., Sakai, Y., Furuichi, M., Iwai, S., et al. (2003) An oxidized purine nucleoside triphosphatase, MTH1, suppresses cell death caused by oxidative stress. *J. Biol. Chem.* **278,** 37965–37973.
8. Ohno, M., Miura, T., Furuichi, M., Tominaga, Y., Tsuchimoto, D., Sakumi, K., et al. (2006) A genome-wide distribution of 8-oxoguanine correlates with the preferred regions for recombination and single nucleotide polymorphism in the human genome. *Genome Res.* **16,** 567–575.
9. Yamaguchi, H., Kajitani, K., Dan, Y., Furuichi, M., Ohno, M., Sakumi, K., et al. (2006) MTH1, an oxidized purine nucleoside triphosphatase, protects the dopamine neurons from oxidative damage in nucleic acids caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Cell Death Differ.* **13,** 551–563.
10. Alam, T. I., Kanki, T., Muta, T., Ukaji, K., Abe, Y., Nakayama, H., et al. (2003) Human mitochondrial DNA is packaged with TFAM. *Nucleic Acids Res.* **31,** 1640–1645.
11. Torisu, K., Tsuchimoto, D., Ohnishi, Y. and Nakabeppu, Y. (2005) Hematopoietic

Tissue-Specific Expression of Mouse Neil3 for Endonuclease VIII-Like Protein. *J. Biochem.* **138,** 763–772.

12. Sakumi, K., Tominaga, Y., Furuichi, M., Xu, P., Tsuzuki, T., Sekiguchi, M., et al. (2003) Ogg1 knockout-associated lung tumorigenesis and its suppression by Mth1 gene disruption. *Cancer Res.* **63,** 902–905.
13. Oka, S., Ohno, M., Tsuchimoto, D., Sakumi, K., Furuichi, M. and Nakabeppu, Y. (2008) Two distinct pathways of cell death triggered by oxidative damage to nuclear and mitochondrial DNAs. *EMBO J.* **27,** 421–432.

Chapter 14

In Vitro Measurement of DNA Base Excision Repair in Isolated Mitochondria

Melissa M. Page and Jeffrey A. Stuart

Abstract

Mitochondrial DNA (mtDNA) is in relatively close proximity to reactive oxygen species (ROS) arising from spontaneous superoxide formation during respiration. As a result, it sustains oxidative damage that may include base modifications, base loss, and strand breaks. mtDNA replication past sites of oxidative damage can result in the introduction of mutations. mtDNA mutations are associated with various human diseases and can manifest as loss of bioenergetic function. DNA repair processes exist in mitochondria from apparently all metazoans. A fully functional DNA base excision repair (BER) pathway is present in mitochondria of vertebrates. This pathway is catalyzed by a number of DNA glycosylases, an AP endonuclease, polymerase γ, and a DNA ligase. This chapter outlines the step-by-step protocols for isolating mitochondrial fractions, from a number of different model organisms, of sufficient purity to allow mtDNA repair activities to be measured. It details in vitro assays for the measurement of BER enzyme activities in lysates prepared from isolated mitochondria.

Key words: Mitochondria, mtDNA, base excision repair, BER, glycosylase, AP endonuclease, Saccharomyces cerevisiae, DNA repair.

1. Introduction

Mitochondrial DNA (mtDNA) exists in a highly oxidative environment, where it is vulnerable to being damaged by reactive oxygen species (ROS) produced during respiration. A unique aspect of mtDNA is that thousands of copies of the genome are often present within a single cell (reviewed in Ref. *(1)*). Even in post-mitotic cells, some copies are continually being replicated *(2, 3)* while others are being degraded during the process of organelle autophagy *(4)*. mtDNA replication past sites of oxidative

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_14 Springerprotocols.com

modification can lead to the introduction of mutations *(5)*, which perhaps most commonly are substitutions and deletions. While such events would be associated initially with a heteroplasmic state, subsequent selection of mutant genomes (or random intracellular drift *(6)*) can lead to a homoplasmic mutant state in individual cells *(7)*. Recently, homoplasmic mtDNA mutations associated with the loss of both mitochondrial function and cell viability have been identified in substantia nigra neurons of humans afflicted with Parkinson's disease *(8, 9)*. Thus, mtDNA mutagenesis secondary to high levels of mtDNA oxidative damage may be an important mechanism underlying mitochondrial dysfunction and cell death *(10)*.

There are several possible cellular responses to oxidative mtDNA damage that could potentially limit its deleterious impact. Complementation *(11)* between thousands of unaffected mtDNA molecules might adequately suppress the manifestation of lesions as dysfunction. It is clear from the studies outlined above that the efficacy of this in an individual cell might decline with time. Alternatively, the selective degradation of highly damaged mtDNA molecules would eliminate them from the mtDNA pool. However, data from Anson et al. *(12)* suggest that the disappearance of oxidative damage following an oxidative stress event is not due to selective degradation of damaged genomes. Finally, mtDNA damage may be repaired. There is now abundant evidence indicating that DNA repair occurs in mitochondria from all organisms that have been studied to date and that such repair is important in maintaining the levels of mtDNA oxidative damage at relatively low levels *(13)*. Indeed, in some cell types oxidative DNA damage appears to be repaired more rapidly in mitochondria than in the nucleus *(14)*.

While it is now clear that DNA repair occurs in mitochondria, it is much less clear which specific repair activities are present. A fully functional DNA base excision repair (BER) pathway, catalyzed by a number of DNA glycosylases, an AP endonuclease, polymerase γ, and a DNA ligase, has been demonstrated in all vertebrate mitochondria *(15–17)*. Studies of individual gene knockout mice have highlighted the important roles of oxoguanine DNA glycosylase (Ogg1) and uracil DNA glycosylase (UNG) in the removal of 8-oxodeoxyguanine *(13)* and uracil *(18, 19)*, respectively.

In most cases, the presence of DNA repair enzymes in mitochondria is due either to alternative splicing generating a unique mitochondrial isoform *(20)* or the presence of ambiguous signaling sequences causing the same protein to be targeted to the nucleus and mitochondrial compartments *(21)*. Thus, as mitochondrial DNA repair enzymes may be identical, or nearly so, to their nuclear counterparts, it is necessary to account for potential nuclear contamination.

In this chapter we outline the basic protocols for isolating highly pure mitochondria from a number of different sources, assessing the purity of the mitochondrial fraction, and carrying out in vitro assays of BER in mitochondrial lysates.

2. Materials

2.1. Isolation of Mitochondria and Preparation of Lysates

2.1.1. From Mammalian Tissues and Cultured Cells

1. Phosphate-buffered saline (PBS).
2. MSHE buffer: 210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EGTA, 2 mM EDTA, 0.15 mM spermine, 0.75 mM spermidine, 5 mM dithiothreitol (DTT), 2 μg/ml leupeptin, 2 μg/ml benzamide, 1 μM E-64, and 1 mM phenylmethylsulfonyl fluoride (PMSF) (*see* **Note 1**), pH 7.4.
3. Cell scrapers (for adherent cultured cells).

2.1.2. From Saccharomyces cerevisiae

1. Pre-spheroplasting (PSB) buffer: 100 mM Tris, 10 mM dithiothreitol (DTT) (*see* **Note 2**), pH 9.4.
2. Spheroplasting (SP) buffer: 1.2 M sorbitol, 25 mM potassium phosphate, pH 7.4.
3. Mitochondrial isolation (MI) buffer: 0.6 M sorbitol, 20 mM HEPES–KOH, pH 7.4.
4. Complete® protease inhibitor tablets (Amersham).

2.1.3. From All Cells and Tissues

1. Percoll gradient buffer: 1:1 (v:v) 2X MSHE and Percoll.
2. Mitochondrial lysis buffer: 20 mM HEPES, 1 mM EDTA, 5% glycerol, 0.2% Triton X-100, 300 mM KCl, 5 mM DTT, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml chymostatin A, 2 μg/ml leupeptin, 2 μM benzamide, 1 mM PMSF, and 1 μM E-64 (*see* **Note 1**).
3. Proteinase K.
4. Centrifuge tubes, 15 ml.
5. Potter-Elvehjem homogenizer.
6. Vortexer.

2.2. Assessing Nuclear Contamination

1. Antibodies to nuclear lamin B, proliferating cell nuclear antigen (PCNA), polymerase β, or other sensitive markers of nuclear contamination.
2. Antibody to cytochrome oxidase subunit IV or other specific marker of mitochondrial fraction.
3. Secondary antibodies.
4. Electrophoresis unit with electroblotting accessory.

5. Odyssey fluorescence scanner for visualizing and quantifying Western blots.

2.3. DNA Repair Assays

1. Pure DNA repair enzymes (positive controls): Uracil DNA glycosylase (UDG); oxoguanine DNA glycosylase (OGG1); apurinic/apyrimidinic endonuclease (APE1); polymerase γ.
2. Oligonucleotides used in mtDNA repair assays (**Table 14.1**).

^{32}P-labeling of oligonucleotide substrates:

3. Oligonucleotide (*see* **Table 14.1**).
4. 10X T4 polynucleotide kinase buffer.
5. [γ-^{32}P]ATP.
6. T4 polynucleotide kinase enzyme.

Reaction constituents:

7. **UDG activity assay:** 90 fmol ^{32}P-end-labeled uracil-containing oligonucleotides (UDG; **Table 14.1**), 70 mM HEPES–KOH (pH 7.5), 1 mM EDTA, 1 mM DTT, 75 mM NaCl, 0.05% BSA, 4 ng of recombinant endonuclease IV.
8. **OGG1 activity assay:** 90 fmol ^{32}P-end-labeled annealed oxoguanine-containing oligonucleotides (OG; **Table 14.1**), 40 mM HEPES–KOH (pH 7.6), 5 mM EDTA, 1 mM DTT, 75 mM KCl, 10% glycerol.
9. **AP endonuclease assay:** 1 pmol ^{32}P-end-labeled annealed THF-containing oligonucleotides (THF; **Table 14.1**), 50 mM HEPES–KOH (pH 7.5), 50 mM KCl, 100 μg/ml BSA, 10 mM $MgCl_2$, 10% glycerol, 0.05% Triton X-100.
10. **Polymerase γ gap-filling assay:** 1 pmol gapped annealed oligonucleotide (GAP; **Table 14.1**), 40 mM HEPES–KOH (pH 7.5), 0.1 mM EDTA, 5 mM $MgCl_2$, 0.2 mg/ml BSA, 50 mM KCl, 1 mM DTT, 40 mM phosphocreatine, 100 μg/ml creatine phosphokinase, 2 mM ATP, 40 μM dCTP, 4 μCi [α-^{32}P]dCTP, 3% glycerol.
11. **Assay termination solution:** 5 mg/ml proteinase K, 10% SDS.
12. **DNA precipitation:** 95% ethanol, 1 μg/μl glycogen, 11 M ammonium acetate, 70% ethanol, formamide loading buffer.
13. **Oligonucleotide gel loading dye:** formamide loading buffer (80% formamide, 10 mM EDTA, 1 mg/ml xylene cyanol FF, 1 mg/ml bromophenol blue).

2.4. Gel Electrophoresis of Oligonucleotide Products

1. Denaturing acrylamide solution (20% acrylamide; 19:1 acrylamide:bisacrylamide), 7 M urea.

Table 14. 1
Oligonucleotides used to measure activities of mitochondrial DNA base excision repair in vitro

Assay	Name	Sequence
Uracil DNA glycosylase	UDG	5′-ATATACCGCGG(**U**)CGGCCGATCAAGCTTATT-3′
	UDG complement (*see* **Note 8**)	5′-AATAAGCTTGATCGGCCGGCCGCGGTATAT-3′
	UDG control	5′-ATATACCGCGGCCGGCCGATCAAGCTTATT-3′
Oxoguanine DNA glycosylase	OG	5′-GAACGACTGT(**OG**)ACTTGACTGCTACTGAT-3′
	OG complement	5′-ATCAGTAGCAGTCAAGTCACAGTCGTTC-3′
	OG control	5′-GAACGACTGTCACTTGACTGCTACTGAT-3′
AP endonuclease	Tetrahydrofuran (THF)	5′-ATATACCGCGG(**THF**)CGGCCGATCAAGCTT ATT-3′
	THF complement	5′-AATAAGCTTGATCGGCCGGCCGCGGTATAT-3′
	THF control	5′-ATATACCGCGGCCGGCCGATCAAGCTTATT-3′
Polymerase γ	GAP 15mer and 19mer complements	15mer – 5′-CTGCAGCTGATGCGC-OH-3′ 19mer – 5′-P-GTACGGATCCCCGGGTTAC-3′
	GAP complement	5′-GTAACCCGGGGATCCGTACGGCGCAT CAGCTGCAG-3′
	GAP control	5′-CTGCAGCTGATGCGCCGTACGGATCCCCGGGTTAC-3′

2. 10X TBE stock solution (Tris-base, boric acid, 0.5 M EDTA (pH 8.0)).
3. 40% Acrylamide solution.
4. 0.2-μm Pore size vacuum filter.
5. Filter vacuum system.
6. *Denaturing gel:* Denaturing acrylamide solution, 10% ammonium persulfate, TEMED.
7. Electrophoresis buffer: 0.5X TBE (45 mM Tris–borate, 1 mM EDTA).
8. 3 Heat blocks, set to 37, 55, and 90°C.
9. Radiation containment: minimum 7-mm thick plexiglass shielding for all work areas and storage containers.
10. Gel electrophoresis apparatus.

2.5. Gel Imaging and Analysis

1. Storage phosphor screens.
2. Autoradiography cassettes.
3. Plastic wrap.
4. Phosphorimager (Fujifilm FLA-3000 or Molecular Dynamics Storm 860).
5. ImageGauge™ or ImageQuant™ software.
6. Light box for erasing storage phosphor screens.

3. Methods

3.1. Isolation of Mitochondria

Different protocols are required for the isolation of mitochondria from different organisms and tissues *(22, 23)*. In all cases, it is necessary to begin with fresh (not frozen) material and maintain the integrity of the organelle throughout the separation process. Also, it is essential to separate the mitochondrial fraction from potential nuclear contamination (*see* **Fig. 14.1** for an overview). The methods described below for isolating mitochondrial fractions essentially free from nuclear contamination for the measurement of mitochondrial base excision repair are of course applicable also to studies of other mitochondrial DNA transactions.

3.1.1. Isolation of Crude Mitochondrial Fraction from Mouse Liver (All Steps on Ice or at 4°C)

1. Excise liver and mince with a razor blade on a cold (4°C) surface.
2. Place minced liver into 10 volumes (w/v) of MSHE buffer in a 15-ml centrifuge tube (*see* **Note 3**).
3. Centrifuge at 1,000 × g for 10 min at 4°C.
4. Discard supernatant (containing blood).

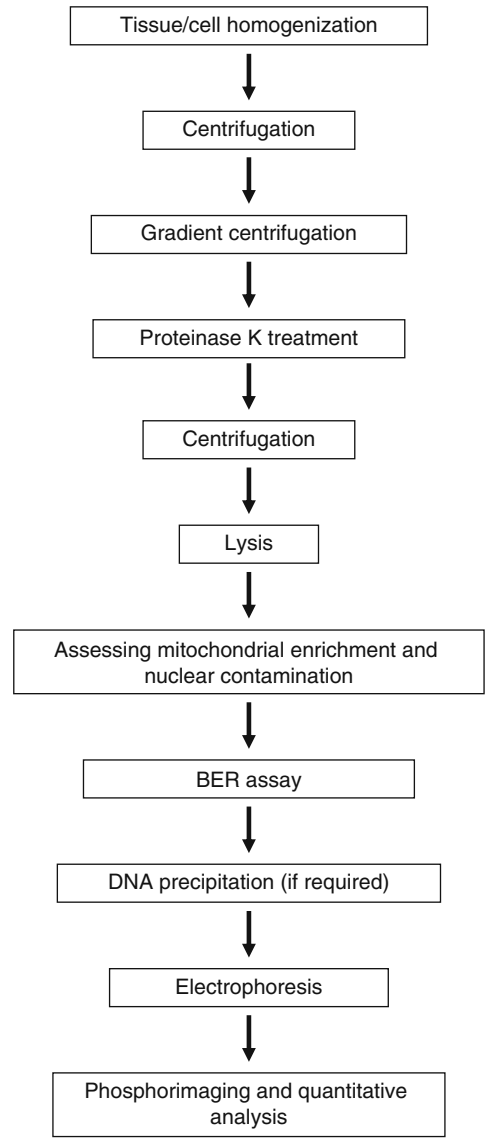


Fig. 14.1. Steps involved in preparing mitochondrial lysates for measuring base excision repair activity in isolated mitochondria.

5. Add 10 volumes of ice-cold MSHE buffer and transfer to a pre-cooled Potter-Elvehjem tissue homogenizer (*see* **Note 4**).
6. Homogenize using 20 passes of the pestle.
7. Transfer to 15-ml tubes and centrifuge at 500 × g for 10 min (*see* **Note 5**).
8. Transfer supernatant to a fresh, pre-cooled 15-ml centrifuge tube. Discard pellet.
9. Centrifuge again at 500 × g for 10 min.

10. Transfer supernatant to a fresh, pre-cooled 15-ml centrifuge tube. Discard pellet.
11. Centrifuge supernatant at 10,000 × g for 10 min.
12. Discard supernatant. Resuspend mitochondrial pellet in 250 µl MSHE buffer.
13. Go to **Section 3.1.4.**

3.1.2. Isolation of Crude Mitochondrial Fraction from Cultured Fibroblasts

1. Harvest cells by scraping into 15-ml centrifuge tubes. Add 20 volumes of PBS.
2. Centrifuge at 250 × g for 3 min. Discard supernatant.
3. Add 20 volumes of fresh PBS and repeat centrifugation. Discard supernatant.
4. Add cells to 20 volumes of ice-cold MSHE buffer and transfer to a Potter-Elvehjem homogenizer.
5. Homogenize using 20 passes of the pestle.
6. Transfer homogenate to a 15-ml centrifuge tube and centrifuge at 500 × g for 10 min.
7. Transfer supernatant to fresh 15-ml centrifuge tube and store on ice.
8. Resuspend pellet in 20 volumes of ice-cold MSHE buffer and transfer to a Potter-Elvehjem homogenizer.
9. Repeat Steps 5 and 6. Pool the supernatant with the previous supernatant that has been stored on ice.
10. Centrifuge supernatants at 500 × g for 10 min.
11. Discard pellet and repeat Step 10.
12. Transfer supernatant to a fresh 15-ml centrifuge tube. Discard pellet.
13. Centrifuge supernatant at 10,000 × g for 10 min.
14. Go to **Section 3.1.4**

3.1.3. Isolation of Crude Mitochondrial Fraction from S. cerevisiae

1. Transfer liquid cultures to 250-ml centrifuge jars and centrifuge at 2,500 × g for 10 min.
2. Discard supernatant and wash by adding 250 ml of Milli-Q grade water. Repeat centrifugation.
3. Repeat Step 2.
4. Suspend cells in PSB and incubate at 30°C with gentle shaking for 10 min.
5. Centrifuge at 2,500 × g for 5 min. Discard supernatant.
6. Resuspend cells in 50 ml spheroplasting buffer and centrifuge as in Step 5.
7. Repeat Step 6.

8. Resuspend cells in 50 ml spheroplasting buffer and add lyticase at 3 mg/ml.
9. Incubate at 30°C with periodic mixing for 10–30 min, while regularly checking for spheroplasting (*see* **Note 6**).
10. When cells have spheroplasted, centrifuge at 2,500 × g at 4°C for 10 min.
11. Resuspend in SP buffer and repeat centrifugation.
12. Repeat Step 11.
13. Discard supernatant. Resuspend mitochondrial pellet in 10 ml ice-cold MI buffer. Add one Complete Protease Inhibitor tablet per 50 ml MI buffer (*see* **Note 7**).
14. Homogenize with 20 passes of a Potter-Elvehjem homogenizer.
15. Transfer to a 15-ml centrifuge tube and centrifuge at 1,000 × g for 10 min.
16. Transfer supernatant to a fresh 15-ml centrifuge tube and store on ice.
17. Resuspend pellet in 10 ml MI buffer and repeat Steps 14–16.
18. Pool supernatant with stored supernatant. Centrifuge at 10,000 × g for 10 min.
19. Discard supernatant. Resuspend pellet in 10 ml MI buffer and repeat centrifugation at 10,000 × g.
20. Discard supernatant. Resuspend pellet in 250 µl of MI buffer and further purify through Percoll gradient centrifugation, as outlined below (but *see* **Note 8**).

3.1.4. Further Purification of Mitochondria to Remove Nuclear Contamination

Most crude mitochondrial fractions will be substantially contaminated with nuclear proteins, though this varies with tissue type. Thus, it is usually essential to further purify the mitochondrial fraction. This can be done by density gradient centrifugation combined with the use of proteases. It is essential to maintain the integrity of the mitochondrial membranes (particularly inner) so that mitochondrial BER enzymes within the matrix are protected from protease degradation, while nuclear contaminants adhered to the outer surface of the outer membrane will be digested.

1. Layer the mitochondrial suspension on top of 10 ml of Percoll gradient buffer in a centrifuge tube and centrifuge at 50,000 × g for 1 h at 4°C.
2. Remove the beige mitochondrial band from near the bottom third of the gradient, and place into a fresh, pre-cooled 15-ml centrifuge tube.
3. Add 20 volumes of MSHE buffer, mix well (by hand), and wash by centrifugation at 10,000 × g for 10 min. Discard supernatant.

4. Repeat Step 3.
5. Resuspend mitochondrial pellet in 100 µl of MSHE in a 1.5-ml centrifuge tube.
6. Add proteinase K to a final concentration of 50 units/ml and incubate suspension on ice for 45 min.
7. Add PMSF to 1 mM to inhibit proteinase K.
8. Add MSHE to 1.5 ml.
9. Centrifuge at 10,000 × g for 10 min.
10. Repeat Steps 8 and 9 twice to remove all traces of proteinase K.
11. Resuspend mitochondrial pellet in 100 µl of MSHE.

3.1.5. Lysis of Mitochondria

1. To 50 µl of mitochondrial suspension add 200 µl of mitochondrial lysis buffer (*see* **Note 13**).
2. Mix by inversion and incubate on ice for 30 min, occasionally vortexing at lowest speed.
3. Centrifuge at 50,000 × g for 20 min.
4. Transfer supernatant to a new 1.5-ml centrifuge tube. Discard pellet.
5. Measure protein concentration of supernatant. Aliquot and use immediately or store at –80°C.

3.2. Assessing Mitochondrial Enrichment and Nuclear Contamination

Sensitive probing of mitochondrial enrichment and nuclear contamination can be done using Western blot. Cytochrome oxidase subunit IV antibody provides a useful means to gauge mitochondrial enrichment. The nuclear lamin B *(13)*, proliferating cell nuclear antigen (PCNA) *(19)*, and polymerase β *(24)* have all been used successfully to detect and quantify nuclear contamination of mitochondrial preparations. Generally, any nuclear marker that can be sensitively detected and quantified in the mitochondrial fraction can be used for this purpose.

3.3. Measurement of DNA BER Enzyme Activities

The activities of individual BER enzymes are measured by incubating mitochondrial lysates with a ^{32}P-labeled oligonucleotide containing a single-base modification, abasic site, or nucleotide gap at a defined position. The assay is done in 1.5-ml centrifuge tubes incubated in heat blocks, and terminated at different times so that a reaction rate can be calculated. The oligonucleotide substrates and products are separated electrophoretically and then identified and quantified by phosphorimaging. For DNA glycosylases and AP endonuclease, enzyme activities are calculated as the slope of the line describing % substrate incision over time. Below the protocols used to measure the activities of the DNA glycosylases OGG1 and UDG, as well as AP endonuclease are described.

3.3.1. Preparation of Denaturing Acrylamide Solution (20%)

1. Weigh out 210.12 g of 7 M urea.
2. Add 50 ml of 10X TBE solution, 250 ml of 40% acrylamide solution, and ddH_2O to 500 ml and stir until fully dissolved.
3. Filter using 0.2-μm pore size vacuum filter.
4. Store at rt up to 1 month, cover with tinfoil to reduce light exposure.

3.3.2. Preparation of Formamide Loading Buffer

1. 20 g Resin (Bio-Rad AG 501-X8 (D)).
2. 40 ml Formamide.
3. Stir until resin is golden brown.
4. Filter through 0.22-μm pore size syringe filter.
5. Add 0.8 ml of 0.5 M EDTA, 6 mg of xylene cyanol, and 6 mg of bromophenol blue.

3.3.3. 5′-End-Labeling of Oligonucleotide Size Marker with [γ-^{32}P]ATP

1. Add the following to a 1.5-ml centrifuge tube for a 10 μl reaction: 1 μ l oligonucleotide size marker (8–32 bases) for a final concentration of 10%, 5 μ l of ddH_2O, 1 μ l of 10X T4 polynucleotide kinase buffer, 1 μ l of 10U T4 polynucleotide kinase enzyme, 1 μ l of 2 μ Ci [γ-^{32}P]ATP (*see* **Note 10**).
2. Spin down briefly and incubate at 37°C for 30 min.
3. Add 20 μl of formamide loading buffer and incubate at 90°C for 10 min.
4. Store at –20°C (good for 1 month).

3.3.4. DNA Glycosylase Assays

3.3.4.1. 5′-End-Labeling ssDNA Substrate with [γ-^{32}P]ATP for DNA Glycosylase Activity

1. Add the following to a 1.5-ml centrifuge tube for a 10 μl reaction: 4,500 fmol of OG or UDG oligonucleotide (**Table 14.1**), 6 μl of ddH_2O, 1 μl of 10X polynucleotide kinase buffer, 1 μl of T4 polynucleotide kinase enzyme, and 1 μl of 10 μCi [γ-^{32}P]ATP (*see* **Note 10**).
2. Spin down and incubate at 37°C for 30 min.
3. Incubate at 90°C for 10 min.
4. Add 2X OG or UDG complement oligonucleotide (**Table 14.1**, but *see* **Note 9**) and ddH_2O for a total of 10 μl.
5. Incubate at 90°C for 3 min.
6. Remove heat block from dry incubator and allow the reaction to reach rt.
7. Store at –20°C. Final annealed oligonucleotide concentration is 225 fmol/μl.
8. Repeat Steps 1–7 for a control oligonucleotide.

3.3.4.2. OGG1 Activity

1. A 10 μl reaction contains 40 mM HEPES–KOH (pH 7.6), 5 mM EDTA, 1 mM DTT, 75 mM KCl 10% glycerol, 90 fmol annealed 5′ [γ-^{32}P]ATP-OG oligonucleotide (or annealed control oligonucleotide) and 10 μg mitochondrial protein (*see* **Note 11**).

2. Incubate each reaction at 37°C for 1, 2, and 4 h (activity will be calculated as the slope of the line from zero to 4 h).
3. Terminate the reactions with 5 μg of proteinase K and 1 μl of 10% SDS and incubate at 55°C for 30 min.
4. Precipitate the DNA with 1 μg of glycogen, 4 μl of 11 M ammonium acetate, 30 μl of ice-cold 90% ethanol. Incubate overnight at –20°C.
5. Centrifuge at 16,000 × g at 4°C for 60 min.
6. Remove supernatant.
7. Add 500 μl of 70% ice-cold ethanol and centrifuge at 16,000 × g at 4°C for 12 min.
8. Remove supernatant to approximately 20 μl. Dry in a speed vacuum for 45 min or until dry.
9. Add 20 ml formamide loading dye and incubate at 90°C for 10 min.
10. Load samples onto a 20% denaturing acrylamide gel.
11. Go to **Section 3.3.7**.

3.3.4.3. UDG Activity

1. A 10 μl reaction contains 70 mM HEPES–KOH (pH 7.5), 1 mM EDTA, 1 mM DTT, 75 mM NaCl, 0.05% BSA, 4 ng recombinant endonuclease IV, 90 fmol annealed 5′ [γ-^{32}P]ATP–UDG oligonucleotide (or annealed control oligonucleotide), and 1 μg mitochondrial protein (*see* **Note 11**).
2. Incubate each reaction at 37°C for 10, 20, and 30 min (activity will be calculated as the slope of the line from 0 to 30 min).
3. Terminate the reactions with 20 μl of formamide loading dye and incubate at 90°C for 10 min.
4. Load samples onto a 20% denaturing acrylamide gel.
5. Go to **Section 3.3.7**.

3.3.5. AP Endonuclease Assay

3.3.5.1. 5′-End-Labeling ssDNA Substrate with [γ-^{32}P]ATP for AP Endonuclease Activity

1. Add the following to a 1.5-ml centrifuge tube for a 10 μl reaction: 20 pmol of THF oligonucleotide (**Table 14.1**), 5 μl of ddH_2O, 1 μl of 10X T4 polynucleotide kinase buffer, 1 μl of T4 polynucleotide kinase enzyme, 1 μl of 2 μCi [γ-^{32}P]ATP.
2. Spin down and incubate at 37°C for 30 min.
3. Incubate at 90°C for 10 min.
4. Add 2X THF complement oligonucleotide (**Table 14.1**) and ddH_2O for a total of 10 μl.
5. Incubate at 90°C for 3 min.
6. Remove heat block from dry incubator and allow the reaction to reach rt.
7. Store at –20°C. Final dsDNA oligonucleotide concentration is 1 pmol/μl.
8. Repeat Steps 1–7 for a control oligonucleotide.

3.3.5.2. AP Endonuclease Activity

1. A 10 μl reaction contains 50 mM HEPES–KOH (pH 7.5), 50 mM KCl, 100 μg/ml BSA, 10 mM $MgCl_2$, 10% glycerol, 0.05% Triton X-100, 1 pmol annealed 5′ [γ-^{32}P]ATP–THF oligonucleotide (or annealed control oligonucleotide), and 50 ng mitochondrial protein (*see* **Note 11**).
2. Incubate each reaction at 37°C for 5, 10, and 15 min (activity will be calculated as the slope of the line from 0 to 15 min).
3. Terminate the reactions with 10 μl of formamide loading dye and incubate at 90°C for 10 min.
4. Load samples onto a 20% denaturing acrylamide gel.
5. Go to **Section 3.3.7**

3.3.6. Polymerase γ Gap-Filling Assay

The rate of incorporation of a ^{32}P-labeled nucleotide into a short oligonucleotide (**Table 14.1**) containing a single-nucleotide gap is measured by incubating the mitochondrial lysate with the GAP oligonucleotide. The reaction is completed in a series of 1.5-ml centrifuge tubes, with termination at different times. The reaction products are identified and quantified by electrophoresis and phosphorimaging, respectively. Two bands, one representing nucleotide incorporation without ligation, and the other incorporation and ligation, will be discerned. Activity can be calculated from the lower band, representing incorporation without ligation, which under the conditions outlined below is much more prominent. Alternatively, excess T4 DNA ligase can be added to the assay to promote the completion of repair synthesis, in which case a single 35-nt band is prominent. Activity is calculated from the slope of the line obtained by plotting the gain in signal intensity of incorporated nucleotide with time. The removal of uracil followed by strand repair can also be measured essentially the same way, but using the duplex oligonucleotide containing uracil (**Table 14.1**). The basic protocol for both assays is outlined below.

3.3.6.1. Preparation of Duplexed Oligonucleotide with Single-Nucleotide Gap

1. Add the following to a 1.5-ml centrifuge tube to anneal at 50 pmol for a 10 μl reaction: 10 pmol of 34mer GAP oligonucleotide, 10 pmol of 35mer GAP complement oligonucleotide, 10 pmol of 19mer GAP oligonucleotide (**Table 14.1**), and 7 μl of ddH_2O.
2. Incubate at 90°C for 3 min.
3. Remove heat block from dry incubator and allow the reaction to reach rt.
4. Store at –20°C. Final dsDNA oligonucleotide concentration is 1 pmol/μl.
5. Repeat Steps 1–4 for control oligonucleotide, using 10 pmol of 35mer GAP complement oligonucleotide instead of 15mer and 19mer oligonucleotides.

3.3.6.2. Polymerase γ Activity

1. A 10 µl reaction contains 40 mM HEPES–KOH (pH 7.5), 0.1 mM EDTA, 5 mM $MgCl_2$, 0.2 mg/ml BSA, 50 mM KCl, 1 mM DTT, 40 mM phosphocreatine, 100 µg/ml creatine phosphokinase, 2 µM ATP, 40 µM dCTP, 4 µCi [α-^{32}P]dCTP, 3% glycerol, 1 pmol annealed GAP oligonucleotide, and 2 µg mitochondrial protein (*see* **Note 11**).
2. Incubate each reaction at 37°C for 30, 60, and 90 min (activity will be calculated as the slope of the line from 0 to 90 min).
3. Terminate the reactions with 5 µg of proteinase K and 1 µl of 10% SDS and incubate at 55°C for 30 min.
4. Precipitate the DNA with 1 µg of glycogen, 4 µl of 11 M ammonium acetate, 30 µl of ice-cold 90% ethanol. Incubate overnight at –20°C **(***see* **Note 11).**
5. Centrifuge at 16,000 × g at 4°C for 1 h.
6. Remove supernatant.
7. Add 500 µl of 70% ice-cold ethanol and centrifuge at 16,000 × g at 4°C for 12 min.
8. Remove supernatant to approximately 20 µl. Dry in a speed vacuum for 45 min or until dry.
9. Add 20 ml of formamide loading dye and incubate at 90°C for 10 min.
10. Load samples onto a 20% denaturing acrylamide gel.

3.3.7. Electrophoresis of DNA

The substrates and products of all activity assays are electrophoresed for 2 h at 20 W. Immediately prior to loading samples onto the gel, it is important that the wells are thoroughly rinsed with 0.5X TBE to clear accumulated urea that will negatively affect resolution. Following electrophoresis, gel(s) are removed from the apparatus, covered with plastic wrap to contain radiation, and placed on a storage phosphor screen in an autoradiography cassette. Prior to exposing a storage phosphor screen to the gel overnight at 4°C ensure that the screen has been blanked for at least 30 min on an appropriate light box.

3.3.8. Phosphorimaging and Analytical Quantification

After scanning the screens, ImageGauge^{TM} software can be used to analyze the substrate and product-band intensities. Band intensities are corrected for background intensity. Incision assays (DNA glycosylases and AP endonuclease activities) are quantified as the increase in intensity of the product bands relative to the combined intensity of the substrate (higher molecular weight band) and product band (lower molecular weight band; *see* **Fig. 14.2**).

Sample	Signal intensity[a]	Sum	% Incision	Time (min)
			0	0
5 min Substrate	757.7	794.44	4.62	5
5 min Product	36.74			
10 min Substrate	555.11	618.61	10.26	10
10 min Product	63.5			
15 min Substrate	644.71	726.42	11.24	15
15 min Product	81.71			
			Slope	0.7877
			Activity ($\mathbf{pmol \cdot min^{1} \cdot \mu g^{1}}$)	0.1574

[a]Corrected for background signal intensity.

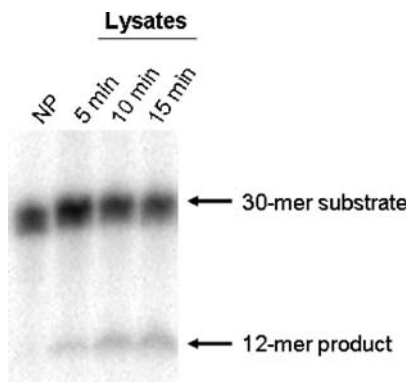


Fig. 14.2. Sample gel showing measurement of AP endonuclease incision activity. A 1 pmol double-stranded oligonucleotide (30mer) containing THF at position 12 (*see* **Table 14.1**) is incubated for 5, 10, or 15 min with mitochondrial lysate. No protein (NP).

Percent incision is plotted against time, and activity is calculated as the slope of this line (*see* **Note 12**). Sample calculations are shown below for an AP endonuclease reaction:

To calculate the % incision for the 5 min reaction

$$\begin{aligned} \%\ \text{Incision} &= \text{product}/(\text{product} + \text{substrate}) \\ &= 36.74/(36.74 + 757.7) \\ &= 4.62\% \end{aligned}$$

The activity of AP endonuclease of this sample over the three time-points can be calculated as:

Sample	Signal intensity[a]	Time (min)
	0	0
Sample 1	262.54	10
	451.34	20
	523.74	30
	Slope	17.6
	Activity (Δ signal intensity·min^{1}·μg^{1})	8.8

[a]Corrected for background signal intensity.

$$\begin{aligned}\text{Activity} &= (\text{ slope }\%) \times ([\text{labeled oligonucleotide}]/\text{amount mitochondrial protein}).\\ \text{Activity} &= (0.7877/100) \times (1\text{ pmol}/0.05\mu\text{g})\\ &= (0.007877) \times (20)\\ &= 0.15754\text{ pmol} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}\end{aligned}$$

Polymerase γ gap-filling activity can be calculated as the increase in signal intensity of the band representing incorporation of [^{32}P]dCTP. The signal intensity of the incorporation of [^{32}P]dCTP is plotted against time, and the activity is calculated as the slope of this line (*see* **Note 12**). A sample calculation of a polymerase γ reaction is shown below:

$$\begin{aligned}\text{Gap}-\text{filling activity} &= \text{slope}/\text{amount mitochondrial protein}\\ &= 17.6/2\mu\text{g}\\ &= 8.8\end{aligned}$$

If polymerase γ activity is measured over several days, the polymerase γ activity must be corrected for the decay of [^{32}P]dCTP by multiplying signal intensities measured on subsequent days by the following correction:

$$2^{n}, \text{where } n = (\text{elapsed time in days})/(14.3)$$
$$(14.3\text{ is the half}-\text{life of}^{32}\text{P})$$

4. Notes

1. Protease inhibitors must be added to the buffer immediately prior to use.
2. A 1 M stock of DTT can be prepared and stored in frozen aliquots at –20°C.

3. Choose tubes designed to withstand centrifugation up to 15,000 × g.
4. The tightness of fit of the homogenizer is important. The entire unit should be pre-cooled. When the homogenizer is filled with water or buffer, the pestle should slide easily without "rattling." This will give the appropriate clearance between the vessel and the pestle to disrupt cells while minimizing damage to mitochondria.
5. Unless otherwise indicated centrifugation is carried out at room temperature.
6. To check for spheroplasting, pipette 10 μl of suspension into each of: 1 ml water and 1 ml SP buffer and observe under a microscope or measure absorbance at 600 nm. If spheroplasted, the cells placed in water will swell and lyse, clarifying the solution, whereas cells in SP buffer will remain intact and either be visible under a microscope or scatter light at 600 nm.
7. Add the protease inhibitor tablet just prior to use. Prepare 50 ml of MI buffer in a beaker or 50-ml conical tube. Add the tablet and mix well. Store on ice until use (within 2 h).
8. For yeast mitochondria, substitute 2X MI buffer for 2X MSHE. Similarly, substitute MI buffer for MSHE buffer throughout protocol.
9. Uracil DNA glycosylase activity can be measured using either ssDNA or dsDNA.
10. Add T4 polynucleotide kinase enzyme and [γ-^{32}P]ATP to the reaction last. Also all work with radiolabeled nucleotides and oligonucleotides should be conducted in a certified radioactive location.
11. For all reactions, the following controls should be run: (1) all reaction constituents, but substrate oligonucleotide replaced with control oligonucleotide (without damage) (*see* **Table 14.1**); (2) all reaction constituents but no mitochondrial lysate; and (3) all reaction constituents, but mitochondrial lysate protein replaced with pure enzyme.
12. *R*-square values for the slopes in AP endonuclease and polymerase γ activity assays should be between 0.95 and 0.99.
13. A relatively high ionic strength (KCl) is necessary to dissociate BER enzymes from a particulate fraction that will sediment at the centrifugation speed used here *(23)*.

Acknowledgments

The authors gratefully acknowledge the support of the Natural Sciences and Engineering Research Council of Canada, the Canada Foundation for Innovation, and the Ontario Innovation Trust.

References

1. Moraes, C.T. (2001) What regulates mitochondrial DNA copy number in animal cells? Trends Genet **17**, 199–205.
2. Gross, N.J., Getz, G.S., Rabinowitz, M. (1969) Apparent turnover of mitochondrial deoxyribonucleic acid and mitochondrial phospholipids in the tissues of the rat. J Biol Chem **244**, 1552–1562.
3. Menzies, R.A., Gold, P.H. (1971) The turnover of mitochondria in a variety of tissues of young adult and aged rats. J Biol Chem **246**, 2425–2429.
4. Kim, I., Rodriguez-Enriquez, S., Lemasters, J.J. (2007) Selective degradation of mitochondria by mitophagy. Arch Biochem Biophys **462**, 245–253.
5. Pinz, K.G., Shibutani, S., Bogenhagen, D.F. (1995) Action of mitochondrial DNA polymerase gamma at sites of base loss or oxidative damage. J Biol Chem **270**, 9202–9206.
6. Elson, J.L., Samuels, D.C., Turnbull, D.M., Chinnery, P.F. (2001) Random intracellular drift explains the clonal expansion of mitochondrial DNA mutations with age. Am J Hum Genet **68**, 802–806.
7. Mambo, E., Gao, X., Cohen, Y., Guo, Z., Talalay, P., Sidransky, D. (2003) Electrophile and oxidant damage of mitochondrial DNA leading to rapid evolution of homoplasmic mutations. Proc Natl Acad Sci USA **100**, 1838–1843
8. Bender, A., Krishnan, K.J., Morris, C.M., Taylor, G.A., Reeve, A.K., Perry, R.H., Jaros, E., Hersheson, J.S., Betts, J., Klopstock, T., Taylor, R.W., Turnbull, D.M. (2006) High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. Nat Genet **38**, 515–517.
9. Kratysberg, T., Kudryavtseva, E., McKee, A.C., Geula, C., Kowall, N.W., Khrapko, K. (2006) Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons. Nat Genet **38**, 518–520.
10. Krisham, K.J., Reeve, A.K., Samuels, D.G., Chinnery, P.F., Blackwood, J.K., Taylor, R.W., Wanrooij, S., Spelbrink, J.N., Lightowlers, R.N., Turnbull, D.M. (2008) What causes mitochondrial DNA deletions in human cells? Nat Genet **40**, 275–279.
11. Gilkerson, R.W., Schon, E.A., Hernandez, E., Davidson, M.M. (2008) Mitochondrial nucleoids maintain genetic autonomy but allow for functional complementation. J Cell Biol **181**, 1117–1128.
12. Anson, R.M., Croteau, D.L., Stierum, R.H., Filburn, C., Parsell, R., Bohr, V.A. (1998) Homogenous repair of singlet oxygen-induced DNA damage in differentially transcribed regions and strands of human mitochondrial DNA. Nucleic Acids Res **26**, 662–668.
13. de Souza-Pinto, N.C., Eide, L., Hogue, B.A., Thybo, T., Stevnsner, T., Seeberg, E., Klungland, A., Bohr, V.A. (2001) Repair of 8-oxodeoxyguanosine lesions in mitochondrial DNA depends on the oxoguanine DNA glycosylase (OGG1) gene and 8-oxoguanine accumulates in the mitochondrial DNA of OGG1-defective mice. Cancer Res **61**, 5378–5381.
14. Thorslund, T., Sunesen, M., Bohr, V.A., Stevnsner, T. (2002) Repair of 8-oxoG is slower in endogenous nuclear genes than in mitochondrial DNA and is without strand bias. DNA Repair **1**, 261–273.
15. LeDoux, S.P., Wilson, G.L., Beecham, E.J., Stevnsner, T., Wassermann, K., Bohr V.A. (1992) Repair of mitochondrial DNA after various types of DNA damage in Chinese hamster ovary cells. Carcinogenesis **13**, 1967–1973.
16. Pinz, K.G., Bogenhagen, D.F. (1998) Efficient repair of abasic sites in DNA by mitochondrial enzymes. Mol Cell Biol **18**, 1257–1265

17. Nyaga, S.G., Bohr, V.A. (2002) Characterization of specialized mtDNA glycosylases. Methods Mol Biol **197**, 227–244.
18. Endres, M., Biniszkiewicz, D., Sobol, R.W., Harms, C., Ahmadi, M., Lipski, A., Katchanov, J., Mergenthaler, P., Dirnagl, U., Wilson, S.H., Meisel, A., Jaenisch, R. (2004) Increased postischemic brain injury in mice deficient in uracil-DNA glycosylase. J Clin Invest. **113**, 1711–1721.
19. Akbari, M., Visnes, T., Krokan, H.E., Otterlei, M. (2008) Mitochondrial base excision repair of uracil and AP sites takes place by single-nucleotide insertion and long-patch DNA synthesis. DNA Repair **7**, 605–616.
20. Nakabeppu, Y. (2001) Regulation of intracellular localization of human MTH1, OGG1, and MYH proteins for repair of oxidative DNA damage. Prog Nucleic Acid Res Mol Biol **68**, 75–94.
21. Mitra, S., Izumi, T., Boldogh, I., Bhakat, K.K., Chattopadhyay, R., Szczesny, B. (2007) Intracellular trafficking and regulation of mammalian AP-endonuclease 1 (APE1), an essential DNA repair protein. DNA Repair **6**, 461–469.
22. Stuart, J.A., Karahalil, B., Hogue, B.A., Souza-Pinto, N.C., Bohr, V.A. (2004) Mitochondrial and nuclear DNA base excision repair are affected differently by caloric restriction. FASEB J **18**, 595–597.
23. Stuart, J.A., Maynard, S., Hashiguchi, K., Souza-Pinto, N.C., Bohr, V.A. (2005) Localization of mitochondrial base excision repair to an inner membrane-associated particulate fraction. Nucleic Acids Res **33**, 3722–3732.
24. Hansen, A.B., Griner, N.B., Anderson, J.P., Kujoth, G.C., Prolla, T.A., Loeb, L.A., Glick, E. (2006) Mitochondrial DNA integrity is not dependent on DNA polymerase-β activity. DNA Repair **5**, 71–79.

Chapter 15

Targeting Repair Proteins to the Mitochondria of Mammalian Cells Through Stable Transfection, Transient Transfection, Viral Transduction, and TAT-Mediated Protein Transduction

Christopher A. Koczor, Janet W. Snyder, Inna N. Shokolenko, Allison W. Dobson, Glenn L. Wilson, and Susan P. LeDoux

Abstract

The mitochondrial genome represents a target for exogenous and endogenous damage. Its necessity for successful electron transport makes its repair valuable to the cell. Previous work from our lab has shown that mitochondrial DNA (mtDNA) can be repaired in mammalian cells, and the use of mitochondrial-targeted repair proteins can augment repair to enhance viability following genotoxic stress. In addition, it has also been shown that other repair enzymes that are targeted to the mitochondria can sensitize the cell to DNA damaging agents, thereby aiding the effectiveness of certain chemotherapeutic agents. The methods herein describe the development of mitochondrial-targeted proteins using plasmids or protein transduction domains. It includes the utilization of these constructs to create stably transfected cell lines, transiently transfected cell lines, viral-mediated transduction, and protein transduction domain-mediated mitochondrial protein localization. The end result will be a mammalian cell that expresses the mitochondrial-targeted protein of interest.

Key words: mtDNA, base excision repair, MTS, transfection, protein transduction, tat.

1. Introduction

Mitochondrial DNA (mtDNA) represents a sensitive target for alkylation and oxidative DNA damage. Our lab has shown that compounds causing both types of damage can cause extensive damage to the mtDNA *(1–9)*. However, our lab has shown that mtDNA can be repaired in various cell types, and this repair was through the short batch base excision repair pathway. While other

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_15 Springerprotocols.com

repair pathways are either non-existent or still debated, the base excision repair pathway in the mitochondria has been characterized and proven to be functional *(10–11)*.

Previous work has shown that repair proteins can be targeted to the mitochondria using a mitochondrial-targeting sequence (MTS) upstream of the protein of interest *(12–20)*. The original method utilized stable transfection of a cell line to obtain a clone that would express the protein of interest *(21)*. However, advances in protein targeting have allowed new methods to be utilized to obtain the end result. Transient transfection has been used in a similar manner to stable transfection to rapidly determine the benefits of the targeted protein. Viral vectors have been utilized with success to target repair proteins to the mitochondria. Finally, the protein transduction domain Tat from the HIV virus has been utilized to transduce proteins into the mitochondria of cell lines without the need for gene targeting *(22)*. Each of these methods will be discussed below.

For a protein to be targeted to the mitochondria, an MTS must be utilized. The MTS is usually a positively charged amino acid chain that forms an amphipathic α-helix on the N-terminal of the protein of interest. Our lab has utilized the MTS from manganese superoxide dismutase, which has been characterized as a strong MTS *(23–24)*. The MTS will localize the protein to the mitochondria, where localization and activity of the targeted protein are then verified. However, introduction of the mitochondrial-targeted protein into a mammalian cell represents another challenge.

As discussed, previous work has used lipofection to create stably transfected cell lines. Yet, the ability to deliver a protein into a cell without the need for gene delivery presents obvious benefits. The protein transduction domain of Tat, which is the HIV transcriptional activator, is an 86 amino acid protein whose ability to cross the cell membrane has been localized to the region containing amino acids 47–57 *(25–26)*. The mechanism of TAT transduction involves several steps. Initially, the protein transduction domain (PTD) interacts with the cell membrane in a receptor-independent fashion, which stimulates uptake by macropinocytosis. This is followed by a pH drop, whereby the macropinosome is destabilized and the protein is released *(27)*. For Tat-mediated protein transduction to be effective, the Tat sequence can be located either on the N-terminal of the protein or on the C-terminal. However, possibly due to the MTS sequence localized to the N-terminal, our experiments have shown that the Tat sequence works more effectively on the C-terminus of the protein. In addition, a hemagglutanin (HA) tag can be inserted for antibody labeling.

The experimental design will be described in four parts (**Fig.15.1**). First, the addition of a mitochondrial-targeting sequence to the N-terminal of the protein of interest will be detailed, thereby providing a means to stably or transiently transfect mammalian cell lines (**Sections 3.1**, **3.2**, and **3.3**). Secondly, the

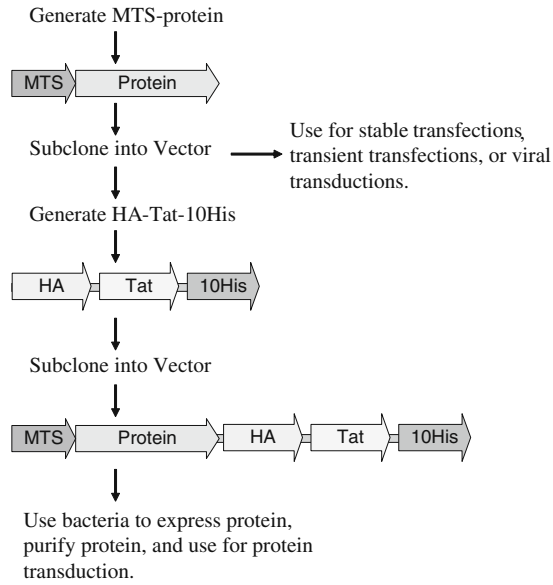


Fig. 15.1. Flow chart of the experimental design.

addition of the Tat sequence to the C-terminus of the MTS-protein sequence will be discussed, along with the expression and purification of the MTS-Tat repair protein (**Sections 3.4**, **3.5**, **3.6**, and **3.7**). Thirdly, validation of the repair enzyme's mitochondrial targeting and enzymatic activity will be discussed (**Sections 3.8** and **3.9**). And finally, the experiments to determine the effects of the mitochondrial DNA repair protein on mtDNA integrity and cell viability will be detailed (**Sections 3.10**, **3.11**, and **3.12**).

2. Materials

2.1. Creation of the Plasmid for the MTS-Targeted Protein

1. Oligonucleotides designed to serve as primers to amplify the DNA repair enzyme of choice.
2. pcDNA3.1neo vectors or other vector of choice.

2.2. Transfection

1. Fugene 6 transfection reagent (Roche).
2. Serum-free media. (Different for each cell type. For HeLa cells: DMEM, 50 μg/ml penicillin/streptomycin, and 2 mM L-glutamine.)
3. Complete media. (Different for each cell type. For HeLa cells: DMEM, 10% fetal bovine serum, 50 μg/ml penicillin/streptomycin, and 2 mM L-glutamine.)
4. Plasmid DNA.

2.3. Selection of Stable Clones

1. G-418 geneticin antibiotic or other appropriate selection antibiotic.
2. Complete media.

2.4. Creation of the MTS-Targeted Protein with a C-Terminal HA-Tat-HIS Tag

1. Synthetic oligonucleotides for overlapping PCR.
2. Oligonucleotides designed to serve as primers to amplify the HA-Tat-10His C-terminus.

2.5. MTS-Protein-Tat Protein Expression

1. pBluescriptII (Stratagene) vector or other derivative vector for MTS-protein-Tat expression in a bacterial culture.

2.6. MTS-Protein-Tat Protein Purification

1. Purification buffer: 20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 1 mM PMSF (no EDTA), 1X protease inhibitor cocktail EDTA-free, 5 mM imidazole (PMSF has a half-life of 30 min so it needs to be added right before lysing).
2. Sonicator for lysing bacteria.
3. Wash buffer: 20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 1 mM PMSF, 30 mM imidazole, 5% glycerol. (The imidazole concentration may need to be adjusted to obtain optimal protein binding, and this concentration will depend on the protein.)
4. Elution buffer: 20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 1 mM PMSF, 300 mM imidazole.
5. Ni-NTA agarose (Nickel Nitrilo-Triacetic Acid) (Qiagen).
6. Disposable polystyrene columns (Pierce).

2.7. MTS-Protein-Tat Transduction

1. Desalting, buffer exchange column (Bio-Rad).

2.8. Isolating Cellular Fractions for Protein Localization

1. Digitonin solution: 325 mM digitonin, 2.5 mM EDTA, 250 mM mannitol, and 17 mM 3-[*N*-morpholino]propanesulfonic acid (MOPS) (pH 7.4).
2. 2.5X Mannitol–sucrose buffer: 525 mM mannitol, 175 mM sucrose, 12.5 mM EDTA, 12.5 mM Tris–HCl (pH 7.5).
3. Organelle buffer: 20 mM HEPES (pH 7.6), 1 mM EDTA, 5 mM dithiothreitol (DTT), 300 mM KCl, 5% glycerol.
4. Protease inhibitors (for mammalian cell extracts): 100 mM 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF), 4 mM bestatin, 1.4 mM E-64, 2.2 mM leupeptin, 1.5 mM pepstatin, and 80 mM aprotinin (Sigma).
5. At least 10 million cells per sample.

2.9. Repair Enzyme Activity Assays

1. Single-stranded oligonucleotide with specific damage at one position.
2. γ-^{33}P ATP.

3. T4 polynucleotide kinase and 10X buffer (Promega).
4. Complementary oligonucleotide (to the damaged one).
5. 10X Reaction buffer (may differ depending on the targeted enzyme): 1 M NaCl, 250 mM phosphate buffer (pH 7.5), 20 mM Na-EDTA, 10 mM $MgCl_2$.
6. 3X Bromophenol blue loading dye: 300 mM NaOH, 97% formamide, 0.2% bromophenol blue.
7. Bio-Rad Mini-Protean II or similar vertical gel apparatus.

2.10. In Vivo Repair Assay

1. Menadione (Sigma) or other DNA damaging agent.
2. Serum-free media.
3. Hanks' balanced salt solution (HBSS).
4. Lysis buffer: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA (pH 8.0), 0.5% sodium dodecyl sulfate (SDS), 0.3 mg/ml proteinase K.

2.11. Quantitative Southern Blots

1. 5 M NaCl (unless otherwise stated, the solvent used for the reagents in this section is double-distilled water – ddH_2O).
2. SEVAG (24 parts chloroform:1 part isoamyl alcohol).
3. 10 M Ammonium acetate.
4. 95–100% Ethanol and 75% ethanol.
5. RNase.
6. *Xho*1 restriction endonuclease.
7. TE: 10 mM Tris–HCl (pH 8.0), 1 mM EDTA (pH 8.0).
8. 6X Alkaline loading dye: 0.5 M NaOH, 10 mM EDTA, 25% Ficoll, 0.25% bromocresol purple.
9. Large horizontal gel electrophoresis system and transfer apparatus.
10. HCl wash: 0.25 M HCl.
11. NaOH wash: 0.5 M NaOH, 1.5 M NaCl.
12. Tris-buffer wash: 0.5 M Tris–HCl, 1.5 M NaCl, pH 7.5.
13. Zeta-probe GT nylon membrane (Bio-Rad).
14. 10X SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0.
15. Hybridization solution: 0.25 M sodium phosphate buffer, pH 7.2; 7% SDS.
16. 5% SDS wash: 20 mM sodium phosphate buffer, pH 7.2; 5% SDS.
17. 1% SDS wash: 20 mM sodium phosphate buffer, pH 7.2; 1% SDS.

2.12. Viability Assay

1. Trypan blue reagent.
2. Hemocytometer.

3. Methods

3.1. Creation of the Plasmid for the MTS-Targeted Protein

1. Design oligonucleotides to serve as primers to amplify the desired repair enzyme from cDNA.
2. Prepare the 5′ primer, containing an *Eco*RI site (underlined in the sequence below) or another restriction enzyme site that will be used for subcloning into the vector of choice, the mitochondrial targeting sequence (the MTS from MnSOD in bold: *GGAATTC***ATGTTGAGCCGGGCAGTGTGC GGCACCAGCAGGCAGATGCCTGCCGCGCGCTTC TG**-aa1-aa2-aa3-aa4) and the sequence corresponding to the first three to four amino acids (aa1-aa4) of the enzyme you are planning to clone. The MTS sequence is 24 amino acids in length.
3. Prepare the 3′ primer appropriate to the enzyme sequence that contains another restriction enzyme site of choice at the 5′-end of the primer.
4. Amplify the cDNA using a high-fidelity thermostable DNA polymerase by polymerase chain reaction (PCR) in a thermocycler under the following conditions: 5 min start (94°C), then 30 cycles of 30 s denaturing (94°C), 1 min annealing (55°C), 2 min extension (72°C), followed by a 10 min end (72°C). The resulting PCR fragment should consist of an *Eco*RI site, the MTS, the enzyme coding region, and another restriction site.
5. Use the PCR product in a double restriction enzymatic digest with *Eco*RI and the other restriction enzyme overnight at 37°C.
6. Subclone the restriction fragment into the *Eco*RI and other enzymatic sites of pcDNA3.1neo or another vector of choice.
7. Sequence to confirm fidelity.
8. At this point, the methods leading to the final mitochondrial-targeted protein will diverge depending on the method of delivery. If you wish to establish a transient transfection or a stable transfection in a cell line, proceed to **Section 3.2**. If you wish to use viral-mediated transduction, the gene product from above can be ligated into a viral shuttle vector to utilize viral transduction at this point. Once you have established a viral vector, proceed to **Section 3.8**. Finally, if you wish to utilize the Tat protein transduction domain, proceed to **Section 3.4**.

3.2. Transfection

1. Grow the cells in 75-cm^2 flasks until they reach 75% confluency.
2. Add Fugene 6 reagent dropwise to serum-free media (ratio of 3 μl Fugene for 1 μg DNA to be used), flick tube to mix gently, and incubate for 10 min.

3. Add DNA dropwise to the mixture in Step 2, flick the tube gently, and incubate for 30 min.
4. Add the entire mixture (media, Fugene, and DNA) dropwise to cells in normal culture media, swirl vessel, and return the cells to the incubator.
5. Allow transfection to occur for 24 h.
6. Following this incubation, a transiently transfected cell line has been created, wherein not all of the cells have been efficiently transfected but the repair enzyme's effects can be quickly determined. To determine the activity and effects of the protein in the transiently transfected cells, proceed to **Section 3.8**. However, to create a stably transfected cell line that expresses the protein of interest more efficiently than the transiently transfected cells, proceed to **Section 3.3**.

3.3. Selection of Stable Clones

1. Following the 24 h incubation, replace the media with fresh complete media containing 0.6 mg/ml G-418.
2. Passage the cells normally, including G-418 (7–10 days). During the selection period, significant cell death should occur. Reduce the culture vessel size if necessary. Keep 0.6 mg/ml G-418 in the media.
3. After 2 weeks of selection, maintain the cells in complete media supplemented with 0.4 mg/ml G-418.
4. Isolate individual cells for clonogenic expansion (*see* **Note 1**).
5. Following expansion, proceed to **Section 3.8** to assay protein localization and activity.

3.4. Creation of the MTS-Targeted Protein with a C-Terminal HA-Tat-HIS Tag

1. The common C-terminal extension for chimeras, HA-TAT-10His, is generated from synthetic oligonucleotides using an overlapping PCR technique. The oligonucleotides are listed in **Table 15.1**. The structure of the C-terminal extension is shown in **Fig. 15.2** (*see* **Note 2**).

Table 15.1
Oligonucleotides used to generate C-terminal HA-TAT-10His domain

HATat10H-1	ccatatgggcggatccctgtacaaggagctcggctatccgtatgatgtgccggattatgc
HATat10H-2	gcggcgctggcggcgttttttgcggccatagcccaggctcgcataatccggcacatcata
HATat10H-3	ccagcgccgccgcggccatcaccatcaccatcaccatcaccatcactaagaattcc
PCRcTerF	ccatatgggcggatccctg
PCRcTerR	ggaattcttagtgatggtg

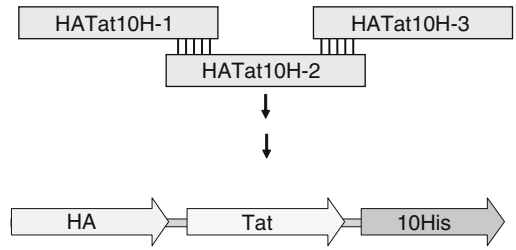


Fig. 15.2. Overlapping PCR using the HA-TAT-10His primers to generate the C-terminal HA-TAT-10His domain.

3.5. MTS-Protein-Tat Protein Expression

1. The final construct is assembled in the pBluescriptII (Stratagene) derivative vector. The expression is driven by the Plac promoter. This vector is introduced in *Escherichia coli* BL21 (DE3) cells. These cells have DE3 prophase carrying T7 RNA polymerase under the control of IPTG-inducible lac promoter integrated into their chromosome.

3.6. MTS-Protein-Tat Protein Purification

1. Grow the bacteria in liquid culture to an OD_{600}=0.6 and induce with 1 mM IPTG for 3 h to promote the production of the protein (*see* **Note 3**).
2. Pellet the bacteria by centrifugation at 1800 × g for 15 min. Resuspend the pellet in purification buffer.
3. Lyse the bacterial suspension by sonication. We have had success using a Branson Sonifier 250 using the following settings: output control – 5, duty cycle – 50. Sonicate for 15 s with 45 s in between. Repeat for five times.
4. Place the bacterial lysates in ultracentrifuge tubes, carefully balance the tubes, and centrifuge at 22,500 × g for 30 min at 4°C.
5. Mix the clear lysates with 1 ml of Ni-NTA agarose suspension and incubate for 1 h at 4°C on a slow orbital shaker. If the binding is not strong between the protein of interest and the Ni-NTA slurry, then the suspension will need to be incubated overnight.
6. Prepare a polystyrene column for the lysate suspension.
7. The formed Ni-NTA–agarose column is washed with 2–3 volumes of wash buffer.
8. Take a sample of the flow through for OD measurement using a spectrophotometer. The OD_{280} should be <0.05 before the elution buffer is applied.
9. Apply the elution buffer and begin collecting timed fractions of eluting protein from the column in 1.5-ml Eppendorf tubes.

10. Measure the amount of protein using a spectrophotometer. The purity of the eluted protein should be assessed using SDS-PAGE and Coomassie staining. A Western blot can also be performed to confirm the correct size of the protein.
11. N-terminal sequencing will need to be performed to verify the identity and integrity of the purified protein.

3.7. MTS-Protein-Tat Transduction

1. Cells to be transduced should be grown under normal growth conditions to approximately 50–75% confluency.
2. Run the purified protein on a desalting, buffer exchange column (*see* **Note 4**).
3. Add 25–50 μg/ml of protein directly to the media (*see* **Note 5**).
4. Allow the cells to incubate overnight with the protein solution. This should be enough time for the cells to be transduced and for the protein to localize to the mitochondria. To verify localization of the transduced protein, proceed to **Section 3.8** (*see* **Note 6**).

3.8. Isolating Cellular Fractions for Protein Localization

1. Grow at least 10 million cells for each cell type. The number of cells required will depend on the number of mitochondria per cell. For HeLa cells, this is approximately three 75-cm^2 flasks at near-confluency.
2. Dislodge cells from monolayers with trypsin and collect each cell type into a single pellet by centrifugation at 1000 × g for 10 min.
3. Resuspend each cell pellet in ice-cold digitonin solution for 80 s. Pipette cells on ice until no clumps are observed, thereby effectively lysing the cellular membrane.
4. Add the lysed cell mixture to 2.5X mannitol–sucrose buffer for a final strength of 1X (210 mM mannitol, 70 mM sucrose, 5 mM EDTA, 5 mM Tris–HCl, pH 7.5).
5. Centrifuge the ice-cold suspension at 4°C for 10 min at 800 × g to pellet nuclei.
6. Save the supernatant, resuspend the pelleted nuclear material in 1X mannitol–sucrose, and repeat the centrifugation.
7. Repeat Step 6 three additional times (*see* **Note 7**).
8. Combine the saved supernatants and centrifuge at 800 × g for 10 min to pellet any remaining nuclei, and very carefully draw off the resulting supernatant. Save the nuclei on ice.
9. Centrifuge the new supernatant at 10,000 × g for 20 min to pellet mitochondria. You should see a small pellet.
10. Decant supernatant carefully and save (this is the cytosolic fraction). Add 5 μl/ml protease inhibitors (*see* **Note 8**).

11. Resuspend isolated mitochondria and nuclei in organelle buffer + 5 µl/ml protease inhibitors (add just before use).
12. Pulse-sonicate (1 s) the resuspended organelles on ice (*see* **Note 9**).
13. Centrifuge the sonicated organelles at 5,000 × g for 10 min to pellet remaining cell debris, and carefully draw off the supernatants.
14. Determine the protein concentration for each sample (Bio-Rad/Bradford method) (*see* **Note 10**).

3.9. Repair Enzyme Activity Assays

1. End-label the damage-containing oligonucleotide at 37°C for 30 min in the following reaction: 20 pmol oligonucleotide, 20 pmol γ-^{33}P ATP, 1 µl (10U) T4 polynucleotide kinase, and 2 µl 10X buffer in a total volume of 20 µl. Heat to 90°C for 2 min to inactivate the kinase (*see* **Note 11**).
2. Add 20 pmol of complementary oligonucleotide, heat to 70°C, and cool slowly to anneal.
3. In 0.5-ml microtubes on ice, put equal amounts of organelle protein from the samples to be compared. Protein extracts should contribute to less than 30% of the 20 µl total reaction volume. Purified enzyme, if available, should be used as a positive control, and distilled water (dH_2O) as a negative control (*see* **Note 12**).
4. Prepare a "Master Mix" of labeled oligonucleotide and 10X reaction buffer (*see* **Note 13**).
5. On ice, add equal amounts of the Master Mix to each protein sample and incubate at 37°C for 30 min (*see* **Note 14**).
6. Stop the reactions by removing samples from 37°C and placing on ice, and add 3X bromophenol blue loading dye to a 1X final concentration (*see* **Note 15**).
7. Prepare 20% polyacrylamide, 7 M urea, 1X TBE vertical gels in a Bio-Rad Mini-Protean II apparatus. Use 1X TBE for electrophoresis buffer.
8. Flush urea from wells with 1X TBE before loading samples. Subject to electrophoresis for 1 h at 200 V (*see* **Note 16**).
9. Leave the gel on one glass plate and wrap in clear plastic. Expose to film overnight (*see* **Note 17**).

3.10. In Vivo Repair Assay

1. The choice of a chemical to determine the repair capacity of the targeted enzyme in vivo will determine the type of damage repaired by the targeted repair enzyme. In the case of 8-oxoguanine, menadione will be used as the chemical to induce 8-oxoguanine lesions.

2. Dissolve menadione in sterile serum-free media to make a 10 mM stock solution. Menadione is light sensitive, so work quickly in low light or keep solutions covered.
3. Prepare the serial dilutions of menadione from the stock solution and additional serum-free media (*see* **Note 18**).
4. Rinse cultured cells with HBSS and replace media with menadione solution for 1 h (5% CO_2 at 37°C). Control cells should be treated with serum-free media only (*see* **Note 19**).
5. Remove menadione, and rinse samples with HBSS. Lyse cells immediately on the culture surface with lysis buffer. Be sure to tilt the culture vessel such that lysis buffer spreads over the entire surface. It will become viscous very rapidly. For timed repair of sample mtDNA, fresh complete media should be placed on those samples until their designated time-point and then lysed in lysis buffer.
6. Return the lysed cells to the incubator for 5 min and then use a cell lifter to transfer lysates into 15-ml conical tubes. Incubate the sample at 37°C overnight.

3.11. Quantitative Southern Blot

1. Add 0.2 volumes of 5 M NaCl to each lysate (*see* **Note 20**).
2. To extract the DNA, add an equal volume of SEVAG, invert the tube gently and gently agitate for 10 min to mix thoroughly, and centrifuge for 10 min at 2,500 × g to separate the aqueous phase from the organic phase. The aqueous phase will be on the top.
3. Using a wide-mouth pipette, carefully draw off the aqueous phase, avoiding the protein at the interface (white) as a much as possible (*see* **Note 21**).
4. Repeat Steps 2 and 3 two to three times (*see* **Note 22**).
5. Precipitate the total DNA with 0.2 volumes of 10 M ammonium acetate, followed by 2.2 volumes of 100% ice-cold ethanol. Centrifuge for 10 min at 3,000 × g or higher to pellet DNA (*see* **Note 23**).
6. Rinse the DNA pellet with 70% ethanol and centrifuge again at 3,000 × g for 10 min. Pour off the ethanol and allow the pellet to dry inverted for 5–30 min.
7. Resuspend the DNA in dH_2O and restrict the samples with *Xho*1 overnight (this cuts the human mitochondrial genome once, but other restriction enzymes may be used). Treat with RNase (1 mg/ml).
8. Precipitate the digested samples with ammonium acetate and ethanol as in Step 5.
9. Prepare a horizontal 0.6% alkaline agarose gel: Weigh 1.2 g agarose into 200 ml dH_2O and boil to dissolve agarose. Cool to 55°C and add 400 µl of 0.5 M EDTA and 600 µl of 10 N

NaOH. Allow at least 2 h for this gel to set and remove the comb very slowly. Use extreme caution handling this gel in later steps, as it slides and breaks very easily!

10. Resuspend the DNA in TE buffer and determine a precise and accurate concentration. We use a Hoefer DyNA Quant 200 Fluorometer and Hoechst 33258 dye.
11. For each sample, bring up 5 μg of total DNA to 20 μl in TE.
12. Heat each sample for 15 min at 70°C and cool to room temperature (about 10 min).
13. Add NaOH to a final concentration of 0.1 N NaOH and incubate at 37°C for 20 min.
14. Add alkaline loading dye to each sample.
15. Load the samples and subject to electrophoresis at 30 V (1.5 V/cm gel length) for 16 h.
16. In Tris–HCl wash, stain the gel with ethidium bromide to confirm even loading and DNA integrity. Take a picture for a permanent record.
17. Wash the gel two times for 10 min each with HCl wash.
18. Wash the gel two times for 10 min each with NaOH wash.
19. Wash the gel two times for 10 min each with Tris-buffer wash (*see* **Note 24**).
20. Soak Zeta-probe membrane in 10X SSC.
21. Transfer the DNA to the Zeta-probe membrane with vacuum transfer in 10X SSC (approx. 1 h). Add 10X SSC periodically to top of gel during transfer (*see* **Note 25**).
22. Rinse membrane in 2X SSC briefly after transfer is complete.
23. Crosslink the membrane.
24. Hybridize the membrane overnight with ^{32}P-labeled human mtDNA-specific PCR-generated probe. This 672-bp probe corresponding to part of the cytochrome- *c* oxidase, subunit 3 gene and part of the ATP synthase F0, subunit 6 gene is made by PCR amplification (94°C, 5 min followed by 30 cycles of: 95°C, 30 s; 60°C, 30 s; 72°C, 1 min) from human genomic DNA with the following primers: 5′-CACAACTAACCTCCTCG-3′ and 5′-CTTTTTGGACAGGTGGTG-3′.
25. Wash the membrane twice with 5% SDS wash and twice with 1% SDS wash (15 min per wash).
26. Expose the membrane to film overnight.

3.12. Viability Assay

1. Allow cells to grow to 50–75% confluency, and expose to menadione or other chemical of choice as done in **Section 3.10**. Following exposure, remove menadione, wash one time with HBSS, and put complete media on the cells for 24 h (*see* **Note 26**).

2. After 24 h, collect the cells by trypsinization, and centrifuge cells ($\sim$200 $\times$ g for 5–10 min) to form a pellet.
3. Resuspend the cells into a specific volume (which can be from 1 ml up to 15 ml), which is referred to as the resuspension volume.
4. Remove a sample of the resuspended cells, and add to an equal volume of trypan blue, creating a dilution factor of 2.
5. Pipette 10 µl of the cell/trypan blue mixture onto each side of the hemocytometer.
6. Count the cells on a hemocytometer. Trypan blue will stain cells that have a permeabilized membrane blue, while cells that do not have a permeabilized membrane will remain clear. To determine viability, counts will be required for both live and dead cells.
7. Once the cells have been counted in each of the four main quadrants, use the following equation to obtain the total number of cells counted:Total number of cells = CPQ $\times$ dilution factor $\times$ resuspension volume $\times$ 10^4.

where

$$\text{CPQ} = \frac{\text{Total cells counted}}{\text{Number of quadrants counted.}}$$

8. The viability of the cells can be found by the following equation:

$$\text{Viability} = \frac{\text{total viable cells}}{\text{total viable cells} + \text{total dead cells.}}$$

4. Notes

1. Isolation of single cells can be accomplished several ways. For example, a cell sorter can be used to deposit one cell in each well of a 96-well plate.
2. The restriction sites utilized for the creation of the HA-TAT-10His C-terminus should correspond with the restriction sites used in the MTS-protein construct to ensure proper subcloning.
3. The first step of purification varies depending on the best method chosen to grow the bacteria with the protein of interest. For example, MTS-GFP-TAT grows best in *E. coli* on agar plates for 37°C the first day and at room temperature the following day, whereas MTS-ExoIII-TAT grows best in liquid cultures of *E. coli* overnight.

4. The protein needs to be in a buffer that is optimal for the protein and for your cell type.
5. The optimal amount of protein may vary.
6. The HA-tag presents an immunohistochemical marker that is not present in mammalian cells. Therefore, antibody labeling with an HA antibody will only label the MTS-Tat protein, and this can be localized with one of many mitochondrial markers to show proper targeting of the protein to the mitochondria *(22)*.
7. These additional centrifugations are only necessary to optimize the mitochondrial yield. If a very large number of cells are used, omit this step.
8. The cytosolic components may need to be concentrated. There are many commercial concentrators available if necessary.
9. Sonication is one of several ways to rupture organelle membranes. We use a Branson Sonifier Cell Disruptor 185 with the output control set to 6, and sonicate for 1 s. Some foam is then visible at the surface of the sample. Alternatively, Triton X-100 can be added to the organelle lysis buffer to 0.2% and after resuspension, the organelles can be incubated at 37°C for 30 min. This may interfere with determination of protein concentration, however.
10. This protein may also be used in Western blots to confirm localization of the protein of interest and proper organelle isolation.
11. The total amount of labeled oligonucleotide required will depend on the enzyme. Some DNA repair enzymes are optimally active at higher substrate concentrations than others. Human 8-oxoguanine glycosylase (OGG) activity, for example, is best detected when the substrate concentration is 100 nM or higher.
12. The total reaction volume can be varied, but in general smaller volumes tend to yield better results.
13. The use of a master mix minimizes the human error introduced by additional pipetting steps; that is, it ensures more even distribution of 10X buffer and substrate oligonucleotide to each reaction. Also, the 10X buffer should be designed to optimize the reaction conditions for the enzyme being studied. Be sure to calculate the amount of 10X buffer based on the final concentration in the total reaction.
14. The desired reaction time will also depend on the particular enzyme being studied.
15. Again, this step will depend on the enzyme being studied. A stop solution may be necessary in some cases. For example, glycosylases that only remove the damaged base will not

cleave the labeled oligonucleotide. In such cases, it is necessary to heat the sample to 95°C, which will cause strand breaks at abasic sites, before electrophoresis.

16. Electrophoresis will depend on the length of the damaged oligonucleotide before and after cleavage. As a general rule, the bromophenol blue dye should be one-half to two-thirds down the gel.
17. Film exposure time will depend on the amount of radiolabeled substrate used in each reaction and the amount of the reaction loaded on the gel. We have used exposure times ranging from 1 h to 3 days.
18. The appropriate concentrations of menadione will vary greatly from one cell type to another. For HeLa cells, 300–400 μM menadione induces a level of mtDNA damage that can be readily detected and repaired to some extent within 6 h.
19. Menadione exposure time can be altered. Because menadione is a redox cycler, damage is generated for the entire duration of the exposure. However, the stability of menadione in solution for long periods must also be considered.
20. Some of the commercial DNA isolation kits may work just as well.
21. Using normal pipettes can shear the DNA. If wide-mouth pipettes are not available, you can also cutoff the end of a 1-ml micropipette tip (about 0.25 cm.).
22. The number of extractions required may depend on the cell type. When the aqueous phase is clear and no protein is observed at the interface of the two phases, proceed to the DNA precipitation steps.
23. In some situations, small amounts of DNA may be present. To maximize precipitation of the DNA, store the samples at –20°C overnight.
24. The gel may remain in the Tris–HCl wash for up to 2 h.
25. Other transfer methods may be used, but vacuum transfer is preferred.
26. Methods other than trypan blue may be used if cells do not readily form single-cell suspensions. Clonogenic assays have been used with much success *(21)*.

References

1. Driggers WJ, LeDoux SP, Wilson GL. (1993) Repair of oxidative damage within the mitochondrial DNA of RINr 38 cells. *J Biol Chem* **268,** 22042–5.
2. Grishko V, Solomon M, Wilson GL, LeDoux SP, Gillespie MN. (2001) Oxygen radical-induced mitochondrial DNA damage and repair in pulmonary vascular endothelial

cell phenotypes. *Am J Physiol Lung Cell Mol Physiol* **280,** L1300–8.

3. Hollensworth SB, Shen C, Sim JE, Spitz DR, Wilson GL, LeDoux SP. (2000) Glial cell type-specific responses to menadione-induced oxidative stress. *Free Radic Biol Med* **28,** 1161–74.
4. LeDoux SP, Patton NJ, Avery LJ, Wilson GL. (1993) Repair of N-methylpurines in the mitochondrial DNA of xeroderma pigmentosum complementation group D cells. *Carcinogenesis* **14,** 913–7.
5. Ledoux SP, Shen CC, Grishko VI, Fields PA, Gard AL, Wilson GL. (1998) Glial cell-specific differences in response to alkylation damage. *Glia* **24,** 304–12.
6. LeDoux SP, Wilson GL, Beecham EJ, Stevnsner T, Wassermann K, Bohr VA. (1992) Repair of mitochondrial DNA after various types of DNA damage in Chinese hamster ovary cells. *Carcinogenesis* **13,** 1967–73.
7. Pettepher CC, LeDoux SP, Bohr VA, Wilson GL. (1991) Repair of alkali-labile sites within the mitochondrial DNA of RINr 38 cells after exposure to the nitrosourea streptozotocin. *J Biol Chem* **266,** 3113–7.
8. Shen CC, Wertelecki W, Driggers WJ, LeDoux SP, Wilson GL. (1995) Repair of mitochondrial DNA damage induced by bleomycin in human cells. *Mutat Res* **337,** 19–23.
9. Wilson GL, Patton NJ, LeDoux SP. (1997) Mitochondrial DNA in beta-cells is a sensitive target for damage by nitric oxide. *Diabetes* **46,** 1291–5.
10. Mandavilli BS, Santos JH, Van Houten B. (2002) Mitochondrial DNA repair and aging. *Mutat Res* **509,**127–51.
11. Wilson DM, 3rd, Bohr VA. (2007) The mechanics of base excision repair, and its relationship to aging and disease. *DNA Repair*(Amst) **6,** 544–59.
12. Dobson AW, Grishko V, LeDoux SP, Kelley MR, Wilson GL, Gillespie MN. (2002) Enhanced mtDNA repair capacity protects pulmonary artery endothelial cells from oxidant-mediated death. *Am J Physiol Lung Cell Mol Physiol* **283**, L205–10.
13. Dobson AW, Xu Y, Kelley MR, LeDoux SP, Wilson GL. (2000) Enhanced mitochondrial DNA repair and cellular survival after oxidative stress by targeting the human 8-oxoguanine glycosylase repair enzyme to mitochondria. *J Biol Chem* **275,** 37518–23.
14. Druzhyna NM, Hollensworth SB, Kelley MR, Wilson GL, Ledoux SP. (2003) Targeting human 8-oxoguanine glycosylase to mitochondria of oligodendrocytes protects against menadione-induced oxidative stress. *Glia* **42,** 370–8.
15. Harrison JF, Hollensworth SB, Spitz DR, Copeland WC, Wilson GL, LeDoux SP. (2005) Oxidative stress-induced apoptosis in neurons correlates with mitochondrial DNA base excision repair pathway imbalance. *Nucleic Acids Res* **33,** 4660–71.
16. Ho R, Rachek LI, Xu Y, Kelley MR, LeDoux SP, Wilson GL. (2007) Yeast apurinic/apyrimidinic endonuclease Apn1 protects mammalian neuronal cell line from oxidative stress. *J Neurochem* **102,** 13–24.
17. Rachek LI, Grishko VI, Alexeyev MF, Pastukh VV, LeDoux SP, Wilson GL. (2004) Endonuclease III and endonuclease VIII conditionally targeted into mitochondria enhance mitochondrial DNA repair and cell survival following oxidative stress. *Nucleic Acids Res* **32,** 3240–7.
18. Rachek LI, Grishko VI, Musiyenko SI, Kelley MR, LeDoux SP, Wilson GL. (2002) Conditional targeting of the DNA repair enzyme hOGG1 into mitochondria. *J Biol Chem* **277,** 44932–7.
19. Rachek LI, Thornley NP, Grishko VI, LeDoux SP, Wilson GL. (2006) Protection of INS-1 cells from free fatty acid-induced apoptosis by targeting hOGG1 to mitochondria. *Diabetes* **55,** 1022–8.
20. Shokolenko IN, Alexeyev MF, Robertson FM, LeDoux SP, Wilson GL. (2003) The expression of Exonuclease III from E. coli in mitochondria of breast cancer cells diminishes mitochondrial DNA repair capacity and cell survival after oxidative stress. *DNA Repair*(Amst) **2,** 471–82.
21. Dobson AW, Kelley MR, Wilson GL, LeDoux SP. (2002) Targeting DNA repair proteins to mitochondria. *Methods Mol Biol* **197,** 351–62.
22. Shokolenko IN, Alexeyev MF, LeDoux SP, Wilson GL. (2005) TAT-mediated protein transduction and targeted delivery of fusion proteins into mitochondria of breast cancer cells. *DNA Repair*(Amst) **4,** 511–8.
23. Shimoda-Matsubayashi S, Matsumine H, Kobayashi T, Nakagawa-Hattori Y, Shimizu Y, Mizuno Y. (1996) Structural dimorphism in the mitochondrial targeting sequence in the human manganese superoxide dismutase gene. A predictive evidence for conformational change to influence mitochondrial transport and a study of allelic association in Parkinson's disease. *Biochem Biophys Res Commun* **226,** 561–5.

24. Tamura T, McMicken HW, Smith CV, Hansen TN. (1996) Mitochondrial targeting of glutathione reductase requires a leader sequence. *Biochem Biophys Res Commun* **222,** 659–63.
25. Vives E, Brodin P, Lebleu B. (1997) A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J Biol Chem* **272,** 16010–7.
26. Ho A, Schwarze SR, Mermelstein SJ, Waksman G, Dowdy SF. (2001) Synthetic protein transduction domains: enhanced transduction potential in vitro and in vivo. *Cancer Res* **61,** 474–7.
27. Wadia JS, Stan RV, Dowdy SF. (2004) Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat Med* **10,** 310–5.

Chapter 16

Construction and Characterization of a Cell Line Deficient in Repair of Mitochondrial, but Not Nuclear, Oxidative DNA Damage

Sugako Oka, Mizuki Ohno, and Yusaku Nakabeppu

Abstract

Oxidative base lesions, such as 8-oxoguanine, accumulate in nuclear and mitochondrial DNAs under oxidative stress, resulting in cell death. However, it is not known whether only oxidative lesion accumulated in mitochondrial DNA is involved in such cell death. By introducing human cDNA encoding a nuclear form of 8-oxoG DNA glycosylase (hOGG1-1a) into immortalized mouse embryo fibroblasts lacking *Ogg1* gene, we established a cell line which selectively accumulates 8-oxoguanine in mitochondrial DNA under oxidative stress. Selective accumulation of 8-oxoguanine in mitochondrial DNA in this cell line causes degradation of mitochondrial DNA followed by ATP depletion, mitochondrial membrane permeability transition, and Ca^{2+} efflux, which in turn activates calpains to execute cell death. Knockdown of MUTYH which excises adenine opposite 8-oxoG in DNA prevents degradation of mitochondrial DNA and activation of calpain, thus suppressing the cell death induced by menadione.

Key words: 8-Oxoguanine, mitochondrial DNA, cell death, mitochondrial degeneration, calpain, single-strand break, MUTYH.

1. Introduction

8-Oxoguanine (8-oxoG) is a major form of oxidative base damage in DNA *(1)*, and is known to be a potent pre-mutagenic lesion because it can pair with adenine as well as cytosine during DNA replication *(2)*. 8-OxoG has been shown to accumulate in both nuclear and mitochondrial DNAs under conditions of oxidative stress and also during aging, and significantly increased accumulation of 8-oxoG has been reported in patients with various neurodegenerative diseases *(3, 4)*, suggesting that an accumulation of

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_16 Springerprotocols.com

oxidative DNA lesions may cause cell death. However, because 8-oxoG accumulates in both nuclear and mitochondrial DNAs (mtDNAs) under many conditions, it is not known whether its accumulation specifically in mtDNA is involved in cell death.

8-OxoG DNA glycosylase-1 (OGG1) excises 8-oxoG paired with cytosine in DNA *(5, 6)*. Several isoforms of human OGG1 (hOGG1) are generated by alternative splicing *(7)* (**Fig. 16.1**). To elucidate the effects on cell death of 8-oxoG accumulated in mtDNA, we have developed an expression vector carrying a cDNA encoding the nuclear isoform of human (hOGG1-1a) that can be used to express hOGG1-1a in immortalized *Ogg1*-knockout mouse embryo fibroblasts (MEFs). Characterization of this cell line (Nu-hOGG1 MEFs) shows selective accumulation of 8-oxoG in mtDNA under conditions of oxidative stress, as only 8-oxoG in nuclear DNA is effectively repaired by hOGG1-1a which localizes and functions exclusively in the nucleus *(8)* Nu-hOGG1 MEFs exhibit an increased rate of cell death under oxidative stress in comparison to MEFs expressing both nuclear and mitochondrial forms of hOGG1. Electron microscopy reveals that swollen mitochondria with electron dense deposits appear under conditions of oxidative damage *(9)*. In addition, selective accumulation of 8-OxoG in mtDNA induces degradation of mtDNA followed by ATP depletion, mitochondrial membrane permeability transition (MPT), and Ca^{2+} efflux, which in turn activates calpains to execute cell death. Knockdown of adenine DNA glycosylase (encoded by *Mutyh* gene), which excises adenine inserted opposite

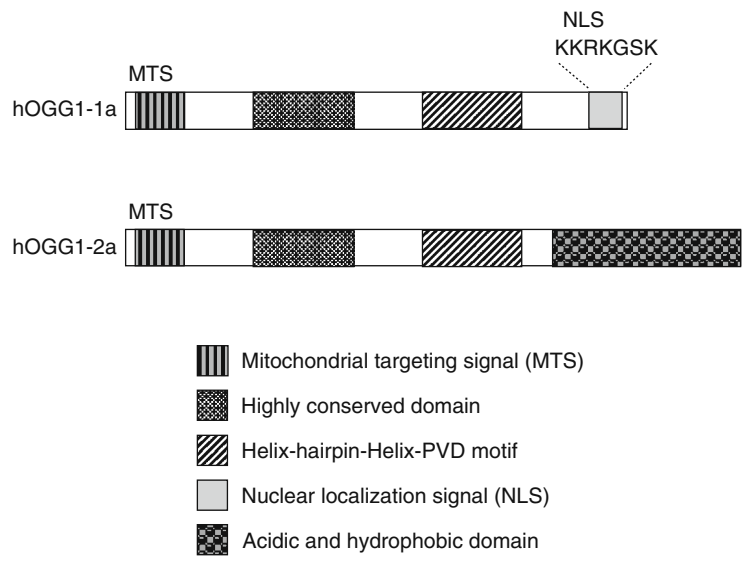


Fig. 16.1. **Structural comparison of hOGG1 proteins.** hOGG1-1a is a nuclear form with a C-terminal NLS, while hOGG1-2a is a major mitochondrial form with a C-terminal acidic and hydrophobic domain. (Adapted from Ref. *(8)*).

8-oxoG in template DNA during DNA replication, significantly attenuates mtDNA degradation as well as calpain activation, thus suppressing cell death. These observations demonstrate that the excision of adenine inserted opposite 8-oxoG selectively accumulated in mtDNA results in the degradation of mtDNA under oxidative stress, which in turn causes mitochondrial dysfunction and cell death.

Here we describe this novel experimental system for studying the effects of 8-oxoG accumulation specifically in mtDNA. We include detailed descriptions of both the design and the characterization of mtDNA repair deficient cells. While this approach has been applied to the study of 8-oxoG accumulation, it should be broadly applicable to the study of other mtDNA lesions by similarly manipulating different DNA repair enzymes.

2. Materials

2.1. Cell Lines

1. Wild-type mouse embryo fibroblasts (MEFs): Wild-type MEFs isolated from embryos (13.5 days postcoital) obtained by mating *Ogg1*$^{+/-}$ mice are spontaneously immortalized *(8)*.
2. OGG1-null MEFs: *Ogg1*$^{-/-}$ MEFs immortalized as above are transfected with a control vector pcDNA3.1Hyg(–) *(8)*.
3. Nu-hOGG1 MEFs: *Ogg1*$^{-/-}$ MEFs immortalized as above are transfected with a vector pcDNA3.1Hyg(–): hOGG1-1a encoding the nuclear isoform of hOGG1 *(8)*. hOGG1-1a with 8-oxoG DNA glycosylase activity is localized only in nuclei of Nu-hOGG1 MEFs and there is no detectable 8-oxoG DNA glycosylase activity in their mitochondria (**Fig. 16.2**) (*See* **Note 1**).

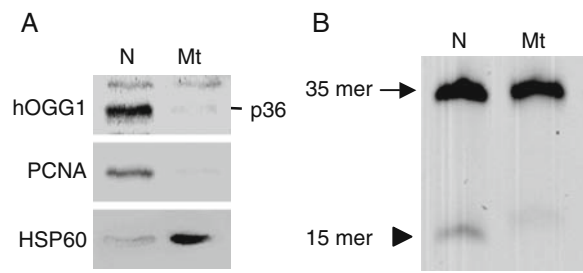


Fig. 16. 2. **Characterization of Nu-hOGG1 MEFs.** (**A**) Detection of hOGG1-1a (p36) in nuclear extracts in Nu-hOGG1 MEFs. PCNA or HSP60 was detected as a nuclear or mitochondrial marker, respectively. Nuclear (N) and mitochondrial (Mt) extracts (5 μg of protein). (**B**) 8-OxoG DNA glycosylase activity in nuclear extracts in Nu-hOGG1 MEFs. (**A**, **B**: all adapted from Ref. *(8)*).

4. Nu/Mt-hOGG1 MEFs: *Ogg1*$^{-/-}$ MEFs immortalized as above are first transfected with a vector pcDNA3.1Hyg (–):hOGG1-2a encoding the mitochondrial form of hOGG1, and then with pcDNA3.1PURO:hOGG1-1a encoding the nuclear form of hOGG1 *(8)*.

2.2. Cell Culture and Exposure to Oxidative Stress

1. Growth medium: Dulbecco's Modified Eagle's Medium (DMEM, low glucose) supplemented with 10% heat-inactivated fetal bovine serum, with penicillin–streptomycin.
2. Hygromycin B: dissolved in PBS at 100 mg/ml, stored in aliquots at –30°C, and then added to the medium as required.
3. Puromycin: dissolved in PBS at 10 mg/ml, stored in aliquots at –30°C, and then added to the medium as required.
4. Trypsin/EDTA solution: trypsin (0.25%) and ethylenediamine tetraacetic acid (EDTA, 1 mM). Working solution: 0.3X dilution of the stock solution with PBS (*see* **Note 2**).
5. 2-Methyl-1.4-naphthoquinone (menadione): dissolved in PBS at 500 mM, stored in single use aliquots at –30°C. For use, dilute with DMEM without FBS and penicillin–streptomycin and filter through 0.2-μm filter.
6. MDL28170 (Calpain inhibitor; Biomol Research Laboratories, Inc., Plymouth Meeting, PA, USA): dissolved in DMSO at 200 mM, stored at –30°C.
7. Cyclosporin A (mitochondrial permeability transition inhibitor): dissolved in DMSO at 1 mM, stored at –30°C.

2.3. Morphological Examination of Cells

1. Hoechst33342: dissolved in distilled water at 1 mM, stored at 4°C, and then added to culture dishes as required.
2. Propidium iodide (PI): dissolved in distilled water at 10 mg/ml, stored at –30°C, and then added to culture dishes as required.

2.4. Cell Viability Assay

0.3% Trypan blue in PBS, stored at 4°C.

2.5. Electron Microscopy

1. 2.5% Glutaraldehyde containing 0.1 M cacodylate (pH 7.4, TAAB), 3.4% NaCl, 0.1 M saccharose, and 3 mM $CaCl_2$.
2. 1% OsO_4 containing 0.1 M cacodylate (pH7.4), 3.4% NaCl, 0.1 M saccharose, and 3 mM $CaCl_2$.
3. Epoxy resin: Epon812:DDSA:MNA:DMP30 (48.5:18.5:33.0:1.5; TAAB).
4. 2% Uranyl acetate (Merck, Darmstadt, Germany).
5. Lead acetate: $Pb(NO_3)_2$ (1 g), $Pb(H_3COO)_2 \bullet 3H_2O$ (1 g), $Pb3(C_6H_5O_7)_2 \bullet 3H_2O$ (1 g) (TAAB), and trisodium citrate dihydrate (2.0 g) are dissolved in 8.2 ml distilled water, and the solution is slowly mixed with 18 ml of 4% NaOH with gentle shaking.
6. Transmission Electron Microscope JEM2000EX (JEOL, Tokyo).

2.6. Determination of Mitochondrial Membrane Potential and Ca^{2+} Levels

1. 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Molecular Probes, Eugene, OR, USA).
2. Rhod-2 AM (Molecular Probes).
3. A fluorescence microscope (Axioskop2) equipped with a CCD camera (AxioCam) (Carl Zeiss).
4. Digital image processing and camera control: AxioVision software (Carl Zeiss).
5. Image processing: Image Gauge V4.0 (Fuji Film).

2.7. Intracellular ATP Levels and Calpain Activity

1. CellTiter-Glo^{R}Assay Kit (Promega Corp., Madison, WI, USA).
2. Calpain Activity Assay Kit (BioVision, Inc., Mountain View, CA, USA).
3. A spectrofluorometer, Wallac 1420 ARVOsx Multi Label Counter (Perkin–Elmer).

2.8. Southern Blotting

1. DNAzol reagents (Invitrogen).
2. RNase solution (stock): 10 mg/ml RNase A in 10 mM Tris–HCl (pH 7.5), 15 mM NaCl. RNase solution is boiled at 98°C for 15 min to inactivate residual DNase and then stored at –30°C. Working solution: 1:2 dilution of stock solution with same buffer.
3. Glyoxal reaction mixture: 6 ml of DMSO, 2 ml of glyoxal dimethylsulfate, 0.6 ml of 80% glycerol, 0.2 ml of 10 mg/ml ethidium bromide, 1.2 ml of 10X BPTE buffer. Store at –30°C.
4. BPTE buffer: prepare a 10X stock solution with 100 mM PIPES, 300 mM Bis-Tris, and 10 mM EDTA (pH 8.0). Dilute 100 ml with 900 ml water for use.
5. Nylon membrane (Hybond-N^{+}; GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA).
6. AlkPhosDirect Kit (GE Healthcare).
7. CDP-Star chemiluminescent reagent (GE Healthcare).

2.9. Amplification of mt-Co1 DNA Encoding Cytochrome c Oxidase I

1. Primer set for the *mt-Co1* gene:
   mCOX1s: 5′-TGATTATTCTCAACCAATCAC-3′.
   mCOX1a: 5′-TGGTGGAGGGCAGCCATGAAG-3′.
2. ISOGEN (Nippon Gene, Co., Ltd., Tokyo, Japan).
3. Primer set for *Gapdh* gene:
   mGA5-1: 5′-CTGCCATTTGCAGTGGCAAAG-3′.
   mGA3-1: 5′-TGGTATTCAAGAGAGTAGGGA-3′.

4. PCR reaction mixture: 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.4 U recombinant *Taq* DNA polymerase (Takara Bio., Inc., Siga, Japan), 0.2 μM each primer, and 200 μM each deoxynucleoside triphosphate.

2.10. siRNA and Transfection

1. siRNAs (# 86583, # 86769) against mouse *Mutyh* (*see* **Note 3**) and a scrambled-siRNA (# 4603G) as a control siRNA (from Ambion).
2. siPORT Amine, Silencer TM siRNA Transfection Kit (Ambion).

3. Methods

3.1. Quantification of 8-OxoG in Nuclear and Mitochondrial DNA

The content of 8-oxoG in nuclear DNA is determined by HPLC–MS/MS (*see* Ref. *(8)*). The contents in OGG1-null and Nu-hOGG1 MEFs, as well as in wild-type MEFs, are immediately and significantly increased during the exposure to menadione (0 h in **Fig. 16.3**, 50 μM), and the increased contents in wild-type and Nu-hOGG1 MEFs returned to the basal level 24 h after the exposure. In contrast, the levels remain high in OGG1-null MEFs 24 h after exposure (**Fig. 16.3**, **left panels**).

The 8-oxoG index in mtDNA is determined according to the method described in the chapter 13 by Ohno et al. Mitochondrial 8-oxoG index increased during the exposure to menadione (0 h in **Fig. 16.3**, 50 μM), are gradually increased in all three cell lines, and the levels remain high in Nu-hOGG1 MEFs and OGG1-null MEFs, but not in wild-type MEFs 8 h after the exposure (**Fig. 16.3**, **right panels**).

3.2. Morphological Examination of Cells

1. MEFs cultured to 60–70% confluence in a 10-cm dish are exposed to medium containing menadione for 60 min, then cultured in fresh growth medium for 24 h.
2. The cells are incubated with 10 μM Hoechst 33342 and 2 μg/ml PI for 30 min and examined under a fluorescence microscope. To determine the percentage of dead cells, the number of dead cells stained with both Hoechst33342 and PI is divided by the total number of cells stained with Hoechst (**Fig. 16.4**).

3.3. Cell Viability Assay

1. MEFs cultured to 60–70% confluence in a 10-cm dish are incubated in a medium containing menadione for 60 min, then cultured in fresh growth medium for 24 h.
2. The cells are treated with 0.3X trypsin/EDTA and harvested by centrifugation.

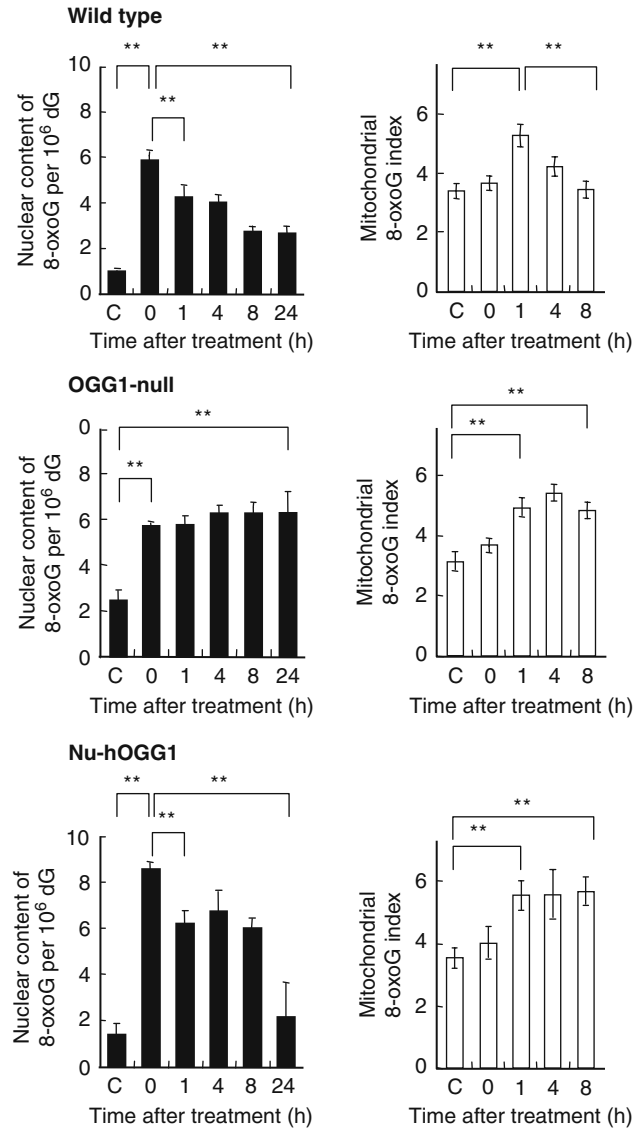


Fig. 16.3. **Selective accumulation of 8-oxoG in mitochondrial DNA in Nu-hOGG1 MEFs after exposure to menadione.** *Left panels* show 8-oxoG accumulation in nuclear DNA after exposure to menadione. The content of 8-oxoG in nuclear DNA (8-oxoG residues per 10^6 residues of guanine) was determined by HPLC–MS/MS analysis at certain time-points (0–24 h) after a 60-min exposure to 50 μM menadione. Results from one of the two independent experiments are presented (mean ± SD, $n = 3$ per experiment). C: control with no exposure. Student's *t*-test, $^{**}p < 0.01$. *Right panels* show the 8-oxoG index representing mitochondrial 8-oxoG immunoreactivity in each cell (mean ± SEM, 30 cells). Student's *t*-test, $^{**}p < 0.01$. (Adapted from Ref. *(8)*).

3. The cells are suspended in an appropriate volume of growth medium, mixed with an equal volume of 0.3% trypan blue solution, and subjected to phase contrast microscopy. Numbers of unstained living cells are counted, and cell viability in each experiment is determined as a percentage of the untreated control.

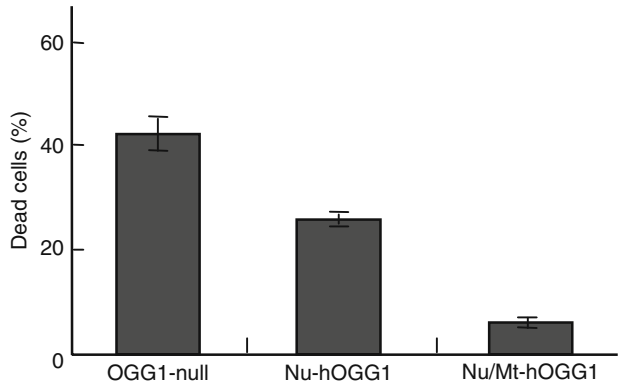


Fig. 16.4. **The selective accumulation of 8-oxoG in mitochondrial DNA causes cell death.** Dead cells stained with Hoechst/PI were counted and their percentage in each culture was determined (mean ± SEM, $n = 3$). Results from one of the two independent experiments are presented.

3.4. Electron Microscopy

For electron microscopic observation, MEFs cultured to 60–70% confluence in a 14-cm dish are exposed to medium containing menadione for 60 min and then cultured in fresh growth medium for 6–24 h. The cells are fixed with 2.5% glutaraldehyde for 30 min, and harvested using a cell scraper. Cell pellets are fixed with 1% OsO_4 on ice for 1 h and embedded in epoxy resin. Ultrathin sections (80–90 nm) prepared from these samples are stained with uranyl acetate and lead acetate, then observed using a Transmission Electron Microscope JEM2000EX *(9)*. Examples of the results are shown in **Fig. 16.5**.

3.5. Determination of Mitochondrial Membrane Potential and Levels of Ca^{2+} in Mitochondria

1. MEFs cultured to 60–70% confluence are incubated in a medium containing menadione for 60 min, then cultured in fresh growth medium for various periods.
2. At various time-points, MEFs are incubated in the presence of 5 μg/ml JC-1 to visualize mitochondrial membrane potential, or 4.5 μM rhod-2 AM to visualize mitochondrial Ca^{2+} levels, for 20 min at 37°C.

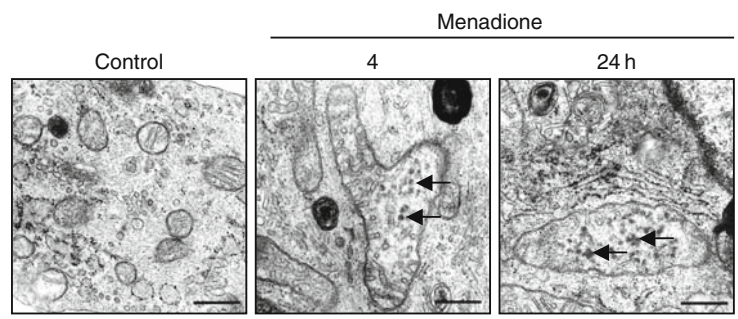


Fig. 16.5. **Menadione-induced death of Nu-hOGG1 MEFs is accompanied by mitochondrial degeneration.** Morphological alterations of mitochondria after exposure to menadione in Nu-hOGG1 MEFs observed under electron microscopy. Control: no exposure. Bar: 0.5 μm. The *arrow* indicates electron dense deposits.

3. MEFs are observed under an Axioskop2-equipped AxioCam after washing twice with PBS. Signal intensities of JC-1 (red-fluorescent) and rhod-2 are measured using Image Gauge V4.0. Examples of the results are shown in **Fig. 16.6**.

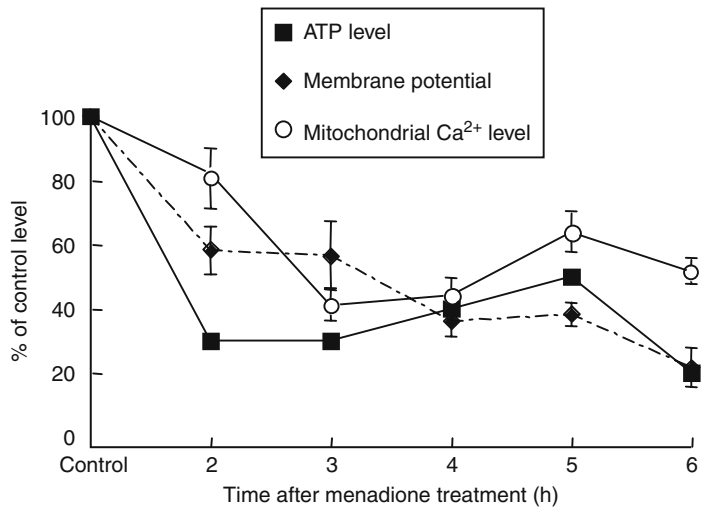


Fig. 16.6. **Menadione-induced death of Nu-hOGG1 MEFs is accompanied by ATP depletion, mitochondrial membrane permeability transition, and Ca^{2+}efflux.**The graph shows time-dependent alterations in the intracellular ATP level, mitochondrial membrane potential, and mitochondrial Ca^{2+}level, in Nu-hOGG1-MEFs after exposure to menadione. Fluorescence intensities of JC-1 (red fluorescence) and rhod-2 of 30 and 50 cells, respectively (mean ± SEM), were examined. (Adapted from Ref. *(8)*).

3.6. Intracellular ATP Levels

1. MEFs cultured to 60–70% confluence in two sets of 96-well plates for each experiment are incubated in a medium containing menadione for 60 min, then cultured in fresh growth medium for various periods.
2. One set of plates is subjected to the luciferase reaction to monitor the intracellular ATP level using a Wallac 1420 ARVOsx multilabel counter.
3. The other set of plates is subjected to staining with Hoechst33342 and PI. The cells are incubated with 10 μM Hoechst 33342 and 2 μg/ml PI for 30 min and examined under a fluorescence microscope.
4. To determine the ATP index representing the relative amount of ATP per cell, the luminescence signal is divided by the number of living cells stained only with Hoechst33342. Examples of the results are shown in **Fig. 16.6**.

3.7. Calpain Activity

Calpain activity is measured using a Calpain Activity Assay Kit according to the manufacturer's instructions. The calpain substrate (Ac-LLY-AFC) is incubated with cell lysates (10 μg

protein) for 60 min at 37°C in the dark. Fluorescence of released free AFC is monitored using a Wallac 1420 ARVOsx multilabel counter.

Examples of the results are shown in **Fig. 16.7.**

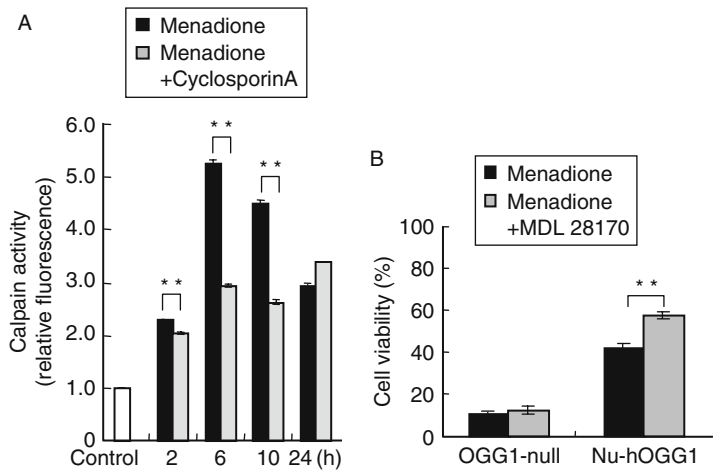


Fig. 16.7. **Selective accumulation of 8-oxoG in mitochondrial DNA causes cell death dependent on calpain activation**(**A**) Activation of calpain and its suppression by cyclosporin A. Nu-hOGG1 MEFs, pre-incubated for 60 min in the presence or absence of 100 nM cyclosporin A, were exposed to 50 μM menadione for 60 min, then calpain activity was determined periodically (mean ± SD, $n = 3$ per experiment). Relative calpain activities normalized by that of an untreated control (control) are shown. Student's *t*-test, $^{**}p < 0.01$. (**B**) Suppression of cell death by calpain inhibitor. MEFs pre-incubated in the presence or absence of 20 μM MDL28170 for 60 min were exposed to 50 μM menadione for 60 min. Cell viability at 24 h after the exposure was determined by the trypan blue exclusion test. Results from one of the two independent experiments are presented (mean ± SEM, $n = 3$). Student's *t*-test, $^{**}p < 0.01$ (**A**, **B**: all adapted from Ref. *(8)*).

3.8. Southern Blotting

Southern blotting of mtDNA is performed according to the method with some modifications *(10)*.

1. MEFs cultured to 60–70% confluence are incubated in a medium containing menadione for 60 min, then cultured in fresh growth medium for 24 h.
2. The cells are treated with 0.3X trypsin/EDTA and harvested by centrifugation.
3. The total DNA is extracted with DNAzol reagents according to the manufacturer's instruction.
4. The extracted DNA is solubilized in distilled water and incubated with 5 mg/ml RNase and *Pst*I at 37°C for overnight, and the digested DNA is phenol-extracted, ethanol-precipitated, and solubilized in 0.1X TE. The concentration of DNA is determined by measuring absorbance at 260 nm.

5. The DNA is denatured in glyoxal reaction mixture for 60 min at 55°C *(11)*, the total DNA (1 μg) is electrophoresed on a 0.7% agarose gel in BPTE buffer for 1.5 h at 70 V.
6. Transfer onto the nylon membrane.
7. The membrane is hybridized with the mtDNA probe overnight at 55°C with gentle shaking.
8. The signals are visualized using CDP-Star chemiluminescent reagent and quantified with Las1000Plus (Fuji Film, Tokyo, Japan). Examples of the result produced are shown in **Fig. 16.8**.

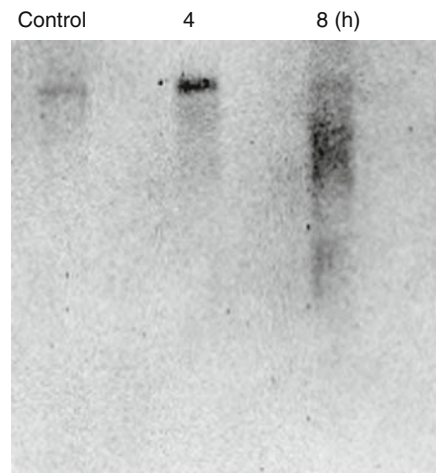


Fig. 16.8. **Degradation of mitochondrial DNA after exposure to menadione.** Menadione-induced mitochondrial DNA fragmentation. Total cellular DNA was prepared from Nu-hOGG1 MEFs 4 or 8 h after a 60-min exposure to 50 μM menadione, and *Pst* I-digested DNA was subjected to Southern blotting for mitochondrial DNA. Control: no exposure. (Adapted from Ref. *(8)*).

3.9. Amplification of mt-Co1 DNA

1. MEFs cultured to 60–70% confluence in a 10-cm dish are incubated in a medium containing menadione for 60 min, then cultured in fresh growth medium for 24 h.
2. The cells are treated with 0.3X trypsin/EDTA and harvested by centrifugation.
3. The total cellular DNA is prepared using ISOGEN according to the manufacturer's instructions.
4. Using a primer set for the *mt-Co1* gene a 1,438-bp fragment of mtDNA (residues 5,393 to 6,831) is amplified from total cellular DNA (500 ng).
5. The relative amount of *mt-Co1* DNA is normalized with nuclear *Gapdh* DNA. Examples of the results are shown in **Fig. 16.9A.**

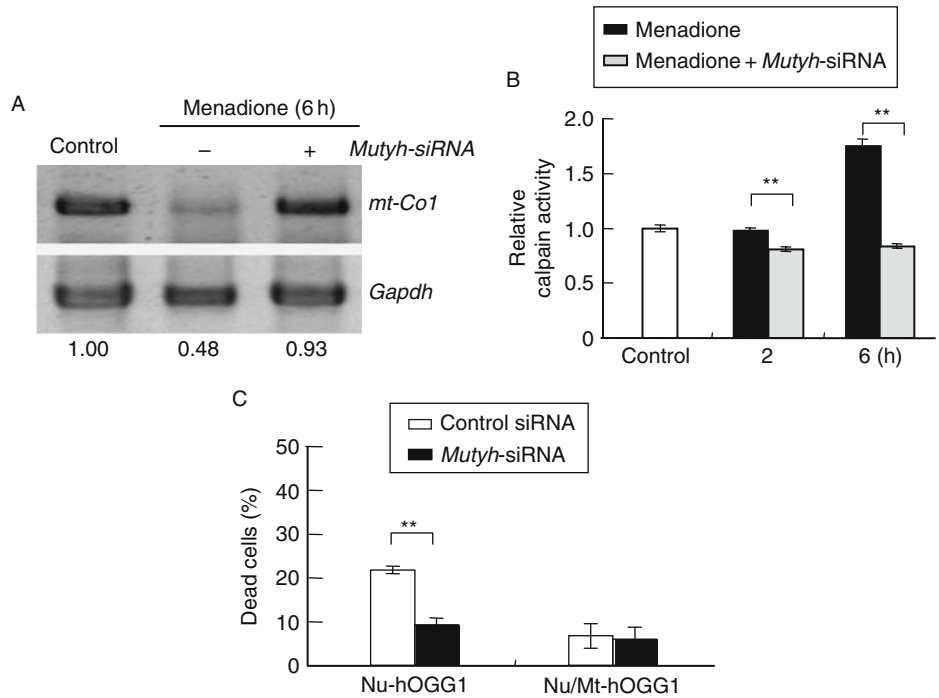


Fig. 16.9. **Suppression of mitochondrial 8-oxoG-induced cell death by *Mutyh*-siRNA.** (**A**) Suppression of menadione-induced depletion of mitochondrial DNA by *Mutyh*-siRNA. Total cellular DNA was prepared from Nu-hOGG1 MEFs 6 h after a 60-min exposure to 50 μM menadione, and *mt-Co1* and *Gapdh* DNA were amplified. Control: no exposure. The intensity of *mt-Co1* DNA was normalized by that of nuclear *Gapdh* DNA, and the relative intensity of each sample to that of the control sample is shown at the *bottom* of each lane. (**B**) Suppression of menadione-induced activation of calpain in Nu-hOGG1 MEFs by *Mutyh*-siRNA. Calpain activity was determined in Nu-hOGG1 MEFs exposed to 50 μM menadione for 60 min in the presence or absence of *Mutyh*-siRNA. Control: no exposure. Relative calpain activities normalized by that of an untreated control are shown. Results from one of the two independent experiments are presented (mean ± SD, $n = 3$ per experiment). Student's *t*-test, $^{**}p < 0.01$. (**C**) Suppression of menadione-induced cell death by *Mutyh*-siRNAs. MEFs were transfected with *Mutyh*-siRNAs or control-siRNA, cultured for 24 h, and then exposed to 25 μM menadione. Percentages of dead cells were determined 24 h after the exposure. Results from one of the two independent experiments are presented (mean ± SD, $n = 3$ per experiment). Student's *t*-test, $^{**}p < 0.01$ (**A**, **B**, **C**: all adapted from Ref. *(8)*).

3.10. siRNA and Transfection

1. MEFs cultured to 40–50% confluence in a 10-cm dish are transfected with *Mutyh*-siRNA or control-siRNA using siPORT Amine, Silencer TM siRNA Transfection Kit.
2. Next day, MEFs are incubated in a medium containing menadione for 60 min, then cultured in fresh growth medium for 24 h.
3. The cells are treated with 0.3X trypsin/EDTA and harvested by centrifugation.
4. The total cellular DNA is prepared using ISOGEN, and subjected to PCR for *mt-Co1*.
5. Examples of the result produced are shown in **Fig. 16.9**.

4. Notes

1. For subcellular fractionation, avoid freezing cell pellets before fractionation. Cells on culture dishes are gently removed using a cell scraper, and the harvested cells are immediately subjected to subcellular fractionation. To measure DNA glycosylase activity, the fractionated extracts can be stored at –80°C in small aliquots to avoid repeated freezing and thawing.
2. We usually use 0.3X trypsin/EDTA for established MEFs; however, we recommend using 0.2X trypsin/EDTA for cultures maintained with hygromycin B. Since hygromycin B-selected cells are rather sensitive to trypsin treatment, trypsin should be completely removed from the culture by changing medium after centrifugation.
3. MUTYH: MutY homolog (MUTYH) is an adenine DNA glycosylase that excises adenine inserted opposite 8-oxoG in template DNA. During replication, adenine can be inserted opposite 8-oxoG accumulated in template DNA as a result of an OGG1 deficiency, thereby forming a considerable number of A:8-oxoG pairs. After excision of adenine opposite 8-oxoG by MUTYH, an abasic site is generated and the abasic site is further converted to a DNA single-strand break by AP endonuclease or AP lyase.

Acknowledgments

We extend our special thanks to all other members of our laboratory for useful comments on the manuscript. We thank M. Sasaki in the Laboratory for Technical Support of our institute for their expertise in performing the EM analysis. This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (grant numbers: 18013038, 20013034), and the Japan Society for the Promotion of Science (grant numbers: 16390119, 18300124).

References

1. Kasai, H. and Nishimura, S. (1984) Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents. *Nucleic Acids Res* **12,** 2137–2145.
2. Maki, H. (2002) Origins of spontaneous mutations: specificity and directionality of base-substitution, frameshift, and sequence-substitution mutageneses. *Annu. Rev. Genet.* **36,** 279–303.
3. Shimura-Miura, H., Hattori, N., Kang, D., Miyako, K., Nakabeppu, Y. and Mizuno, Y. (1999) Increased 8-oxo-dGTPase in the

mitochondria of substantia nigral neurons in Parkinson's disease. *Ann. Neurol* **46,** 920–924.

4. Nunomura, A., Perry, G., Aliev, G., Hirai, K., Takeda, A., Balraj, E. K., et al. (2001) Oxidative damage is the earliest event in Alzheimer disease. *J. Neuropathol. Exp. Neurol* **60,** 759–767.
5. Nakabeppu, Y., Sakumi, K., Sakamoto, K., Tsuchimoto, D., Tsuzuki, T. and Nakatsu, Y. (2006) Mutagenesis and carcinogenesis caused by the oxidation of nucleic acids. *Biol. Chem* **387,** 373–379.
6. Boiteux, S. and Radicella, J. P. (2000) The human OGG1 gene: structure, functions, and its implication in the process of carcinogenesis. *Arch. Biochem. Biophys* **377,** 1–8.
7. Nishioka, K., Ohtsubo, T., Oda, H., Fujiwara, T., Kang, D., Sugimachi, K., et al. (1999) Expression and differential intracellular localization of two major forms of human 8-oxoguanine DNA glycosylase encoded by alternatively spliced OGG1 mRNAs. *Mol. Biol. Cell* **10,** 1637–1652.
8. Oka, S., Ohno, M., Tsuchimoto, D., Sakumi, K., Furuichi, M. and Nakabeppu, Y. (2008) Two distinct pathways of cell death triggered by oxidative damage to nuclear and mitochondrial DNAs. *EMBO J* **27,** 421–432.
9. Yoshimura, D., Sakumi, K., Ohno, M., Sakai, Y., Furuichi, M., Iwai, S., et al. (2003) An oxidized purine nucleoside triphosphatase, MTH1, suppresses cell death caused by oxidative stress. *J. Biol. Chem* **278,** 37965–37973.
10. Miyako, K., Takamatsu, C., Umeda, S., Tajiri, T., Furuichi, M., Nakabeppu, Y., et al. (2000) Accumulation of adenine DNA glycosylase-sensitive sites in human mitochondrial DNA. *J. Biol. Chem* **275,** 12326–12330.
11. Ogden, R. C. and Adams, D. A. (1987) Electrophoresis in agarose and acrylamide gels. *Methods Enzymol* **152**, 61–87.

Chapter 17

Mitochondrial DNA Oxidative Damage and Mutagenesis in *Saccharomyces cerevisiae*

Lyra M. Griffiths, Nicole A. Doudican, Gerald S. Shadel, and Paul W. Doetsch

Abstract

Mutation of human mitochondrial DNA (mtDNA) has been linked to maternally inherited neuromuscular disorders and is implicated in more common diseases such as cancer, diabetes, and Parkinson's disease. Mutations in mtDNA also accumulate with age and are therefore believed to contribute to aging and age-related pathology. Housed within the mitochondrial matrix, mtDNA encodes several of the proteins involved in the production of ATP via the process of oxidative phosphorylation, which involves the flow of high-energy electrons through the electron transport chain (ETC). Because of its proximity to the ETC, mtDNA is highly vulnerable to oxidative damage mediated by reactive oxygen species (ROS) such as hydrogen peroxide, superoxide, and hydroxyl radicals that are constantly produced by this system. Therefore, it is important to be able to measure oxidative mtDNA damage under normal physiologic conditions and during environmental or disease-associated stress. The budding yeast, *Saccharomyces cerevisiae*, is a facile and informative model system in which to study such mtDNA oxidative damage because it is a unicellular eukaryotic facultative anaerobe that is conditionally dependent on mitochondrial oxidative phosphorylation for viability. Here, we describe methods for quantifying oxidative mtDNA damage and mutagenesis in *S. cerevisiae*, several of which could be applied to the development of similar assays in mammalian cells and tissues. These methods include measuring the number of point mutations that occur in mtDNA with the erythromycin resistance assay, quantifying the amount of oxidative DNA damage utilizing a modified Southern blot assay, and measuring mtDNA integrity with the "petite induction" assay.

Key words: Mitochondrial disorder, oxidative DNA damage, oxidative stress, reactive oxygen species, mitochondria, mitochondrial DNA, *Saccharomyces cerevisiae*, petite induction, erythromycin resistance.

1. Introduction

Oxidative DNA damage is thought to be the most frequently occurring spontaneous DNA damage, and it is estimated that 10,000 oxidative hits occur per cell per day on the mammalian genome *(1)*. Mitochondrial DNA (mtDNA) is housed in the

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_17 Springerprotocols.com

mitochondrial matrix and encodes many of the protein components of the mitochondrial oxidative phosphorylation system comprising the electron transport chain (ETC) and the ATP synthase *(2)*. Reactive oxygen species (ROS) are generated as byproducts of the ETC, and these ROS can damage DNA, RNA, lipids, and proteins *(3, 4)*. It is thought that the proximity of mtDNA to ETC-generated ROS results in an increased vulnerability of mtDNA to oxidative damage and a higher rate of mtDNA mutagenesis compared to nuclear DNA *(5)*. Additionally, mtDNA is not associated with histones or packaged into nucleosomes, but instead is coated with other DNA-binding proteins. While these proteins may shield mtDNA from endogenous and environmental DNA damaging agents *(6)*, it has been suggested that the lack of histones and nucleosomal packaging may make mtDNA more susceptible to such insults *(7)*. The vulnerability of mtDNA to oxidative damage can be illustrated by describing the number of lesions that occur spontaneously and in response to exogenous ROS. For example, in rat liver it is estimated that the number of spontaneous 8-oxoguanine lesions in nuclear DNA (2.75 billion bp) is one in 130,000 bases; while lesions in mtDNA (16.3 thousand bp) are estimated to occur at a level of one in 8,000 bases *(7)*. Furthermore, in human and yeast cells, mtDNA contains two to three times more oxidative lesions than nuclear DNA following oxidative stress induced by various agents *(8, 9)*.

Necessarily, all cells have evolved the ability to repair DNA damage. Various repair proteins have been shown to localize to mitochondria; however, to what extent and how these proteins recognize and repair mtDNA damage is largely unknown. In 1974, it was shown that UV light-induced cyclobutane pyrimidine dimers (CPDs), substrates of nucleotide excision repair (NER), are not repaired in mammalian mitochondria *(10)*. Since then, no evidence has emerged indicating that NER occurs in mitochondria. Mismatch repair and recombination have been demonstrated in yeast mitochondria *(11, 12)*; however, this remains a controversial area with regard to mammalian mtDNA *(13)*. Oxidative DNA damage is primarily repaired by base excision repair (BER) and it has been clearly demonstrated that this repair pathway exists in mitochondria from yeast to humans *(14–19)*. A list of DNA repair and damage-resistance proteins that localize to yeast mitochondria is presented in **Table 17.1**.

Compromised DNA repair in mitochondria results in mtDNA instability. mtDNA instability corrupts the efficiency of the ETC which, in turn, further compromises the ETC via ROS-mediated damage leading to greater increases in ROS in a process termed the "vicious cycle" *(20, 21)*. ROS-mediated mtDNA damage and mutagenesis are thought to play a critical role in the process of aging in humans. Additionally, mtDNA mutations have been implicated in many human diseases including a large number of

Table 17. 1
Mitochondrial DNA repair proteins in *Saccharomyces cerevisiae*. Known repair proteins that localize to mitochondria and allow repair of mtDNA and the known or proposed in vivo and/or in vitro function(s) for each

Protein name	Human homolog	Repair pathway	Function	Reference
Apn1p	hHAP1; hREF1	BER	AP endonuclease, 3′ diesterase	*(47)*
Ogg1p	hOGG1	BER	DNA glycosylase, AP lyase	*(17)*
Ogg2p	–	BER	DNA glycosylase, AP lyase	*(17, 48)*
Ntg1p	hNTH1	BER	DNA glycosylase, AP lyase	*(14, 25)*
Pif1p	hPIF	Recombination	5′–3′ DNA helicase; works with Ntg1p to reduce oxidative damage in mtDNA	*(16, 49)*
Rrm3p	hPIF	Recombination	DNA helicase	*(49, 50)*
Abf2p	h-mtTFA	Recombination	High mobility group (HMG) protein; maintains rho status of mtDNA	*(51)*
Msh1p	hMSH-2	MMR	*E. coli* MutS homolog	*(11)*
Cce1p	–	Recombination	Cruciform cutting endonuclease	*(52)*
Nuc1p	EndoG	Recombination	Mitochondrial nuclease, DNase, RNase	*(53)*
Mhr1p	–	Recombination	Maintains rho status of mtDNA	*(54)*

BER: base excision repair; MMR: mismatch repair.

maternally inherited neuromuscular disorders, cancer, diabetes, and Parkinson's disease *(22)*. In yeast, a decrease in mtDNA stability over time is revealed by an increase in the number of petite colonies *(23)*. In this situation, the mtDNA deteriorates from a normal, organized sequence to forms containing multiple mutations, deletions, amplifications, and recombined segments *(24)*; ultimately, the mtDNA is lost altogether. Respiration is no longer possible for yeast cells with disorganized or lost mtDNA (more information regarding petite formation can be found in **Section 3.3**). Thus, it is apparent that management of oxidative stress and mtDNA damage in mitochondria is important in mammalian and yeast cells.

Over the years, numerous methods have been employed to study the mitochondrial and nuclear oxidative DNA damage. Various studies have employed microscopy to detect localization of DNA repair proteins to mitochondria in order to elucidate the players in mitochondrial oxidative DNA repair *(17, 25, 26)*. In 1988, HPLC was utilized for the detection of a specific oxidative

DNA base damage product, 8-oxoguanine, in order to establish the ratio of oxidative DNA damage in the nucleus to that in mitochondria *(7)*. In 1998, Helbock et al., updated the protocol for HPLC detection of 8-oxoguanine and described some limitations of the original procedure, including the demonstration that DNA extraction with phenol causes the introduction of adventitious oxidative damage *(27)*. In 2000, Anson et al., compared the HPLC detection assay with a new method employing a formamidopyrimidine glycosylase (Fpg) digestion in combination with Southern blotting to assess oxidative DNA damage throughout the entire mitochondrial genome *(28)*. The results of the Fpg method suggested that the HPLC-based method over-estimated the number of oxidative lesions in a cell by approximately 10-fold. An advantage to the modified Southern assay is that Fpg not only detects 8-oxoguanine, but also other types of DNA lesions (such as 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG), FapyA, Me-FaPy, and AP sites *(29-31)*), therefore allowing detection of a broader range of DNA base damage products. More recently, the modified Southern blot assay has been expanded by the employment of various repair enzymes, further expanding the number of lesions that can be analyzed *(14, 32)*. Other methods for the detection of oxidative damage in DNA include a quantitative polymerase chain reaction (PCR) assay based on the property that DNA polymerase stalls at damaged bases, and thus, the quantity of completed PCR product is inversely proportional to the amount of damage in the DNA sequence *(33)*.

The budding yeast, *Saccharomyces cerevisiae,* is an ideal model system for studying mtDNA expression, replication, and repair *(34)*. The primary utility of yeast for studying oxidative mtDNA damage is the conditional dependence on mitochondrial oxidative phosphorylation for survival, due to the fact that they are facultative anaerobes. That is, if provided with glucose (or other fermentable carbon sources), yeast can live in the complete absence of respiration and therefore can tolerate mutation and even complete loss of mtDNA as well as nuclear gene mutations that compromise mtDNA expression or maintenance. Additionally, yeast are capable of forming petites when their mtDNA is compromised, making the measurement of mtDNA integrity straightforward. It is also important to emphasize for the purpose of studying oxidative mtDNA damage that *S. cerevisiae* shares similar nuclear and mtDNA repair pathways with humans, making them a highly manageable and informative model system in this regard. Using this system, one can study mtDNA repair and damage-resistance pathways that impact mtDNA stability under normal growth conditions as well as in response to exogenous sources of damage and stress. For example, oxidative stress conditions can be studied by simply exposing cells to chemicals such as hydrogen peroxide to increase cellular ROS levels or hydrogen

peroxide in combination with antimycin *(35)*, alloxan *(36)*, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) *(37)*, or doxorubicin *(38)* in order to target oxidative stress to mitochondria.

2. Materials

2.1. Measurement of Mitochondrial Mutation Frequency Based on Erythromycin Resistance

2.1.1. Media and Plates

1. YPG medium: 1% yeast extract, 2% peptone, 3% (v/v) glycerol; autoclave and store at room temperature.
2. YPG plates: 1% yeast extract, 2% peptone, 3% (v/v) glycerol, 2% agar; store at room temperature.
3. YPG plates containing 1 mg/ml erythromycin (first dissolved in absolute ethanol and added post-autoclave after agar cools to approximately 50°C); store plates at 4°C.

2.2. Mitochondrial Oxidative DNA Damage Assay Based on Modified Southern Blot Procedure

2.2.1. Isolation of Yeast DNA

1. YPG medium (as in **Section 2.1.1**).
2. SorEDTA: 0.9 M sorbitol, 0.1 M EDTA, pH 7.4; store at room temperature.
3. 1 M Dithiothreitol: make 1 ml aliquots in MilliQ grade water, and store at –20°C.
4. 10 mg/ml Zymolyase 20T (US Biological): store lyophilized at 4°C until use; make 10 mg/ml solution from lyophilized zymolyase resuspended in SorEDTA.
5. Tris–EDTA: 50 mM Tris–HCl, pH 7.4, 20 mM EDTA; store at room temperature.
6. Proteinase K: 20 mg/ml solution, made in sterile water; store at –20°C.
7. 10% Sodium dodecyl sulfate (respiratory hazard); store at room temperature.
8. 5 M Potassium acetate in water; store at room temperature.
9. Chloroform.
10. 3 M Sodium acetate, pH 5.2; store at room temperature.
11. Isopropanol.
12. Absolute ethanol.
13. 70% Ethanol.
14. TE buffer: 10 mM Tris, 1 mM EDTA, pH 8.0; store at room temperature.
15. 10 mg/ml RNase A: Make 1 ml aliquots; store at –20°C.

2.2.2. DNA Treatment with Enzyme

1. DNA quantification kit (Sigma, product no. DNA-QF).
2. *Nde*I restriction enzyme; store at –20°C.
3. 4 M Sodium acetate, pH 5.2; store at room temperature.
4. Absolute ethanol.
5. 70% Ethanol.
6. Recombinant GST-tagged Ntg1p (*see* **Note 1**); stored in 20% glycerol at –20°C.
7. Ntg1p digestion buffer: 15 mM KH_2PO_4 (pH 6.8), 10 mM EDTA, 10 mM 2-mercaptoethanol, 40 mM KCl; stocks of KH_2PO_4, EDTA, and KCl, made up in water and stored at room temperature. Digest buffer should be prepared just before use.

2.2.3. Southern Blot Procedure

1. 1.1% Alkaline agarose gel containing 30 mM NaOH, 1 mM EDTA; make on the day of the experiment.
2. Alkaline electrophoresis buffer: 30 mM NaOH, 2 mM EDTA; make on the day of the experiment.
3. Denaturing loading dye: 18% Ficoll (w/v) (type 400, Pharmacia), 0.15% (w/v) bromocresol green, 0.25% (w/v) xylene cyanol, 50 mM NaOH, 1 mM EDTA; stocks of each component can be stored at room temperature and mixed just before use. Dye will change to a bright blue color following mixing.
4. 20X Sodium chloride–sodium citrate buffer (SSC), pH 7.0 (3.0 M NaCl, 0.3 M sodium acetate); store at room temperature.
5. Nylon membrane (Osmonics, MagnaGraph, Nylon, Catalog# NJ0HYB0010).
6. Alkaline transfer buffer (20X SSC, 0.4 M NaOH); make on the day of the experiment.
7. Southern transfer apparatus (*see* Sambrook and Russell *(39)*).
8. Wash buffer: 2X SSC; make on the day of the experiment from 20X SSC.
9. UV crosslinker (UV Stratalinker, Stratagene).
10. Hybridization buffer (Amersham Biosciences).
11. PFU turbo polymerase for making the probe PCR product (Stratagene).
12. *COB1* primers: 5′-GGCATTTAGAAAATCAAATGTG-3′ and 5′-CTGTCCATAAACACAACAATAACC-3′ *(40)*.
13. Klenow buffer: 500 mM Tris–HCl, pH 7.2, 100 mM $MgSO_4$, 1 mM DTT, Klenow enzyme (Promega); make on the day of the experiment.

14. Random hexamers: 25 μM stock (Integrated DNA Technologies).
15. dNTP mix (without dATP): Mix 2.5 mM of each dCTP, dGTP, and dTTP in 50 μl total volume; store at –20°C.
16. [α-^{32}P]dATP, 10–15 μCi/μl; store at 4°C.
17. Micro Bio-Spin 6 Chromatography Columns (Bio-Rad, Catalog# 732-6200); refrigerate.
18. Wash buffer: 2X SSC, 0.1% SDS, 500 ml; make on the day of the experiment.
19. Phosphorimager.

2.3. Measurement of mtDNA Integrity Based on "Petite-Mutant Induction" Assay

1. YPG medium (as in **Section 2.1.1**).
2. YPG plates (as in **Section 2.1.1**).
3. YPD medium: 1% yeast extract, 2% peptone, and 2% dextrose.
4. YPD plates: 1% yeast extract, 2% peptone, 2% dextrose, and 2% agar.
5. SD medium: 2% dextrose, 0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, and 2% agar.

3. Methods

3.1. Measurement of Mitochondrial Mutation Frequency Based on Erythromycin Resistance

Erythromycin specifically inhibits mitochondrial translation by inhibiting elongation and causing dissociation of tRNAs from the ribosome *(41-43)*. Resistance to erythromycin can be acquired by specific point mutations in the mitochondrial *RIB2* and *RIB3* genes. Because only point mutations in mtDNA can confer resistance, mtDNA point mutations can be scored by colony growth on plates containing erythromycin. Growth on glycerol plates containing erythromycin requires respiratory proficiency. One would expect a greater number of erythromycin-resistant colonies in strains where the ability to acquire point mutations is increased, such as in strains containing mutations in genes responsible for managing or preventing the accumulation of oxidative mtDNA base damage (modified from Doudican et al. *(14)*).

To determine the number of point mutations acquired in mtDNA, the number of erythromycin-resistant yeast is scored. First, yeast are grown in YPG media, which require mitochondrial respiration; then, yeast are plated on YPG containing erythromycin in order to determine the number of cells with point mutations (**Fig. 17.1**).

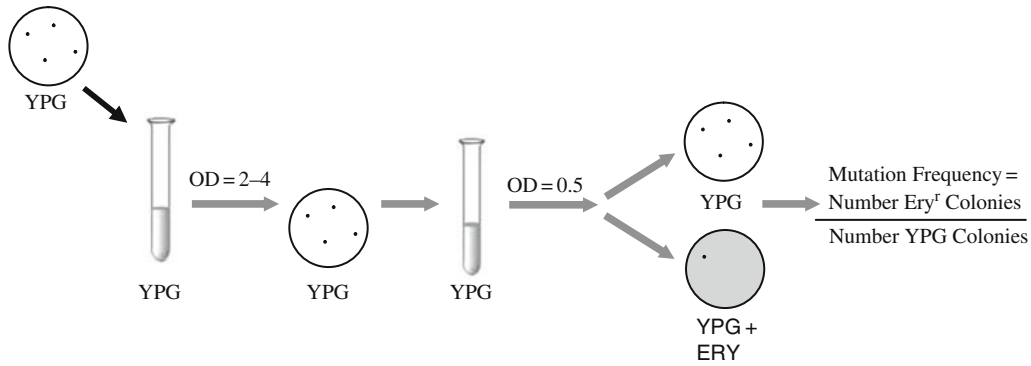


Fig. 17.1. Measurement of mtDNA mutant frequencies. First, yeast are grown in YPG media, requiring mitochondrial respiration. Next, yeast are placed on YPG plates or YPG plates containing erythromycin. Yeast containing point mutations in the mitochondrial genes, *RIB2* and *RIB3*, will grow on plates containing erythromycin; while all respiration-competent yeast cells will grow on the YPG plates. From the number of colonies on each plate, the mutation frequency can be calculated.

1. Grow isogenic yeast strains in 5 ml of YPG medium from single colonies. Cultures should be grown at 30°C to an OD_{600} between 2 and 4.
2. Cultures should be diluted such that approximately 100 colonies grow on each plate (*see* **Note 2**).
3. Plate diluted cultures on YPG, and grow at 30°C for 4–5 days.
4. Inoculate approximately 15 separate 10-ml cultures of YPG with individual colonies grown on the YPG plates. Grow cultures at 30°C for 48 h.
5. Dilute and plate cultures on YPG and YPG containing 1 mg/ml erythromycin. For YPG plating, remove a small aliquot of the culture and dilute it in sterile water before plating (*see* **Note 3**). For YPG plus erythromycin, cells from approximately 5 ml of culture should be plated by spinning down the culture and resuspending in a small volume of water prior to plating. The culture is enough to plate two YPG plates and two YPG plus erythromycin plates.
6. Count the number of colonies on YPG plates (for total number of respiration-competent cells) and YPG plus erythromycin (for total number of cells harboring point mutations in the mitochondrial *RIB2* and *RIB3* genes).
7. Calculate mutation frequencies: number of erythromycin-resistant (Ery^r) colonies/number of colonies grown on YPG = cells with *RIB2 RIB3* point mutations/total number of respiration-competent cells (*see* **Note 4**). **Table 17.2** presents an example of mutation frequencies found in an experiment using erythromycin resistance to quantify point mutations in mtDNA. The results indicate that *NTG1*, *ABF2*, and *PIF1* are

Table 17. 2
Erythromycin resistance frequencies for yeast mutants with compromised mtDNA stability functions. Mutation frequency is high in cells lacking the gene, *PIF1*, or combinations of genes, *PIF1* and *NTG1* or *ABF2* and *NTG1*

Strain	Average no. of cells plated (10^8)	Average no. of erythromycin-resistant mutants	Mutant frequency[a]	Fold increase[b]
NTG1	7.71	0.7	1.13×10^{-9}	1
*ntg1*Δ	9.13	1.9	2.59×10^{-9}	2.3
*pif1*Δ	5.69	16	3.34×10^{-8}	29
*pif1*Δ *ntg1*Δ	5.28	31	6.35×10^{-8}	56
*abf2*Δ	6.19	1.2	2.09×10^{-9}	1.8
*abf2*Δ *ntg1*Δ	5.22	25	5.68×10^{-8}	50

From O'Rourke et al. *(16)*, reprinted with permission.
[a]Calculated from the results of plating 25–30 independent cultures.
[b]Calculated relative to the wild-type (*NTG1*) strain.

important for the maintenance of mtDNA integrity in yeast, and when mutated, particularly in combination, the number of point mutations in yeast mtDNA is increased by greater than 25-fold *(14)*.

3.2. Mitochondrial Oxidative DNA Damage Assay Based on Modified Southern Blot Procedure

This assay employs BER proteins, which recognize and cleave DNA containing oxidative base damage present in a specific sequence of mtDNA, allowing estimation of the number of oxidative DNA lesions within the mtDNA genome *(14, 28, 44)*. Ntg1p-recognizable lesions in a 4.4-kb fragment of the mitochondrial genome containing the cytochrome *b* (*COB1)* gene are quantified to estimate the total number of oxidative lesions in mtDNA. The technique employs alkaline gel electrophoresis to resolve mtDNA which, if containing oxidative DNA damage, has been recognized and cleaved by Ntg1p (producing a smear of single-stranded DNA products) from uncut DNA (4.4-kb band). The number of lesions is estimated by comparing the intensity of the band in an untreated sample (**Fig. 17.2B**, odd lanes) to the full-length fragment that remains after incubation with Ntg1p (**Fig. 17.2B**, even lanes) and is visualized via Southern blot analysis (**Fig. 17.2**, Table **17.3**). This method allows one to determine the genes that are involved in managing oxidative mtDNA damage by testing strains mutant for specific repair or repair-related genes. It can also be used to measure the amount of oxidative mtDNA damage caused by exogenous sources of ROS (for example, hydrogen peroxide plus

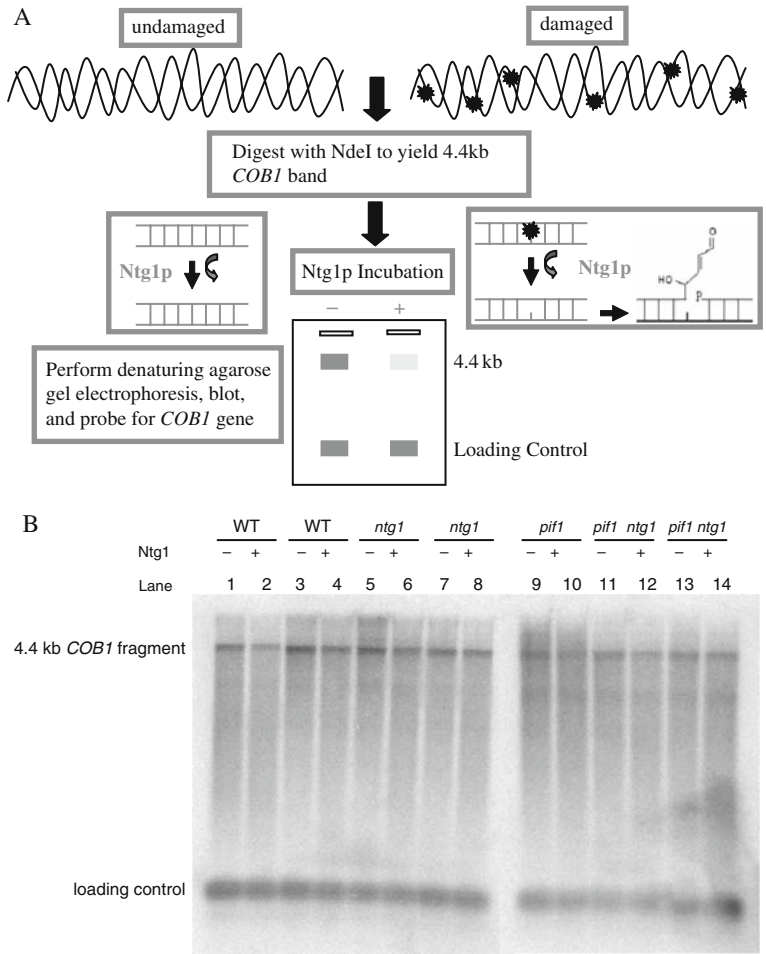


Fig. 17.2. Oxidative DNA damage assay. (**A**) Protocol for quantification of Ntg1p-recognizable lesions in mtDNA. (**B**) Southern blot result using DNA from WT or mutant cells with or without Ntg1p treatment.

antimycin). Finally, the assay can be employed to determine the effects of increased ROS levels resulting from mutations in ROS scavenging pathways such as those mediated by mitochondrial superoxide dismutase (*SOD2)* on mtDNA *(45)* (modified from Doudican et al. *(14)*).

The mitochondrial oxidative DNA damage assay is used to approximate the number of oxidative lesions in the mtDNA population of yeast cells. This method involves the isolation of mtDNA, cleavage with the DNA glycosylase/AP lyase, Ntg1p, and a Southern hybridization step in order to determine the number of oxidative DNA lesions per cell.

Table 17. 3
Oxidative DNA damage frequencies. Ntg1p-recognized lesions in the COB1 locus and overall mitochondrial genome. Mutation frequency is high in cells lacking *NTG1* and *PIF1*

Strain	Ratio of band intensities of Ntg1p-treated to untreated samples[a]	Lesions per 4.4-kb *COB1* fragment[b]	Lesions per genome[c]
WT	0.96 ± 0.05	0.043	1
ntg1	0.90 ± 0.04	0.110	2
pif1	0.71 ± 0.02	0.357	7
pif1 ntg1	0.52 ± 0.02	0.654	12
sod2	0.87 ± 0.06	0.154	3
ntg1 sod2	0.65 ± 0.03	0.430	8

From Doudican et al. *(14)*, reprinted with permission.
[a]Values represent the average of ratios derived from a minimum of four independent blots plus or minus the SEM.
[b]Values calculated using the Poisson expression: $S = -\ln P_0$ (S, lesions per 4.4-kb *COB1* fragment; P_0, ratio of band intensities of Ntg1p-treated to untreated samples).
[c]Values were extrapolated from calculation of lesions per 4.4-kb *COB1* fragment.

3.2.1. Isolation of Yeast DNA

1. Centrifuge log phase yeast culture at 5,000 x g to collect approx. 2×10^9 cells per tube in ten tubes. Follow Steps 2–14 for each of the ten tubes.
2. Resuspend in 0.8 ml SorEDTA, and transfer to an Eppendorf tube.
3. Add 100 µl of 10 mg/ml zymolyase 20T and 25 µl of 1 M DTT. Incubate cells overnight at 37°C.
4. Pellet spheroplasts at 2,500 × g for 5 min, and resuspend pellet in 0.5 ml Tris–EDTA containing 50 µl of 20 mg/ml of proteinase K. Incubate at 55°C for 1 h.
5. Add 100 µl of 10% SDS, and incubate at 65°C for 20 min.
6. Add 300 µl of 5 M potassium acetate, and incubate on ice for 30 min.
7. Centrifuge in microfuge at maximum speed (15,000 × g) at room temperature for 5 min, and transfer the supernatant to a new tube.
8. Add 500 µl of chloroform. Mix and centrifuge for 2 min. Remove upper aqueous layer (contains DNA). Repeat chloroform extraction twice on the aqueous layer.

9. Add an equal volume of isopropanol to precipitate the DNA. Centrifuge for 10 min.
10. Remove isopropanol, and air-dry the pellet.
11. Resuspend the pellet in 300 µl of TE with 5 µl of 10 mg/ml RNase A. Incubate at 37°C for 1 h.
12. Add 30 µl of 3 M sodium acetate. Add 1 ml of absolute ethanol.
13. Place in –20°C freezer overnight.
14. Centrifuge at maximum speed (15,000 × g) at 4°C for 30 min.
15. Wash twice in 70% ethanol, and air-dry the pellet.
16. Resuspend each tube in 10–30 µl of water. Consolidate ten tubes, and incubate at 42°C for 10 min (*see* **Note 5**).

3.2.2. DNA Treatment with Enzymes

1. Quantify DNA using the fluorescent dye bisbenzimide, as per instructions supplied by Sigma.
2. Digest 10 µg aliquots of genomic DNA with 50 U of *Nde*I in a 50 µl reaction for 4 h at 37°C (*see* **Note 6**).
3. Precipitate DNA by adding 11 µl of 4 M sodium acetate and 1 ml of absolute ethanol. Vortex for 5 s at medium speed, and place at –20°C overnight. Centrifuge in 4°C microfuge at highest speed for 15 min. Remove supernatant. Wash pellet with 1 ml of 70% ethanol. Allow wash to sit at room temperature for 5 min. Centrifuge at maximum speed (15,000 × g) for 2 min at room temperature. Remove supernatant. Remove all remaining liquid. (Caution: if drying pellet by vacuum centrifugation, do not over-dry the pellet.) Add 25 µl of water to resuspend the pellet. Place at 37°C for 1 h to completely dissolve DNA. It is very important to ensure that the DNA is fully dissolved.
4. Digest DNA with Ntg1p. In a 25 µl reaction containing Ntg1p digestion buffer, mix 10 µg of *Nde*I-digested genomic DNA and 5 µg of recombinant GST-tagged Ntg1p. Digest at 37°C for 30 min. Identical control reactions lacking Ntg1p should be performed for the determination of the level of total *COB1* DNA fragment loaded onto the gel (*see* **Note 1**).
5. Terminate the reaction by heating at 60°C for 5 min.

3.2.3. Southern Blot

1. Generate probe complementary to the restriction fragment of interest containing the *COB1* gene. First, amplify a region of the *COB1* gene using primers specific to *COB1*. Mix the following: 2.5 µl of PFU 10X buffer, 2.5 µl of dNTP mix (2 mM), 0.5 µM of forward primer, 0.5 µM of reverse primer, 0.2 µl of PFU polymerase, 1 µl of (approx. 125 ng) DNA from yeast genomic preparation, 17.8 µl of water. Perform PCR using the following conditions: 95°C, 5 min; 25 cycles of the following: 95°C, 1 min; 58°C, 1 min; 72°C, 1 min; and

finally 72°C, 8 min. Hold at 4°C. Pool 5 × 25 µl reactions, and gel purify using the Qiagen PCR purification kit (follow kit directions, and elute with water). Verify expected product size (400 bp) on a 1% agarose gel.

Label the PCR fragment with [α-^{32}P]dATP using random primed synthesis. Mix 1 µl of probe (25–50 ng) with 27 µl of water. Heat to 90°C for 10 min. Cool on ice. Centrifuge to collect liquid. Add 5 µl of Klenow buffer, 5 µl of random hexamers, 5 µl of dNTP mix without dATP, 5 µl of [α-^{32}P]dATP, 2 µl of Klenow (Promega). Mix with pipette. Allow to sit at room temperature for 1 h. Add 50 µl of TE buffer. Measure the labeling efficiency in a scintillation counter (count 1). Purify sample over a Micro Bio-Spin 6 column. Centrifuge for 3 min at 3.5 rpm, collecting sample in Eppendorf tube. Count active sample again in scintillation counter (count 2). Percent radiolabel incorporation is calculated as count 2/count 1 × 100; 30–50% incorporation is adequate labeling. The probe can be prepared several days before use. Store at –20°C until use.

2. Electrophorese restricted DNA on a 1.1% denaturing, alkaline agarose gel using alkaline electrophoresis buffer. Mix DNA, denaturing loading dye, and add *COB1* unlabeled PCR product (identical to the probe) to each sample as a loading control (0.15 ng per µg of genomic DNA). Run gel at 40 V for 30 min and then overnight (15 h) at 18 V (*see* **Note 7**).
3. Remove gel from electrophoresis. If desired, take a picture of the gel using gel imager. Soak the gel in 100 ml alkaline transfer buffer for 15 min. Change buffer. Soak gel in alkaline transfer buffer again for 10 min.
4. Perform Southern transfer in alkaline transfer buffer as described by Sambrook and Russell *(39)*. Assemble the Southern transfer apparatus. Place a glass plate support over a glass dish such that there are gaps on either end of the glass plate. Lay a piece of thick Whatman 3 MM paper (cut to be the length of the glass dish) over the glass plate, and insert the ends of the paper into the gaps at the ends of the glass plate. Pour alkaline transfer buffer over the thick Whatman 3 MM paper, filling the glass dish approximately half way. Place the gel on top of the Whatman paper. Surround, but do not cover the gel with cut pieces of parafilm, placing the parafilm on top of the Whatman paper. Place pre-wet nylon membrane (cut to be the size of the gel) over the gel, smoothing out any bubbles. Place two pre-wet Whatman 3 MM paper (cut to be the size of the gel) on top of the nylon membrane. Place a 2.5 in. stack of paper towels (cut to be the size of the gel) on top of the Whatman paper. Place another glass plate on top of the paper towels, and place a weight (250–300 g) on top of the plate.

5. Following 24 h of transfer, gel can be washed with 100 ml of 2X SSC for a few seconds, and DNA should be crosslinked to the nylon membrane using a UV crosslinker set at 2400 J, and the blot should be pre-hybridized at 65°C for 2–3 h in rapid-hyb buffer (Amersham Biosciences).
6. Following the pre-hybridization for 2–3 h, 50 µl of labeled probe should be boiled in a screw cap tube for 10 min, placed on ice briefly to cool, and added directly to the pre-hybridization buffer. Rotate for approximately 3 h at 65°C. Wash with approximately 20 ml of wash buffer for 10 min, and repeat washes until the center of the blot contains approx. 4 mR/h when scanned with a Geiger counter held at approx. 1 in. distance from the blot and the edges are less than 4 mR/h. Wrap the blot in plastic wrap, and place in phosphorimager plate. Develop for 2 h or overnight at room temperature. Placing the cassette at –20°C can allow greater resolution of bands.
7. Band intensity should be determined using phosphorimager analysis. The number of Ntg1p-recognized lesions can be determined by normalizing the density of each *COB1* band (top band in gel) to the loading control (*COB1* PCR product at bottom of gel; *see* **Note 7**). Next, the number of Ntg1p-recognizable lesions within the *COB1* fragment is calculated by the following equation, derived from the Poisson equation: $-\ln [A/B]$, where A is the normalized density of Ntg1-treated 4.4-kb fragment, and B is the normalized density of untreated 4.4-kb fragment. The number of lesions per mitochondrial genome can be calculated with the following equation: $-\ln[A/B] \times 85{,}799/4{,}400$ (the number of bases in *S. cerevisiae* mtDNA divided by the number of bases in the probed fragment) (*see* **Note 8**). **Table 17.3** illustrates an example of quantification of lesions by the oxidative DNA damage assay *(14)*. The results of the experiment show an increase in the number of lesions in *pif1* mutants that is similar to that of *ntg1 sod2* double mutants. In double mutants for *ntg1 pif1*, the number of lesions is increased synergistically. Therefore, in yeast, *NTG1* and *PIF1* are important in the repair of oxidative mtDNA damage.

3.3. Measurement of mtDNA Integrity Based on "Petite-Mutant Induction" Assay

mtDNA integrity can be determined by evaluating a yeast cell's respiratory competency. Yeast that cannot respire due to mtDNA or nuclear gene mutations are called petite mutants due to small colony formation when grown on medium with limited amounts of dextrose *(24)*. All laboratory yeast strains are capable of spontaneously producing petite mutants resulting from mutation or loss of mtDNA, and so the petite-induction rates vary dramatically depending on strain background. Additionally, insult to the

mtDNA by DNA damaging agents, including oxidative DNA damage, can induce yeast to become petite. A respiration-competent (rho^+, rho plus) yeast cell contains intact mtDNA; whereas rho^0 (rho zero) and rho^- (rho minus) cells are unable to respire and are termed petite *(23)*. Rho^- mutants are incapable of carrying out normal mitochondrial respiratory function due to point mutations and large deletions of most of the mtDNA sequence along with amplification of specific sequences seen as head-to-tail repeats; whereas, rho^0 mutants have no mtDNA at all *(24)*. **Figure 17.3** illustrates the relationship between mtDNA integrity and respiration competency. The rho status of a cell can be ascertained by determining the survival on plates containing dextrose versus glycerol. Both spontaneous and induced petite formations are commonly used measures of mtDNA stability, and it is an important phenotype to monitor and quantify when studying mtDNA damage and repair pathways *(34)*. One can study the genes involved in maintaining mitochondrial genomic integrity by determining the genes required for maintaining rho^+ status of mitochondria.

The basis for petite-mutant induction is that yeast must respire in order to grow on YPG plates; whereas, all yeast can grow on YPD plates regardless of rho status. Therefore, yeast lacking mtDNA (rho^0) or containing multiple rearrangements and mutations (rho^-) are unable to respire and will not grow on YPG, but will grow on YPD (modified from Doudican et al. *(14)*).

1. Grow yeast strains in YPG medium for approx. 36 h to near-saturation (stationary phase) at 30°C.
2. Dilute cultures to approx. 5×10^4 cells/ml into 100 ml SD medium with appropriate nutritional supplements as required by each strain or in YPD if no selection is necessary.
3. Remove samples from the cultures at times 0 and 24 h (after eight generations).

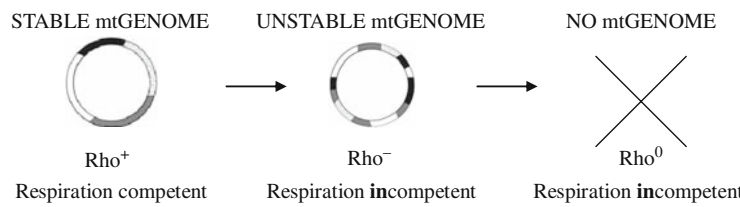


Fig. 17.3. The relationship between the mtDNA integrity and respiration competency. Respiration-competent yeast contain an intact mtDNA genome and are designated Rho^+. These yeast can grow on YPD and YPG media. Yeasts with unstable mtDNA or no mtDNA are respiration incompetent and can only grow on YPD plates, not YPG plates. Those yeast with unstable, corrupted mtDNA are designated rho^-, and those with no mtDNA are designated rho^0. Rho^0 cells are distinguished from rho^- cells by fluorescence microscopy of DAPI stained mtDNA.

4. Dilute samples in sterile water and plate on YPD and YPG plates. Dilutions should be done in order to allow between 100 and 1,000 colonies per plate (*see* **Note 9**). Grow at 30°C for 2–5 days.
5. Determine the number of colonies on each plate, and calculate the ratio of respiration-competent cells (number of colonies on YPG/number of colonies on YPD). **Figure 17.4** displays an example of a petite-induction assay. The results of the experiment show that Pif1p and Ntg1p are important for the maintenance of the mtDNA genome, particularly under circumstances of high oxidative stress. In the presence of exogenous oxidative stress, *ntg1*Δ*pif1*Δ strains accumulate a significantly number of petite cells.

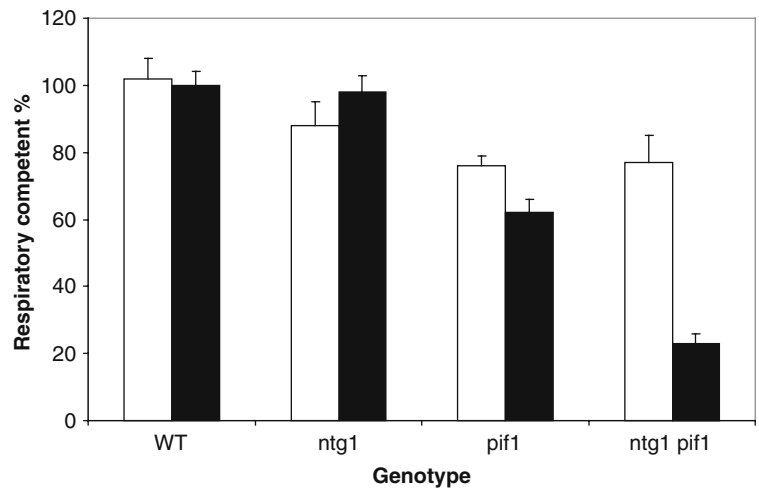


Fig. 17.4. "Petite induction" assay. Petite-mutation induction assays with WT, *ntg1*Δ, *pif1*Δ, and *ntg1*Δ *pif1*Δ mutations before (*open bars*) and after (*closed bars*) exposure to exogenous antimycin (1 mg/ml) and hydrogen peroxide (10 mM) for 1 h. From Doudican et al. *(14)*, reprinted with permission.

4. Notes

1. Activity should be tested on each purified aliquot prior to use. This can be done by incubating with oligonucleotides containing lesions recognized by Ntg1p as in Meadows et al. *(46)*. Other oxidative DNA damage recognition enzymes can be employed to detect the presence of oxidative damage. Many enzymes are available from vendors. These include

E. coli Fpg (NEB: M0240L; Trevigen: 4040-100-01); hOGG1 (NEB: M0241L; Trevigen: 4130-100-01); *E. coli* Endo III (NEB: M0268L); *E. coli* Endo IV (NEB: M0304L).

2. A suggested starting dilution factor is 10^5.
3. A suggested starting dilution factor is 10^6.
4. It is best to grow 30–45 independent YPG starter cultures in order to obtain a good approximation of the number of point mutations caused by a genetic background.
5. It is important that the DNA is completely dissolved at this step. It may be necessary to incubate at 4°C overnight in order to completely dissolve the DNA before digestion.
6. Digestion with this enzyme leads to a 4.4-kb fragment containing the *COB1* gene.
7. A 1.1% agarose gel is sufficient for mtDNA entry onto the gel. The gel and buffer should contain ethidium bromide at a concentration of 0.6 and 0.3 μg/ml, respectively, so that the DNA can be visualized prior to transfer. It is also advisable to verify that the DNA entered the gel and appears to be the correct size. For this purpose, it is useful to include a DNA ladder in one of the lanes. The loading control is simply the probe, which will run at 400 bp and will be probed during hybridization. During phosphorimaging, this band will be used to normalize for any error in the loading of the DNA. ImageQuant is a useful computer program for analyzing band intensity. A simple volume report for each band will give a band intensity that can be used to calculate the number of lesions.
8. It is advisable that at least four independent experiments be performed to determine the levels of oxidative damage in mtDNA to account for any variability between samples and experiments. Considering its size compared to nuclear DNA (12,500,000 bp), an equivalent fold change in oxidative DNA damage is more significant when found in mtDNA (85,799 bp) than in nuclear DNA.
9. Suggested starting dilutions are 10^3 or 10^4.

Acknowledgments

Funding provided by the Program Project Grant P01 ES011163. Emory University for use of facilities and funding. Dr. Barbara Evert for creating the figure from which Figure 1A was modified. Dr. Tina Saxowsky and Dr. Natasha Degtyareva for comments and suggestions regarding the writing of the chapter.

References

1. Beckman, K.B. and Ames, B.N. (1997) Oxidative decay of DNA. *J Biol Chem*, **272**, 19633–19636.
2. Lecrenier, N. and Foury, F. (2000) New features of mitochondrial DNA replication system in yeast and man. *Gene*, **246**, 37–48.
3. Cooke, M.S., Evans, M.D., Dizdaroglu, M. and Lunec, J. (2003) Oxidative DNA damage: mechanisms, mutation, and disease. *Faseb J*, **17**, 1195–1214.
4. Slupphaug, G., Kavli, B. and Krokan, H.E. (2003) The interacting pathways for prevention and repair of oxidative DNA damage. *Mutat Res*, **531**, 231–251.
5. Miquel, J. (1991) An integrated theory of aging as the result of mitochondrial-DNA mutation in differentiated cells. *Arch Gerontol Geriatr*, **12**, 99–117.
6. Caron, F., Jacq, C. and Rouviere-Yaniv, J. (1979) Characterization of a histone-like protein extracted from yeast mitochondria. *Proc Natl Acad Sci U S A*, **76**, 4265–4269.
7. Richter, C., Park, J.W. and Ames, B.N. (1988) Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc Natl Acad Sci U S A*, **85**, 6465–6467.
8. Santos, J.H., Mandavilli, B.S. and Van Houten, B. (2002) Measuring oxidative mtDNA damage and repair using quantitative PCR. *Methods Mol Biol*, **197**, 159–176.
9. Yakes, F.M. and Van Houten, B. (1997) Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. *Proc Natl Acad Sci U S A*, **94**, 514–519.
10. Clayton, D.A., Doda, J.N. and Friedberg, E.C. (1974) The absence of a pyrimidine dimer repair mechanism in mammalian mitochondria. *Proc Natl Acad Sci U S A*, **71**, 2777–2781.
11. Chi, N.W. and Kolodner, R.D. (1994) Purification and characterization of MSH1, a yeast mitochondrial protein that binds to DNA mismatches. *J Biol Chem*, **269**, 29984–29992.
12. Foury, F. and Lahaye, A. (1987) Cloning and sequencing of the PIF gene involved in repair and recombination of yeast mitochondrial DNA. *Embo J*, **6**, 1441–1449.
13. Mason, P.A., Matheson, E.C., Hall, A.G. and Lightowlers, R.N. (2003) Mismatch repair activity in mammalian mitochondria. *Nucl. Acids Res.*, **31**, 1052–1058.
14. Doudican, N.A., Song, B., Shadel, G.S. and Doetsch, P.W. (2005) Oxidative DNA damage causes mitochondrial genomic instability in *Saccharomyces cerevisiae*. *Mol Cell Biol*, **25**, 5196–5204.
15. Pinz, K.G. and Bogenhagen, D.F. (1998) Efficient repair of abasic sites in DNA by mitochondrial enzymes. *Mol Cell Biol*, **18**, 1257–1265.
16. O'Rourke, T.W., Doudican, N.A., Mackereth, M.D., Doetsch, P.W. and Shadel, G.S. (2002) Mitochondrial dysfunction due to oxidative mitochondrial DNA damage is reduced through cooperative actions of diverse proteins. *Mol Cell Biol*, **22**, 4086–4093.
17. Singh, K.K., Sigala, B., Sikder, H.A. and Schwimmer, C. (2001) Inactivation of *Saccharomyces cerevisiae* OGG1 DNA repair gene leads to an increased frequency of mitochondrial mutants. *Nucleic Acids Res*, **29**, 1381–1388.
18. Bohr, V.A. (2002) Repair of oxidative DNA damage in nuclear and mitochondrial DNA, and some changes with aging in mammalian cells. *Free Radic Biol Med*, **32**, 804–812.
19. Mandavilli, B.S., Santos, J.H. and Van Houten, B. (2002) Mitochondrial DNA repair and aging. *Mutat Res*, **509**, 127–151.
20. Lenaz, G. (1998) Role of mitochondria in oxidative stress and ageing. *Biochim Biophys Acta*, **1366**, 53–67.
21. Wei, Y.H. (1998) Oxidative stress and mitochondrial DNA mutations in human aging. *Proc Soc Exp Biol Med*, **217**, 53–63.
22. Wallace, D.C. (1992) Diseases of the mitochondrial DNA. *Annu Rev Biochem*, **61**, 1175–1212.
23. Ferguson, L.R. and von Borstel, R.C. (1992) Induction of the cytoplasmic 'petite' mutation by chemical and physical agents in *Saccharomyces cerevisiae*. *Mutat Res*, **265**, 103–148.
24. Ephrussi, B., de Margerie-Hottinguer, H. and Roman, H. (1955) Suppressiveness: a new factor in the genetic determinism of the synthesis of respiratory enzymes in yeast. *Proc Natl Acad Sci U S A*, **41**, 1065–1071.
25. You, H.J., Swanson, R.L., Harrington, C., Corbett, A.H., Jinks-Robertson, S., Senturker, S., Wallace, S.S., Boiteux, S., Dizdaroglu, M. and Doetsch, P.W. (1999) *Saccharomyces cerevisiae* Ntg1p and Ntg2p: broad specificity N-glycosylases for the repair of oxidative DNA damage in the

nucleus and mitochondria. *Biochemistry*, **38**, 11298–11306.

26. Alseth, I., Eide, L., Pirovano, M., Rognes, T., Seeberg, E. and Bjoras, M. (1999) The *Saccharomyces cerevisiae* homologues of endonuclease III from *Escherichia coli*, Ntg1 and Ntg2, are both required for efficient repair of spontaneous and induced oxidative DNA damage in yeast. *Mol Cell Biol*, **19**, 3779–3787.
27. Helbock, H.J., Beckman, K.B., Shigenaga, M.K., Walter, P.B., Woodall, A.A., Yeo, H.C. and Ames, B.N. (1998) DNA oxidation matters: the HPLC-electrochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guanine. *Proc Natl Acad Sci U S A*, **95**, 288–293.
28. Anson, R.M., Hudson, E. and Bohr, V.A. (2000) Mitochondrial endogenous oxidative damage has been overestimated. *Faseb J*, **14**, 355–360.
29. Coste, F., Ober, M., Carell, T., Boiteux, S., Zelwer, C. and Castaing, B. (2004) Structural basis for the recognition of the FapydG lesion (2,6-diamino-4-hydroxy-5-formamidopyrimidine) by formamidopyrimidine-DNA glycosylase. *J Biol Chem*, **279**, 44074–44083.
30. Gilboa, R., Zharkov, D.O., Golan, G., Fernandes, A.S., Gerchman, S.E., Matz, E., Kycia, J.H., Grollman, A.P. and Shoham, G. (2002) Structure of formamidopyrimidine-DNA glycosylase covalently complexed to DNA. *J Biol Chem*, **277**, 19811–19816.
31. Frosina, G. (2006) Prophylaxis of oxidative DNA damage by formamidopyrimidine-DNA glycosylase. *Int J Cancer*, **119**, 1–7.
32. Radyuk, S.N., Michalak, K., Rebrin, I., Sohal, R.S. and Orr, W.C. (2006) Effects of ectopic expression of Drosophila DNA glycosylases dOgg1 and RpS3 in mitochondria. *Free Radic Biol Med*, **41**, 757–764.
33. Ayala-Torres, S., Chen, Y., Svoboda, T., Rosenblatt, J. and Van Houten, B. (2000) Analysis of gene-specific DNA damage and repair using quantitative polymerase chain reaction. *Methods*, **22**, 135–147.
34. Shadel, G.S. (1999) Yeast as a model for human mtDNA replication. *Am J Hum Genet*, **65**, 1230–1237.
35. Potter, V.R. and Reif, A.E. (1952) Inhibition of an electron transport component by antimycin A. *J Biol Chem*, **194**, 287–297.
36. Bromme, H.J., Ebelt, H., Peschke, D. and Peschke, E. (1999) Alloxan acts as a prooxidant only under reducing conditions: influence of melatonin. *Cell Mol Life Sci*, **55**, 487–493.
37. Seo, B.B., Nakamaru-Ogiso, E., Flotte, T.R., Matsuno-Yagi, A. and Yagi, T. (2006) In vivo complementation of complex I by the yeast Ndi1 enzyme. Possible application for treatment of Parkinson disease. *J Biol Chem*, **281**, 14250–14255.
38. Hixon, S.C., Ocak, A., Thomas, J.E. and Daugherty, J.P. (1980) Resistance to adriamycin cytotoxicity among respiratory-deficient mutants in yeast. *Antimicrob Agents Chemother*, **17**, 443–449.
39. Sambrook, J. and Russell, D.W. (2001) *Molecular cloning: a laboratory manual of research procedures.* 3rd ed. Cold Springs Harbor Laboratory Press, Cold Springs, NY.
40. Rodeheffer, M.S., Boone, B.E., Bryan, A.C. and Shadel, G.S. (2001) Nam1p, a protein involved in RNA processing and translation, is coupled to transcription through an interaction with yeast mitochondrial RNA polymerase. *J. Biol. Chem.*, **276**, 8616–8622.
41. Clark-Walker, G.D. and Linnane, A.W. (1967) The biogenesis of mitochondria in *Saccharomyces cerevisiae*. A comparison between cytoplasmic respiratory-deficient mutant yeast and chlormaphenicol-inhibited wild type cells. *J Cell Biol*, **34**, 1–14.
42. Cui, Z. and Mason, T.L. (1989) A single nucleotide substitution at the rib2 locus of the yeast mitochondrial gene for 21S rRNA confers resistance to erythromycin and cold-sensitive ribosome assembly. *Curr Genet*, **16**, 273–279.
43. Tenson, T. and Ehrenberg, M. (2002) Regulatory nascent peptides in the ribosomal tunnel. *Cell*, **108**, 591–594.
44. Evert, B.A., Salmon, T.B., Song, B., Jingjing, L., Siede, W. and Doetsch, P.W. (2004) Spontaneous DNA damage in *Saccharomyces cerevisiae* elicits phenotypic properties similar to cancer cells. *J Biol Chem*, **279**, 22585–22594.
45. Liu, X.F., Elashvili, I., Gralla, E.B., Valentine, J.S., Lapinskas, P. and Culotta, V.C. (1992) Yeast lacking superoxide dismutase. Isolation of genetic suppressors. *J Biol Chem*, **267**, 18298–18302.
46. Meadows, K.L., Song, B. and Doetsch, P.W. (2003) Characterization of AP lyase activities of *Saccharomyces cerevisiae* Ntg1p and Ntg2p: implications for biological function. *Nucl. Acids Res.*, **31**, 5560–5567.
47. Vongsamphanh, R., Fortier, P.K. and Ramotar, D. (2001) Pir1p mediates

translocation of the yeast Apn1p endonuclease into the mitochondria to maintain genomic stability. *Mol Cell Biol*, **21**, 1647–1655.

48. Bruner, S.D., Nash, H.M., Lane, W.S. and Verdine, G.L. (1998) Repair of oxidatively damaged guanine in *Saccharomyces cerevisiae* by an alternative pathway. *Curr Biol*, **8**, 393–403.
49. Bessler, J.B., Torredagger, J.Z. and Zakian, V.A. (2001) The Pif1p subfamily of helicases: region-specific DNA helicases? *Trends Cell Biol*, **11**, 60–65.
50. O'Rourke, T.W., Doudican, N.A., Zhang, H., Eaton, J.S., Doetsch, P.W. and Shadel, G.S. (2005) Differential involvement of the related DNA helicases Pif1p and Rrm3p in mtDNA point mutagenesis and stability. *Gene*, **354**, 86–92.
51. MacAlpine, D.M., Perlman, P.S. and Butow, R.A. (1998) The high mobility group protein Abf2p influences the level of yeast mitochondrial DNA recombination intermediates in vivo. *Proc Natl Acad Sci U S A*, **95**, 6739–6743.
52. Kleff, S., Kemper, B. and Sternglanz, R. (1992) Identification and characterization of yeast mutants and the gene for a cruciform cutting endonuclease. *Embo J*, **11**, 699–704.
53. Zassenhaus, H.P. and Denniger, G. (1994) Analysis of the role of the NUC1 endo/exonuclease in yeast mitochondrial DNA recombination. *Curr Genet*, **25**, 142–149.
54. Ling, F., Morioka, H., Ohtsuka, E. and Shibata, T. (2000) A role for MHR1, a gene required for mitochondrial genetic recombination, in the repair of damage spontaneously introduced in yeast mtDNA. *Nucleic Acids Res*, **28**, 4956–4963.

Chapter 18

Determination of DNA Mutation Load in Human Tissues Using Denaturing HPLC-Based Heteroduplex Analysis

Kok Seong Lim, Robert K. Naviaux, and Richard H. Haas

Abstract

Since its introduction more than a decade ago, denaturing HPLC has been widely used to screen nuclear and mitochondrial DNA for mutations. We recently developed a quantitative method based on denaturing HPLC to measure DNA mutation load, using tRNA Leu(UUR) region of the mitochondrial DNA as an example. The mutation load is determined based on the quadratic function that governs DNA reannealing and the formation of heteroduplex and homoduplex DNAs. We have used this assay to measure heteroplasmy level for several mitochondrial mutations in DNAs obtained from human tissue samples. This quantitative DHPLC assay is well suited for simultaneous detection and quantification of DNA mutations.

Key words: DHPLC, DNA mutation, mitochondrial DNA, heteroplasmy, genetic analysis, heteroduplex, homoduplex, tRNA Leu(UUR), human tissues, buccal DNA.

1. Introduction

Many methods have been developed to study DNA mutations including the most commonly used PCR-RFLP assay, PCR/allele-specific oligonucleotide dot blot, single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), temporal TGGE (TTGE) analysis, pyrosequencing, ligation-mediated PCR, real-time fluorescent PCR, and peptide nucleic acid binding assays. Most of these methods are either simple with low sensitivity or laborious and overly sensitive. Some modifications have also been applied to the existing assays to improve the sensitivity, e.g., radiolabeled PCR-RFLP. In recent years, denaturing HPLC (DHPLC) has become a popular technology in the study of mutation detection *(1–9)*. It relies on the use of a

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_18 Springerprotocols.com

hydrophobic column based on ion-pair reverse-phase liquid chromatography for the separation of heteroduplex and homoduplex DNAs. Heteroduplex DNA contains mismatched base pairs, and it forms during hybridization when two DNAs differing in one nucleotide are denatured. The heteroduplex DNA, which is thermally less stable than the corresponding homoduplex DNA, is resolved at elevated column temperatures, typically in the range of 50–70°C (partially denaturing temperatures) (For detailed reviews on the DHPLC technology, please *see* refs. *(10, 11)*.)

DHPLC has been used either to screen the whole mitochondrial genome *(7–9, 12, 13)* or to study specific regions of the genome for mutation *(7, 14, 15)*, followed by DNA sequencing to identify the mutation sites. Most of the studies that used this technology to study mitochondrial DNA (mtDNA) mutations have measured the mutation load (heteroplasmy level) in the mtDNA using PCR-RFLP–PAGE *(7)* or pyrosequencing *(9)*. We recently investigated the possibility of using DHPLC as a quantitative tool and developed a method to accurately measure the mutation load in DNA using this technology *(16)*. We have also improved the existing published methods *(12, 13)* in determining the mutation in low-level heteroplasmic DNA sample *(17)*.

1.1. Applications of DHPLC Analysis

DHPLC has been used extensively to study nuclear and mitochondrial DNA mutations. We demonstrate in this chapter the use of DHPLC to quantitatively study mutations in the tRNA Leu(UUR) region of the mitochondrial genome. This method can be modified to study other types or regions of DNA provided that suitable primers are designed to amplify the DNA region of interest. The accuracy of the mutation load determined by this quantitative DHPLC relies on a good separation of heteroduplex and homoduplex peaks.

DHPLC has also been used to purify heteroduplex DNA. Most DNA sequencing procedures are unable to detect mutation loads below 20%. We as well as other labs have reported methods to improve the low detection limit that involves the use of DHPLC to collect elution fractions containing heteroduplex for DNA fragment amplification before DNA sequencing (*12, 13, 17)*. Using this method, we have been able to detect mutation present at levels as low as 1% *(17)*.

1.2. Limitation of DHPLC

The use of DHPLC elution profiles has been suggested as a method that allows the correlation of a characteristic chromatographic profile with a specific sequence alteration (*2, 6, 18–21)*, however, we observed no characteristic DHPLC signatures for several common mtDNA mutations *(17)*. Therefore, DNA sequencing is necessary for the identification of the mutation even if the profiles showed very similar characteristic patterns.

2. Materials

2.1. DNA Extraction

1. RBC Lysis Solution (Qiagen, cat. no. 158904, former Gentra Puregene cat. no. D-50K1) (used with the whole blood sample).
2. Cell Lysis Solution (Qiagen, cat. no. 158908, former Gentra Puregene cat. no. D-50K2).
3. Protein Precipitation Solution (Qiagen, cat. no. 158912, former Gentra Puregene cat. no. D-50K3).
4. Oragene DNA Self-Collection Kit (DNA Genotek, cat. no. OG-250).
5. RNase A (Roche, cat. no. 109169).
6. Proteinase K (Roche, cat. no. 3115879).
7. Isopropanol (Sigma–Aldrich, cat. no. I9516).
8. Ethanol (BDH, cat. no. 101074F).

2.2. PCR

1. 2.5 Unit/μl Optimase DNA Polymerase and 10X Optimase reaction buffer (containing $MgSO_4$) (Transgenomic, cat. no. 703030).
2. 100 m*M* premixed dNTPs (25 m*M* of each dNTP) (Transgenomic, cat. no. 705010). A solution of 10 m*M* of total dNTPs is prepared and stored as aliquots of 100 μl at –20°C.
3. 100 μ*M* primer stocks [forward primer: *5′*-CTCACTGTCAA CCCAACACAGG-*3′ (2*,415–2,436 bp) and reverse primer: 5′-TGTGTTGTGATAAGGGTGGAGAG-*3′* (3,790–3,812 bp)] are stored at –80°C. These primers can be obtained from the MitoScreen Assay Kit (Transgenomic, cat. no. 707001) and have been used before (*8, 16)*. In our laboratory, we routinely store the diluted primers (at a concentration of 10 μ*M*) as small aliquots at –20°C. When it is in use, the diluted aliquot is kept at 4°C (s*ee* **Notes 1** and **2**).

2.3. Restriction Enzyme Digestion and Heteroduplex Formation

1. 10,000 Units/ml *Dde*I (New England Biolabs, cat. no. R0175S).

2.4. DHPLC Analysis

1. DNA Sizing Standard (pUC18 *Hae*III digest) (Transgenomic, cat. no. 560078).
2. 2 M Triethylammonium acetate (TEAA) solution (Transgenomic, cat. no. 553303).
3. Acetonitrile (Sigma–Aldrich, cat. no. 34851).

4. DHPLC buffers were prepared using the 2 *M* TEAA, acetonitrile, and water. Buffer A consists of 0.1 *M* TEAA, buffer B consists of 0.1 *M* TEAA and 25%(v/v) acetonitrile, and buffer C consists of 75%(v/v) acetonitrile.
5. DNASep cartridge (4.6 mm × 50 mm) (Transgenomic, cat. no. DNA-99-3510).
6. WAVE 3500 system equipped with a Hitachi D-7000 interface, L-7100 pump, L-7200 autosampler, L-7300 oven, L-7400 UV detector, and Navigator software (version 1.6.2) (Transgenomic).

3. Methods

3.1. DNA Extraction

We have extracted total DNAs (*see* **Note 3**) from human urine, saliva, blood, and muscle for DHPLC analysis. The extraction of DNA from human urine, whole blood, and muscle is performed as described in the Gentra Puregene DNA Extraction Protocol (Qiagen). We collect human saliva using Oragene DNA Self-Collection Kit and extract DNA according to the manufacturer's instruction with modifications (*see* **Section 3.1.1**).

3.1.1. Saliva

1. Incubate the Oragene/DNA mixture (4–6 ml, including the 1.9 ml Oragene solution) at 50°C overnight (if necessary, store the mixture at –20°C until the next step).
2. Add 1/4 volume Cell Lysis Solution, vortex on high speed for 10 s, incubate at room temperature for 15 min.
3. Add 1/100 volume 20 mg/ml proteinase K to the lysate. Incubate at 55°C for at least 1 h.
4. Add 1/40 volume 2 mg/ml RNase A to the lysate. Incubate at 37°C for 15 min, cool sample to room temperature.
5. Add 1/3 volume Protein Precipitation Solution to the lysate, vortex vigorously for 20 s, incubate on ice for 10 min.
6. Centrifuge at 6,000 × g for 10 min.
7. Collect supernatant, add 3/4 volume of isopropanol, invert for > 20 times (to increase yield, incubate at 4°C for > 1 h).
8. Centrifuge at 10,000 × g for 20 min to pellet DNA, wash the DNA pellet with 70%(v/v) ethanol.
9. Dissolve the DNA pellet in <50 µl sterilized water, rehydrate it at 65°C for at least 1 h before storing it at –20°C.
10. Determine the DNA concentration by measuring the absorbance at 260 nm (A_{260}) (*see* **Note 4**).

3.2. PCR

1. Prepare PCR mixture (total volume = 70 µl) as follows (*see* **Note 5**):

Components	Volume added (µl)
10X Optimase reaction buffer (containing $MgSO_4$)	7.0
10 m*M* premixed dNTPs	5.6
2.5 Unit/µl Optimase DNA polymerase	0.7
Sterilized H_2O	49.5
10 µ*M* Forward primer	2.1
10 µ*M* Reverse primer	2.1
50 ng/µl DNA (*see* **Note 6**)	3.0

2. Use the following conditions for PCR: 95°C for 2 min, 35 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 3 min, followed by a final extension step of 72°C for 5 min, and 4°C infinitely.

3.3. Restriction Enzyme Digestion and Heteroduplex Formation

1. Digest the PCR products using 1 µl of 10,000 Units/ml *Dde*I at 37°C for 6 h (*see* **Note 7**).
2. Denature the digested PCR products at 95°C for 5 min and then slowly cool them to 25°C at a rate of 1°C/min to induce heteroduplex formation (*see* **Note 8**).

3.4. DHPLC Analysis

1. Set the DHPLC temperature to 50°C, flow rate at 0.9 ml/min, and equilibrate the column for at least 20 min using 50% Buffer A and 50% Buffer B. UV detection was set at 260 nm.
2. Use the following linear gradient for sample analysis:

Time interval	Buffer A (%)	Buffer B (%)	Buffer C (%)
0.0 min	60	40	0
0.1 min	55	45	0
11.1 min	33	67	0
11.2 min	0	0	100
12.2 min	0	0	100
12.3 min	60	40	0
14.8 min	60	40	0

3. Inject 5 µl DNA Sizing Standard followed by 5 µl of each of the digested PCR products (generated from control DNA or patient DNA) into the DHPLC.
4. Analyze these samples at 50°C to estimate the restriction fragment sizes in the PCR products based on the DNA Sizing Standard (*see* first panel in **Fig. 18.1** and **Note 9**).

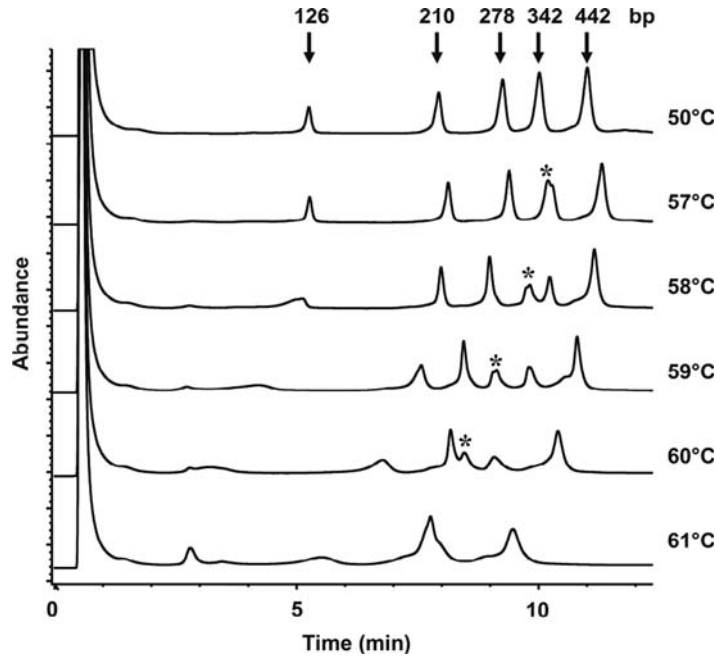


Fig. 18.1. DHPLC profiles of 40% A3260G mutation at several different temperatures. The heteroduplex peaks are marked with *.

5. Measure the peak areas for the restriction fragment of interest (in this case, the 342 bp restriction fragment) in the digested PCR products generated from control DNA and from patient DNA. This allows the estimation of the relative concentration of the control DNA and patient DNA (this is required for Step 8).
6. Analyze the samples at 59°C to check for mutations [this temperature is pre-determined using the software (*see* **Note 10**) and confirmed experimentally (*see* **Fig. 18.1**)].
7. Measure the peak areas for heteroduplex peak ($A1_{HET}$) and homoduplex peak ($A1_{HOM}$) that correspond to the 342-bp restriction fragment (*see* **Fig. 18.2** and **Note 11**). Calculate the mutation load (x_1) using $A1_{HET}$ and $A1_{HOM}$ (*see* Steps 2–4 in **Section 3.5**) [as two different mutation loads may yield the same peak area (for example, in **Fig. 18.3A**, both 10 and 90% mutations have the same heteroduplex and

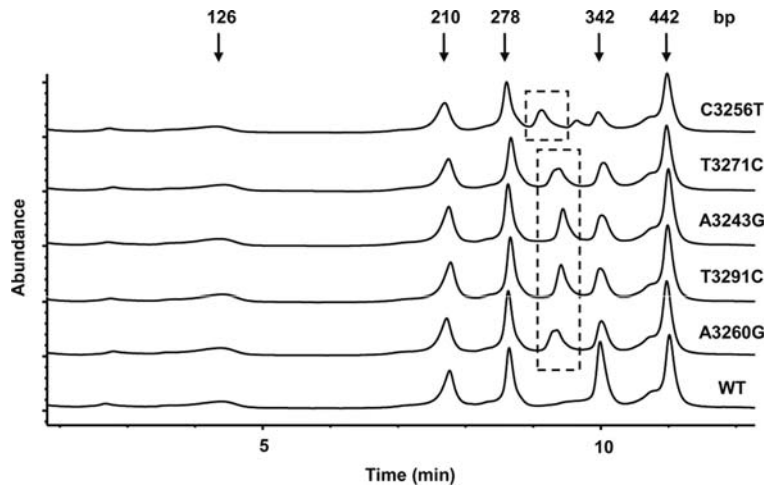


Fig. 18.2. DHPLC profiles of several mutant DNAs at 59°C. Samples containing 40% of C3256T, T3271C, A3243G, T3291C, and A3260G mutations were prepared by mixing the PCR products of respective mutations with wild-type (WT) DNA before DHPLC analysis. The *last panel* represents the DHPLC profile for wild-type (WT) DNA. Heteroduplex peaks are boxed. Note that for the C3256T mutation, the two homoduplex peaks (3256C and 3256T) were resolved using our DHPLC condition. Reprinted from *(17)* with permission from the American Society for Investigative Pathology and the Association for Molecular Pathology.

homoduplex peak area), further procedures (*see* Steps 8–12*)* are necessary in order to determine the exact mutation load] (*see* **Note 12**).

8. Mix the digested PCR products generated from patient DNA with equal concentrations (based on concentration estimation in Step 4) of digested PCR product generated from the control DNA.
9. Denature the digested PCR products mixture at 95°C for 5 min and then slowly cool them to 25°C at a rate of 1°C/min to induce heteroduplex formation.
10. Inject 5 µl of samples into the DHPLC.
11. Analyze the samples at 59°C.
12. Measure the peak areas for heteroduplex peak ($A2_{HET}$) and homoduplex peak ($A2_{HOM}$) that correspond to the 342-bp restriction fragment. Confirm the exact mutation load (x_2) using $A2_{HET}$ and $A2_{HOM}$ (*see* Steps 5–6 in **Section 3.5**).

3.5. Calculation of DNA Mutation Load

1. Determine the peak areas for both heteroduplex peak ($A1_{HET}$) and homoduplex peak ($A1_{HOM}$) for both control and patient DNAs using Navigator software.
2. Determine the heteroduplex proportion (y) using the formula below *(16)* :

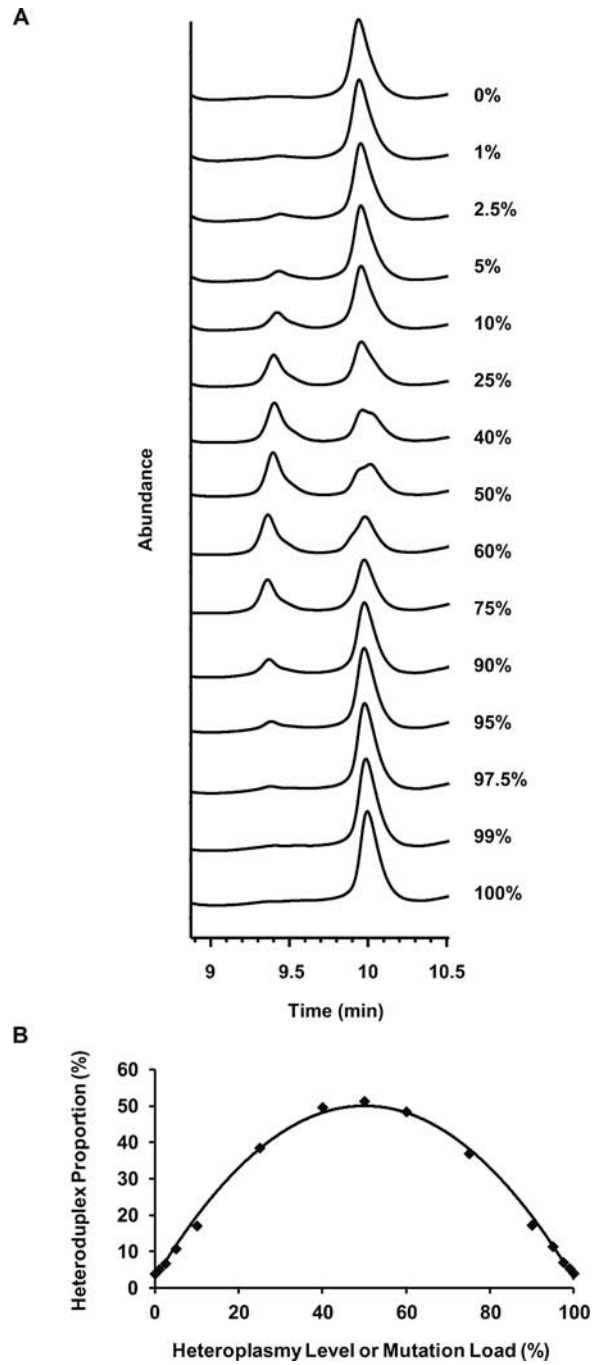


Fig. 18.3. (**A**) Detection of A3243G mutations by DHPLC in a series of samples with increasing mutation loads. (**B**) Parabolic relationships between heteroduplex proportions and heteroplasmy levels. Reprinted from *(16)* with permission from the American Association for Clinical Chemistry.

$$y = \frac{A1_{HET}}{A1_{HET} + A1_{HOM}} \times 100\%$$

3. Determine the corrected heteroduplex proportion (y_1) by subtracting the background level (heteroduplex proportion in control DNA) from the heteroduplex proportion in patient DNA.
4. Determine the possible mutation load (x_1) using the formula below:

$$x1 \approx 50 \pm \sqrt{2500 - 50y1}$$

5. After the analysis of PCR products containing equal mix of patient and control DNA (*see* Steps 8–12 in **Section 3.4**), determine the peak areas for both heteroduplex peak ($A2_{HET}$) and homoduplex peak ($A2_{HOM}$). Determine the heteroduplex proportion (y_2) using the formula below:

$$y_2 = \frac{A2_{HET}}{A2_{HET} + A2_{HOM}} \times 100\%$$

6. Determine the mutation load (x_2) using the formula below:

$$x2 \approx 100 - \sqrt{10000 - 200y_2}$$

7. Identify the exact mutation load from the two possible values obtained for x_1 based on the value obtained for x_2 (*see* **Note 13**).

4. Notes

1. Unless stated otherwise, all solutions should be prepared in water that has a resistivity of 18.2 MΩ cm. This standard is referred to as "water" in this chapter.
2. This protocol demonstrates the analysis of tRNA Leu(UUR) region of the mitochondrial genome, which is a hotspot for disease-causing mutations. The PCR primers used were 5′-CTCACTGTCAACCCAACACAGG-3′ (2,415–2,436 bp) and 5′-TGTGTTGTGATAAGGGTGGAGAG-3′ (3,790–3,812 bp) and this method allows for the detection of mutations, e.g., A3243G, C3256T, A3260G, T3271C, T3291C, and T3308C, which are among the more commonly reported mutations found in this region of the mitochondrial genome (J01415.2) (www.mitomap.org).
3. The presence of nuclear DNA in the mtDNA sample does not affect the quantitation of heteroplasmy, therefore we always use total DNA for the PCR.

4. The DNA yield we obtain with saliva is usually 20–50 μg DNA/ml saliva.
5. Control (wild-type) DNA is always used in the PCR with each batch of patient DNA analysis. Control DNA is used for confirmation of the mutation load (*see* Steps 8–12 in **Section 3.4**) and for background correction (*see* Step 3 in **Section 3.5**). This control DNA can be obtained from a healthy volunteer confirmed by DNA sequencing to be 100% identical in the region studied to the published mitochondrial genome sequence (J01415.2).
6. We have shown that the mutation loads determined by this DHPLC method remain constant over a wide range of DNA amounts (10–300 ng) used in the PCR *(16)*.
7. We have previously digested the PCR products using *Dde* I for 6, 12, and 24 h and observed that 6 h is adequate for complete digestion *(16)*. We observed neither an increase in restriction fragment peak area nor appearance of additional fragment peaks after 6 h.
8. These conditions produce maximal heteroduplex formation – approx. 50% heteroduplex in samples containing 50% heteroplasmy, indicating that the slow reannealing procedure is optimal *(16)*.
9. Restriction digestion of the PCR product generates five fragments of different sizes: 442, 342, 278, 210, and 126 bp (*see* **Fig. 18.1**). Restriction fragment of size 342 bp corresponds to the region of the mitochondrial genome that contains tRNA Leu(UUR) (position 3,193 –3,534 bp).
10. Temperature is the most important parameter that affects the sensitivity of DHPLC in the DNA mutation detection. We predicted temperatures for sample analysis using Navigator software (version 1.6.2) (Transgenomic) *(16)* and confirmed them experimentally to be 59°C. Alternatively, prediction of melting temperatures could be performed using the DHPLC Melt Program created by Stanford Genome Technology *(4)*. This temperature results in the separation of heteroduplex and homoduplex DNA species in the 342-bp restriction fragments (*see* **Fig. 18.1**). The resolution of the heteroduplex and homoduplex peaks is critical in the determination of the exact mutation load. Good peak separation can be achieved with the selection of optimal temperature and acetonitrile gradient. Theoretically, four peaks are resolved in a reannealed heteroplasmic DNA mixture, which produces two homoduplex peaks and two heteroduplex peaks, but in most cases the two homoduplex peaks elute at almost the same time.

11. Although Optimase DNA Polymerase is a proofreading thermostable polymerase that has been shown to effectively minimize the mis-incorporation of nucleotides during DNA amplification by PCR compared with other DNA polymerases, e.g., AmpliTaq and Pfu polymerase *(12, 22)* , we consistently observed a small peak that precedes the homoduplex peak in all the control DNA (100% wild-type) *(16)*. Such a low-level peak can affect the correct identification of heteroduplex peaks (real mutations), especially in DNA with low mutation loads. Therefore, we always use a control DNA (*see* **Note 5**) in the DHPLC analysis as a negative control and this background level is subtracted from the all the patient DNA.
12. A parabolic relationship exists between the measured heteroduplex proportion and heteroplasmy level (*see* **Fig. 18.3B**) *(16)*. Since only the vertex of the parabola at 50% heteroduplex has a single solution for the heteroplasmy level (i.e., the 50% heteroduplex is found only in 50% heteroplasmy) and all other heteroduplex proportions have two solutions, the mutation load determined from the heteroduplex and homoduplex peak areas usually has two possible values. Therefore, we always confirmed the correct levels by mixing the patient PCR products with equal concentrations of control PCR products (*see* **Note 5**) and subjected them to additional slow reannealing process before DHPLC analysis.
13. The value obtained for x_2 should be close to one of the values obtained for x_1. Although x_2 can theoretically be used to represent the exact mutation load in the DNA, we do not use it for this purpose as the concentration estimation and mixing of wild-type and control PCR product might introduce variation in the measurement. We routinely determined the exact mutation load using one of the values obtained for x_1.

Acknowledgments

This work was supported by the United Mitochondrial Disease Foundation (UMDF) grant awarded to RHH and generous grants from Rita and Steve Achard and the William Wright Family Foundation. Patient samples were obtained utilizing the resources of the UCSD GCRC provided under NCRR grant M01RR 000827. RKN is supported by grants from the UCSD Christini Fund and the Lennox Foundation.

Abbreviations

DHPLC: denaturing HPLC
mtDNA: mitochondrial DNA
TEAA: triethylammonium acetate

References

1. Underhill, P. A., Jin, L., Lin, A. A., et al. (1997) Detection of numerous Y chromosome biallelic polymorphisms by denaturing high-performance liquid chromatography. *Genome Res.* **7,** 996–1005.
2. O'Donovan, M. C., Oefner, P. J., Roberts, S. C., et al. (1998) Blind analysis of denaturing high-performance liquid chromatography as a tool for mutation detection. *Genomics* **52,** 44–49.
3. Liu, W., Smith, D. I., Rechtzigel, K. J., Thibodeau, S. N., and James, C. D. (1998) Denaturing high performance liquid chromatography (DHPLC) used in the detection of germline and somatic mutations. *Nucleic Acids Res.* **26,** 1396–1400.
4. Jones, A. C., Austin, J., Hansen, N., et al. (1999) Optimal temperature selection for mutation detection by denaturing HPLC and comparison to single-stranded conformation polymorphism and heteroduplex analysis. *Clin. Chem.* **45,** 1133–1140.
5. Wagner, T., Stoppa-Lyonnet, D., Fleischmann, E., et al. (1999) Denaturing high-performance liquid chromatography detects reliably BRCA1 and BRCA2 mutations. *Genomics* **62,** 369–376.
6. Gross, E., Arnold, N., Pfeifer, K., Bandick, K., and Kiechle, M. (2000) Identification of specific BRCA1 and BRCA2 variants by DHPLC. *Hum. Mutat.* **16,** 345–353.
7. van Den Bosch, B. J., de Coo, R. F., Scholte, H. R., et al. (2000) Mutation analysis of the entire mitochondrial genome using denaturing high performance liquid chromatography. *Nucleic Acids Res.* **28,** E89.
8. Bayat, A., Walter, J., Lamb, H., Marino, M., Ferguson, M. W., and Ollier, W. E. (2005) Mitochondrial mutation detection using enhanced multiplex denaturing high-performance liquid chromatography. *Int. J. Immunogenet* **32,** 199–205.
9. Biggin, A., Henke, R., Bennetts, B., Thorburn, D. R., and Christodoulou, J. (2005) Mutation screening of the mitochondrial genome using denaturing high-performance liquid chromatography. *Mol. Genet. Metab.* **84,** 61–74.
10. Xiao, W., and Oefner, P. J. (2001) Denaturing high-performance liquid chromatography: a review. *Hum. Mutat.* **17,** 439–474.
11. Oefner, P. J., and Huber, C. G. (2002) A decade of high-resolution liquid chromatography of nucleic acids on styrene-divinylbenzene copolymers. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **782,** 27–55.
12. Meierhofer, D., Mayr, J. A., Ebner, S., Sperl, W., and Kofler, B. (2005) Rapid screening of the entire mitochondrial DNA for low-level heteroplasmic mutations. *Mitochondrion* **5,** 282–296.
13. Wulfert, M., Tapprich, C., and Gattermann, N. (2006) Optimized PCR fragments for heteroduplex analysis of the whole human mitochondrial genome with denaturing HPLC. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **831,** 236–247.
14. Liu, M. R., Pan, K. F., Li, Z. F., et al. (2002) Rapid screening mitochondrial DNA mutation by using denaturing high-performance liquid chromatography. *World J. Gastroenterol.* **8,** 426–430.
15. Conley, Y. P., Brockway, H., Beatty, M., and Kerr, M. E. (2003) Qualitative and quantitative detection of mitochondrial heteroplasmy in cerebrospinal fluid using denaturing high-performance liquid chromatography. *Brain Res. Protoc.* **12,** 99–103.
16. Lim, K. S., Naviaux, R. K., and Haas, R. H. (2007) Quantitative mitochondrial DNA mutation analysis by denaturing HPLC. *Clin. Chem.* **53,** 1046–1052.
17. Lim, K. S., Naviaux, R. K., Wong, S., and Haas, R. H. (2008) Pitfalls in the denaturing high-performance liquid chromatography analysis of mitochondrial DNA mutation. *J. Mol. Diagn.* **10,** 102–108.

18. Su, Y. N., Lee, C. N., Hung, C. C., et al. (2003) Rapid detection of beta-globin gene (HBB) mutations coupling heteroduplex and primer-extension analysis by DHPLC. *Hum. Mutat.* **22,** 326–336.
19. Nickerson, M. L., Warren, M. B., Zbar, B., and Schmidt, L. S. (2001) Random mutagenesis-PCR to introduce alterations into defined DNA sequences for validation of SNP and mutation detection methods. *Hum. Mutat.* **17,** 210–219.
20. Nickerson, M. L., Weirich, G., Zbar, B., and Schmidt, L. S. (2000) Signature-based analysis of MET proto-oncogene mutations using DHPLC. *Hum. Mutat.* **16,** 68–76.
21. Ezzeldin, H., Okamoto, Y., Johnson, M. R., and Diasio, R. B. (2002) A high-throughput denaturing high-performance liquid chromatography method for the identification of variant alleles associated with dihydropyrimidine dehydrogenase deficiency. *Anal. Biochem.* **306,** 63–73.
22. Bayat, A., Walter, J. M., Lamb, H., Ferguson, M. W., and Ollier, W. E. (2003) Rapid denaturing high-performance liquid chromatography (DHPLC) for mutation scanning of the transforming growth factor beta3 gene using a novel proofreading polymerase. *Eur. J. Immunogenet.* **30,** 335–340.

Chapter 19

Rapid Identification of Unknown Heteroplasmic Mitochondrial DNA Mutations with Mismatch-Specific Surveyor Nuclease

Sylvie Bannwarth, Vincent Procaccio, and Véronique Paquis-Flucklinger

Abstract

Identification of mitochondrial DNA (mtDNA) mutations is essential for diagnosis and genetic counseling of mitochondrial diseases. In this chapter, we describe a strategy for the rapid identification of heteroplasmic mtDNA mutations that can be used routinely in molecular genetic laboratories. This protocol involves the following three steps: (i) PCR amplification of the entire human mitochondrial genome with 17 overlapping PCR products, (ii) localization of mtDNA mismatch(es) after digestion of the 17 amplicons by Surveyor Nuclease, a member of a family of plant DNA endonucleases that cleave double-strand DNA at any mismatch site, and (iii) identification of the mutation by sequencing the region containing the mismatch.

Key words: mtDNA, heteroplasmic mutation, Surveyor Nuclease, CEL nuclease family, respiratory chain defects, molecular screening.

1. Introduction

Several hundreds of different mitochondrial DNA mutations have been reported with a variety of human disorders (http://mitomap.org). The mutation may affect all the mtDNA molecules within a cell or a tissue (homoplasmy) or may co-exist with wild-type mitochondrial genomes (heteroplasmy). The mtDNA mutations are frequently divided into two groups, i.e., rearrangements (insertion, deletion) and point mutations. Most mtDNA rearrangements can be detected easily by long-range PCR amplification *(1)* or by Southern blot analysis *(2)*. Nevertheless, when common

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_19 Springerprotocols.com

point mutations have been ruled out, the identification of rare or unknown mtDNA mutations remains challenging. Several techniques, such as single-strand conformation polymorphism (SSCP) *(3)*, denaturing gradient gel electrophoresis (DGGE) *(4)*, or denaturing high-performance liquid chromatography (DHPLC) *(5, 6)* have been used. But, DNA sequencing remains the gold standard with multi-capillary electrophoresis and re-sequencing arrays providing platforms for high-throughput genetic analysis *(7, 8)*. Nevertheless, DNA sequencing fails to detect mutations that are present at a low heteroplasmic level (< 20%) *(9, 10)*.

Surveyor Nuclease, a DNA endonuclease isolated from celery (CEL 1), cleaves heteroduplex DNA with high specificity at sites of base-substitution mismatch and insertion/deletion up to at least 12 nucleotides *(11, 12)*. CEL I cuts both strands of a DNA heteroduplex on the 3′-side of the mismatch site in a manner independent of sequence context *(13)*. Surveyor Nuclease has been used for the identification of mutations in nuclear genes *(14, 15)*. Here we used this enzyme for the rapid and effective identification of heteroplasmic mtDNA mutations. The entire mitochondrial genome is amplified with 17 overlapping PCR products (**Fig. 19.1**). After digestion of the PCR products with Surveyor Nuclease, cleavage products are separated and analyzed

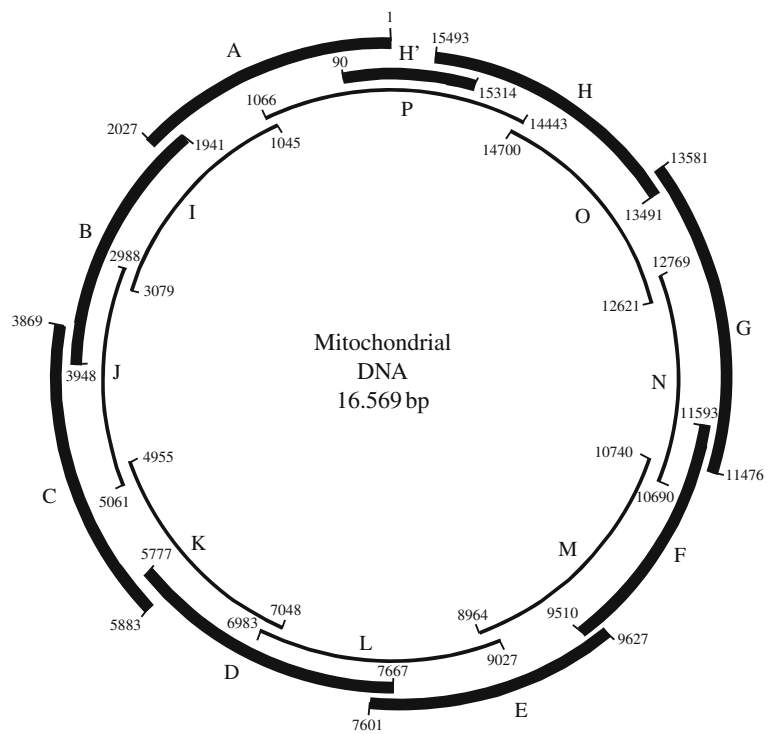


Fig. 19.1. Human mtDNA map and location of the 17 amplicons (GenBank J01415.2).

by agarose gel electrophoresis. The size of the digestion products indicates the location of the mutation, which is then confirmed and characterized by DNA sequencing.

In mitochondrial disorders, the clinical and biochemical phenotypes appear when the percentage of mutant molecule exceeds a critical value (usually in the range of 70–90%). Nevertheless, it has been shown recently that a mutation in a tRNA gene (m.5545C>T in $tRNA^{Trp}$) can cause a severe multisystem disorder and respiratory chain deficiency even at low levels of heteroplasmy in affected tissues *(16)*. This study illustrates the importance to use strategies that are able to detect low levels of heteroplasmic mutations. We have shown that Surveyor Nuclease can detect mutants present at as low as 3% heteroplasmy *(17)*. Nevertheless, when mutant load is low, for example, in DNA extracted from leukocytes, sequencing may yield ambiguous results not clearly distinguishable from background noise. In this case, the use of further methods to identify a mutation, suspected after Surveyor digestion but not clearly resolved by sequencing, is needed. Restriction fragment length polymorphism (RFLP) analysis can be used *(18)*. Another possibility is to use DHPLC coupled to a fraction collector module to elute the heteroduplex-specific fraction before amplification and sequencing. Pyrosequencing, which has been shown to be able to detect samples with 5% mutant load, could also be used *(19)*. Last, when it is possible, a good solution is to analyze different tissues harboring a higher level of heteroplasmy (e.g., urinary cells or muscle biopsy).

The limitation of this Surveyor strategy is that it cannot detect homoplasmic mutations. However, most homoplasmic base substitutions are usually found to be neutral (rather than deleterious) polymorphisms. Furthermore, it is possible to detect mtDNA somatic mutations, described in numerous cancers and tumoral cell lines *(20)*, which are usually found in homoplasmy. In this case, it is possible to mix equimolar amounts of DNA extracted from non-tumoral and tumoral tissues from the same patient to create heteroduplexes which can be detected by Surveyor Nuclease. We also have successfully detected homoplasmic mutations in children by mixing equimolar amounts of genomic DNA extracted from child tissues and mother leukocytes.

2. Materials

2.1. DNA Extraction

Genomic DNA is extracted from blood sample or tissue biopsy by classical phenol/chloroform procedure (*see* **Note 1**) *(21)*. Other procedures can also be used if the absence of DNA degradation is verified by gel agarose analysis.

2.2. PCR Reagents

1. Optimase Polymerase (Transgenomic, Omaha, NB, USA) for high-fidelity PCR. The kit includes Optimase DNA polymerase (250 U; 2.5 U/μl), 10X Optimase PCR buffer (Mg-free), and 25 mM $MgSO_4$. These reagents are stored at –20°C.
2. Deoxyribonucleotide triphosphates (dNTPs) mixture containing 10 mM dATP, 10 mM dTTP, 10 mM dCTP, 10 mM dGTP, purchased from Roche Diagnostics. dNTP mixture is stored at –20°C.
3. Lyophilized primers are resuspended in sterile deionized water at 10 μM stock solution. Aliquots of primer solution are maintained at –20°C.
4. Plasmid G and plasmid C, provided in the Surveyor Mutation Kit (Transgenomic), are used as "Surveyor positive control."

2.3. Gel Electrophoresis

1. Mini-agarose gel electrophoresis apparatus system for analysis of PCR products.
2. Agarose gel electrophoresis apparatus system for analysis of PCR products after Surveyor digestion.
3. Agarose, molecular grade.
4. 10X TBE: 1 M Tris, 0.9 M boric acid, and 0.01 M EDTA.
5. 1-kb DNA ladder and high-mass DNA ladder.
6. 6X Loading buffer containing 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, and 30% (v/v) glycerol.
7. 10 mg/ml Propidium Iodide (*see* **Note 2**).

2.4. Surveyor Digestion

Surveyor mutation kit for WAVE and WAVE HS Systems (Transgenomic). The kit includes 10X Surveyor Nuclease reaction buffer, Surveyor Enhancer W, Surveyor Nuclease W, Stop Solution, control C, and control G plasmids. These reagents are stored at –20°C.

2.5. Identification of the mtDNA Mutation Corresponding to the Mismatch

1. PCR reagents: Taq DNA Polymerase, dNTP mix (Roche Diagnostics), lyophilized primers resuspended in sterile deionized water at 10 μM stock solution. These reagents are stored at –20°C.
2. Gel electrophoresis equipment: *see* **Section 2.3.**
3. ExoSAP-IT (GE Healthcare). ExoSAP-IT and treated PCR products may be stored at –20°C.
4. ABI PRISM dRhodamine Terminal Cycle Sequencing Ready Reaction Kit (Applied Biosystems). These reagents are stored at –20°C.
5. Montage SEQ_{96} sequencing reaction cleanup kit (Millipore).
6. ABI PRISM 3130 Genetic Analyzer or other sequencing equipment.

3. Methods

3.1. Genomic DNA Extraction

High-quality DNA is essential for the PCR amplification before Surveyor digestion. Classical phenol/chloroform extraction procedure gives very stable genomic DNA, yielding comparable amplification over long periods of storage. DNA quantification is performed by spectrophotometric method (*see* **Note 3**).

3.2. PCR Amplification of mtDNA and Control Samples

The human mitochondrial genome is amplified in 17 overlapping PCR fragments. **Table 19.1** shows the sequences and the localization of the oligonucleotides that are used to amplify human mtDNA.

3.2.1. Primers

Using the high-fidelity Optimase Polymerase Kit (*see* **Note 4**).

3.2.2. PCR

1. PCRs are prepared on ice, as follows:
   a. 1X Optimase reaction buffer (Mg^{2+}-free buffer).
   b. 200 nM Final concentration of each dNTP.
   c. 1.5 mM Final concentration of $MgSO_4$.
   d. 200 nM Final concentration of each primer.
   e. 1.25 U Optimase Polymerase.
   f. 50 ng Genomic DNA (*see* **Note 5**) or 0.5 µl of plasmid G (10 ng/µl) and 0.5 µl of plasmid C (10 ng/µl) (*see* **Note 6**).
   g. Water to a total volume of 50 µl (*see* **Note 7**).
2. Mix the contents of the tubes by flicking and spin briefly.
3. Only one PCR program is used to amplify the 17 overlapping and "Surveyor control" PCR products even though their sizes are different (*see* **Table 19.1**). Run the PCR under the following conditions: initial denaturation at 95°C for 4 min, amplification with 35 cycles (95°C for 30 s, 57°C for 30 s, 72°C for 4 min), final elongation at 72°C for 5 min.
   PCR products are stored at –20°C until used.

3.3. Analysis of PCR Products by Agarose Gel Electrophoresis

1. Prepare a 1% (w/v) agarose gel in 0.5X TBE containing 1 µg/ml of propidium iodide.
2. Add 5 µl of each PCR product to 6 µl of 6X loading buffer.
3. Load each PCR product into a lane of the gel. Add 4 µl (520 ng) and 6 µl (780 ng) of high-mass DNA ladder in two further lanes as size reference.
4. Run the gel in 0.5X TBE at 5 V/cm until the bromophenol blue has migrated 2/3 of the length of the gel.
5. Visualize the DNA bands using UV transilluminator and photograph the gel.

Table 19.1
Primers used for PCR amplification of the 17 amplicons

Amplicon name	Amplicon size (bp)	Forward primer (5′-3′)	Forward primer position	Reverse primer (5′-3′)	Reverse primer position
A	2027	GATCACAGGTCTATCACCCTA	nt 1-21	TTGGACAACCAGCTATCACCA	nt 2027-2007
B	2007	GCACACCCGTCTATGTAGCA	nt 1941-1960	TTCGATGTTGAAGCCTGAGAC	nt 3948-3928
C	2014	CCACACTAGCAGAGACCAAC	nt 3869-3888	GGCTGAGTGAAGCATTGGACT	nt 5883-5863
D	1890	GAAGCTGCTTCTTCGAATTTGC	nt 5777-5798	GGGCGTGATCATGAAAGGTG	nt 7667-7648
E	2026	CAAGTAGGTCTACAAGACGCT	nt 7601-7621	CTGATGCGAGTAATACGGATG	nt 9627-9607
F	2083	TACCACTCCAGCCTAGCCC	nt 9510-9528	TCGTAGGCAGATGGAGCTTG	nt 11593-11574
G	2105	CGGCTATGGTATAATACGCCT	nt 11476-11496	AGCGATGAGAGTAATAGATAGG	nt 13581-13560
H	2002	CCTCACAGGTTTCTACTCCAA	nt 13491-13511	GAGGTCTGGTGAGAATAGTGT	nt 15493-15473
H’	1345	GCAGCCCTAGCAACACTCC	nt 15314-15332	CAATGCTATCGCGTGCATACC	nt 90-70
I	2034	GAACACACAATAGCTAAGACCC	nt 1045-1066	CGGTCTGAACTCAGATCACGTA	nt 3079-3058
J	2073	CGATGTTGGATCAGGACATCC	nt 2988-3008	GGTTGTACGGTAGAACTGCTA	nt 5061-5041
K	2093	CATAGCAGGCAGTTGAGGTG	nt 4955-4974	GATAGGACATAGTGGAAGTGG	nt 7048-7028
L	2044	CTCATCACTAGACATCGTACTA	nt 6983-7004	GCCTGCAGTAATGTTAGCGG	nt 9027-9008
M	1776	CATCAGCCTACTCATTCAACC	nt 8964-8984	GTACGTAGTCTAGGCCATATG	nt 10740-10720
N	2079	GGCTAGCCCTACTAGTCTCAA	nt 10690-10710	CTCAGCCGATGAACAGTTGG	nt 12769-12750
O	2079	CGTTACATGGTCCATCATAGAA	nt 12621-12642	GTCGTGGTTGTAGTCCGTGC	nt 14700-14681
P	3192	CTCCTCAATAGCCATCGCTG	nt 14443-14462	GGGTCTTAGCTATTGTGTGTTC	nt 1066-1045

nt: nucleotide.

6. Estimate the quantity of PCR products by comparison of band intensities between the quantitative DNA ladder (high-mass DNA ladder) and the bands corresponding to each amplicon. PCR yield must be sufficiently high (>15 ng/μl) without background for successful digestion with Surveyor Nuclease (*see* **Note 8**).

3.4. Performing Heteroduplex Formation

Before the Surveyor Nuclease digestion, perform heteroduplex formation using a thermocycler. PCR products are denatured at 95°C and annealed by a slow temperature decreasing to ensure complete annealing of complementary strands.

Place PCR products in a thermocycler and run the following program:

$$95°\text{C for } 2\,\text{min}, 95 - 85°\text{C}(-2°\text{C/S}),$$
$$85 - 25°\text{C}(-0.1°\text{C/S}), \text{ and } 10°\text{C hold.}$$

3.5. Surveyor Nuclease Digestion

Prepare a master mix for 19 tubes containing all components except PCR products (*see* **Note 9**). The master mix should be used immediately after the preparation and should not be stored because reducing agent in the Enhancer storage buffer will inactivate Surveyor Nuclease over time (*see* **Notes 10 and 11**).

1. Using the Surveyor Nuclease digestion kit, prepare the master mix on ice to protect enzyme activities:
   a. 114 μl of 10X Surveyor Nuclease reaction buffer (6 μl for one tube).
   b. 19 μl Surveyor Enhancer W (1 μl for one tube).
   c. 19 μl Surveyor Nuclease W (1 μl for one tube).
   d. 703 μl Water (37 μl for one tube).
2. Aliquot 45 μl of master mix into 18 tubes. Add 15 μl of each PCR product to a separate tube of master mix (60 μl of total volume in each tube). For Surveyor positive control, add 5 μl of "Surveyor control" PCR product and 10 μl of water to the final tube of master mix.
3. Mix gently by pipetting up and down using a micropipettor.
4. Incubate at 42°C for 20 min using a thermocycler or a heat block (*see* **Note 12**).
5. During the incubation at 42°C, "undigested tubes" can be prepared. Mix 15 μl of each PCR product or 5 μl of "Surveyor control" PCR product with water in a final volume of 60 μl (*see* **Note 13**).
6. Place the digestion products on ice, immediately add 6 μl of Stop Solution and mix to stop the digestion (*see* **Note 14**).

3.6. Analysis of Surveyor Digestion DNA Products by Agarose Gel Electrophoresis

The approximative size of the fragments obtained after surveyor digestion is determined on an 1.5% agarose gel.

1. Prepare a 1.5% agarose gel in 0.5X TBE containing 1 μg/ml of propidium iodide.
2. Add 3 μl (1/6 volume) of a 6X loading buffer to 20 μl of each Surveyor-digested PCR product and each undigested PCR product and mix.
3. Load the totality of each mixture (23 μl) into a well with 500 ng of 1-kb DNA ladder as a size reference marker in the first and last wells of each comb to facilitate the sizing of digestion products. Load corresponding undigested and digested products side-by-side to facilitate interpretation (*see* **Note 15**).
4. Run the gel in 0.5X TBE at 5 V/cm for 1 h 30 min (*see* **Note 16**).
5. Visualize the DNA bands using a UV transilluminator and photograph the gel.

3.7. Localization of mtDNA Mismatch

Mismatch-specific cleavage products are seen as two supplementary bands in the Surveyor Nuclease-digested sample that are absent in the corresponding undigested sample. Compare the migrating bands between the undigested and the corresponding Surveyor-digested sample to detect mismatches. Using the mtDNA map (**Fig. 19.1**), verify the correspondence between overlapping amplicons as each mismatch is represented in two overlapping amplicons (**Fig. 19.2**). Digested products can be observed in one amplicon only, when the cleavage site is too close to the end of the overlapping amplicon (**Fig. 19.2**). Calculate the approximate localization of the mismatch based on DNA fragment overlap.

3.8. Identification of the Heteroplasmic mtDNA Mutation

3.8.1. PCR

Once the mismatch is localized, PCR amplify the fragment containing the mismatch. Using a proof-reading polymerase enzyme is not essential.

1. For example, our laboratory uses Taq DNA Polymerase Kit (Roche Diagnostics) as follows: mix 1X Taq DNA polymerase reaction buffer (containing 10 mM Mg^{2+}), 200 nM final concentration of each dNTP, 200 nM final concentration of each primer, 0.1 U of Taq DNA Polymerase, 100 ng of genomic DNA.
2. Mix the contents of the tubes by vortexing and spin briefly.
3. Run the PCR under the following conditions: initial denaturation at 95°C 4 min, amplification with 35 cycles (95°C for 30 s, 60°C for 30 s, 72°C for 2 min), final elongation at 72°C for 5 min.

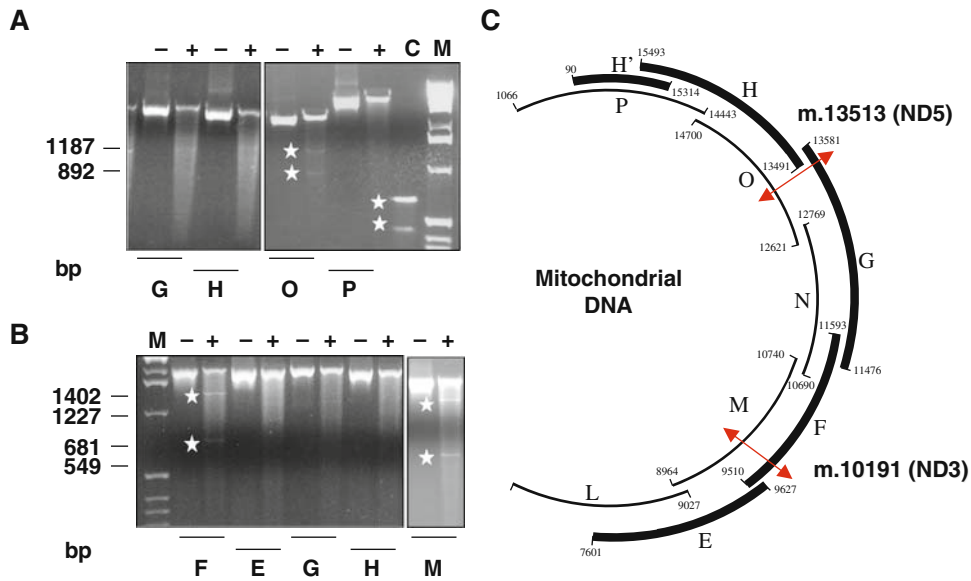


Fig. 19.2. Identification of mtDNA mismatches with Surveyor Nuclease digestion. (**A**) m.13513G>A mutation was seen with amplicon O only because the cleavage site is too close to the end (nt 13,581) of the overlapping amplicon G. Stars indicate the location of cleavage fragments: amplicon O (1,187 and 892 bp). Stars in lane C correspond to the "Surveyor control" digestion products. M: size marker; nt: nucleotide. (**B**) m.10191T>C mutation was seen with overlapping amplicons F and M. Stars indicate the location of cleavage fragments: amplicon F (1,402 and 681 bp) and amplicon M (1,227 and 549 bp). (–) undigested products, (+) digested products. (**C**) *Arrows* indicate the location of the m.13513G>A and m.10191T>C mutations in *ND5* and *ND3* genes, respectively.

3.8.2. PCR Product Analysis and Purification

1. Analyze PCR product quality by agarose gel electrophoresis as described in **Section 3.3**.
2. To degrade remaining primers and nucleotides, purify 10 µl of the remaining PCR product. Mix 10 µl of PCR product with 2 µl of ExoSAP-IT for a combined 12 µl reaction volume (*see* **Note 17**).
3. Mix the contents of the tubes by vortexing and spin briefly.
4. Incubate at 37°C for 30 min.
5. Incubate at 80°C for 15 min to inactivate ExoSAP-IT.

3.8.3. Sequencing PCR Product

1. Design a set of nested primers to sequence the mtDNA region containing the mismatch on both the strands. Primers should be ~21 bp in length, with an annealing temperature of ~56°C, a G+C content of ~50%, and should be localized within ~300 bp of the mismatch. Both forward and reverse primers should be used for the identification of the mtDNA mutation (*see* **Note 18**).
2. Using the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit, the sequences are prepared as follows:

a. 2 µl dRhodamine Terminator.

b. 2 µl Purified PCR product.

c. 3.2 pmol Primer.

d. Water to a final volume of 10 µl.

3. Run the sequence under the following conditions: Initial denaturation at 95°C for 5 min, amplification with 35 cycles (95°C for 30 s, 50°C for 30 s, 60°C for 2 min), final elongation at 60°C for 5 min.
4. Sequencing reactions are purified on the Montage SEQ_{96} sequencing reaction cleanup kit (Millipore). The protocol for purification reaction is performed according to the manufacturer's instructions. Samples that cannot be purified immediately after sequencing should be stored at +4°C for 2–3 days or at –20°C for extended periods (several weeks).
5. Sequence the purified PCR product using dRhodamineTerminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 3130 genetic analyzer, according to the supplier's instructions (*see* **Note 19**).
6. Identify the mutation by comparison of sense and reverse sequences. Its localization must correspond to the size of Surveyor digestion products (*see* **Note 20**).

4. Notes

1. Blood and tissue samples must be obtained from patients with their informed consent.
2. Propidium iodide is an intercalating agent, irritant but less toxic than ethidium bromide. When handling this chemical, wear gloves.
3. The quality of genomic DNA can be analyzed by agarose gel electrophoresis. Genomic DNA appears as a unique band, with any smear, which migrates higher than the first band (40 kb) of the 1-kb DNA ladder on the gel.
4. Success of Surveyor Nuclease digestion depends on the quality of PCR product. To avoid unspecific mismatches that will increase the background after Surveyor Nuclease treatment, prepare the PCR reaction on ice and use a proof-reading DNA polymerase enzyme. This PCR protocol is written for Optimase Polymerase, and may require modifications if another proof-reading DNA polymerase is used.
5. An excess of genomic DNA inhibits Optimase Polymerase. To amplify the multicopy circular mtDNA, 50 ng is enough.

6. The "Surveyor controls" consist as two control plasmids that differ at a single base pair. PCR amplification of these two plasmids mixed together generates heteroduplexes DNA fragments that are used as positive control for Surveyor Nuclease digestion. Several tubes of "Surveyor control" PCR products can be prepared, aliquoted, and stored at –20°C for several months.
7. For each PCR reaction, always perform a "no template" negative control tube containing ddH_2O instead of genomic DNA to ensure that contaminating DNA does not act as a template.
8. Do not digest PCR products if the yield is too low or additional bands or primer dimers are present.
9. Prepare a master mix for 19 tubes to compensate pipetting errors.
10. KCl concentrations above 75 mM inhibit Surveyor Nuclease. Because PCR products are hybridized and digested with Surveyor Nuclease directly in 1X PCR buffer salt, composition of 1X PCR buffer must be considered. Reaction buffers used for most PCR DNA polymerase do not require additional salt (including 10–20 mM Tris–HCl or Tris–SO_4 (pH 8.3–9.3), 50–75 mM KCl, 1–3 mM $MgCl_2$ or $MgSO_4$, 10–20 mM $(NH_4)_2SO_4$, 0.1–1% nonionic detergent, and BSA or gelatin). Only when PCR products are prepared with a low salt PCR buffer, sufficient 0.5 M KCl should be added to adjust the final KCl concentration within the range of 50–75 mM.
11. PCR additives such as DMSO (>5%), glycerol (>10%), betaine (>1 M), and 1X PCR_X Enhancer (Invitrogen) inhibit Surveyor Nuclease. If any of these or other additives are present in the PCR reaction, they must be removed before Surveyor treatment. Ethanol precipitation can be used to remove such additives. The precipitated DNA should be dissolved in a 1X PCR buffer compatible with Surveyor Nuclease digestion, such as 10 mM Tris–HCl (pH 8.8), 1.5 mM $MgCl_2$, and 50 mM KCl.
12. The use of a heated-lid thermocycler is recommended.
13. In "undigested" tubes, it is not necessary to add 10X Surveyor Nuclease reaction buffer and Stop Solution. The presence or the absence of these reagents does not modify the gel migration profile.
14. For optimal results, Surveyor digestion DNA products should be analyzed immediately. If absolutely necessary, store the digestion reaction mixtures after the addition of Stop Solution at –20°C, but not for more than 3 days before analysis.
15. The remaining digestion products should be stored at –20°C (for not more than 3 days) for another analysis if necessary.

16. Small Surveyor Nuclease digestion products (~100 bp) are more easily detected early during a gel electrophoresis run. Stop the run after 45 min, photograph the gel, and continue the electrophoresis process during 45 min.
17. Other PCR product purification kits/methods that remove unincorporated dNTPs, primers, and buffer may alternatively be used.
18. To choose appropriate primers to sequence a mutation-containing region it is possible to use an available online primer list which covers the entire mtDNA: http://insertion.stanford.edu/dhplc.html *(18)*.
19. Other sequencing methods may alternatively be used.
20. The mismatch localization must correspond to the size of Surveyor digestion products. Because the entire mtDNA genome is amplified with overlapping PCR amplicons, in most cases, mismatch generates Surveyor digestion products in two different amplicons.

Acknowledgements

We thank B. Chafino, S. Charabot and A. Figueroa for technical help. This work was made possible by grants from CHU Nice (CPR UF 699), France.

References

1. Paul, R., Santucci, S., Saunières, A., Desnuelle, C. and Paquis-Flucklinger, V. (1996) Rapid mapping of mitochondrial DNA deletions by large fragment PCR. *TIG,* **12,** 131–132.
2. Moraes, C.T., Dimauro, S., Zeviani, M., Lombes, A., Shanske, S., Miranda, A.F., Nakase, H., Bonilla, E., Werneck, L.C., Servidei, S. et al. (1989) Mitochondrial DNA deletions in progressive external ophthalmoplegia and Kearns-Sayre syndrome. *New Engl J Med,* **320,** 1293–1299.
3. Barros, F., Lareu, M.V., Salas, A. and Carracedo, A. (1997) Rapid and enhanced detection of mitochondrial DNA variation using single-strand conformation analysis of superposed restriction enzyme fragments from polymerase chain reaction-amplified products. *Electrophoresis,* **18,** 52–54.
4. Sternberg, D., Danan, C., Lombes, A., Laforet, P., Girodon, E., Goossens, M. and Amselem, S. (1998) Exhaustive scanning approach to screen all the mitochondrial tRNA genes for mutation and its application to the investigation of 35 independent patients with mitochondrial disorders. *Hum Mol Genet,* **7,** 33–42.
5. van de Bosch, B.J.C., de Coo, R.F.M., Scholte, H.R., Nijland, J.G., van den Bogaard, R., de Visser, M., de Die-Smulders, C.E.M. and Smeets, H.J.M. (2000) Mutation analysis of the entire mitochondrial genome using denaturing high performance liquid chromatography. *Nucleic Acids Res,* **28,** e89.
6. Biggin, A., Henke, R., Bennetts, B., Thorburn, D.R. and Christodoulou, J. (2005) Mutation screening of the mitochondrial genome using denaturing high-performance liquid chromatography. *Mol Genet Metab,* **84,** 61–74.

7. Kheterpal, I. and Mathies, R.A. (1999) Capillary array electrophoresis for DNA sequencing. *Anal Chem,* **71,** 31A–37A.
8. Hacia, J.G. (1999) Resequencing and mutational analysis using oligonucleotide microarrays. *Nat Genet,* **21,** 42–47.
9. Moraes, C.T., Atencio, D.P., Oca-Cossio, J. and Diaz, F. (2003) Techniques and pitfalls in the detection of pathogenic mitochondrial mutations. *J Mol Diagn,* **5,** 197–208.
10. Wong, L.J., Liang, M.H., Kwon, H., Park, J., Bai, R.K. and Tan, D.J. (2002) Comprehensive scanning of the entire mitochondrial genome for mutations. *Clin Chem,* **48,** 1901–1912.
11. Oleykowski, C.A., Bronson Mullins, C.R., Godwin, A.K. and Yeung, A.T. (1998) Mutation detection using a novel plant endonuclease. *Nucleic Acids Res,* **26,** 4597–4602.
12. Qiu, P., Shandilya, H., D'Alessio, J.M., O'Connor, K., Durocher, J. and Gerard, G.F. (2004) Mutation detection using Surveyor™ nuclease. *BioTechniques,* **36,** 702–707.
13. Kulinski, J., Besack, C.A., Oleykowski, C.A., Godwin, A.K. and Yeung, A.T. (2000) CEL enzymatic mutation detection assay. *BioTechniques,* **29,** 44–48.
14. Yeung, A.T., Hattangadi, D., Blakesley, L. and Nicolas, E. (2005) Enzymatic mutation detection technologies. *BioTechniques,* **38,** 749–758.
15. Jänne, P.A., Borras, A.M., Kuang, Y., Rogers, A.M., Joshi, V.A., Liyanage, H., Lindeman, N., Lee, J.C., Halmos, B., Maher, E.A. et al. (2006) A rapid and sensitive enzymatic method for epidermal growth factor receptor mutation screening. *Clin Cancer res,* **12,** 751–758.
16. Sacconi, S., Salviati, L., Nishigaki, Y., Walker, W.F., Hernandez-Rosa, E., Trevisson, E., Delplace, S., Desnuelle, C., Shanske, S., Hirano, M. et al. (2008) A functionally dominant mitochondrial DNA mutation. *Hum Mol Genet,* **17,** 1814–1820.
17. Bannwarth, S., Procaccio, V. and Paquis-Flucklinger, V. (2005) Surveyor™ nuclease: a new strategy for a rapid identification of heteroplasmic mitochondrial DNA mutations in patients with respiratory chain defects. *Hum Mutat,* **25,** 575–582.
18. Bannwarth, S., Procaccio, V. and Paquis-Flucklinger, V. (2006) Rapid identification of unknown heteroplasmic mutations across the entire human mitochondrial genome with mismatch-specific Surveyor Nuclease. *Nat Protoc,* **1,** 2037–2047.
19. Andreasson, A., Asp, A., Alderborn, A., Gyllensten, U. and Allen, M. (2002) Mitochondrial sequence analysis for forensic identification using pyrosequencing technology. *BioTechniques,* **32,** 124–126.
20. Brandon, M., Baldi, P. and Wallace, D.C. (2006) Mitochondrial mutations in cancer. *Oncogene,* **25,** 4647–4662.
21. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning: A laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Chapter 20

Collection of Isolated Cells for Studying Mitochondrial DNA Mutations Within Individual Cells

Yevgenya Kraytsberg, Natalya Bodyak, Susan Myerow, Alexander Nicholas, Konstantin Ebralidze, and Konstantin Khrapko

Abstract

Mitochondrial genome integrity is an important issue in somatic mitochondrial genetics. Development of quantitative methods is indispensable to somatic mitochondrial genetics as quantitative studies are required to characterize heteroplasmy and mutation processes, as well as their effects on phenotypic developments. This chapter outlines the methods for collecting individual cells appropriate for analysis of mtDNA mutations by single-molecule PCR. In addition, we describe the protocols for respiratory complexes II and IV in these cells. Together, the identification of respiratory deficiency and mtDNA mutations within single cells provides a powerful means of evaluating the importance of these events in disease and aging.

Key words: Laser capture microdissection, single-molecule PCR (smPCR), mtDNA mutation, SDH staining, COX staining.

1. Introduction

Analyses of mitochondrial DNA mutations and polymorphisms are essential for characterizing hereditary and sporadic mitochondrial diseases with their numerous clinical syndromes. Such analyses are also important for understanding the pathophysiology of wide spectrum diseases, whose development can be correlated with mitochondrial functionality, including endocrine, neurological, and nephrological diseases, and cancer *(1–15)*. Quantification of mtDNA mutations is also important for understanding: aging and the pathophysiology of age-related diseases *(3, 4, 6, 16–36)*;

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_20 Springerprotocols.com

questions related to co-existing nuclear and mitochondrial genomes *(37–39)*; and mitochondrial DNA recombination *(40)*, apoptosis, and homeostatic regulation *(11, 30, 41, 42)*.

Numerous works and reviews concern themselves with characterizing mtDNA mutations and their clonal expansions in different tissues and cell types as well as the relevance of these mutations to functional cell characteristics and their pathophysiological significance in disease development. Since mtDNA clonal expansions result in tissue/cell genomic and functional mosaicism *(3, 4, 16, 21, 25, 31)*, mtDNA analysis should be performed on various levels of tissue organization while focusing on individual cell analysis. Single-cell analysis provides a way to link the mtDNA mutation process with cell and tissue functions and to address questions of genotype–phenotype correlations and nuclear–mitochondrial genome interrelationships *(11, 30, 41, 42)*. Furthermore, single-cell studies are helpful for mtDNA mutation analysis since they often identify clonal mutations. Employment of clonal mutations in analysis avoids inclusion of PCR-derived errors *(36)*. Therefore, the development of single-cell collection techniques and quantification methods to characterize the mtDNA mutation spectrum and mutation load is of increasing significance in somatic mitochondrial genetics. There are various collection procedures ranging from limiting suspension dilution aspiration *(43)* and needle microdissection *(44)* to state-of-the-art lab-on-a-chip (LOC) technologies *(45)* and laser dissection with its different variants *(46, 47)*. For a review of methods employed in mitochondrial genetics *see (48)*. In this chapter, we describe several methods to obtain individual cells from various biological samples and assess mitochondrial respiratory characteristics of these cells. These single cells can then be used to analyze the occurrence and clonal spread of mtDNA mutations, using single-molecule PCR (smPCR) (*see* **Chapter 21** in this volume).

2. Materials

1. Microscope: Fisher Micromaster.
2. Lysis buffer: 0.5% SDS, 10 mM EDTA, 0.2% proteinase K.
3. Humidified chamber (37°C).

2.1. Collection from Suspension

1. Inverted microscope.
2. Evans blue dye.
3. Collagenase (Worthington).
4. Krebs–Henseleit buffer solution (Sigma).

5. Solution for cell dissociation: 0.01% collagenase, 10 mM EDTA, pH 8, 0.03% Evans blue in Krebs–Henseleit buffer solution.
6. TE buffer: 10 mM Tris, pH 8.0, 1 mM EDTA.
7. Mouth-controlled glass pipette system for cell collection, handmade out of a glass collection pipette, tubing, and a mouthpiece (*see* **Note 1**).

2.2. Laser Microdissection

1. Cryostat.
2. Fisher Plus microscope slides.
3. System for warming slides to 37°C (can be made from any heating module).
4. Ethanol (absolute) Series: 50%, 75%, 90%, and 100% dilutions with ddH_2O. Histological jars for the ethanol series should be refilled daily.

2.2.1. Arcturus Laser Capture Microdissection (LCM)

1. Cap Sure LCM caps LC M0201.
2. Xylene.

2.2.2. P.A.L.M. Laser Microdissection and Pressure Catapulting (LMPC)

1. PALM AdhesiveCaps (opaque).
2. PALM Membrane Slide (1 mm), PEN (polyethylene-naphthalate)-membrane.
3. Liquid Blocker super pap pen for staining procedures (Daido Sangyo Co. Ltd).
4. 50% Glycerol in ddH_2O (or PBS).

2.2.3. COX Staining

1. Catalase (Sigma).
2. Cytochrome *c* (Sigma).
3. DAB – 3′-diaminobenzidine tetrahydrochloride (Sigma) (CARCINOGENIC).
4. Sucrose.
5. 0.2 M Phosphate buffer, pH 7.2.
6. COX buffer: 50 mM sodium phosphate, pH 7.2, 200 mg/ml sucrose (*see* **Note 2**).
7. COX reaction mixture: 50 mM sodium phosphate, pH 7.2, 10 mg/ml cytochrome *c* (oxidized), 10 mg/ml diaminobenzidine, 200 mg/ml sucrose, catalase, 1,600 units/ml (*see* **Note 2**).

2.2.4. SDH Staining

1. Sodium succinate.
2. NBT – nitro blue tetrazolium.
3. NBT 100X stock solution (100 mg/ml) (*see* **Note 2**).

4. SDH reaction mixture: 30 mM Tris–HCl, pH 7.5, 10 mg/ml sodium succinate, 1 mg/ml NBT, 5 mM EDTA (*see* **Note 2**).

2.2.5. Cresyl Violet Staining

1. Cresyl Violet (Sigma).
2. Cresyl Violet staining solution: Cresyl Violet 0.1% in 5 mM Tris–HCl, pH 7.5.

3. Methods

The condition of mtDNA after collection should be taken into consideration (*see* **Note 5**). With the procedures described below we were able to perform very sensitive to mtDNA condition smPCR analysis (*see* **Chapter 21** in this volume). All tissue samples are snap-frozen (*see* **Note 3**). Cryostat sectioning is standard (*see* **Note 4**). Collection and staining methods must be optimized for each experimental goal (*see* **Note 5**). Two types of collection procedures are described below: aspiration from suspension (**Section 3.1.1**) and two variants of laser dissection (**Section 3.1.2**), LCM (**Section 3.1.2.1**) and LMPC (**Section 3.1.2.2**). Histological procedures including staining for the detection of cells with respiratory chain deficiency (COX and SDH) are also described.

3.1. Collection from Cell Suspension

In many cases single-cell isolation can be done from a cell suspension. This method is cost effective, with major equipment requirements being an inverted compound microscope and a dissecting microscope. We employed the method described in *(49)*. This method, with minor modifications, has been successfully used on frozen skeletal muscle, heart, and buccal mucosal cells *(22)*. It can also be adapted for use with other tissues.

1. Slice tissue with a razor blade into ~0.1 mm thick slices and transfer into a Petri dish containing the solution for cell dissociation with Evans blue for visualization.
2. Place on an orbital shaker at 100 rpm for 1 h at room temperature.
3. After tissue is dissociated into single cells, place Petri dish with the cell suspension onto ice.
4. Collect single cells under an inverted microscope with the handheld glass pipette of the glass pipette system. Use finely controlled pressure to let individual cells in and out of the fine glass piece. Capture the chosen cell.
5. Transfer captured cell to a 0.5 ml PCR tube. After brief centrifugation, circle the area of the tube containing the cell with a fine marker. Carefully remove supernatant.

6. Apply 1 µl of lysis buffer directly onto the marked area with the cell.
7. Place in a humid chamber at 37°C for 30 min.
8. Add 9 µl of ddH_2O. Incubate under the same conditions for an additional 30 min.
9. Mix gently by pipetting.
10. Dilute 20-fold in 1X TE for an initial dilution of $10^{-2.3}$. For example dilute 3 µl of stock in 57 µl of 1X TE.
11. Freeze and store lysate with dilutions at –70°C. (Keep all dilutions on ice when not in –70°C freezer.)

3.2. Laser Dissection

Laser dissection allows for the precise collection of individual or groups of cells. One of the great advantages of laser dissection is that sections can be stained prior to collection using histochemical, histoimmune, or other staining techniques. Two inverted laser dissections systems Arcturus *(46)* and P.A.L.M. *(47)* are described below (also *see* **Notes 3–8**). P.A.L.M. (LMPC – Laser Microdissection and Pressure Catapulting) uses a focused UV laser to cut out the desired area of tissue section along with the PEN-membrane supporting it as a backbone. Subsequently, a defocused laser pulse is used to catapult the tissue-membrane complex into a collection cap. Arcturus (LCM – Laser-capture microdissection) uses a focused infrared laser beam pulse to melt the thermal plastic of a special cup membrane onto a selected area of section (*see* **Note 11**). Following this step, the selected section area can be lifted from the slide. Other laser dissection technologies are available (*see* **Note 12**).

3.2.1. Arcturus Laser Capture Microdissection (LCM)

1. Mount 10-µm cryostat sections on PLUS slides.
2. Incubate sections in ethanol series followed by xylene as follows:
   Ethanol 75% 1 min
   Ethanol 95% 1 min
   Ethanol 100% 2 min
   Place sections in xylene for a minimum of 5 min (*see* **Note 13**).
3. Collect cells using an Arcturus laser dissection microscope following the manufacturer's protocol. Before collection, the slide should be given time for xylene evaporation. It is better not to exceed 1–2 h maximum collection time depending on the humidity of the air.

3.2.2. P.A.L.M. Laser Microdissection and Pressure Catapulting (LMPC)

1. Mount 10 µm cryostat sections on PEN-membrane-covered slides for laser dissection.
2. Air-dry tissue sections for 15–30 min as preparation for future histochemical staining.

3. Proceed with planned procedure or freeze at –80°C until needed.
4. Take fresh dried sections or thawed sections and incubate them in ethanol series followed by glycerol saturation (*see* **Section 3.2.2.1**) or stain beforehand (*see* **Section 3.3**). Sections can be kept at –70°C until collection.
5. Collect cells using P.A.L.M. laser dissection microscope according to manufacturer's protocol.

3.3. Histological Procedures

3.3.1. Ethanol Series and Glycerol Saturation

1. Ethanol 50% 1 min.
2. Ethanol 75% 1 min.
3. Ethanol 95% 1 min.
4. Ethanol 100% 2 min .
   For glycerol saturation, go back to 50% ethanol (1 min each).
5. Apply 50% glycerol on horizontal slide.
6. Maintain for 10–15 min at room temperature.
7. Remove excess glycerol by gently wiping.
8. Freeze at –70°C.

The method of staining should be adopted according to the goals of the experiment and the tissue samples used (*see* **Note 5**).

3.3.2. Nissl Staining for Neurons

1. Take fresh dried sections or warm frozen sections on a 37°C platform until thawed.
2. Apply Cresyl Violet staining solution on horizontal slides for 1 min. Note that staining can be observed under the microscope (*see* **Note 14**).
3. Conduct ethanol series followed by glycerol saturation (*see* **Section 3.2.2.1**).

3.3.3. COX Staining

1. Take fresh dried sections or warm frozen sections on a 37°C platform until thawed.
2. Encircle sections with liquid blocker.
3. Apply COX buffer for 15 min.
4. Replace COX buffer with COX reaction mixture.
5. Incubate at 37°C for 40 min while gently rocking in a humidified incubator. Time depends on tissue used (*see* **Note 5**).
6. Wash three times with the COX buffer for 3 min per wash.
7. Conduct ethanol series followed by glycerol saturation (**Section 3.2.2.1**).
8. Store at –70°C.

3.3.4. Cresyl Violet/COX Staining

1. Take fresh dried sections or warm frozen sections on a 37°C platform until thawed.
2. Encircle sections with liquid blocker.
3. Apply Cresyl Violet staining solution on a horizontally placed slide for 1 min.
3. Wash horizontally placed slide with COX buffer for 10 min.
4. Continue by following the same procedure as in **Section 3.3.2** (Steps 3–8).

3.3.5. SDH Staining

1. Take fresh dried sections or warm frozen sections on a 37°C platform until thawed.
2. Encircle sections with liquid blocker.
3. Apply SDH reaction mixture.
4. Incubate at 37°C for 1 h while gently rocking in a humidified incubator.
5. Wash three times in PBS
6. Conduct ethanol series followed by glycerol saturation (**Section 3.2.2.1**).
7. Store at –70°C.

3.3.6. COX/SDH Staining

1. COX staining: follow the same procedure as in **Section 3.3.2** (Steps 1–5).
2. Wash three times with PBS.
3. SDH staining: follow the same procedure as in **Section 3.3**.4 (Steps 3–7).

3.4. Single-Cell Lysis After Laser Dissection

This method is applicable to the cells melted into the membrane of the cap (Arcturus) or attached to the adhesive part of the cap (P.A.L.M.).

1. Find the cell by viewing the cap under a vertical microscope using 10X objective and 15X ocular.
2. Add 1 µl of lysis buffer directly onto the cell.
3. Cover the cap with a 0.5-ml Eppendorf tube that has had its cap removed.
4. Incubate for 1 h in a humidified 37°C chamber.
5. Under the microscope, add 5 µl of ddH_2O and mix by gently pipetting.
6. Incubate for an additional 30 min in a humidified 37°C chamber.
7. Apply 14 µl of 1X TE buffer and mix by gently pipetting.
8. Spin (*see* **Note 8**).

9. Dilute further resulting in a $10^{-1.3}$cell lysate dilution (1/20th of the cell) in 1X TE for dilution first to $10^{-2.3}$. For example, dilute 6 µl of stock into 54 µl of 1X TE (*see* **Note 10**).
10. Freeze and store lysate with dilutions at –70°C.
11. Continue to make serial dilutions in 1X TE as needed for experiments. (Keep all dilutions on ice when not in –70°C freezer).

4. Notes

1. To make, heat the end of a glass Pasteur pipette in a flame and pull with forceps until the collection aperture is around 50–100 µm. This should be followed by siliconization. For aerosol protection a cotton ball is placed in the upper part of Pasteur pipette. The tubing connecting the pipette to the mouthpiece should be of sufficient length for easy pipette manipulation. The length is usually about 1 m long. A mouthpiece can be made from the tubing.
2. Solutions can be made in batches, aliquoted, and stored at–70°C.
3. Laser dissection systems are very expensive. Therefore, they are usually shared among several research facilities. The manufacturers' representatives generally have information regarding the closest facilities that have the equipment for use or evaluation. Note that for Arcturus system it is preferable to work under controlled humidity condition, since this ensures better collection reproducibility.
4. There are several ways to freeze samples. Our preferred method is to place samples on glass slides and submerge them in an isopropanol bath cooled with dry ice. Placing pieces on slides before freezing helps to preserve morphology for future histological procedures. Once frozen, all samples should be stored at –70°C and freeze–thawing and desiccation should be avoided.
5. Examples of optimization of collection and staining methods. When working with *substantia nigra* (SN) neurons, it was necessary to acquire mtWG-length fragments after staining with a neuronal marker and staining to evaluate mitochondrial function. There are numerous variants and optimizations of staining, for example *see* Ref. *(50)*. The methods of staining often need to be modified according to the type of

tissue used in experiment, for example *see* Ref. *(51)*. When we estimated the condition of the mtDNA (*see* Chapter 21 in this volume), acceptable mtDNA condition was seen using Cresyl Violet (CV) for Nissl staining as the neuron-specific marker, and for the assessment of mitochondrial respiratory function using variants of histochemical staining for COX and SDH that are presented above. We have tested different combinations of these stains including a COX/SDH combination *(52)* and found that CV/COX staining preserved mtDNA better and marked the greatest number of cells. It appears that CV/COX staining marks cells with lowered COX activity, while COX/SDH staining reveals cells with further lowered COX activity possibly below the threshold for up-regulation of SDH activity. We therefore chose the first combination to screen SN-pigmented neurons for mitochondrial respiratory function, combined with laser dissection to collect cells for mtDNA deletion quantitative analysis *(35)*. For some other experiments such as mtWG smPCR cloning, it was necessary to acquire full length fragments from individual templates without evaluating the mitochondrial function. Collection from suspension is adequate in such cases. Estimating the condition of the mtDNA samples is required in any case.

6. For both LCM and LMPC laser dissection techniques we primarily use 10-µm sections obtained by standard cryostat sectioning. For tissues such as that of the brain and liver we recommend using a sectioning temperature of around –14°C. We also recommend numbering the slides while sectioning, which, if necessary, allows for the determination of the spatial relationship of the sections. Also, starting all of the picture sets with a view of the entire collected area and proceeding to pictures of individual cell collections before and after the dissections helps in resolving questions concerning cell type, conditions, etc., that may arise after analysis. When performing LCM, it is helpful to apply ethanol before making pictures in order to improve the image quality.
7. We have adopted several ways to make collection cheaper. The various reagents and labware necessary for collection and lysis can be purchased commercially. We have tried to minimize the cost by using less expensive procedures where possible. For P.A.L.M. collection we prefer to use adhesive caps in lieu of liquid droplets for capturing catapulted cells for collection. We prefer opaque to clear ones as they are better for cell identification under the microscope. We have not seen any problems with freezing the collected material on the caps at –70°C before cell lysis.

8. Centrifugation of collected caps after lysis should not exceed recommended speeds. Maximum speed may cause the adhesive part of the cap (P.A.L.M.) or thermoplastic membrane of the cap (Arcturus) to detach from the cap.
9. We thaw frozen sections on a 37°C warmer made from a heating module.
10. We keep our stocks in 10^{-1}, $10^{-1.3}$, and $10^{-2.3}$ in a specially designated area at –70°C as it is the main stock to which we return if something happens with less concentrated template. We adapted an unusual way to numerate dilution concentrations. We started to use it after laser dissections, when we decided that our main stock will be 1/20 of a cell. This is a convenient volume to handle, while still having some protective substances, and also 1/200 was the template concentration which we were mainly using for analytical PCR, so it is an "historical" system which can be easily changed if it is not convenient, but we are using it for the presentation of the method. Also, the high extent of dilution implies that the method of DNA isolation is not important for the success of the procedure, as long as it preserves amplifiable DNA molecules of the appropriate length. That is why we usually use dilutions of native lysates. If more then usually concentrated samples are needed proteinase K can be heat-deactivated at 95°C. Note that other components of the lysation buffer can be removed only by cleaning, but all readily available cleaning procedures decrease concentration or quality of DNA.
11. For our purposes, LMPC (P.A.L.M.) was preferable to LCM (Arcturus) (without the Laser Cutting System), since it is less dependent on the condition of the sections, allows more accurate collection of specific areas, and gives a very wide array of options for collection. Because P.A.L.M. can be used to collect from wet sections, the user can manipulate a section with an array of different staining procedures. Finally, it also allowed us to apply a glycerol saturation procedure. The glycerol saturation method improves frozen section visualization and preservation.
12. These include the Veritas Microdissection System which combines Arcturus Laser Capture Microdissection and the Laser Cutting System, also Leica AS LMD upright laser microdissection system that uses UV pulse laser to excise tissue attached to the supporting plastic matrix. Excised section drops (by gravity) into the cap of a microcentrifuge tube. Our laboratory has not had the opportunity to evaluate these methods.
13. Sections in xylene can be kept in the dark for days if necessary.

14. Preliminary fixation by ethanol series, or at least incubation in 75% ethanol, followed by rehydration before staining, will improve the outcome.

Acknowledgments

This work was supported in part by the NIH grant AG019787 and a United Mitochondrial Disease Foundation grant to K.K.

References

1. Wallace, D.C., G. Singh, M.T. Lott, J.A. Hodge, T.G. Schurr, A.M. Lezza, L.J. Elsas, 2nd and E.K. Nikoskelainen. 1988. Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. Science *242*:1427–1430.
2. Holt, I.J., A.E. Harding and J.A. Morgan-Hughes. 1988. Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. Nature *331*:717–719.
3. Wallace, D.C. 1993. Mitochondrial diseases: genotype versus phenotype. Trends Genet *9*:128–133.
4. Shoubridge, E.A. 1994. Mitochondrial DNA diseases: histological and cellular studies. J Bioenerg Biomembr *26*:301–310.
5. Sciacco, M., E. Bonilla, E.A. Schon, S. DiMauro and C.T. Moraes. 1994. Distribution of wild-type and common deletion forms of mtDNA in normal and respiration-deficient muscle fibers from patients with mitochondrial myopathy. Hum Mol Genet *3*:13–19.
6. Marcelino, L.A. and W.G. Thilly. 1999. Mitochondrial mutagenesis in human cells and tissues. Mutat Res *434*:177–203.
7. DiMauro, S. and E.A. Schon. 2001. Mitochondrial DNA mutations in human disease. Am J Med Genet *106*:18–26.
8. Pogozelski, W.K., C.J. Hamel, C.F. Woeller, W.E. Jackson, S.J. Zullo, N. Fischel-Ghodsian and W.F. Blakely. 2003. Quantification of total mitochondrial DNA and the 4977-bp common deletion in Pearson's syndrome lymphoblasts using a fluorogenic 5'-nuclease (TaqMan) real-time polymerase chain reaction assay and plasmid external calibration standards. Mitochondrion *2*:415–427.
9. Linnartz, B., R. Anglmayer and S. Zanssen. 2004. Comprehensive scanning of somatic mitochondrial DNA alterations in acute leukemia developing from myelodysplastic syndromes. Cancer Res *64*:1966–1971.
10. Maitra, A., Y. Cohen, S.E. Gillespie, E. Mambo, N. Fukushima, M.O. Hoque, N. Shah, M. Goggins, et al. 2004. The Human MitoChip: a high-throughput sequencing microarray for mitochondrial mutation detection. Genome Res *14*:812–819.
11. Wallace, D.C. 2005. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. Annu Rev Genet *39*:359–407.
12. Schapira, A.H. 2006. Mitochondrial disease. Lancet *368*:70–82.
13. McFarland, R., P.F. Chinnery, E.L. Blakely, A.M. Schaefer, A.A. Morris, S.M. Foster, H.A. Tuppen, V. Ramesh, et al. 2007. Homoplasmy, heteroplasmy, and mitochondrial dystonia. Neurology *69*:911–916.
14. McFarland, R., R.W. Taylor and D.M. Turnbull. 2007. Mitochondrial disease – its impact, etiology, and pathology. Curr Top Dev Biol *77*:113–155.
15. Pieczenik, S.R. and J. Neustadt. 2007. Mitochondrial dysfunction and molecular pathways of disease. Exp Mol Pathol *83*:84–92.
16. Soong, N.W., D.R. Hinton, G. Cortopassi and N. Arnheim. 1992. Mosaicism for a specific somatic mitochondrial DNA mutation in adult human brain. Nat Genet *2*:318–323.
17. Zhang, C., A. Baumer, R.J. Maxwell, A.W. Linnane and P. Nagley. 1992. Multiple mitochondrial DNA deletions in an elderly human individual. FEBS Lett *297*:34–38.
18. Wallace, D.C., J.M. Shoffner, I. Trounce, M.D. Brown, S.W. Ballinger, M. Corral-

Debrinski, T. Horton, A.S. Jun, et al. 1995. Mitochondrial DNA mutations in human degenerative diseases and aging. Biochim Biophys Acta *1271*:141–151.

19. Khrapko, K., H.A. Coller, P.C. Andre, X.C. Li, J.S. Hanekamp and W.G. Thilly. 1997. Mitochondrial mutational spectra in human cells and tissues. Proc Natl Acad Sci U S A *94*:13798–13803.
20. Michikawa, Y., F. Mazzucchelli, N. Bresolin, G. Scarlato and G. Attardi. 1999. Aging-dependent large accumulation of point mutations in the human mtDNA control region for replication. Science *286*:774–779.
21. Zhang, C., A. Lee, V.W. Liu, S. Pepe, F. Rosenfeldt and P. Nagley. 1999. Mitochondrial DNA deletions in human cardiac tissue show a gross mosaic distribution. Biochem Biophys Res Commun *254*:152–157.
22. Khrapko, K., N. Bodyak, W.G. Thilly, N.J. van Orsouw, X. Zhang, H.A. Coller, T.T. Perls, M. Upton, et al. 1999. Cell-by-cell scanning of whole mitochondrial genomes in aged human heart reveals a significant fraction of myocytes with clonally expanded deletions. Nucleic Acids Res *27*:2434–2441.
23. Wang, Y., Y. Michikawa, C. Mallidis, Y. Bai, L. Woodhouse, K.E. Yarasheski, C.A. Miller, V. Askanas, et al. 2001. Muscle-specific mutations accumulate with aging in critical human mtDNA control sites for replication. Proc Natl Acad Sci U S A *98*:4022–4027.
24. Bodyak, N.D., E. Nekhaeva, J.Y. Wei and K. Khrapko. 2001. Quantification and sequencing of somatic deleted mtDNA in single cells: evidence for partially duplicated mtDNA in aged human tissues. Hum Mol Genet *10*:17–24.
25. Coller, H.A., N.D. Bodyak and K. Khrapko. 2002. Frequent intracellular clonal expansions of somatic mtDNA mutations: significance and mechanisms. Ann N Y Acad Sci *959*:434–447.
26. Fayet, G., M. Jansson, D. Sternberg, A.R. Moslemi, P. Blondy, A. Lombes, M. Fardeau and A. Oldfors. 2002. Ageing muscle: clonal expansions of mitochondrial DNA point mutations and deletions cause focal impairment of mitochondrial function. Neuromuscul Disord *12*:484–493.
27. Lin, M.T., D.K. Simon, C.H. Ahn, L.M. Kim and M.F. Beal. 2002. High aggregate burden of somatic mtDNA point mutations in aging and Alzheimer's disease brain. Hum Mol Genet *11*:133–145.
28. Nekhaeva, E., N.D. Bodyak, Y. Kraytsberg, S.B. McGrath, N.J. Van Orsouw, A. Pluzhnikov, J.Y. Wei, J. Vijg, et al. 2002. Clonally expanded mtDNA point mutations are abundant in individual cells of human tissues. Proc Natl Acad Sci U S A *99*:5521–5526.
29. Nekhaeva, E., Y. Kraytsberg and K. Khrapko. 2002. mtLOH (mitochondrial loss of heteroplasmy), aging, and 'surrogate self'. Mech Ageing Dev *123*:891–898.
30. Chomyn, A. and G. Attardi. 2003. MtDNA mutations in aging and apoptosis. Biochem Biophys Res Commun *304*:519–529.
31. Khrapko, K., E. Nekhaeva, Y. Kraytsberg and W. Kunz. 2003. Clonal expansions of mitochondrial genomes: implications for in vivo mutational spectra. Mutat Res *522*:13–19.
32. Coskun, P.E., M.F. Beal and D.C. Wallace. 2004. Alzheimer's brains harbor somatic mtDNA control-region mutations that suppress mitochondrial transcription and replication. Proc Natl Acad Sci U S A *101*:10726–10731.
33. Bender, A., K.J. Krishnan, C.M. Morris, G.A. Taylor, A.K. Reeve, R.H. Perry, E. Jaros, J.S. Hersheson, et al. 2006. High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. Nat Genet *38*:515–517.
34. Khrapko, K., Y. Kraytsberg, A.D. de Grey, J. Vijg and E.A. Schon. 2006. Does premature aging of the mtDNA mutator mouse prove that mtDNA mutations are involved in natural aging? Aging Cell *5*:279–282.
35. Kraytsberg, Y., E. Kudryavtseva, A.C. McKee, C. Geula, N.W. Kowall and K. Khrapko. 2006. Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons. Nat Genet *38*:518–520.
36. Kraytsberg, Y., A. Nicholas and K. Khrapko. 2007. Are somatic mitochondrial DNA mutations relevant to our health? A challenge for mutation analysis techniques. Expert Opin Mol Diagn *1*:1–8.
37. Jenuth, J.P., A.C. Peterson and E.A. Shoubridge. 1997. Tissue-specific selection for different mtDNA genotypes in heteroplasmic mice. Nat Genet *16*:93–95.
38. Battersby, B.J., J.C. Loredo-Osti and E.A. Shoubridge. 2003. Nuclear genetic control of mitochondrial DNA segregation. Nat Genet *33*:183–186.
39. Antonicka, H., F. Sasarman, N.G. Kennaway and E.A. Shoubridge. 2006. The molecular basis for tissue specificity of the oxidative phosphorylation deficiencies in

patients with mutations in the mitochondrial translation factor EFG1. Hum Mol Genet *15*:1835–1846.

40. Kraytsberg, Y., M. Schwartz, T.A. Brown, K. Ebralidse, W.S. Kunz, D.A. Clayton, J. Vissing and K. Khrapko. 2004. Recombination of human mitochondrial DNA. Science *304*:981.
41. Chowdhury, I., B. Tharakan and G.K. Bhat. 2006. Current concepts in apoptosis: the physiological suicide program revisited. Cell Mol Biol Lett *11*:506–525.
42. Wallace, D.C. 2007. Why do we still have a maternally inherited mitochondrial DNA? Insights from evolutionary medicine. Annu Rev Biochem *76*:781–821.
43. Jeffreys, A.J., V. Wilson, R. Neumann and J. Keyte. 1988. Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells. Nucleic Acids Res *16*:10953–10971.
44. Zhuang, Z., P. Bertheau, M.R. Emmert-Buck, L.A. Liotta, J. Gnarra, W.M. Linehan and I.A. Lubensky. 1995. A microdissection technique for archival DNA analysis of specific cell populations in lesions < 1 mm in size. Am J Pathol *146*:620–625.
45. Sims, C.E. and N.L. Allbritton. 2007. Analysis of single mammalian cells on-chip. Lab Chip *7*:423–440.
46. Emmert-Buck, M.R., R.F. Bonner, P.D. Smith, R.F. Chuaqui, Z. Zhuang, S.R. Goldstein, R.A. Weiss and L.A. Liotta. 1996. Laser capture microdissection. Science *274*:998–1001.
47. Vogel, A., V. Horneffer, K. Lorenz, N. Linz, G. Huttmann and A. Gebert. 2007. Principles of laser microdissection and catapulting of histologic specimens and live cells. Methods Cell Biol *82*:153–205.
48. Williams, S.L. and C.T. Moraes. 2007. Microdissection and analytical PCR for the investigation of mtDNA lesions. Methods Cell Biol *80*:481–501.
49. Moraes, C.T. and E.A. Schon. 1996. Detection and analysis of mitochondrial DNA and RNA in muscle by in situ hybridization and single-fiber PCR. Methods Enzymol *264*:522–540.
50. Old, S.L. and M.A. Johnson. 1989. Methods of microphotometric assay of succinate dehydrogenase and cytochrome c oxidase activities for use on human skeletal muscle. Histochem J *21*:545–555.
51. Vermulst, M., J. Wanagat, G.C. Kujoth, J.H. Bielas, P.S. Rabinovitch, T.A. Prolla and L.A. Loeb. 2008. DNA deletions and clonal mutations drive premature aging in mitochondrial mutator mice. Nat Genet *40*:392–394.
52. Cottrell, D.A., E.L. Blakely, M.A. Johnson, P.G. Ince and D.M. Turnbull. 2001. Mitochondrial enzyme-deficient hippocampal neurons and choroidal cells in AD. Neurology *57*:260–264.

Chapter 21

Quantitative Analysis of Somatic Mitochondrial DNA Mutations by Single-Cell Single-Molecule PCR

Yevgenya Kraytsberg, Natalya Bodyak, Susan Myerow, Alexander Nicholas, Konstantin Ebralidze, and Konstantin Khrapko

Abstract

Mitochondrial genome integrity is an important issue in somatic mitochondrial genetics. Development of quantitative methods is indispensable to somatic mitochondrial genetics as quantitative studies are required to characterize heteroplasmy and mutation processes, as well as their effects on phenotypic developments. Quantitative studies include the identification and measurement of the load of pathogenic and non-pathogenic clonal mutations, screening mitochondrial genomes for mutations in order to determine the mutation spectra and characterize an ongoing mutation process. Single-molecule PCR (smPCR) has been shown to be an effective method that can be applied to all areas of quantitative studies. It has distinct advantages over conventional vector-based cloning techniques avoiding the well-known PCR-related artifacts such as the introduction of artificial mutations, preferential allelic amplifications, and "jumping" PCR. smPCR is a straightforward and robust method, which can be effectively used for molecule-by-molecule mutational analysis, even when mitochondrial whole genome (mtWG) analysis is involved. This chapter describes the key features of the smPCR method and provides three examples of its applications in single-cell analysis: di-plex smPCR for deletion quantification, smPCR cloning for clonal point mutation quantification, and smPCR cloning for whole genome sequencing (mtWGS).

Key words: Single-molecule PCR (smPCR), smPCR cloning, mtDNA mutation analysis, quantification, somatic mutations, point mutations, deletions.

1. Introduction

The development of single-cell collection techniques and quantification methods to characterize the mtDNA mutation spectra and mutation load is of increasing significance in somatic mitochondrial genetics. These methods are necessary to answer the central questions of mitochondrial genetics, since somatic mitochondrial biology is based on statistical genetics and requires quantitative

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_21 Springerprotocols.com

diagnostics *(1)*. The study of somatic mtDNA genetics usually entails an analysis of complex mixtures composed of mitochondrial genomes with multiple mutations and polymorphisms that are often unknown a priori *(2)*. Therefore, highly sensitive techniques with the possibility to cover the whole mitochondrial genome (mtWG) are in high demand. Here we present the single-molecule PCR (smPCR) method, which is a variant of the "limiting dilution" PCR approach. Approximately two decades ago, it was shown that PCR can be efficiently performed on a single DNA template *(3)*. Since then, the approach has been reinvented under different names and used for a variety of applications *(3–6)*. More recently, the breadth and sophistication of applications using single-template PCR have expanded significantly *(7–13)*; for further reviews of nanoscale genome sequencing and digital quantification *see* Refs. *(14–16)*. Methods using single-molecule analysis can recognize single nucleotide changes, avoiding a number of pitfalls that limit other techniques and providing more flexibility for analysis. For instance, they eliminate various PCR artifacts that are a major concern when analyzing data after PCR amplifications *(17–19)* and create digital output that is helpful for quantitative analysis.

smPCR method can be easily performed without special equipment or reagents and for the length of the whole mitochondrial genome. With the use of smPCR, it is possible to bypass pre-cloning PCR and the vector cloning step of conventional vector cloning techniques. Therefore, PCR amplification of a single molecule results in cloning of PCR fragments that is immune to errors such as template jumping and allelic preference. It also provides ways to sort out erroneous nucleotide introduction by polymerase. Consequently, employing smPCR lessens the inclusion of PCR-derived errors in analysis *(2, 20)*. Additionally, only a small amount of high-quality DNA is required for starting material. smPCR can be used instead of conventional cloning and some quantitative techniques in all areas of genetics. It is particularly suitable for mtDNA mutational analysis at any level of tissue organization, including the single-cell level. smPCR can be used to determine point mutations and deletion spectra by screening long-PCR products, as well as to quantify known point mutations and deletion loads in its short variant. The methodological aspects of smPCR as applied to somatic mutation analysis have been described recently in Refs. *(2)* and *(20)*.

The combination of single cell and smPCR requires special sample handling and careful techniques for preparing the PCR. The main aspects necessary for executing single-cell smPCR experiments are discussed and presented in this chapter. By adhering to the described methods, we have successfully performed smPCR at the single-cell level. We have employed these methods with a variety of lengths of PCR fragments using materials which have undergone histochemical staining involving long incubations

at 37°C. All experiments employing single-cell smPCR share common procedural stages that are shown in **Fig. 21.1** and presented in **Sections 3.1**, **3.2**, **3.3** and **3.4.** Three experiments involving human mtDNA are described in detail to demonstrate the applications and highlight the different features of this technique (**Sections 3.5**, **3.6** and **3.7**).

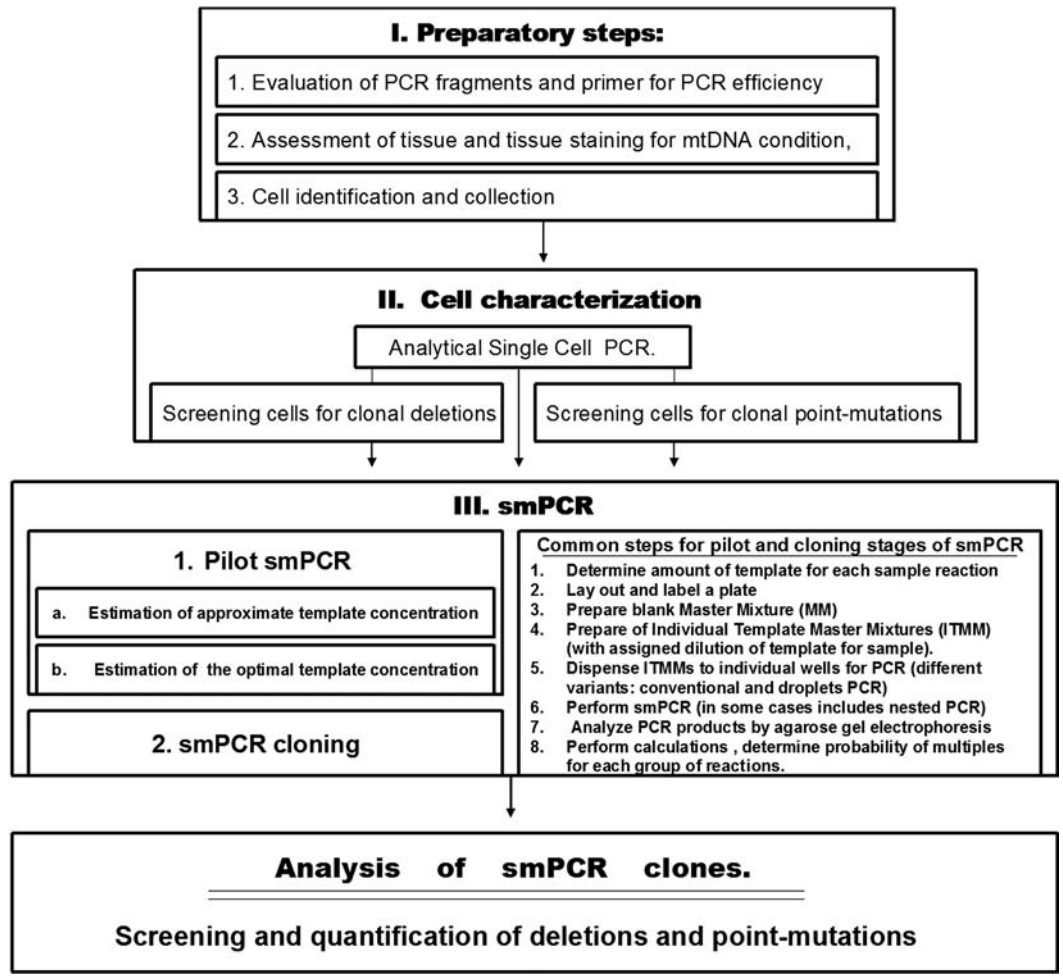


Fig. 21.1. **Organizational strategy for single-cell single-molecule PCR experiments.** I Preparatory steps; II cell characterization; III smPCR cloning stages. Each experiment is concluded with an analysis of the resulting smPCR clones.

2. Materials

2.1. Equipment and General PCR Materials

1. PCR machine with high ramping rates such as Primus (MWG Biotech) or equivalent.
2. Polymerases (TaKaRa Bio USA):

Ex Taq polymerase kit (ExTaq 5 U/μl; dNTPs 2.5 mM each; 10X ExTaq Buffer with 25 mM $MgCl_2$).

LA Taq polymerase kit (LA Taq 5 U/μl; dNTPs 2.5 mM each; 10X LA Taq Buffer with 20 mM $MgCl_2$).

3. 96-Well plates (ABgene ThermoFast non-Skirted AB-0600Y).
4. Plate sealers (Denville Sealing Film B-1212-5 or equivalent).
5. Automatic electronic pipettes: 10 and 100 μl (Eppendorf (EDP3 C0400571E; K0202495E or equivalent)) with sterile filter tips.
6. Automatic multi-channel electronic pipettes (Eppendorf Research Pro series): at least 0.5μl–10 μl (or equivalent) with sterile filter tips.
7. Oil for PCR.
8. Bromophenol blue: 1 mg/ml in 59 mM Tris–HCl pH 8.0.
9. Primers (may be ordered from MWG Biotech). Primers are named F and R for forward and reverse with length by underscore and with numbered position on reference sequence for 5′-end of the primer.
10. Primer dilutant: 5 mM Tris–HCl, pH 8.0, 0.03 mg/ml bromophenol blue.
11. 100 μM Primer stocks prepared with dilutant, store at –20 or –70°C.
12. 2 μM Working primer solutions. Dilute from 100 μM stock with ddH_2O and store at –20 or +2°C.
13. Restriction enzymes: BsTNI (10,000 units/ml); *Dra*I (20,000 units/ml); *Ava*I (10,000 units/ml); *Bcl*I (15,000 units/ml).
14. Product purification kits: Qiagen QIAquick PCR purification kit (18104), Denville Isopure-10 PCR Products purification kit (CM-0100-50).

2.2. Gel Equipment and Materials

1. 4X Loading buffer: 50% glycerol, 0.3 mg/ml bromophenol blue, 10 mM Tris–HCl, pH 8.0.
2. Ethidium bromide: 10 mg/ml (TOXIC, MUTAGENIC).
3. DNA markers: 1-kb DNA ladder (NEB, GIBCO), 100-bp ladder (NEB), pBR322 DNA *Msp*I digest (NEB).
4. 50X TAE buffer (Qiagen).

2.2.1. Agarose Gel

5. Gel apparatus: CBS High-Throughput Horizontal Gel Electrophoresis System (SGU 2640T-02).
6. The Owl Gator^{TM} horizontal A2 large gel electrophoresis system or equivalent.
7. Agarose (American Analytical Agarose GPG/LE ultra pure GAS: 9012-36-6 or equivalent).

2.2.2. Native Polyacrylamide Gel

8. Vertical electrophoresis system (BRL1071 modelVIG-2 or equivalent).
9. Duracryl:30% acrylamide, 0.8% BIS (Oxford Glycosystems).
10. TEMED ultrapure (BRL).
11. Ammonium persulfate (APS): 10% in ddH_2O.

3. Methods

A general scheme of single-cell smPCR experiments is presented in **Fig. 21.1**. It includes the main stages of the experiment, i.e., PCR optimization, cell characterization, and smPCR per se.

3.1. Optimization of smPCR

It is important to establish and use a standardized PCR protocol to facilitate comparison of data from different experiments. After selecting an amplification system, the most efficient way to optimize PCR for many different fragments is to evaluate a number of primer sets for PCR efficiency without varying other Parameters. After choosing primers, the reaction volume and number of cycles can be optimized and the need to use nested PCR determined.

3.1.1. Selection of the Amplification System

When screening for unknown point mutations, selection of a polymerase with high fidelity is important to avoid spontaneous polymerase errors, introduction of damaged dNTPs, or reading through a damaged template and introducing incorrect nucleotides. Both fidelity and processivity are particularly important for long PCR fragments, which acquire more damage, resulting in higher numbers of artificial mutations and lower numbers of amplifiable templates *(17, 21–23)*. Differences in the rate of successful amplification of short and long fragments can be employed to assess mtDNA condition (*see* **Section 3.2.**).

We use the TaKaRa amplification system for smPCR. It is based on a combination of polymerases to improve Taq DNA polymerase processivity, fidelity, and efficiency. *TaKaRa Ex Taq*™ provides both efficient amplification and acceptable fidelity (4.5X better than Taq polymerase). *TaKaRa LA Taq*™ has the additional proof-reading activity. It provides comParable efficiency and somewhat higher fidelity than Ex Taq (6.5X better than Taq polymerase) with similarly good processivity, allowing amplification of longer fragments up to 30 kb (*see* **Note 2**). Therefore, use Ex Taq polymerase for amplifying fragments shorter than 8 kb and LA Taq polymerase for amplifying long fragments up to mtWG.

The following concentrations of reagents in PCR cocktails (master mixtures) are effective:

1. 0.2 μM Primers.
2. 2.5 mM Mg^{2+} in LA Taq PCR buffer.
3. Bromophenol blue at 2–6 μg/ml.
4. Polymerase concentration ranges from 2.5 U/100 μl to 10 U/100 μl, as determined by PCR optimization (*see* below).
5. dNTP concentration depends on the template length: 0.25 mM each for short PCR and 0.375 mM each for long PCR.

Use primers that are 30–40 nucleotides long, with a GC content of at least 50%. This allows the annealing and elongation steps to be combined into a single 68°C step. The time required for the combined annealing and elongation steps depends on the PCR fragment length. As a rule of thumb, use 1 min/1 kb of fragment length.

3.1.2. Evaluation of Primer Sets

Different approaches and programs are available for primer design (*see* **Note 3**). An empirical approach is needed to evaluate PCR efficiency for specific primer sets. Generally, the PCR outcome can be improved by manipulating the concentration of $MgCl_2$, primers, and dNTPs, or employing different additives (*see* **Note 2**). As it is preferable to use standardized PCR conditions, do not modify the PCR cocktail recipe, but rather optimize PCR by evaluating several primer combinations. Primer testing is conducted using the same template subjected to the same PCR procedure, but with different primer sets. For this purpose, it is preferable to use low concentration samples with an amplifiable template of not more than 100 copies per reaction. The results are evaluated for product yield, primer-dimer formation, and the presence of other by products.

Typical results of primer pair evaluation are shown in **Fig. 21.2**. Two out of four primer pairs (3 and 4) presented in the figure are acceptable for first-stage smPCR, as they show good specific product

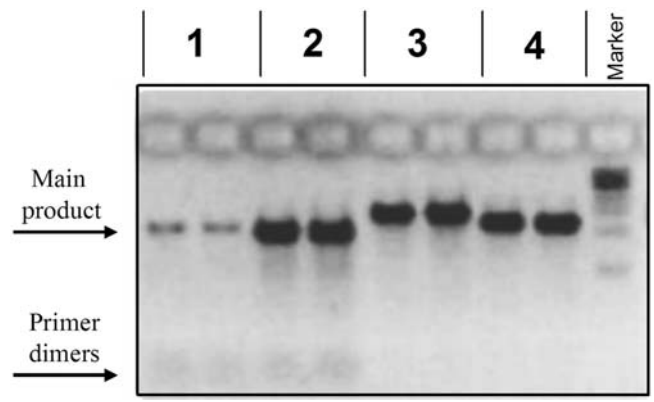


Fig. 21.2. **Evaluation of primers.** Illustrates the outcome of PCR with four different primer sets for the same fragment. Primer sets 3 and 4 show an ideal combination of yield and product specificity. They are the only pairs acceptable for the first stage of smPCR. Set 2 shows good yield, but is accompanied by primer-dimer formation. This set could be used for the second stage of nested PCR. Primer set 1 shows poor yield and primer-dimer formation; therefore, it should be excluded from experiments.

yield with minimal primer-dimer formation. Primer set 2 could be used for the second stage of nested PCR, since it shows a good product yield, but this comes with some primer-dimer formation. Primer set 1 should be excluded from experiments because it shows poor product yield as well as primer-dimer formation. For optimizing WG mtDNA long-range PCR, different fragments should each be tested with several pairs of primers.

3.1.3. Selection of PCR Reaction Volume

smPCR can be performed in volumes ranging from 10 µl per reaction to as small as 0.6–1 µl per reaction added into an oil drop. We have named this small volume variant "droplets PCR." If needed, a second amplification can be performed directly in the same tube by adding a second PCR reaction mixture containing the second set of primers and additional polymerase, over the oil layer of the first reaction. This second reaction mixture should be at least twice the volume of the first reaction.

The optimum volume can be determined while performing **Section 3.1.4** below. When volumes greater than 3 µl are used for first-stage PCR, a common nested PCR technique can be used which will generally result in a higher quality product. For this method, 1µl of the first-stage PCR reaction is dispensed into a new plate, followed by the addition of the second-stage PCR master mixture. For long-range PCR with our PCR setting and amplification system larger reaction volumes in some cases give better results.

3.1.4. Evaluation of Optimal Cycle Number and Necessity for Nested PCR

Typically 40–45 cycles are ideal for one-stage PCR and 50–60 for nested PCR (for example, 30 for the first stage of PCR and 20 cycles for the second). It is difficult to predict the optimal number of cycles a priori or whether using nested or droplets PCR will be optimum. These Parameters should be determined empirically. Factors influencing results include length of the amplifiable DNA; sequence composition; and the particular primer pair. Determination of optimal conditions for smPCR for a new amplicon can be performed with PCR using a template with a known mtDNA copy number of comParable fragment length. Alternatively, optimization can be done simultaneously with the determination of optimum template concentration for the new sample.

It is important that individual positive PCR reactions produce large amounts of high-quality PCR product. This guarantees that single templates are efficiently amplified under the chosen PCR conditions. The PCR reaction should reach or approach a plateau, but the product should not be "overamplified." For example, if the dominant products of the reactions are high molecular weight and produce smears or fail to enter the gel producing "caps," the number of cycles is too high (see **Fig. 21.7**). Conversely, if the PCR product forms a weak band barely visible above the background, then either the number of cycles is too low or the PCR conditions need to be further optimized.

Post-optimization, the result of smPCR in each well should be a clear positive or negative. The absence of a clear difference between the positive and negative reactions may indicate that the "last positive dilution" contains multiple templates and the "first negative dilution" may not be truly negative. False-negative reactions result from the inability of the PCR to amplify a single template into the detectable concentration range and make it clearly visible. Therefore, such a situation is not true smPCR. If increasing the number of cycles and/or polymerase concentration does not improve the outcome, a second round of PCR (nested PCR) should be considered. In some cases, dilution of the first-stage PCR product leads to improved quality of the second-stage PCR. Even where one stage of PCR appears adequate, nested PCR is recommended for all individual reactions of initial experiments to verify results.

If smPCR cannot be successfully accomplished after all the above-mentioned manipulations have been attempted and problems with reagents have been excluded, it is necessary to start over with redesigned primer sets.

3.2. Obtaining smPCR Clones

3.2.1. Estimation of Template Concentration for smPCR (Pilot PCR)

This is the central part of smPCR. Usually at least one (generally two) pilot stage is required (**Figs. 21.1 III (**pilot stages a,b) and **21.6A,B)**. Estimation of the approximate template concentration for smPCR involves serially diluting the template over several orders of magnitude and then subjecting the diluted templates to multiple PCR reactions with the chosen primers. The number of reactions for each dilution may be from 4 to 12 depending on the expected number of successful amplifications (number of positives). Generally, PCR products of the expected length are observed only up to a certain template concentration; higher dilutions will not yield a product. The highest "last positive" dilution should contain one or a few amplifiable DNA templates while the following "first negative" dilution should not contain any DNA templates.

Estimation of the optimum template concentration for smPCR (**Figs. 21.1 III** pilot stage (b) and **21.6B**) is done as follows. The optimal template concentration lies somewhere within the interval between the "last positive" and "first negative" dilutions as described in step (a) above. For optimization an intermediate DNA concentration is calculated within that interval, then used in the next set of reactions. We suggest using sets of 12–24 reactions for this step. The goal is to produce a comparable number of positive and negative reactions. Otherwise an adjustment should be made so that the proportion of positive reactions lies between 0.3 and 0.6. Next, the Poisson distribution table is used to calculate the actual copy number. After applying the correction, the template concentration for smPCR may be calculated (**Fig. 21.6A–C**).

3.2.2. Application of Poisson Distribution and Selecting smPCR Set Size

The Poisson-corrected fraction is equal to $-ln(1-p)$, where p is the observed fraction of positive wells (see **Fig. 21.6C Table 1**). It is critically important to determine the optimal fraction of positive reactions for a particular goal. We usually use positive fractions around 0.5 (multiples of 0.15) for quantitative purposes. For smPCR cloning where individual clones will be analyzed, a fraction of 0.3 or less is preferable. It is particularly important not to significantly exceed a fraction of 0.3 for smPCR in cases where SM clones are screened for mutations, as it corresponds to 0.05 multiples in a reaction (1 multiple in 20). smPCR clones from reaction sets exceeding this proportion will result in too many instances such as the examples shown in **Figs. 21.4C** (smPCR clones 7 and 17) and **21.6D** (smPCR clone 4). PCR from multiple molecules can hinder the interpretation of resulting sequences because of the likeness of wt/real mutation mixtures to PCR-introduced mutations in the early stages *(2)*. The cost of large experiments is worthwhile in order to minimize the probability of having mixed results from multiple molecules.

The number of individual PCR reactions in a set is important for statistically sound data and is dependent on the purpose of the smPCR. It can range from sets of 12 which is typical for rough copy number estimates to sets of up to 96 for cloning experiments or for acquiring adequate statistical power. Generally 24–48 reactions are used in a set. For large cloning experiments, particularly those involving long fragments, a maximum of 48 simultaneous reactions are recommended in order to avoid wasting large amounts of time and materials, as a potential decrease in the number of amplifiable templates cannot be excluded. Such losses sometimes occur for no obvious reason, probably as a result of inadvertent sample mishandling (*see* **Note 10**).

3.3. Rules for Successful smPCR

smPCR follows all the rules of conventional PCR, such as preventing contamination by using filter tips, wearing gloves, and performing positive and negative controls within the experiment. It is important to evaluate mtDNA condition in all experiments. A standard routine with procedures to maximize the success of smPCR is described below.

1. Assess mtDNA condition

   DNA damage acquired in vivo or in vitro may affect PCR polymerase performance. In vitro DNA damage can be diminished by exercising care during all the stages of tissue and reagent handling from tissue preParation through staining and homogenate handling. Precautions should be exercised for PCR reagents handling (*see* **Notes 9 and 10**). Monitoring the condition of mtDNA and using known intact mtDNA samples as controls are very important.

   Since longer fragments acquire more damage that result in fewer amplifiable templates, the condition of mtDNA can be evaluated using PCR with different length amplicons. The

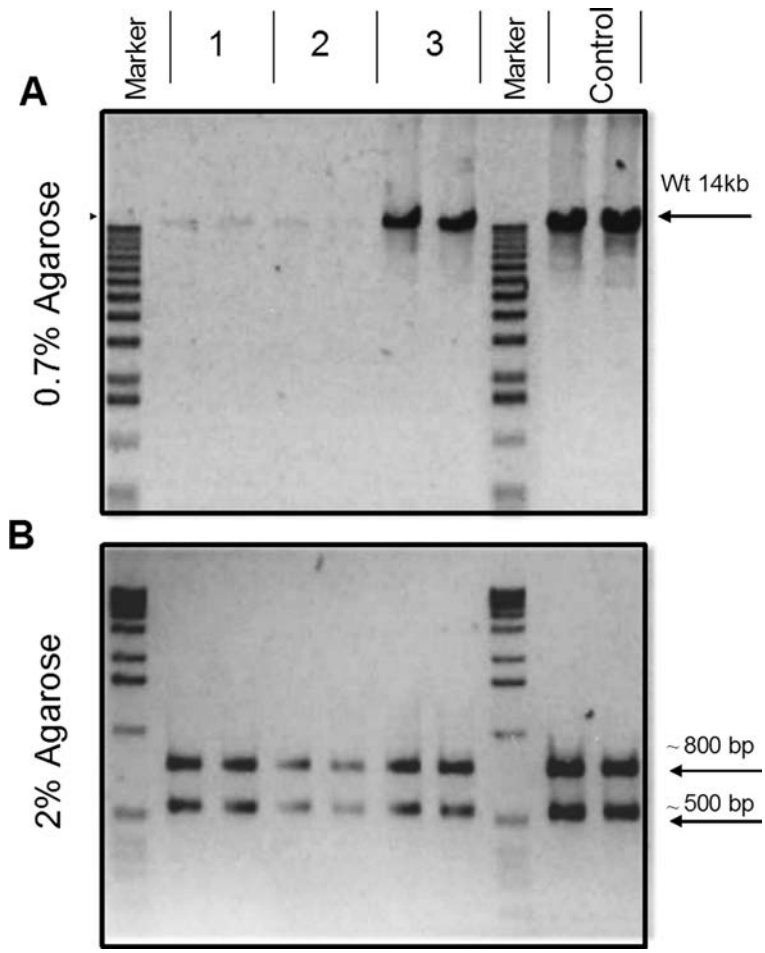


Fig. 21.3. **mtDNA assessment.** Assessment of mtDNA condition following staining procedures is done by comparing PCR yield for fragments of various lengths. (**A**) 14-kb fragment PCR; (**B**) di-plex fragments PCR (~800 and ~500 bp). Staining procedure 3 proves to be suitable for use in long-range PCR analysis; procedures 1 and 2 show extensive loss of long amplifiable fragments and are unacceptable for use in long-range PCR analysis.

evaluation may employ smPCR quantification or may compare homogenate yields as illustrated in **Fig. 21.3**. Different primer sets can be used for this purpose. In the example shown in **Fig. 21.3**, the primer sets are $F_{33}3066/R_{36}780$ for one long fragment and $F_{32}3160/R_{36}3710$ and $F_{31}10161/R_{31}10937$ for two short fragments. Note that if the samples have reverse correspondence between the long and short fragments (yielding relatively fewer for long and more for short fragments), it means that the condition of the mtDNA template is poor. A known homogenate of young tissue with mtDNA in good condition can serve as a standard control for such experiments.

2. Evaluate primer sets for PCR efficiency (**Fig. 21.2**).
3. Track all labware batches, all reagent lots, and aliquot handlings (*see* **Note 10**).

4. Designate laboratory areas for specific functions to prevent contamination.

   Perform all pre-PCR steps including tissue processing, cellular DNA, and PCR mixture preParation in a segregated clean room area, ideally under a laminar flow hood. PCR itself and post-PCR processing such as gel electrophoresis, PCR aliquoting, and PCR purification are conducted in seParate area.

5. Consider human error. Use automatic equipment for all steps when possible, such as automatic electronic pipettes for master mixes and dispensing templates for PCR, automatic multichannel electronic pipettes for dilutions and loadings of PCR.

6. Design control experiments. smPCR minimizes or eliminates many methodological problems as already discussed. Nevertheless, each new development in the applications of smPCR should be checked extensively for possible artifacts. This can be done using pre-characterized templates see Ref. *(24)*, for example.

3.4. PCR and smPCR Product Analysis

Analyzing PCR products with agarose gel electrophoresis is the most time-consuming part of the work. Use of automatic and multi-channel pipettes is recommended whenever possible.

3.4.1. Agarose Gel Electrophoresis

1. Use TAE 0.7% agarose gel for long fragments and 2% agarose gel for short ones.
2. The High-Throughput Horizontal Gel Electrophoresis System enables four 96-well plates for short runs (or two for long runs) to be checked simultaneously. When individual 96-well plates or smaller plates need to be run, the gel can be cut in quarters or smaller pieces and run in smaller horizontal electrophoresis systems.
3. Use 2X loading buffer (1:1 sample:buffer).
4. Run gel at 140 V/l. The running time depends on products length and differences.
5. Stain gel with ethidium bromide, Green Star, or SYBR green.
6. Visualize and image gel on a UV transilluminator equipped with a camera.
7. In the case of long-range smPCR cloning, where reamplification is necessary, a short analytical run is performed initially, followed by full-length side-by-side long run after reamplification of the chosen smPCR products (**Fig. 21.4 B,C**). This is more efficient from both the time and reagents point of view and is more appropriate for longer fragments (*see* **Notes 4–6)**.

3.4.2. Polyacrylamide Gel Electrophoresis (PAGE)

Used for RFLP analysis when necessary: deletion mapping by RFLP, PCR/RFLP analysis.

1. Use TAE 8% native PAGE. Mix reagents in a beaker placed on ice to slow polymerization, thus allowing more time to remove bubbles when making the gel.

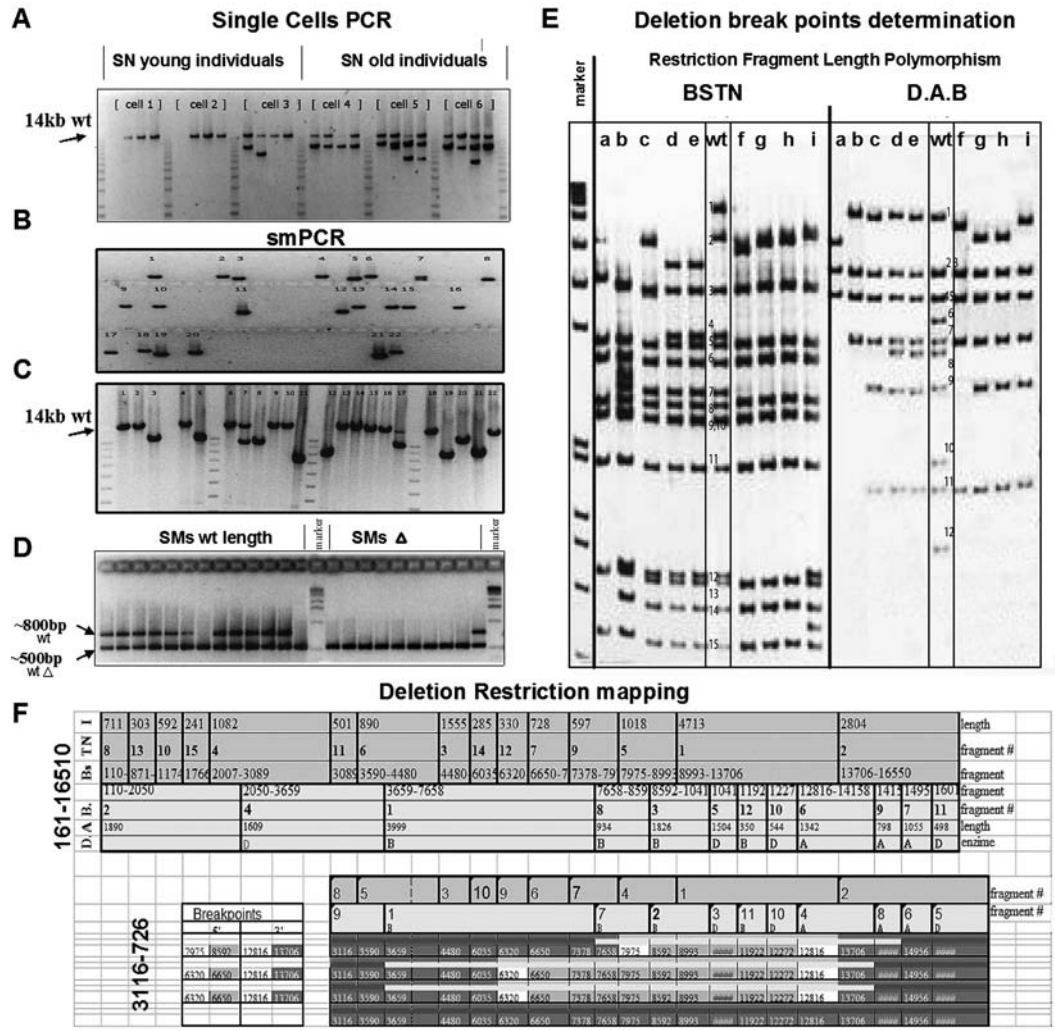


Fig. 21.4. **Single-cell smPCR analysis for screening and quantification of deletions.** (**A**) Multiple long-range PCR from different age adult SN single cells. Cells 4–6 have clonal deletions according to our criteria for first-round evaluation (at least two deleted products of the same length in four reactions). (**B**) First-stage PCR of deletion cloning. Agarose gel is run briefly to find positive PCR reactions (0.7% agarose gel 270 V (140 V/l), 30 min run). Positive PCR reactions are labeled on the plate and subjected to nested PCR. (**C**) Second-stage PCR of deletion cloning was performed only on the positive reactions of the first-stage PCR. The resulting products are run side-by-side for a long period of time to get better resolution for different lengths of molecules (0.7% agarose gel 270 V (140 V/l), 90 min run). (**D**) Di-plex, non-quantitative PCR of smPCR clones from SN neurons. The products of individual SMs are subjected to PCR in order to determine whether △mtDNA SMs are quantifiable with di-plex PCR and to recognize some long deletions that are not distinguishable from wt by the agarose gel electrophoresis already performed. Two groups of SMs are shown. The first has SMs that can appear as wt mtDNA and the second has SMs that can appear as deletions. (**E**) RFLP of deletions using BsTNI and D.A.B. restriction (fragment 161/16,510 restriction maps with fragment size and positions on mtDNA are shown in **F**). Each group has restriction of the same individual **wt** product for reference and all fragments are numbered according to their size. Deletions "d" with "e" and "g" with "h" are good candidates to be clonal as they have the same pattern of RFLP for both restrictions. Sequencing should be done in order to confirm that deletions have the same breakpoints. (**F**) Excel spreadsheet for deletion mapping by restriction of two amplicons; diagram of both amplicons (161/16,510 and 3,116/726) and examples of breakpoint mapping for 3,116/726. All restriction fragments with their number and sizes are placed according to their position on mtDNA (sizes on the diagram are not relative).

2. Use 4X loading buffer to load restricted samples (1:3 sample:buffer).
3. Stain gel with Green Star or SYBR green.

Examples of RFLP analysis are discussed below and shown in **Figs. 21.4E** and **21.6D2**.

3.4.3. PCR/RFLP Analysis

1. PCR-RFLP analysis is one of the traditional ways to determine and quantify heteroplasmy. RFLP can be utilized directly if the difference in sequence of two nucleotide variants creates or destroys a restriction site. If restriction polymorphism is not present, it is possible to create a restriction site difference using primers that are designed to introduce the difference by changing one or several nucleotides based on the existing sequence difference between two variants. This approach has been reinvented several times under different names and the free software "dCAPS" is available on the web to assist in its application (*see* **Note 3**). Quantification can be done by restricting short PCR fragments and resolving the resulting fragments by various methods. While restriction polymorphism is widely used for homogenate quantification, it has important issues that should be considered. Its sensitivity dependant on the detection systems are being employed; for example, for analytical densitometry radioactive detection systems much more sensitive then routine ethidium bromide or other staining such as Gel star, SYBR green. It also poses the specific problem of undercuts that has different reasons. Designing fragments with additional restriction sites to control for unexpected problems with particular restriction reactions can partly address the problem for individual restriction reaction control. Undercuts may occur as a result of annealing different variant strands and the formation of unrestrictable heteroduplexes, restriction difficulties as result of DNA conformation near restriction site, etc. There are different approaches to overcome these problems such as performing additional PCR cycles using PCR cocktails with at least twice the volume of the original PCR reaction or "hot last cycle PCR" *(25)*; using additives to improve restriction *(26)*.
2. Cloning/RFLP analysis for heteroplasmy quantification is straightforward. dCAPS is useful for this application (*see* **Note 3**).

3.4.4. Sequence Analysis

1. Sequencing of homogenates is employed for screening and quantifications of mixtures with proportions starting from about 20 to 30% *(27, 28)*. In order to quantitatively characterize sequences of mixtures of molecules, several issues should be kept in mind. First, each peak's height is affected by adjacent peaks and sequence traces have patterns of peak heights. Second, the height of the peaks decreases when there

is more than one nucleotide in the same position (heteroplasmy) as illustrated in **Fig. 21.6D1**(a good illustration can be found in *(28)* **Fig. 21.4**). In order to more accurately evaluate the level of heteroplasmy on sequence traces, results from pure sequences of each variant should be used for comparison. If the peaks of both variants are of equal height in their pure state, calculations are relatively easy. If the peaks of pure variants are differing in height, it is necessary to make corrections for the difference by creating a coefficient. When measuring the peaks at the heteronucleotide position of first variant and if they are equal in height, the proportion of heteroplasmy is 50%; if the peaks are of unequal heights, it is recommended to measure the height of each peak in the heteronucleotide position and divide the height of one by the sum of both. When measuring peaks at the heteronucleotide position of second variant, for the same operation, it is necessary to use a coefficient to compensate for the difference in pure state. This approach is relatively precise. A number of software packages are available that align sequence traces with heteroplasmy measurement (*see* **Note 3**).

2. Cloning/sequencing analysis for heteroplasmy quantification is straightforward. For software *see* **Note 3**.

3.5. Deletion Quantification by Di-plex smPCR

smPCR small droplets variant protocol as applied to pigmented neurons of substantia nigra (SN) collected by LMPC.

Deletion quantification presents a significant challenge, especially for single-cell analysis. There are numerous approaches to characterize and quantify mtDNA deletion process *(29–36)*. Quantitative di-plex smPCR produces results of a quality comParable to some real PCR methods *(37, 38)* and is readily accomplished in the general PCR laboratory setting. It employs characteristic features of the deletion process for designing multiplex PCR with fragments belonging to differently affected areas of mtDNA. The methods employing this approach, including di-plex smPCR, use two short fragments belonging to a minor arc segment that is usually spared from the deletion process and a major arc segment that is usually deleted, see diagram in **Fig. 21.5A** and in Refs. *(34, 36)*. smPCR involves two fragments of different lengths in order to resolve them on a gel. The deleted (belonging to MTND4) fragment is designated as "wt" and the fragment spared in deletions (belonging to MTND1) is designated as "Δwt." The fragment lengths are ~800 and ~500 bp, respectively. ure **Fig. 21.5** shows the fragment design and a simplified example calculation. The di-plex PCR has the potential to acquire a bias due to minor difference in fragment length and different primer sets. An mtDNA sample in good condition and without deletions can be used to test for such bias. We include this bias control in our experiments, which demonstrates

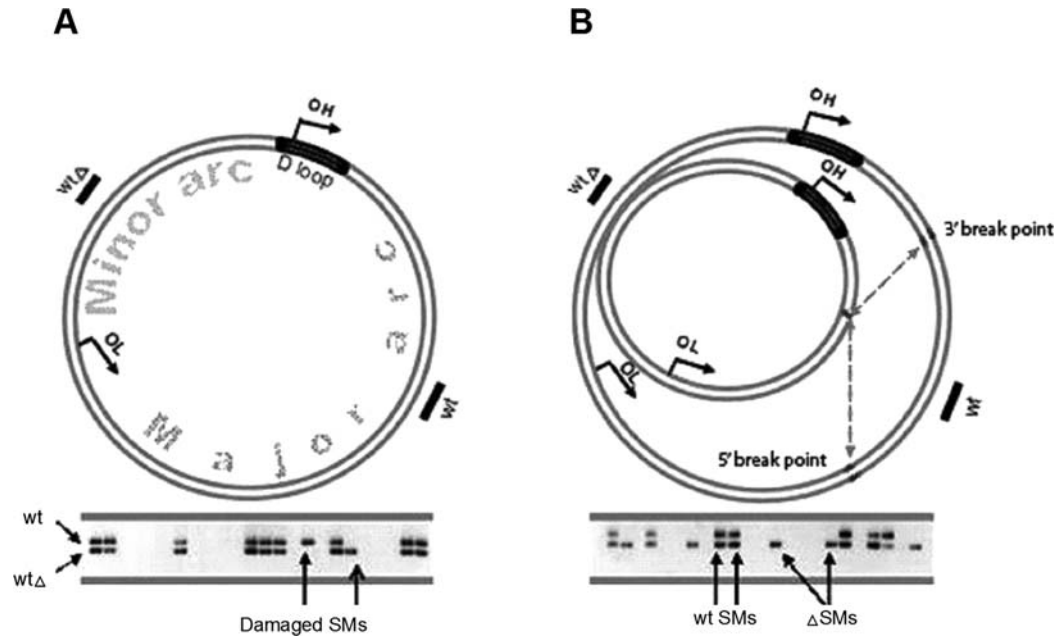


Fig. 21.5. **mtDNA map and di-plex quantitative single-molecule PCR**. **OH** and **OL** are origins of replication for heavy and light strands of mtDNA, respectively; "**wtΔ**" is a PCR fragment in a region that belongs to both wild and deleted molecules (a longer fragment of ~800-bp fragment) and "**wt**" is a PCR fragment in a region that belongs (with few exceptions) only to wt mtDNA molecules amplified by smPCR (a shorter fragment of ~500 bp). Wild-type and deleted SMs are presented as "**wt SM**" and "**ΔSM**". **(A**) Represents a cell with only wt mtDNA. Ideally, both fragmentsshould give an equal number of positives in di-plex quantitative smPCR. In rare instances, an individual smPCR reaction of wt mtDNA may produce only one of the fragments. This is due to template molecule breakage resulting in the seParation of **wtΔ** and **wt** segments of wild-type mtDNA into different wells. (**B**) Represents a cell with both wt and Δ mtDNA types. Fragment **wt**, which is usually affected by the deletion process, will give a lesser number of positives than fragment **wtΔ**, which is usually spared from deletion process. The extent of the difference between two fragments depends on the deletion load. The calculations employ the Poisson distribution. Fragment frequencies are calculated seParately with the Poisson correction. In order to determine the deletion load, the number of upper bands which represent only wt is subtracted from the number of lower bands which represent both deletions and wt molecules; the result is then divided by the number of low bands. Demonstrative calculations for "cell B": the lower **wtΔ** common fragment 12/24 = 0.5 SMs per reaction. According to the Poisson distribution, this corresponds to 0.69 of SMs per well. The upper band fragment belongs only to **wt** molecules 7/24 = 0.29. According to the Poisson distribution it is 0.36 SMs. The deleted molecules load would be (**NwtΔ–Nwt)/NwtΔ** or (0.69–0.35)/0.69=0.49. So Cell B's level of deletions is about 50%. In reality, calculations should be done for larger groups in order to obtain statistically sound data. Also, if there is a PCR bias for one of the fragments, coefficient (*K*) for correction of the bias should be calculated and introduced into the formula (**KNwtΔ–Nwt)/KNwtΔ**.

a bias of approx. 7% in favor of deleted mtDNA molecules. This might be attributed to a slightly shorter length of the "Δwt" PCR fragment compared to the "wt." We recommend that each laboratory, even if using the same fragments, independently determine the bias, which can be influenced by such factors as thermocycler type and reagents.

3.5.1. PreParation for Deletion Quantification of Individual Substantia Nigra (SN) Neurons

1. Check condition of SN cells for mtDNA damage and determine if staining procedure is suitable for the experiment (*see* **above**).
2. Perform preparatory steps for PCR:

   Selected PCR protocol for long-range PCR to characterize mtDNA deletions:

   Chosen primers:

   First PCR stage: $F_{33}3066$, $R_{36}780$;

   Second PCR stage: $F_{39}3116$, $R_{36}780$ (*see* **Note 13**)

   Nested PCR is determined to be optimum.

   PCR program:

Initial denaturing:	94°C 1 min
First stage:	35 cycles. Second stage: 20 cycles
Denaturing:	94°C 30 s
Annealing and elongation step:	68°C 14 min
Final extension:	68°C 5 min

   Reaction volumes:

   10 μl for both PCR stages, as for conventional PCR.

   Selected PCR protocol for quantitative short-fragment PCR:

   Chosen primers:

   First PCR stage: Minor arc (MTND1): $F_{32}3160$, $R_{36}3710$

   Major arc (MTND4): $F_{31}10161$, $R_{31}10937$

   Second PCR stage: Minor arc (MTND1): $F_{34}3204$, $R_{36}3710$

   Major arc (MTND4): $F_{30}10195$, $R_{35}10853$

   Nested PCR is determined to be optimum.

   PCR program:

Initial denaturing:	94°C 1 min
31 Cycles first stage, 16 cycles nested stage	
Denaturing:	94°C 30 s
Annealing and elongation step:	68°C 1 min
Final extension:	68°C 1 min

   Reaction volumes:

   0.6–1 μl for the first stage and 3–6 μl for the second stage, as for small-droplet PCR.
3. Collect cells.

   The PCR described here is applied to the cells collected by P.A.L.M. (*see* **Chapter 20**); 10 μm cryostat sections on PEN-membrane-covered slides are stained by Cresyl Violet/COX staining, then incubated through the ethanol series followed by glycerol. Collected cells are lysed as described in **Chapter 20**.

3.5.2. Cell Characterization: Determining Deletion Clonality

Perform first-stage PCR:

1. Lay out and label PCR plate. We generally use 3–4 reactions per cell.
2. Prepare master mixture for first PCR according to the number of reactions (for example, for eight cells with four reactions per cell and one positive control, prepare a master mixture for 34 reactions with 10 μl per reaction):

ddH_2O:	153 μl
10X LA Taq Buffer:	34 μl
dNTPs:	51 μl
Forward primer $F_{33}3066$:	34 μl
Reverse primer $R_{36}780$:	34 μl
LA Taq polymerase:	3.4 μl

3. Mix thoroughly by vortexing (*see* **Note 14**).
4. Dispense 1 μl of $10^{-2.3}$dilution (1/200th of the cell lysate) from each cell into four wells.
5. Dispense 9 μl of PCR master mixture to each well (*see* **Note 15**).
6. Cover with oil (*see* **Note 16**).
7. Set up and run the thermocycler for first-stage PCR program.

Perform second-stage PCR:

8. Dispense 1 μl of each first PCR product to a new plate (in same order as original plate).
9. Prepare second PCR master mixture for 34 reactions 10 μl each:

ddH_2O:	153 μl
10 × LA Taq Buffer:	34 μl
dNTPs:	51 μl
Forward primer $F_{39}3116$:	34 μl
Reverse primer $R_{36}780$:	34 μl
LA Taq polymerase:	3.4 μl

10. Mix thoroughly by vortexing (*see* **Note 14**).
11. Dispense 9 μl of the second master mixture into each well (*see* **Note 15**).
12. Cover with oil (*see* **Note 16**).
13. Set up and run the thermocycler for second-stage PCR program.
14. Analyze a 2-μl PCR aliquot by agarose gel electrophoresis.

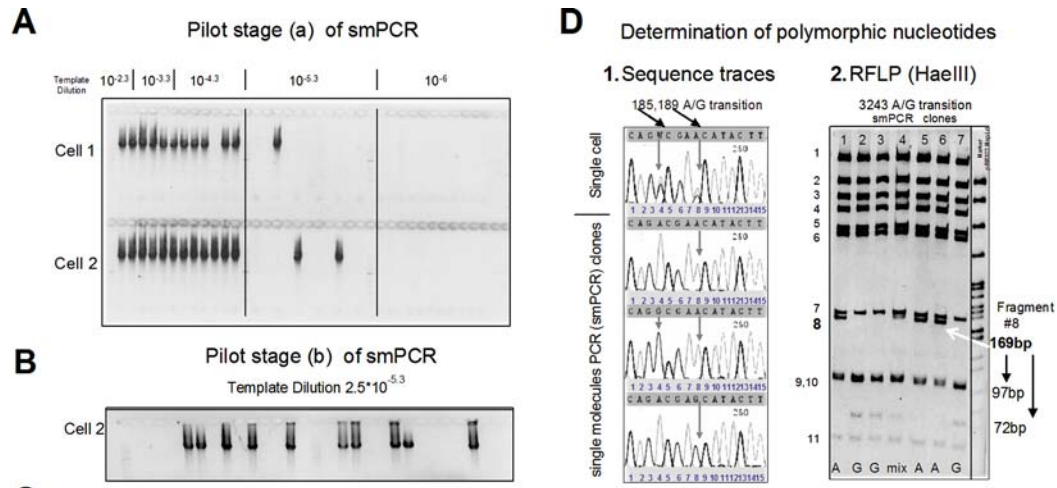
Identified deletions are considered clonal if there are at least two deletions of the same length (see **Fig. 21.4A** showing the long-range PCR for six cells). In **Fig. 21.4**, the last three cells, numbered 4–6, have clonal deletions according to this criterion. Clonality is confirmed by RFLP mapping and breakpoint sequencing when necessary; see below and also **Fig. 21.4E,F**.

3.5.2.1. Deletion smPCR Cloning

In order to obtain smPCR cloned deletions, single-cell lysates are diluted further and used in multiple PCR reactions. The same primer sets and PCR protocol as above can be employed. Gel electrophoresis analysis may be performed in sequential runs. For example, **Fig. 21.4B,C** shows smPCR results from further evaluation of a cell similar to cell 5 in **Fig. 21.4A**. Four reactions of analytical PCR show seven deletions for this cell, four of which may be clonal. Since analytical PCR was performed with a concentration of $10^{-2.3}$of cell lysate and gave at least seven deletions in four reactions for cell 2 (the number is greater because one of deletions appears to be clonal), smPCR can be performed at a concentration of $0.5 \times 10^{-3.3}$or less. Generally between 6 and 18 reactions are enough to obtain several clonal deletions. PCR using 72 reactions is shown in **Fig. 21.4B,C**. All reactions would require 3.6 µl of original $10^{-2.3}$lysate. Note that several of the smPCR reactions have double products, a situation to be avoided. Adjusting for the Poisson distribution gives a number very close to the expected number of doubles $24/72 = 0.33$ (**Fig. 21.6 Table 1**), which corresponds to 0.36 real templates and 0.05 multiples in each positive reaction (or 1 in 20). The presented example has 2 recognizable multiples from 72 reactions. Considering also that some of the doubles could be unrecognizable wt/wt mixtures, the number is close to our prediction. Comparison of deletion lengths from each cell is the first step to determining deletion clonality. In order to more accurately compare deletion lengths, products are diluted in ddH_2O to compensate for the difference in product concentrations. Products should be diluted so that their intensity will be similar to the intensity of the marker bands on the gel. Such evenness is needed to determine fragment length more accurately. The diluted material can later be used in RFLP analysis to determine deletion breakpoints (*see*below in this section) or for PCR to determine whether a deletion is quantifiable by di-plex PCR (see **Fig. 21.4D**).

In principle, WG smPCR cloning can produce quantitative data for both point mutations and rearrangements. However, it has many practical and technical disadvantages for quantification in comparison with short-fragment smPCR, and it is usually not practically feasible to get enough mtWG smPCR clones for quantification. mtWG smPCR cloning also requires costly polymerase, often larger volumes and more time compared to short-fragment smPCR; therefore, short-fragment PCR is usually used for deletion quantification. At the same time, cloning some of deletion "representatives" is necessary to find deletion breakpoints and to accurately answer questions about deletion clonality.

Deletion breakpoints can be identified initially by mapping using RFLP analysis to find the fragment containing the deletion breakpoint. A restriction map of a chosen mtDNA segment can be constructed from the mtDNA reference sequence using software for



C

Table.1. Poisson Distribution. Real copy number for reaction. Fraction of multiple templates.

Fraction of events (positive wells)	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	0.95
Real number of events (templates per well)	0.11	0.22	0.36	0.51	0.69	0.92	1.20	1.61	2.30	3.00
Fraction of multiple events (multiple templates per well)	0.01	0.02	0.05	0.09	0.15	0.23	0.34	0.48	0.67	0.80

Fig. 21.6. **Single-cell smPCR analysis for screening and quantification of clonal point mutations.** (**A–C**) Pilot smPCR. (**A**) Estimating the approximate template concentration. The picture of the gel presents PCR of two single-cell lysates that were serially diluted. Template concentrations that were likely to result in all positive reactions had a lower assigned number of reactions. It provides information that a desirable DNA concentration for both cells is between $10^{-4.3}$ and $10^{-5.3}$ dilutions. Without calculations, the optimum dilution to get 50% positives is around 2 μl of $10^{-5.3}$ dilution (10^{-5}). Necessary calculation steps involving Poisson distribution are in the main text. (**B**) Estimating optimum template concentration. The picture of the gel presents PCR for cell 2 after it was subjected to 24 PCR reactions with a lysate concentration of 2.5 μl of $10^{-5.3}$ that was calculated applying the Poisson correction to the results presented in section (**A**) of this figure. This reaction results in about 50% positives for 24 reactions. That has enough statistical meaning to plan a larger experiment which for smPCR cloning would ideally need template concentration in individual reactions of about 0.3. Further calculations are in the main text. (**C**) **Table 1.** The Poisson distribution is essential for the calculation of required template concentration for the set of reactions. (**D**) Determination of nucleotides in heteroplasmic positions by sequencing and RFLP. (1) Example of sequence traces from the whole cell and its smPCR clones that were aligned with Codon Code Aligner. Two heteroplasmic positions are marked by errors. Note how changes in the nucleotide sequence and heteroplasmy influence the height of the peaks. (2) Example of RFLP. D-loop/MELAS smPCR clones *(1–7)* were subjected to digestion by *Hae*III and seParated using PAGE. The fragment has 10 non-specific restriction sites for *Hae*III, which creates 11 restriction fragments numbered by length. MELAS causing 3243A to G transition is located in fragment 8 of 169 bp long and creates a restriction site for *Hae*III, as a result fragment 169 bp is cut into two fragments which are 97 and 72 bp long (shown on the right-side of the gel). A or G on the bottom of the gel picture indicates the defined nucleotide according to the pattern of digested fragments. Note that smPCR clone 4 has a mixture of molecules, a situation that can and should be avoided.

restriction analysis (*see* **Note 3**). For example, two Parallel restrictions with BsTNI and D.A.B. (*Dra*I, *Ava*I, *Bcl*I) restriction cocktails are presented below. Their maps are shown in **Fig. 21.4G** for two different segments of mtDNA (161/16,510 and 3,116/726) and PAGE results for RFLP of 161/16,510 fragment are shown in **Fig. 21.4E**. A control restriction reaction should be included using known wt DNA of the same mtDNA, since some polymorphisms cause RFLP. Restriction can be prepared in a 4 μl volume using material diluted for agarose gel (*see* above in this section):

1. Lay out and label the plates for restriction. Two different plates are necessary since in this case restriction reactions are performed at two temperatures.
2. Add 0.5 µl of diluted PCR product for each reaction in two PCR plates on ice.
3. Prepare restriction cocktails. The recipes for 18 single-smPCR clones plus one control are shown below:

BsTNI:

H_2O:	51 µl
10X NEB2:	8 µl
10X BSA:	8 µl
BsTNI(10 U/µl):	3 µl

Mix thoroughly by gently pipetting.

D.A.B:

H_2O:	46 µl
10X NEB2:	8 µl
10X BSA:	8 µl
*Dra*I (20 U/µl):	4 µl
*Ava*I (10 U/µl):	2 µl
*Bcl*I(15 U/µl):	2 µl

Mix thoroughly by gently pipetting.

4. Add 3.5 µl of restriction cocktail to each reaction well, then mix by pipetting.
5. Cover with PCR oil.
6. Place the plate with D.A.B restriction at 37°C overnight and the plate with BsTNI restriction at 60°C for 1 h.
7. Add loading buffer and resolve fragments by 8% PAGE.
8. Compile a list of fragments from the two restriction reactions for each molecule.

An Excel file or other spreadsheet application may be useful to organize and interpret restriction mapping results. For example, as shown in **Fig. 21.4F**, one row may contain results from one restriction reaction, a second row may contain results from a second restriction reaction, and a third row may contain compiled data from the first two with the resulting breakpoint areas. These results can be sorted for convenience (**Fig. 21.4F**). Alternatively, the wt RFLP gel picture and diagram can be printed out in order to mark on it all absent fragments for each individual deletion. After identifying areas absent from 3′ to 5′ bordering positions use sequence primers just outside of one of the bordering positions to sequence the area of breakpoint localization. BLAST resulting sequence against database.

Examples of SMs restriction mapping can be seen in **Fig. 21.4E**. Four out of nine deletions are good candidates to be clonal as they have the same pattern of RFLP for both restrictions. Sequencing should be done in order to confirm that deletions have the same breakpoints.

3.5.3. Quantitative Di-plex smPCR

3.5.3.1. Pilot Stage: Determining the Optimal Dilution for smPCR

Groups of 12 reactions can be used to determine the optimal dilution for SM analysis. The optimal dilution for cell lysates from single P.A.L.M. dissected pigmented neurons of human *substantia nigra* is approximately 1/10,000 parts of a cell lysate per reaction. Therefore usually a human SN pigmented neuron lysate concentration of 10^{-4}(0.2 of $10^{-3.3}$(*see* **Note 1**)) prepared as in **Chapter 20** for each reaction is adequate. If a significant difference in long-range PCR results, such as a decreased yield, is seen at the cell characterization stage, either the concentration of lysate for PCR can be adjusted or the number of PCR reactions increased.

1. Lay out and label the PCR plate from the stock with oil drops (*see* **Note 17**). If investigating 8 cells with 12 reactions each, the entire 96-well plate will be used.
2. Label tubes for individual template master mixes (ITMM), one for each cell being analyzed. In this case, eight 0.5ml PCR tubes are used. Dispense 2.6 µl of $10^{-3.3}$dilution of the cell lysate to each tube (this is based on 13 reactions per cell). If the volume between cell lysates is different, normalize using 1X TE.
3. Prepare master mix for a volume of 1 µl per reaction. For example, for 8 prepare 110 µl which would be 13 µl per cell plus adequate dead volume as shown below:

ddH_2O:	40 µl
10X LA Taq Buffer:	11 µl
dNTPs:	11 µl
F_{32}3160:	11 µl
R_{36}3710:	11 µl
F_{31}10161:	11 µl
R_{31}10937	11 µl
Bromophenol blue:	2.2 µl
ExTaq polymerase:	2.2 µl

4. Mix thoroughly by vortexing (*see* **Note 14**).
5. Dispense 10.4 µl to the ITMM tubes for each individual cell and mix by pipetting followed by gently vortexing (*see* **Note 14**).
6. Dispense 1 µl under the oil drop of each tube to the bottom of the well.
7. Set up and run the thermocycler for first-stage PCR program.

8. Prepare master mix for the second PCR. For the entire 96-well plate, prepare materials for 100 reactions, based on a volume of 3 μl for the second-stage PCR or 300 μl total volume:

ddH_2O:	114 μl
10X LA Taq Buffer:	30 μl
dNTPs:	30 μl
Primers:	
$F_{34}3204$:	30 μl
$R_{36}3710$:	30 μl
$F_{30}10195$:	30 μl
$R_{35}10853$:	30 μl
Bromophenol blue:	3 μl
ExTaq polymerase:	3 μl

9. Mix thoroughly by vortexing (*see* **Note 14**).
10. Apply second PCR master mixture to each well (*see* **Note 15**).
11. Follow second-stage PCR protocol.
12. Analyze a 2 μl PCR aliquot by agarose gel electrophoresis.
13. Calculate the optimum template concentration based on the Poisson distribution. Examples of calculations are shown in **Figs. 21.5A**–C and **21.4**. Using this method, the optimal dilution of a template is that which produces the total number of bands (for both the common "**wtΔ**" and the wild-type-specific "**wt**" fragments) equivalent to the number of wells. This maximizes the information about the fraction of deleted mtDNA.

3.5.3.2. Quantification by Di-plex smPCR

After determining the optimal dilution, quantitative PCR for each cell is done in groups of 48 reactions in replicates of at least three. Two PCR machines can be used in tandem with three 48-reaction sets for individual cells, and one 48-reaction set as the control. The control group requires good-quality mtDNA.

1. Lay out and label the plate with oil drops (*see* **Note 17**).
2. Label four tubes for ITMM according to the design of the particular experiment.
3. Add the calculated volume of each cell's dilution to the ITMM for each cell. Human *substantia nigra* pigmented neurons usually need from 1 to 3 μl of $10^{-2.3}$ cell lysate dilution per group of 48 reactions; normalize volumes up to 5 μl using 1X TE.
4. Prepare main master mixture for 200 reactions; 120 μl would be required for 0.6 μl small-droplet PCR:

ddH_2O:	40 µl
10X LA Taq Buffer:	12 µl
dNTPs:	12 µl
$F_{32}3160$:	12 µl
$R_{36}3710$:	12 µl
$F_{31}10161$:	12 µl
$R_{31}10937$:	12 µl
Bromophenol blue:	4 µl
Add ExTaq polymerase:	2.4 µl.

5. Mix thoroughly by vortexing (*see* **Note 14**).
6. Dispense 30 µl to each ITMM tube for all PCR reactions of individual cells and mix by pipetting followed by gentle vortexing (*see* **Note 14**).
7. Dispense 0.6 µl under the oil drop to the bottom of the well (*see* **Note 18**).
8. Perform Steps 8–12, as for previous pilot-stage PCR.
13. Perform calculations in a spreadsheet using the Poisson distribution (**Fig. 21.6C**). Checking and correction for bias toward shorter fragment should be done for each PCR setting. The formula, including correction for bias, is $(KNwt\Delta - Nwt)/KNwt\Delta$, where N is the real number of SMs calculated using the Poisson distribution (as described above) and K is the bias coefficient. Collect data and organize it in a spreadsheet including data about deletion clonality from analytical PCR of the cell characterization step.

3.6. Quantification of Clonal Point Mutations by smPCR

smPCR conventional protocol as applied to skeletal muscle single fibers collected by Arcturus.

Analysis of clonal mutations has two aspects: to determine spectra of clonal mutations and to quantify heteroplasmy. Both are necessary for evaluation of the physiological importance of mutations and polymorphisms. There are several ways to screen for clonal mtDNA point mutations. Often, screening is done by sequencing segments selected according to the goal of the experiment *(27)* to determine clonal mutations at concentrations as low as 20–30% *(27, 28)* and can be done for mtWG *(28)*. Other approaches that can be used to screen for clonal point mutations, including methods to quantify clonal mutations at concentrations below 20%, are mentioned in the next experiment (an example of mtWG single-cell analysis). There are numerous techniques to quantify heteroplasmy and the number is growing due to the need for sensitive and accurate measurements of sequence variations. Different approaches with different detection systems have been employed to quantify single-nucleotide polymorphisms *(6, 13, 25, 39, 50)*. We recommend considering smPCR

cloning for statistically sound heteroplasmy quantification when other resources are not available. It is a strong quantitative approach, but has the disadvantages of being costly and time-consuming if compared with non-cloning techniques. The nucleotide of interest may be determined either by sequencing or by restriction; quantification, however, is done by individual molecule analysis. Determination of molecule number required for statistically valid quantification is discussed below in **Section 3.6.3.3,** Step 3.

We present here the smPCR of a single-fiber section of skeletal muscle, involving a fragment which includes a D-loop and the mitochondrial pathogenic mutations responsible for MELAS (3243 A to G transition). The D-loop has the greatest number of polymorphic sites and is more prone to point mutations than coding regions of mtDNA. Therefore, it is an appropriate area to study relatively neutral clonal expansions of somatic point mutations.

Generally, for quantification purposes it is unnecessary to design long fragments such as those presented in this section. It is more efficient to use shorter fragments which require shorter PCR times. Fragments should be convenient for resolution lengths and additional non-specific restriction site for control. **Figure 21.6D** shows SMs sequence traces and RFLP analysis of the fragment which has ten restriction sites for *Hae*III. The MELAS mutation creates a site for *Hae*III in fragment 8. As a result, the 169-bp fragment is cut into two 97 and 72 bp long fragments that can be distinguished by 8% native PAGE. Seven smPCR clones are shown in **Fig. 21.6D2** of which three are wt molecules, three are mutated molecules, and one is a mixture, the situation to be avoided. The probability of getting multiple templates in one reaction depends on the template concentration in each reaction.

3.6.1. PreParation for smPCR-Cloning of a D-Loop-MELAS Fragment

1. Check selected tissue for mtDNA condition.
2. Perform preParatory steps for PCR:
   Chosen primers:
   Forward – $F_{39}15925$; Reverse – $R_{36}3710$
   One stage of PCR is determined to be satisfactory.
   PCR program:

Initial denaturing:	94°C 1 min
50 Cycles one-stage PCR:	
Denaturing:	94°C 30 sec
Annealing and elongation step:	68°C 3 min
Final extension:	68°C 1.5 min

   Reaction volumes:
   10 μl, as for conventional PCR.
3. Collect cells by Arcturus as in **Chapter 20**; 10 μm cryostat sections on PLUS slides are incubated through the ethanol series followed by xylene clearing. Collected cells are lysed as in **Chapter 20**.

3.6.2. Cell Characterization: Identification of Clonal Point Mutations

1. Lay out and label PCR plate. We generally use 2 reactions per cell.
2. Prepare the PCR master mixture according to the number of cells to be analyzed. For example, to analyze 8 cells and 1 positive control, prepare a master mix for 18 reactions as follows:

ddH_2O:	90 µl
10X LA Taq Buffer:	18 µl
dNTPs:	18 µl
$F_{39}15925$:	18 µl
$R_{36}3710$:	18 µl
Ex Taq polymerase:	1.8 µl

3. Mix thoroughly by vortexing (*see* **Note 14**).
4. Dispense 1 µl of $10^{-2.3}$dilution of each cell in two wells.
5. Dispense 9 µl of PCR master mixture to each well (*see* **Note 15**).
6. Cover with oil (*see* **Note 16**).
7. Set up and run the thermocycler for selected PCR program.
8. Analyze a 2-µl PCR aliquot by agarose gel electrophoresis.
9. Clean with QIAquick PCR purification kit according to the manufacture's protocol.
10. Analyze a 2-µl aliquot by agarose gel electrophoresis.
11. Sequence single-cell PCR with primers covering D-loop. For example, use primers $F_{20}15974$ and $F_{20}16490$.
12. Align and look for heteroplasmic positions. Analyse sequences as suggested in **Section 3.4.4.** or use software (*see* **Note 3**). Save results in spreadsheet.

3.6.3. smPCR Cloning of a D-Loop-MELAS Fragment

If data for the number of amplifiable templates for the fragment of interest is unavailable, begin with several dilutions of the sample to obtain an approximate value. Fewer reactions should be used for the lower dilutions which will have a higher probability of yielding positive reactions. A series might include 2 reactions of $10^{-2.3}$, 4 of $10^{-3.3}$, 6 of $10^{-4.3}$, 12 of $10^{-5.3}$, and 12 of 10^{-6}dilution.

3.6.3.1. Pilot Stage of smPCR (D-Loop-MELAS Fragment)

3.6.3.1.1. Estimation of the Approximate Template Concentration (***Fig. 21.6A***)

1. Lay out and label the plate. For 2 cells it would be 72 reactions.
2. Label tubes for ITMM containing the template for each set. Ten 0.5 ml PCR tubes should be used in this example.

   Dispense 2 µl of $10^{-2.3}$ dilutions to the first tubes for ITMM for each cell.

   Dispense 4 µl of $10^{-3.3}$ dilution to the second tubes.

   Dispense 6 µl of $10^{-4.3}$ dilution to the third tubes.

   Dispense 12 µl of $10^{-5.3}$ dilution to the fourth tubes.

   Dispense 2.4 µl of $10^{-5.3}$ to the fifth tubes.

Add 1X TE to each tube to equalize the volume if necessary (9.6 µl to the fifth tubes).

3. Calculate blank main master mixture for 10 µl for each reaction plus dead volume (10 µl × 36 × 2+10 µl = 730 µl).

ddH_2O:	329 µl
10X LA Taq Buffer:	73 µl
dNTPs:	109.5 µl
$F_{39}15925$:	73 µl
$R_{36}3710$:	73 µl
Bromophenol blue:	7.3 µl
ExTaq polymerase:	7.3 µl.

4. Mix thoroughly by vortexing (*see* **Note 14**).
5. Dispense blank master mixture to the ITMM tubes prepared in Step 2. For 2 reactions ($10^{-2.3}$dilution) dispense18 µl, for 4 reactions ($10^{-3.3}$dilution) dispense 36 µl, for 6 reactions ($10^{-4.3}$dilution) dispense 54 µl, and for 2 12-reaction sets ($10^{-5.3}$ and 10^{-6}dilutions) dispense108 µl.
6. Mix first by pipetting followed by gentle vortexing (*see* **Note 14**).
7. Dispense 10 µl to each well of the group.
8. Apply oil (*see* **Note 16**).
9. Set up and run the thermocycler for selected PCR program.
10. Analyze 2 µl PCR aliquots by agarose gel electrophoresis.
11. Calculate the Poisson distribution to determine the optimum dilution for each cell for the next step. The example in **Fig. 21.6A** for both cells 1 and 2 calculations will not result in an accurate copy number, because only low numbers were available for calculation, therefore the next stage will be necessary. Calculations for demonstrative purpose: (a) 2 positives from 12 reaction results in 2/12 or approx. 0.17 positives per reaction; (b) assuming the Poisson distribution (**Fig. 21.6C**), the approximate template for the reaction is approx. 0.2 copy per well; (c) the desired template number per well is 0.5, which is 2.5X higher; (d) multiply the template concentration used in the example reaction by 2.5. In the example presented in **Fig. 21.6A**, 2.5 µl of $10^{-5.3}$dilution for a single reaction would be used for cell 2.

If this step yields acceptable results for determining the optimal dilution for smPCR cloning (about 50% positive reactions), no additional steps are necessary.

*3.6.3.1.2. Estimation of Optimal Template Concentration (see **Fig. 21. 6B,** for example, of Optimal Dilution Determination)*

1. Calculate sample dilution for 12 or 24 reactions. For cell 2 from the previous section for 24 reactions, this would be 2.5 µl × $10^{-5.3}$ × 24 which is equivalent to 60 µl of $10^{-5.3}$ (6 µl × $10^{-4.3}$).

2. Perform smPCR as described in the previous stage with recalculated volumes and reaction numbers. For cell 2 in **Fig. 21.6A** using a set of 24 reactions this would be 6 μl × $10^{-4.3}$. **Figure 21.6B** shows possible outcome.
3. Calculate sample dilution for smPCR cloning. Use the Poisson distribution to find the real copy number. Note that for cell 2 in **Fig. 21.6B**, some positives will have more than one template; 10 positives out of 24 reactions ∼ 0.5 copies per reaction. This translates to approx. 1 multiple reaction in 10. This is acceptable for some experiments, but usually 0.3 positives per reaction (0.36 copies per reaction) or 1 multiple per 20 smPCR clones is desired. In the case considered here for cell 2, it is preferable to use a less concentrated template. For considered example 0.36/0.51 it would be ∼ 0.7; for 2.5 μl × 0.7 it would be 1.75 μl of $10^{-5.3}$. For subsequent smPCR cloning, the recommended concentration will be 1.75 μl of $10^{-5.3}$ in reaction.

3.6.3.2. Obtaining smPCR Clones (a D-Loop-MELAS Fragment)

After determining the optimal dilution, the desired number of smPCR clones can be obtained by performing PCR with the template concentration chosen for each cell. For the example considered here, the calculation from the previous Paragraph gave 0.175 μl of $10^{-4.5}$foreach reaction.

1. Lay out and label the PCR plate.
2. Label tubes according to the design of the particular experiment. For example, obtaining smPCR clones for 2 cells with 48 reactions per set each would require 96 reactions. Add the calculated volume of each cell's dilution to the ITMM for each cell and then equalize volumes in all tubes with 1X TE. For example, if the maximum volume of the groups is 10 μl, add 1X TE up to 10 μl, if needed. In the case presented above, it would be 0.175 μl of $10^{-4.5}$for each reaction: for 49, it would be 8.75 μl of $10^{-4.5}$. The addition of 1.25 μl of 1X TE is required.
3. Prepare blank main master mixture for 98 reactions:

ddH_2O:	500 μl
10X LA Taq Buffer:	98 μl
dNTPs:	147 μl
$F_{39}15925$:	98 μl
$R_{36}3710$:	98 μl
Bromophenol blue:	9.8 μl
Add ExTaq polymerase:	9.8 μl

4. Mix thoroughly by vortexing (*see* **Note 14**).
5. Dispense assigned volume to each ITMM tube and mix by pipetting followed by gentle vortexing (*see* **Note 14**).

6. Dispense 10 μl to each well.
7. Apply oil (*see* **Note 16**).
8. Set up and run the thermocycler for selected PCR program.
9. Analyze 2 μl PCR aliquots by agarose gel electrophoresis.
10. Mark and number positives (smPCR clones) on the gel picture and on the plate.
11. Prepare products for sequence and restriction if needed (*see* **Note 8**).

3.6.3.3. Analysis of Resulting smPCR Clones and Calculation of Clone Number for Quantification

1. (a) Sequence smPCR products with primers covering the D-loop. Determine nucleotide variants of all heteroplasmic positions found at the cell characterization stage.
   (b) Restriction may be employed. An example is shown in **Fig. 21.6D2**.
2. Align sequences. Helpful software is discussed in **Note 3**. Perform RFLP analysis if applicable. Mine data using a spreadsheet application such as Microsoft Excel.
3. Analyze results. Translating SM experimental data results into measurements of the heteroplasmy is done using statistical techniques for point estimation in a binomial population. The methods involve the determination of confidence levels and intervals for found proportions and the computing of sample sizes (number of SMs) for desirable confidence levels and intervals. The most commonly used formula relies on approximating the binomial distribution with a normal distribution (standard normal (Z) distribution, Wald method). Modified Wald method is considered one of the most adequate for small sample sizes and Laplace method for low proportion level. They use the same formulas as standard z-statistic method:

$$p \pm Z\sqrt{\{\mathrm{pq/n}\}}$$

or

$$E = Z\sqrt{\{\mathrm{pq/n}\}} \text{and} n = Z^2 pq/E^2$$

where $\boldsymbol{n}$ is the number of molecules examined (or to be examined), $\boldsymbol{Z}$ is the z-statistic corresponding to the given percent confidence level **C**. When **C** is 95%, **Z**=1.96, when 90%, **Z**=1.64. $\boldsymbol{p}$ is the proportion of mtDNA copies in the cell with a given nucleotide, and $\boldsymbol{q}$ is the proportion of its counterpart ($\boldsymbol{q=1-p}$). $\boldsymbol{E}$ is the amount of error in the measurement of $\boldsymbol{p}$.

The modified Wald method uses an addition of two counts (½ of Z^2 value for **C**=95%) to each count and Laplace uses an addition of one. Tables 2 and 3 of **Fig. 21.8** demonstrate the dependence of

sample size on heteroplasmy level and measured molecular pool size. Note that the number of SMs needed to satisfy the same confidence interval and level depends on the heteroplasmy level and the number of mtDNA copies in the particular cell type, which allows developing more refined methods. For additional details and useful links *see* **Note 19**.

3.7. Point Mutation Screening by mtWG smPCR

smPCR droplets variant protocol for WG smPCR cloning as applied to heart myocytes collected from suspension.

The mitochondrial genomes of vertebrates range from 16 to 18 kb, making it feasible to amplify them in their entirety by PCR. Since complete human mitochondrial genome amplification was introduced accompanied by RFLP analysis *(51)*, it has been actively employed in mtDNA genetics for both deletion and point mutation analyses. There are numerous methods for mutation screening and for quantifying mutation load, including different cloning methods. A number of them were adapted to screen mtDNA mutations, and some for mtWG *(2, 20, 52–78)*. smPCR cloning and sequencing of mtWG SMs is an accessible way to obtain the most complete and accurate information applicable to single-cell analysis. It is not obligatory to use WG smPCR clones in order to analyze the point mutation process. Shorter fragments designed to serve specific experimental goals may be used in order to more easily and economically acquire the necessary number of smPCR clones. In order to show some of the difficulties of long-range smPCR, **Fig. 21.7A** shows the result of one of the first attempts to clone the whole mitochondrial genome via smPCR, with the eventual goal of sequencing. The pictured smPCR cloning was performed with a new droplet PCR modification using 0.6 μl in oil drop for the first PCR. Recommendations described herein were adopted after the presented experiment.

3.7.1. PreParation for mtWG smPCR Cloning

1. Check tissue for mtDNA condition.
2. Perform preParatory steps for PCR.

   PCR conditions are as follows:

   Chosen primers:

   First-stage PCR: Forward – $F_{30}2999$, Reverse – $R_{32}2949$

   Second stage of PCR: Forward – $F_{33}3066$, Reverse – $R_{32}2949$

   Nested PCR is determined to be optimum,

   PCR program:

   Initial denaturing: 94°C 1 min

   (35 cycles – 1st stage, 22 cycles – nested stage)

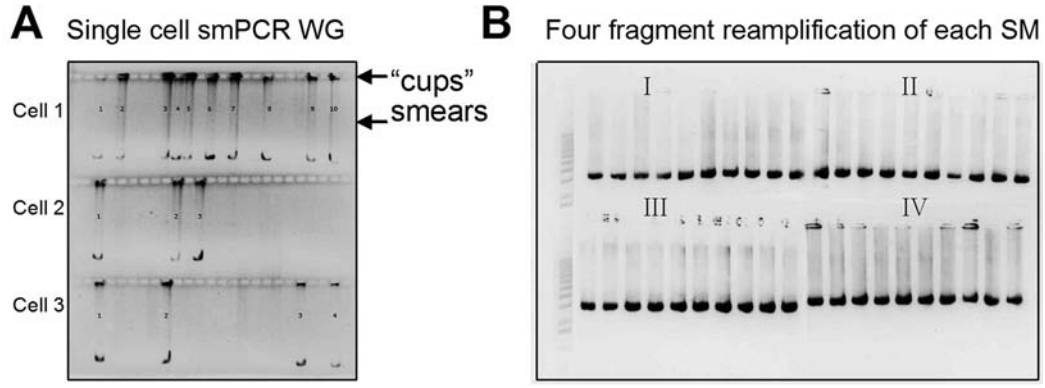
   Denaturing: 94°C 30s

   Annealing and elongation step: 68°C 15 min

   Final extension: 68°C 5 min

   Reaction volumes:

   3 μl for the first stage, 10 μl for the second stage, as in droplets protocol.



C Primer list for WG sequencing

I	F20.3167	F20.3629	F20.4146	F21.4625	F20.5154	F23.5652	F20.6159	F23.6608
II	F20.7105	F20.7627	F20.8105	F20.8618	F20.9049	F20.9687	F23.10203	F20.10694
III	F21.11234	F21.11636	F20.12183	R20.12861	F22.12735	F21.13243	F20.13801	F21.14253
IV	F22.15044	F20.15568	F20.16103	F20.110	F20.615	F20.1170	F20.1709	F21.2257

Fig. 21.7. **Whole genome smPCR cloning for sequence analysis.** (**A**) An example of acceptable but not perfect smPCR (better long smPCR is shown in **Fig. 4(B,C)**. Note weaker bands, upper smear, and "cups" in the wells as described in **Section 3.2.1.** *(4).* All reactions were checked with nested PCR for unseen products to avoid false-negative results. Since no additional positive reactions were seen, the resulting products were used in the experiment, but the procedure was modified. Note that SMs from cells 1, 3, and 4 are less likely than SMs of cell 2 to be the result of amplification of multiple molecules. (**B**) Gel presents the result of reamplification of individual molecules with four overlapping fragments of ~4 kb. Each of the products was subjected to sequencing by eight primers. (**C**) List of possible primers to use for sequencing.

3. Collect cells. The PCR described here is applied to single cells collected from suspension by aspiration (*see* **Chapter 20**)

3.7.2. Cell Characterization by mtWG PCR

Check cells by subjecting lysates to PCR in duplicates. Follow the procedure of the previous experiment, but use primers selected specifically for this application, with a nested PCR protocol and conventional 10 μl volumes. mtWG sequencing can be done as presented below in Section **3.7.2.3.1** but for clonal mutation screening using PCR of concentrated cell lysate, as it is useful to have information about clonal mtDNA mutations present in the cell for interpreting data of SMs analysis.

3.7.2.1. Pilot Stage of smPCR (mtWG)

Determine the optimal dilution for smPCR, as in the previous experiment, but using a PCR program and primers selected for this application and with droplets variant as described above. Use modified reaction volumes, as detailed below.

3.7.2.2. Obtaining smPCR Clones (mtWG)

First obtain smPCR clones, as in the previous experiment, but with droplets variant. **Figure 21.7A** shows an example which produced marginal results for WG smPCR, based on smears, "caps," and uneven bands. The volume used was 0.6 μl for the first PCR and 3 μl for the second PCR. The whole set of reactions for smPCR

cloning was then subjected to third stage using an additional nested PCR in order to check for false-negative reactions. False-negative reactions were not determined. We found that in our setting for this amplicon it is preferable to use larger volumes such as 3–5 μl for the first stage of long PCR and 10 μl for the second stage.

3.7.2.3. Analysis of Resulting mtWG smPCR Clones

3.7.2.3.1. PreParation for mtWGS Analysis

Perform PCR amplification with four fragments for each cloned molecule (**Fig. 21.7B**).

1. Dilute smPCR WG clones 10-fold in ddH_2O.
2. Lay out and label the plate. Aliquot 1 μl of diluted PCR into four wells of the PCR plate. For example, 24 molecules will utilize the entire 96-well plate.
3. Calculate a volume of primerless master mixture of 10 μl for each reaction and individual primer pair for individual primer pair master mixes (IPpMM). This example of 24 molecules uses 10 μl × 24 × 4 + 40 μl. This includes dead volume.
4. Label four tubes for IPpMM containing the primer pair for specific fragments I–IV of all 24 molecules. Dispense 25 μl of each primer to assigned tubes as follows:

Fragment I:	$F_{33}3066$, $R_{35}7405$
Fragment II:	$F_{32}6836$, $R_{33}11417$
Fragment III:	$F_{30}10935$, $R_{34}15148$
Fragment IV:	$F_{32}14678$, $R_{32}2949$

5. Prepare primerless PCR master mixture:

ddH_2O:	440 μl
10X LA Taq Buffer:	100 μl
dNTPs:	150 μl
Add ExTaq polymerase:	10 μl

6. Mix thoroughly by vortexing (*see* **Note 14**).
7. Dispense the primerless master mixture (175 μl for this example) to the tubes prepared in Step 3 to make IPpMM.
8. Mix thoroughly by vortexing (*see* **Note 14**).
9. Dispense 9 μl of IPpMMs to each of the four aliquoted SM templates (*see* **Note 15**).
10. Apply oil (*see* **Note 16**).
11. Set up and run the thermocycler for selected PCR program.
12. Analyze PCR products by agarose gel electrophoresis.
13. Clean resulting amplified fragments of PCR clones with a PCR purification kit (*see* **Note 8**).
14. Analyze PCR products by agarose gel electrophoresis.
15. Sequence each 4-kb fragment with eight primers. Possible primers for all fragments are listed in **Fig. 21.6C**.

3.7.2.3.2. Analysis of mtWG smPCR Clones

1. Perform screening for mutations with selected software (*see* **Note 3**).
2. Analyze results. There are two main potential problems to consider when analyzing sequences. First is the possible existence of double templates in one reaction (rarely more) which appear as 50% heteroplasmy as discussed above. The probability of multiple templates depends on the percent of positives in the set for smPCR from which the particular SM was obtained. Second is the problem of acquiring artificial mutations during PCR amplification. Artificial mutations would appear as not more than a 25% difference or in cases of single-strand amplifications not more than 50% *(2)*. While artificially introduced mutations can usually be distinguished from real ones during the analysis of such mixtures (the existence of several instances on a single molecule helps), it is preferable to avoid this situation by taking precautions upfront. The probability of artificial mutations depends on the condition of the template mtDNA, dNTPs, polymerase intrinsic fidelity, and PCR conditions.
3. We recommend performing data mining with quantification of mutation frequency and type using an Excel spreadsheet. Some software will do this automatically (*see* **Note 3**).

4. Notes

1. We keep our stocks in 10^{-1}, $10^{-1.3}$, and $10^{-2.3}$in a specially designated area at –70°C as it is the main stocks to which we return if something happens with less concentrated template. We adapted an unusual way to numerate dilution concentrations. We started to use it after laser-dissections, when we decided that our main stock will be 1/20 of a cell. This is a convenient volume to handle, while still having some protective substances, and also 1/200 was the template concentration which we were mainly using for analytical PCR, so it is an "historical" system which can be easily changed if it is not convenient, but we are using it for the presentation of the method (*see* **Note 12**). Also, the high extent of dilution implies that the method of DNA isolation is not important for the success of the procedure, as long as it preserves amplifiable DNA molecules of the appropriate length. That is why we usually use dilutions of native lysates. If more than usually concentrated samples are needed proteinase K can be heat-deactivated at 95°C. Note that other components of the lysation buffer can be removed only by cleaning, but all readily available cleaning procedures decrease concentration or quality of DNA.

2. It is very important to choose the right amplification system. Information about polymerases can be found on the web (for Takara enzymes, for example,http://catalog.takara-bio.co.jp/en/product/basic_info.asp?catcd=B1000495&subcatcd=B1000496&unitid=U100005923 http://catalog.takara-andbio.co.jp/en/product/basic_info.asp?catcd=B1000495&subcatcd=B1000496&unitid=U100005868 also http://www.takarabiousa.com/WEBINARS/presentations/ConsumerPresentation/index.html). Phusion polymerase can be adopted for smPCR. It has great fidelity and processivity.http://www.neb.com/nebecomm/products/phusion_overview.asp.

   PCR buffers with additives for optimal PCR conditionsand DNA protection can be considered *(79)*. We have found that an LA Taq Buffer is preferable for both LA Taq and ExTaq polymerases (it preserves DNA better); we have also found that bromophenol blue not only provides better visualization of small volumes but also improves outcome of some PCRs. It was found after certain mistakes in recipes (which were incorporated in the lab book) and which proved so useful that they were introduced into our technique (*see* **Note 10**).

3. Examples of useful software. Data mining.

   There are very useful free, routinely used software available on the web. These include ClustalW for alignments (http://www.ebi.ac.uk/Tools/clustalw/index.html), BLAST, SciTools of IDT (http://www.idtdna.com/SciTools/SciTools.aspx) for primer pair evaluation, and options to run designed primers against nuclear-mtDNA pseudogene (NUMT) database, for restriction analysis TACGhttp://bioweb.pasteur.fr/seqanal/interfaces/tacg.htmland NEB2cutter of NEB (http://tools.neb.com/NEBcutter2). dCAPs website mentioned in **Section 3.4.3** (http://helix.wustl.edu/dcaps/dcaps.html). Reference sequences can be found on the NCBI webpage (http://www.ncbi.nlm.nih.gov/genomes/static/euk_o.html) or specifically for human origin on MITOMAP (http://www.mitomap.org/mitoseq.html). MITOMAP is helpful for human mtDNA research and is constantly being improved. Commercial software may have advantages in terms of analysis time and accuracy of analysis for large data sets. Choosing the optimum software can also be a time-consuming process. We had the opportunity to work with programs such as Mutation Explorer, Codon Code Aligner, and DNASTAR Lasergene for multiple alignment. Each program has its own advantages and limitations, and all of them are constantly being upgraded and improved. The software developers give a trial period with workshops and are often very helpful with finding solutions for specific tasks.

4. It can be helpful to organize and load PCR products to facilitate analysis. Dividing the analyzed groups with DNA markers helps.
5. When using a multi-channel pipette for handling PCR products for aliquoting and loading, it is handy to place the aliquots for the PCR set in two rows (4 neighboring wells for 4 reactions, 2 rows of 12 for 24 reactions, etc.).
6. In order to simplify the PCR loading process, a loading buffer can be added to the PCR reaction wells by dispensing it on the side of the wells, spinning, vortexing, and spinning once more. It saves significant time and the resulting mixture can be used for further PCRs upon dilution in ddH_2O.
7. Sequence primers for Whole Genome Sequencing can be placed in a cassette. For example, a cassette can be made from a 2.2-ml 96-well MARK II (ABgene) storage plate. An automatic multi-channel electronic pipette may be employed for dispensing primers to PCR plates for further sequencing.
8. For sequencing we generally had been using cleaned products. Recently we started using directly diluted PCR products for sequencing without any effect on traces quality. For our sequencing setting we dilute products with yielding near plato in about 100 times with good quality tracing.
9. All plates, reagent tubes, and mixtures should be kept on ice whenever possible. The PCR plates should be lowered and pressed into the ice while making sure that no ice or water gets into the wells while working. We found it convenient to use several small square ice buckets made from styrofoam reagent mailing.
10. Because smPCR is highly sensitive, we recommend keeping track of all reagents and labware batches and variants. For example, we strongly recommend using the particular type of plate described under **Section 2**. We found that for low template concentrations, especially smPCR, the plastic used in the plates can affect the PCR outcome. We also recommend keeping track of tube types used for dilutions, as unexpected decrease in the number of amplifiable templates sometimes might happen possibly as result of adsorption on the tube wall. The lab book should include all the details of the ongoing experiment where there is space to make any corrections or mishandling of the planned procedure. This not only helps to avoid mistakes, but also gives the opportunity to make use of some of them if they cause improvement (*see* **Notes 2** and **12**).
11. Organizing the PCR working area helps to prevent mistakes. For example, when a large number of PCR groups (individual reagents master mixtures) are used, we recommend

placing the mixture tubes in an open-bottom rack in the order in which the PCR groups were laid out on the main PCR plate.

12. The specifications for single-cell analysis in this chapter should be considered as guidelines. For example, in "Analysis of Data for Cell Characterization and Determination of Deletion Clonality" we may say that among the many rearrangements we have observed using smPCR Δ cloning by the fragments presented in **Fig. 21.4B**, only a few were not quantifiable. Therefore, the determination of deletion quantifiability by di-plex PCR may be postponed until a later stage of experimentation when necessary. *See* also **Note 1**.
13. Ideally, nested PCR should be done with another set of primers, but if these are not available, at least one of the primers should be changed (partially nested PCR).
14. All PCR reagent mixtures should be thoroughly mixed. Special care should be given to mixtures with templates, usually called Individual Template Master Mixtures. Template for ITMM can be added before the addition of templateless master mixture, as presented in methods, or after its aliquoting in ITMM tubes. If later used ITMMs should be mixed thoroughly but gently by multiple vortexing (we use vortex speed between 4 and 5 on VORTEX-GENIE 2).
15. A PCR master mixture should be applied carefully to the PCR plate wells with templates approximately two-thirds of the way down the wall of the well. This will help to protect from contamination, and ensure the cooling of the solution as it slides down the cold wall. When the second PCR master mixture is added to the wells with the first PCR reactions approximately half of the way down the wall of the well, gently tap the plate to help the mixture slide down into the oil. Check that all droplets have reached the oil, tapping additionally if necessary. A short spin can be used.
16. We used 1,000-μl pipettes to gently add oil to the side of each well and let it cool against the cold wall.
17. When using a procedure such as small-droplet PCR where the first PCR mixture will be dispensed into oil, plates with oil aliquots may be prepared in advance and stored at 4°C.
18. For droplets PCR, dispense droplets into oil close to the bottom of the well. Spin plates if droplets began to flow.
19. The modified Wald test is most often recommended for this analysis *(80–82)*. The following information could be helpful in order to reduce the number of SMs necessary for the measurement. The possible range of p (heteroplasmy) can reduce the sample size; the farther from 50% is the possible

A

Table.2. Dependence of sample size (n) on the level of heteroplasmy (p) for confidence interval (E) at the confidence level 95%.

E \ p	5%	10%	15%	20%	25%	30%	35%	40%	45%	50%	55%
±0.1	-	35	49	61	72	81	87	92	95	96	95
±0.07	37	71	100	125	147	165	178	188	194	196	194
±0.05	73	138	196	246	288	323	350	369	380	384	380

B

Table.3. Dependence of Sample size (n) on molecular pool level (N) with p=50% for confidence interval (E) at the confidence level 95%.

E \ N	100	200	300	400	500	750	1000	2500	5000	10000	100000	n
±0.1	49	65	73	78	81	85.2	88	93	94	95	96	96
±0.07	66	99	119	132	141	156	164	182	189	192	196	196
±0.05	80	132	169	196	217	254	278	333	357	370	383	384

Fig. 21.8. **Calculation of SMs number for heteroplasmy measurement.** (**A**) **Table 2.** Demonstrates dependency of necessary SMs for measurement on heteroplasmy level. (**B**)**. Table 3.** Demonstrates dependency of necessary SMs for measurement on mtDNA pool size of the cells analyzed.

heteroplasmy level the fewer SMs are required. **Figure 21.8 Table 2** illustrate the dependence of required SM number (n), for a 95% confidence level and a 0.1–0.05 confidence interval (E), on the level of heteroplasmy (p).

The number of molecules required can sometimes be reduced for cells with a low number of mtDNA copies. In such cases the "finite population correction" factor $fpc\,(n,N) = (1 + (n-1)/N)$ applies to the formula, where N would be the expected number of mtDNA copies in the cells and n the required number of SMs for measurements of samples with infinite mtDNA copies (crude homogenates or cells with a high mtDNA copy number). If n/N is even a few percent, this correction can reduce the number of molecules sampled. Therefore in cases of an analysis of single cells with low numbers of mtDNA copies, the following formula can be applied:

$$\mathrm{nc} = \mathrm{n}/[1 + (\mathrm{n} - 1)/\mathrm{N}]$$

where n_c is the corrected number and $N$is the expected number of mtDNA copies in cells of interest. **Figure 21.8** Table 3 illustrate dependence of required SM number (n) for 0.1–0.05 confidence interval (E); a 95% confidence level and a proportion of 50% (which requires the maximum number of SMs) for pool levels from 100 to 100 thousands copies.

The number of SMs that is required for measurement at a desired confidence level may be determined based on preliminary estimation of approximate heteroplasmy level, i.e., to provide a confidence interval for p which, if far away from ½,

would reduce the number *n* of additional molecular samples required. Data obtained earlier from Cell Characterization and Determination of Point Mutation Clonality may be used in the calculation to indicate an approximate level of heteroplasmy or it can be performed in sequential stages. This enables optimizing the determination of the required number of SMs based on the each stage result using the formula, treating the earlier round of data as the first few measurements toward the required sample size ***n*** calculated by formula. Optimized versions of this scheme are available under the rubric of "double sampling" *(83)* or sequential estimation *(84)*. A range of resources is available on the web that can help with the calculations (for example, http://www.dimensionresearch.com/resources/calculators/conf_prop.html; http://www.raosoft.com/samplesize.html; http://statpages.org//; http://www.surveysystem.com/sscalc.htm; http://statpages.org/confint.html).

Acknowledgments

This work was supported in part by the NIH grant AG019787 and a United Mitochondrial Disease Foundation grant to K.K.

References

1. Wallace, D.C. (2007) Why do we still have a maternally inherited mitochondrial DNA? Insights from evolutionary medicine. *Annu Rev Biochem* **76,** 781–821.
2. Kraytsberg, Y. and Khrapko, K. (2005) Single-molecule PCR: an artifact-free PCR approach for the analysis of somatic mutations. *Expert Rev Mol Diagn* **5,** 809–815.
3. Jeffreys, A.J., Neumann, R. and Wilson, V. (1990) Repeat unit sequence variation in minisatellites: a novel source of DNA polymorphism for studying variation and mutation by single molecule analysis. *Cell* **60,** 473–485.
4. Ruano, G., Kidd, K.K. and Stephens, J.C. (1990) Haplotype of multiple polymorphisms resolved by enzymatic amplification of single DNA molecules. *Proc Natl Acad Sci U S A* **87,** 6296–6300.
5. Lukyanov, K.A., Matz, M.V., Bogdanova, E.A., Gurskaya, N.G. and Lukyanov, S.A. (1996) Molecule by molecule PCR amplification of complex DNA mixtures for direct sequencing: an approach to in vitro cloning. *Nucleic Acids Res* **24,** 2194–2195.
6. Vogelstein, B. and Kinzler, K.W. (1999) Digital PCR. *Proc Natl Acad Sci U S A* **96,** 9236–9241.
7. Mitra, R.D., Butty, V.L., Shendure, J., Williams, B.R., Housman, D.E. and Church, G.M. (2003) Digital genotyping and haplotyping with polymerase colonies. *Proc Natl Acad Sci U S A* **100,** 5926–5931.
8. Mitra, R.D., Shendure, J., Olejnik, J., Edyta Krzymanska, O. and Church, G.M. (2003) Fluorescent in situ sequencing on polymerase colonies. *Anal Biochem* **320,** 55–65.
9. Zhu, J., Shendure, J., Mitra, R.D. and Church, G.M. (2003) Single molecule profiling of alternative pre-mRNA splicing. *Science* **301,** 836–838.
10. Dressman, D., Yan, H., Traverso, G., Kinzler, K.W. and Vogelstein, B. (2003) Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations. *Proc Natl Acad Sci U S A* **100,** 8817–8822.

11. Li, M., Diehl, F., Dressman, D., Vogelstein, B. and Kinzler. K.W. (2006) BEAMing up for detection and quantification of rare sequence variants. *Nat Methods* **3,** 95–97.
12. Huse, S.M., Huber, J.A., Morrison, H.G., Sogin, M.L. and Welch, D.M. (2007) Accuracy and quality of massively Parallel DNA pyrosequencing. *Genome Biol* **8,** R143.
13. Jahangir Tafrechi, R.S., van de Rijke, F.M., Allallou, A., Larsson, C., Sloos, W.C., van de Sande, M., Wahlby, C., Janssen, G.M. et al. (2007) Single-cell A3243G mitochondrial DNA mutation load assays for segregation analysis. *J Histochem Cytochem* **55,** 1159–1166.
14. Braslavsky, I., Hebert, B., Kartalov, E. and Quake, S.R. 2003. Sequence information can be obtained from single DNA molecules. *Proc Natl Acad Sci U S A* **100,** 3960–3964.
15. Ryan, D., Rahimi, M., Lund, J., Mehta, R. and Parviz, B.A. (2007) Toward nanoscale genome sequencing. *Trends Biotechnol* **25,** 385–389.
16. Diehl, F. and Diaz, Jr., L.A. (2007) Digital quantification of mutant DNA in cancer patients. *Curr Opin Oncol* **19,** 36–42.
17. Paabo, S., Irwin, D.M. and Wilson, A.C. (1990) DNA damage promotes jumping between templates during enzymatic amplification. *J Biol Chem* **265,** 4718–4721.
18. Kajander, O.A., Kunnas, T.A., Perola, M., Lehtinen, S.K., Karhunen, P.J. and Jacobs, H.T. (1999) Long-extension PCR to detect deleted mitochondrial DNA molecules is compromised by technical artefacts. *Biochem Biophys Res Commun* **254,** 507–514.
19. Pavlov, A.R., Pavlova, N.V., Kozyavkin, S.A. and Slesarev, A.I. (2004) Recent developments in the optimization of thermostable DNA polymerases for efficient applications. *Trends Biotechnol* **22,** 253–260.
20. Kraytsberg, Y., Nekhaeva, E., Chang, C., Ebralidse, E. and Khrapko, K. (2004) Analysis of somatic mutations via long-distance single molecule PCR. *In* V.V. Demidov and N.E. Broude (ed.), DNA AMPLIFICATION: Current Technologies and Applications. *Horizon Bioscience*.
21. Eckert, K.A. and Kunkel, T.A. (1991) DNA polymerase fidelity and the polymerase chain reaction. *PCR Methods Appl* **1,** 17–24.
22. Cline, J., Braman, J.C. and Hogrefe, H.H. (1996) PCR fidelity of pfu DNA polymerase and other thermostable DNA polymerases. *Nucleic Acids Res* **24,** 3546–3551.
23. Sikorsky, J.A., Primerano, D.A., Fenger, T.W. and Denvir, J. (2007) DNA damage reduces Taq DNA polymerase fidelity and PCR amplification efficiency. *Biochem Biophys Res Commun* **355,** 431–437.
24. Kraytsberg, Y., Schwartz, M., Brown, T.A., Ebralidse, K., Kunz, W.S., Clayton, D.A., Vissing, J. and Khrapko, K. (2004) Recombination of human mitochondrial DNA. *Science* **304,** 981.
25. Moraes, C.T., Atencio, D.P., Oca-Cossio, J. and Diaz, F. (2003) Techniques and pitfalls in the detection of pathogenic mitochondrial DNA mutations. *J Mol Diagn* **5,** 197–208.
26. Sugimoto, K., Makihara, T., Saito, A., Ohishi, N., Nagase, T. and Takai, D. (2005) Betaine improved restriction digestion. *Biochem Biophys Res Commun* **337,** 1027–1029.
27. Nekhaeva, E., Bodyak, N.D., Kraytsberg, Y., McGrath, S.B., Van Orsouw, N.J., Pluzhnikov, A., Wei, J.Y., Vijg, J., et al. (2002) Clonally expanded mtDNA point mutations are abundant in individual cells of human tissues. *Proc Natl Acad Sci U S A* **99,** 5521–5526.
28. Taylor, R.W., Taylor, G.A., Durham, S.E. and Turnbull, D.M. (2001) The determination of complete human mitochondrial DNA sequences in single cells: implications for the study of somatic mitochondrial DNA point mutations. *Nucleic Acids Res* **29,** E74–74.
29. Sciacco, M., Bonilla, E., Schon, E.A., DiMauro, S. and Moraes, C.T. (1994) Distribution of wild-type and common deletion forms of mtDNA in normal and respiration-deficient muscle fibers from patients with mitochondrial myopathy. *Hum Mol Genet* **3,** 13–19.
30. Schinogl, P., Muller, M. and Steinborn, R. (2001) Quantification of the 4977-bp deletion in human mitochondrial DNA using real-time PCR. *Forensic Sci Int* **122,** 197–199.
31. Poe, B.G., Navratil, M. and Arriaga, E.A. (2007) Absolute quantitation of a heteroplasmic mitochondrial DNA deletion using a multiplex three-primer real-time PCR assay. *Anal Biochem* **362,** 193–200.
32. Zhang, C., Baumer, A., Maxwell, R.J., Linnane, A.W. and Nagley, P. (1992) Multiple mitochondrial DNA deletions in an elderly human individual. *FEBS Lett* **297,** 34–38.
33. Zhang, C., Lee, A., Liu, V.W., Pepe, S., Rosenfeldt, F. and Nagley, P. (1999) Mitochondrial DNA deletions in human cardiac tissue show a gross mosaic distribution. *Biochem Biophys Res Commun* **254,** 152–157.
34. Bai, R.K. and Wong, L.J. (2005) Simultaneous detection and quantification of

mitochondrial DNA deletion(s), depletion, and over-replication in patients with mitochondrial disease. *J Mol Diagn* **7,** 613–622.

35. Vermulst, M., Wanagat, J., Kujoth, G.C., Bielas, J.H., Rabinovitch, P.S., Prolla, T.A. and Loeb, L.A. (2008) DNA deletions and clonal mutations drive premature aging in mitochondrial mutator mice. *Nat Genet* **40,** 392–394.
36. Bodyak, N.D., Nekhaeva, E., Wei, J.Y. and Khrapko, K. (2001) Quantification and sequencing of somatic deleted mtDNA in single cells: evidence for partially duplicated mtDNA in aged human tissues. *Hum Mol Genet* **10,** 17–24.
37. Bender, A., Krishnan, K.J., Morris, C.M., Taylor, G.A., Reeve, A.K., Perry, R.H., Jaros, E., Hersheson, J.S., et al. (2006) High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. *Nat Genet* **38,** 515–517.
38. Kraytsberg, Y., Kudryavtseva, E., McKee, A.C., Geula, C., Kowall, N.W. and Khrapko, K. (2006) Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons. *Nat Genet* **38,** 518–520.
39. Naini, A. and Shanske, S. (2007) Detection of mutations in mtDNA. *Methods Cell Biol* **80,** 437–463.
40. Piggee, C.A., Muth, J., Carrilho, E. and Karger, B.L. (1997) Capillary electrophoresis for the detection of known point mutations by single-nucleotide primer extension and laser-induced fluorescence detection. *J Chromatogr A* **781,** 367–375.
41. Chinnery, P.F., Brown, D.T., Andrews, R.M., Singh-Kler, R., Riordan-Eva, P., Lindley, J., Applegarth, D.A., Turnbull, D.M., et al. (2001) The mitochondrial ND6 gene is a hot spot for mutations that cause Leber's hereditary optic neuropathy. *Brain* **124,** 209–218.
42. Wong, L.J. and Bai, R.K. (2006) Real-time quantitative polymerase chain reaction analysis of mitochondrial DNA point mutation. *Methods Mol Biol* **335**, 187–200.
43. Gibson, N.J. (2006) The use of real-time PCR methods in DNA sequence variation analysis. *Clin Chim Acta* **363,** 32–47.
44. Lim, K.S., Naviaux, R.K. and Haas, R.H. (2007) Quantitative mitochondrial DNA mutation analysis by denaturing HPLC. *Clin Chem* **53,** 1046–1052.
45. Ragoussis, J., Elvidge, G.P., Kaur, K. and Colella, S. (2006) Matrix-assisted laser desorption/ionisation, time-of-flight mass spectrometry in genomics research. *PLoS Genet* **2,** e100.
46. Mashima, Y., Nagano, M., Funayama, T., Zhang, Q., Egashira, T., Kudho, J., Shimizu, N. and Oguchi, Y. (2004) Rapid quantification of the heteroplasmy of mutant mitochondrial DNAs in Leber's hereditary optic neuropathy using the Invader technology. *Clin Biochem* **37,** 268–276.
47. Cassandrini, D., Calevo, M.G., Tessa, A., Manfredi, G., Fattori, F., Meschini, M.C., Carrozzo, R., Tonoli, E., et al. (2006) A new method for analysis of mitochondrial DNA point mutations and assess levels of heteroplasmy. *Biochem Biophys Res Commun* **342,** 387–393.
48. Singh, R., Ellard, S., Hattersley, A. and Harries, L.W. (2006) Rapid and sensitive real-time polymerase chain reaction method for detection and quantification of 3243A>G mitochondrial point mutation. *J Mol Diagn* **8,** 225–230.
49. White, H.E., Durston, V.J., Seller, A., Fratter, C., Harvey, J.F. and Cross, N.C. (2005) Accurate detection and quantitation of heteroplasmic mitochondrial point mutations by pyrosequencing. *Genet Test* **9,** 190–199.
50. Csako, G. (2006) Present and future of rapid and or high-throughput methods for nucleic acid testing. *Clin Chim Acta* **363,** 6–31.
51. Cheng, S., Higuchi, R. and Stoneking, M. (1994) Complete mitochondrial genome amplification. *Nat Genet* **7,** 350–351.
52. Sauer, S. (2006) Typing of single nucleotide polymorphisms by MALDI mass spectrometry: principles and diagnostic applications. *Clin Chim Acta* **363,** 95–105.
53. Jiang, Y., Hall, T.A., Hofstadler, S.A. and Naviaux, R.K. (2007) Mitochondrial DNA mutation detection by electrospray mass spectrometry. *Clin Chem* **53,** 195–203.
54. Linnartz, B., Anglmayer, R. and Zanssen, S. (2004) Comprehensive scanning of somatic mitochondrial DNA alterations in acute leukemia developing from myelodysplastic syndromes. *Cancer Res* **64,** 1966–1971.
55. Jazin, E.E., Cavelier, L., Eriksson, I., Oreland, L. and Gyllensten, U. (1996) Human brain contains high levels of heteroplasmy in the noncoding regions of mitochondrial DNA. *Proc Natl Acad Sci U S A* **93,** 12382–12387.
56. Michikawa, Y., Mazzucchelli, F., Bresolin, N., Scarlato, G. and Attardi, G. (1999) Aging-dependent large accumulation of

point mutations in the human mtDNA control region for replication. *Science* **286,** 774–779.

57. Fayet, G., Jansson, M., Sternberg, D., Moslemi, A.R., Blondy, P., Lombes, A., Fardeau, M. and Oldfors, A. (2002) Ageing muscle: clonal expansions of mitochondrial DNA point mutations and deletions cause focal impairment of mitochondrial function. *Neuromuscul Disord* **12,** 484–493.
58. Michikawa, Y. and Attardi, G. (2002) Screening for aging-dependent point mutations in mtDNA. *Methods Mol Biol* **197,** 75–92.
59. Wong, L.J., Liang, M.H., Kwon, H., Park, J., Bai, R.K. and Tan, D.J. (2002) Comprehensive scanning of the entire mitochondrial genome for mutations. *Clin Chem* **48,** 1901–1912.
60. Conley, Y.P., Brockway, H., Beatty, M. and Kerr, M.E. (2003) Qualitative and quantitative detection of mitochondrial heteroplasmy in cerebrospinal fluid using denaturing high-performance liquid chromatography. *Brain Res Brain Res Protoc* **12,** 99–103.
61. Biggin, A., Henke, R., Bennetts, B., Thorburn, D.R. and Christodoulou, J. (2005) Mutation screening of the mitochondrial genome using denaturing high-performance liquid chromatography. *Mol Genet Metab* **84,** 61–74.
62. Meierhofer, D., Mayr, J.A., Ebner, S., Sperl, W. and Kofler, B. (2005) Rapid screening of the entire mitochondrial DNA for low-level heteroplasmic mutations. *Mitochondrion* **5,** 282–296.
63. Wulfert, M., Tapprich, C. and Gattermann, N. (2006) Optimized PCR fragments for heteroduplex analysis of the whole human mitochondrial genome with denaturing HPLC. *J Chromatogr B Analyt Technol Biomed Life Sci* **831,** 236–247.
64. Jacobs, L., Gerards, M., Chinnery, P., Dumoulin, J., de Coo, I., Geraedts, J. and Smeets, H. (2007) mtDNA point mutations are present at various levels of heteroplasmy in human oocytes. *Mol Hum Reprod* **13,** 149–154.
65. Bannwarth, S., Procaccio, V. and Paquis-Flucklinger, V. (2005) Surveyor Nuclease: a new strategy for a rapid identification of heteroplasmic mitochondrial DNA mutations in patients with respiratory chain defects. *Hum Mutat* **25,** 575–582.
66. Maitra, A., Cohen, Y., Gillespie, S.E., Mambo, E., Fukushima, N., Hoque, M.O., Shah, N., Goggins, M., et al. (2004). The Human MitoChip: a high-throughput sequencing microarray for mitochondrial mutation detection. *Genome Res* **14,** 812–819.
67. Kraytsberg, Y., Nicholas, A. and Khrapko, K. (2007) Are somatic mitochondrial DNA mutations relevant to our health? A challenge for mutation analysis techniques. *Expert Opin Mol Diagn* **1,** 1–8.
68. Zheng, W., Marcelino, L.A. and Thilly, W.G. (2002) Scanning low-frequency point mutants in the mitochondrial genome using constant denaturant capillary electrophoresis. *Methods Mol Biol* **197,** 93–106.
69. Bielas, J.H. and Loeb, L.A. (2005) Quantification of random genomic mutations. *Nat Methods* **2,** 285–290.
70. Greenberg, B.D., Newbold, J.E. and Sugino, A. (1983) Intraspecific nucleotide sequence variability surrounding the origin of replication in human mitochondrial DNA. *Gene* **21,** 33–49.
71. Monnat, R.J., Jr. and Loeb, L.A. (1985) Nucleotide sequence preservation of human mitochondrial DNA. *Proc Natl Acad Sci U S A* **82,** 2895–2899.
72. Bodenteich, A., Mitchell, L.G. and Merril, C.R. (1991) A lifetime of retinal light exposure does not appear to increase mitochondrial mutations. *Gene* **108,** 305–309.
73. Kovalenko, S.A., Tanaka, M., Yoneda, M., Iakovlev, A.F. and Ozawa, T. (1996) Accumulation of somatic nucleotide substitutions in mitochondrial DNA associated with the 3243 A-to-G tRNA(leu)(UUR) mutation in encephalomyopathy and cardiomyopathy. *Biochem Biophys Res Commun* **222,** 201–207.
74. Simon, D.K., Lin, M.T., Ahn, C.H., Liu, G.J., Gibson, G.E., Beal, M.F. and Johns, D.R. (2001) Low mutational burden of individual acquired mitochondrial DNA mutations in brain. *Genomics* **73,** 113–116.
75. Lin, M.T., Simon, D.K., Ahn, C.H., Kim, L.M. and Beal, M.F. (2002) High aggregate burden of somatic mtDNA point mutations in aging and Alzheimer's disease brain. *Hum Mol Genet* **11,** 133–145.
76. Khaidakov, M., Heflich, R.H., Manjanatha, M.G., Myers, M.B. and Aidoo, A. (2003) Accumulation of point mutations in mitochondrial DNA of aging mice. *Mutat Res* **526,** 1–7.
77. Del Bo, R., Crimi, M. Sciacco, M., Malferrari, G., Bordoni, A., Napoli, L., Prelle, A., Biunno, I., et al. (2003) High mutational burden in the mtDNA control region from

aged muscles: a single-fiber study. *Neurobiol Aging* **24,** 829–838.

78. Cantuti-Castelvetri, I., Lin, M.T., Zheng, K., Keller-McGandy, C.E., Betensky, R.A., Johns, D.R., Beal, M.F., Standaert, D.G., et al. (2005) Somatic mitochondrial DNA mutations in single neurons and glia. *Neurobiol Aging* **26,** 1343–1355.
79. Kurz, M. (2008) Compatible solute influence on nucleic acids: many questions but few answers. *Saline Systems* **4,** 6.
80. Du, Y., Davisson, M.T., Kafadar, K. and Gardiner, K. (2006) A-to-I pre-mRNA editing of the serotonin 2C receptor: comparisons among inbred mouse strains. *Gene* **382,** 39–46.
81. Dawson, B. and Trapp, R.G. 2004. Basic & Clinical Biostatistics. McGraw-Hill, New York.
82. Newcombe, R.G. (1998) Two-sided confidence intervals for the single proportion: comparison of seven methods. *Stat Med* **17,** 857–872.
83. Cochran, W.G. (1977) Sampling Techniques. Wiley, New York.
84. Ghosh, M. (1991) Handbook of Sequential Analysis. CRC, Boca Raton, MA.

Chapter 22

Measuring DNA Precursor Pools in Mitochondria

Christopher K. Mathews and Linda J. Wheeler

Abstract

The ability to measure molar concentrations of deoxyribonucleoside 5′-triphosphates (dNTPs) within the mitochondrial matrix is important for several reasons. First, the spontaneous mutation rate for the mitochondrial genome is much higher than that for the nuclear genome, and dNTP concentrations are known determinants of DNA replication fidelity. Second, several human mitochondrial diseases involve perturbations of nucleotide metabolism, and dNTP pool analysis can help us to understand the consequences of these abnormalities. Third, it is important to understand how mtDNA is supplied with precursors in non-cycling cells, where the cytosolic machinery that supplies dNTPs for nuclear replication is downregulated. Fourth, the toxicity of several antiviral nucleoside analogs involves their metabolic activation within mitochondria, and dNTP pool analyses can help us to understand the processes leading to toxicity. Analyses of dNTP pools in whole-cell extracts from tissues or cultured cells are carried out either by HPLC or by an enzymatic method using DNA polymerase and defined templates. Because dNTP pools are much smaller in mitochondria than in whole cells, HPLC lacks the sensitivity needed for these measurements. The enzymatic method possesses sufficient sensitivity and is the method described in this chapter.

Key words: Deoxyribonucleoside 5′-triphosphates, nucleotides, DNA polymerase, DNA precursor pools, nucleotide metabolism, enzyme-based metabolite assays, mitochondrial dNTP pools, mutagenesis.

1. Introduction

The earliest method used for dNTP pool analysis was introduced in the mid1960s and applied to bacterial extracts. To a bacterial culture was added [^{32}P]orthophosphate at high specific activity. After about three generations of growth, by which time all phosphorylated compounds had reached the same specific radioactivity, the cells were harvested and extracted, followed by thin-layer chromatographic resolution of the radiolabeled dNTPs *(1)*.

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_22 Springerprotocols.com

Radioactive spots corresponding to each nucleotide were cut from the chromatogram and counted, with radioactivity in each spot being directly proportional to the size of the nucleotide pool. Because of the impossibility of equilibrating ^{32}P radioactivity in the complex growth media used for slower-growing mammalian cells, other approaches had to be developed. HPLC came into wide use in the early 1970s, with anion-exchange columns being used to resolve nucleoside triphosphates. A problem was that these columns did not separate deoxyribonucleotides from the far more abundant ribonucleotides. Garrett and Santi *(2)* treated extracts with periodate, which selectively attacks vicinal diols and, hence, selectively destroys ribonucleotides. However, many researchers found the periodate oxidation products to interfere with resolution and analysis of the undamaged dNTPs remaining in the periodate-treated extract. This problem was solved when Shewach *(3)* showed that vicinal diols, including 5′-ribonucleotides, could be selectively and quantitatively removed by passing an extract through a boronate column. More recently it has become possible to separate deoxyribonucleotides from their cognate ribonucleotides by reverse-phase HPLC in the presence of an ion-pairing agent *(4)*.

A more serious problem for mitochondrial dNTP analysis is that the mtDNA content of a cell is but a small fraction of the nuclear DNA content, and dNTP pools in mitochondria were expected to be correspondingly lower. Accordingly, the method of choice became the DNA polymerase-based enzymatic assay, which had been introduced in the early 1970s for analysis of dNTP pools in bacteria and in cultured mammalian cells *(5)*. Although this method is fraught with possible artifacts, it is quite sensitive, and with careful attention to all that can go wrong, it does generate reliable data (*see* **Note 1**). When the assay was first applied in our laboratory, to HeLa cells *(6)*, we confirmed our expectation that the mitochondrial dNTP pools were low, but not as low as expected. In HeLa cells mtDNA amounts to about 1% of total DNA, whereas we found the four mt-dNTP pools to range from 2 to 10% of corresponding whole-cell values.

The enzymatic assay is carried out by incubating an extract containing the four dNTPs in unknown amounts with purified DNA polymerase, a template of known repeating nucleotide sequence, and a radiolabeled "counternucleotide," in considerable excess over the expected amount of the particular dNTP being analyzed *(5)*. For example, in analyzing the dTTP pool, the incubation mixture would contain the d(AT) copolymer, with a regular repeating –AT-sequence, and [^{3}H] dATP in excess. Under these conditions the incorporation of radioactivity into acid-insoluble material is limited by the amount of dTTP in the unknown, and a plot of maximum cpm incorporated vs dTTP in a series of standards should yield a straight line, which can then be used to determine dTTP concentration in an unknown.

In 1989, Sherman and Fyfe *(7)* introduced an important modification of this assay. Instead of using repeating-sequence polymers as the templates for the polymerase reaction, they used chemically synthesized oligonucleotides, each one designed for a specific analysis. For example, an oligonucleotide with a repeating –ATTT– sequence would triple the sensitivity of this assay for dTTP, because three molecules of radiolabeled counternucleotide (dATP in this case) would be incorporated for every molecule of the analyte, dTTP. This modification allows the determination of dNTPs in subpicomole amounts, and that is the protocol used in our laboratory.

2. Materials

2.1. Mitochondrial Isolation

2.1.1. Rat Tissues (Except Skeletal Muscle)

1. Cold 0.9% NaCl.
2. Isolation buffer: 220 mM mannitol, 70 mM sucrose, 5 mM MOPS, pH 7.4, 2 mM EGTA, 0.2% bovine serum albumin.
3. Centrifuge and Sorvall SS34 rotor.

2.1.2. Rat Skeletal Muscle

1. Muscle isolation buffer: 0.1 M KCl, 50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, and 1 mM EGTA.
2. Nagarse (or equivalent protease).
3. Centrifuge and Sorvall SS34 rotor.

2.1.3. Yeast Mitochondria

1. YPG medium (4 g/l yeast extract, 8 g/l peptone, and 12 ml/l glycerol, pH 5.8) or lactate medium (3 g/l yeast extract, 0.5 g/l glucose, 0.5 g/l $CaCl_2 \cdot 2H_2O$, 0.5 g/l NaCl, 0.6 g/l $MgCl_2 \cdot 2\ H_2O$, 1 g/l KH_2PO_4, 1 g/l NH_4Cl, 22 ml/l 90% DL-lactic acid, and 8 g/l NaOH pellets (final pH = 5.5)).
2. Dithiothreitol buffer: 0.1 M Tris–HCl, pH 9.3, and 10 mM dithiothreitol.
3. Buffer A: 1.2 M sorbitol, 20 mM potassium phosphate buffer, pH 7.4.
4. Zymolase 20T (MP Biomedicals).
5. Buffer B: 0.6 M sorbitol, 20 mM Tris–HCl, pH 7.4, 2 mM EDTA.
6. Dounce homogenizer.
7. Sterile water.
8. Ice-cold methanol.

2.2. dNTP Analysis

1. 5X dNTP buffer: 0.5 M HEPES and 0.05 M $MgCl_2$, pH 7.5. Autoclave and store at 4°C.

2. Bovine serum albumin (BSA): 10 mg/ml.
3. Standard dNTP solutions. Prepare 10 μM solutions of each dNTP from the Invitrogen ultra-pure dNTP set (100 mM of each dNTP).
4. Working dNTP standards. Serially dilute the 10 μM stocks prepared previously to working standards of 0.4, 0.2, 0.1, 0.05, 0.02, and 0.01 μM for each dNTP. Store each solution at –20°C.
5. DNA polymerase, Klenow fragment (US Biochemicals; 5 units per μl) (*see* **Note 2**).
6. Oligonucleotide templates: All oligonucleotides are prepared at 0.05 nmol/μl in sterile water and stored at –20°C in 200-μl aliquots (*see* **Note 3**).

   dATP assay template:

   5′-AAATAAATAAATAAATAAATGGCGGTGGAGGCGG-3′.

   dTTP assay template:

   5′-TTATTATTATTATTATTAGGCGGTGGAGGCGG-3′

   dCTP assay template:

   5′-AAAGAAAGAAAGAAAGAAAGGGCGGTGGAGGCGG-3′

   dGTP assay template:

   5′-AAACAAACAAACAAACAAACGGCGGTGGAGGCGG-3′.

   Oligonucleotide primer: 5′-CCGCCTCCACCGCC-3′.
7. [^{3}H]dNTPs: for dATP, dCTP, and dGTP assays: [^{3}H]dTTP (30 Ci/mmol, 1 μCi/μl, GE Healthcare). For dTTP assay: [^{3}H]dATP (30 Ci/mmol, 1 μCi/μl, GE Healthcare).
8. DE81 chromatography paper, cut into 2 × 2 cm^2.

3. Methods

3.1. Isolation of Mitochondria

For mitochondrial isolation we use standard techniques involving differential centrifugation *(8)*. For cultured cells we find that sufficient mitochondria for a complete dNTP analysis can be isolated from about 2 × 10^8 HeLa cells. In our earlier study *(6)*, where considerably more cells were needed, we grew cells in suspension culture, requiring 1 l per analysis. With the increased sensitivity allowed by the Sherman–Fyfe procedure *(7)* we can grow sufficient cells as subconfluent monolayer cultures in eight 150-mm culture dishes *(9)*. For analysis of animal tissues, we find that 1 g of tissue yields more than enough mitochondria for a

complete dNTP analysis. We have recently begun experiments with yeast mitochondria, and we find that a 2-l culture in lactate medium *(10)* yields 2–3 $\times$ 10^{10} exponential-phase cells, which yield in turn enough mitochondria *(10)* for a complete dNTP analysis.

In all cases we monitor our preparations for cytosolic contamination by carrying out lactate dehydrogenase assays, and for functional integrity by carrying out measurements of *P/O* ratio and respiratory control ratio *(11)*.

3.1.1. Rat Tissues (Except Skeletal Muscle)

1. For rat tissues except skeletal muscle we rapidly excise the organ and rinse it several times in cold 0.9% NaCl.
2. A piece of tissue at least 1 g in weight is trimmed and minced, then placed in 5–10 volumes of isolation buffer.
3. The mixture is homogenized in a Teflon homogenizer with a motor-driven pestle.
4. The homogenate is centrifuged for 10 min at 500 $\times$ g in a Sorvall SS34 rotor.
5. The supernatant is recentrifuged at 10,000 $\times$ g.
6. The pelleted mitochondria are resuspended in isolation buffer, then repelleted and either extracted or stored at –20°C.

3.1.2. Rat Skeletal Muscle

1. Minced tissue as described above (**Section 3.1.1**) is suspended in 5–10 volumes of a muscle isolation buffer.
2. Nagarse is added at 5 mg/g of tissue.
3. The mixture is incubated on ice for 5 min with constant stirring.
4. Two volumes of isolation buffer is added and the mixture is homogenized as described above.
5. Centrifuge at 500 $\times$ g for10 min.
6. Filter the supernatant through two layers of cheesecloth.
7. Centrifuge for 10 min at 7,000 $\times$ g
8. Wash the mitochondrial pellet twice by resuspension in muscle isolation buffer containing 0.5 mg/ml bovine serum albumin, followed by recentrifugation.

3.1.3. Yeast Mitochondria

1. We isolate yeast mitochondria following growth in either YPG medium (4 g/l yeast extract, 8 g/l peptone, and 12 ml/l glycerol, pH 5.8) or lactate medium (3 g/l yeast extract, 0.5 g/l glucose, 0.5 g/l $CaCl_2 \cdot 2H_2O$, 0.5 g/l NaCl, 0.6 g/l $MgCl_2 \cdot 2H_2O$, 1 g/l KH_2PO_4, 1 g/l NH_4Cl, 22 ml/l 90% DL-lactic acid, and 8 g/l NaOH pellets (final pH = 5.5)).
2. Cells are pelleted by centrifugation at 4,000 $\times$ g for 10 min.
3. Pellets are resuspended in water, combined, and repelleted.

4. The pellet is suspended in about 1.5 m1 per g yeast in dithiothreitol buffer.
5. The suspension is incubated at 30°C for 15 min with gentle shaking.
6. The cells are centrifuged for 5 min at 2,000 × g.
7. The cells are resuspended in buffer A.
8. The cells are pelleted and resuspended in 2 ml per g of cells of buffer A containing 2.5 mg/g cells of Zymolase 20T (MP Biomedicals).
9. Incubation at 30°C follows for 30 min, with gentle shaking.
10. The cells are pelleted at 3,000 × g for 5 min.
11. Resuspend cells in buffer A.
12. Recentrifuge as above.
13. This and all subsequent steps are carried out at 0–4°C. The cell pellet is resuspended in buffer and the suspension transferred to a Dounce homogenizer on ice.
14. The suspension is homogenized for 15 strokes with a tight-fitting pestle.
15. Centrifuge at 2,000 × g for 5 min.
16. Transfer the supernatant to a fresh tube.
17. Resuspend the pellet in 5 ml buffer B and homogenize as above.
18. Centrifuge as above, and combine the two supernatants.
19. Centrifuge the combined supernatants for 10 min at 12,000 × g.
20. Resuspend the mitochondrial pellet in 1.0 ml of sterile water.
21. Remove an aliquot for total protein analysis.
22. To the remainder add 1.5 ml of ice-cold methanol, to give a final concentration of 60% (*see* **Note 4**).
23. Incubate for 30 min at –20°C.
24. Heat the extract from Step 23 for 3 min in a boiling water bath.
25. Centrifuge for 15 min at 17,000 × g.
26. Dry the supernatant under vacuum.
27. Redissolve the residue in water for the nucleotide analysis.

3.2. Annealing Primer to Template

1. Mix equal volumes of oligonucleotide template and primer (the same primer is used for both templates and all four assays).
2. Place in water at 70°C.

3. When the temperature of the mixture has reached 70°C, remove and allow the solution to cool slowly to room temperature.
4. Store at –20°C in 20-µl aliquots.

3.3. Preparing Reaction Mix

1. For each 25-µl assay reaction mixture prepare the following, fresh, on the day that assays are to be conducted:

5X dNTP buffer	5 µl
Sterile water	13.7 µl
BSA solution	0.5 µl
Template annealed to primer	0.33 µl
[^{3}H]dNTP	0.5 µl
DNA polymerase, Klenow fragment	0.02 µl

3.4. Running Assay Reactions

1. Add 20 µl of reaction mix, prepared as described above, to 5 µl of standard or unknown.
2. Incubate at 37°C for about 45 min (*see* **Note 5** for establishment of correct incubation time).

3.5. Analyzing Reaction Mixtures

1. Spot 20 µl of each reaction mixture, standard, or unknown, onto a 2 × 2 cm^2 of DE81 chromatography paper and allow to dry at room temperature. Use a soft pencil to label each square.
2. Wash squares batchwise with three successive washes of 5% (w/v) Na_2HPO_4 solution (about 2 ml per square). Rock the mixture gently for 10 min during each wash.
3. Wash squares once with water and once with 95% ethanol, rocking the mixture for 10 min after addition of each wash.
4. Allow squares to dry at room temperature, then add 5 ml of Ecolite liquid scintillation cocktail (MP Biochemicals) to each filter in a separate vial, and determine radioactivity in a liquid scintillation counter optimized for counting tritium. As discussed earlier, the radioactivity measured represents the incorporation of counternucleotide into DNA. Provided that DNA polymerase and counternucleotide are in excess and the reactions have been run to completion, then the amount of counternucleotide incorporated is proportional to the concentration of the analyzed nucleotide in the sample, and this is determined by reference to a standard curve (*see* **Notes 6 and 7**).

4. Notes

1. Another complication with the enzymatic assay is the possible presence of DNA polymerase inhibitors in extracts being analyzed. We deal with this by diluting each extract by various factors. We accept data only when we are satisfied that cpm incorporated in an unknown sample is seen to be directly proportional to the amount of sample analyzed. Also we routinely add standards to unknowns and assure ourselves that the entire standard is recovered in the analysis.
2. The source of Klenow fragment used is important. We have experimented with Klenow from four different suppliers, and we find the preparation from US Biochemicals to be the best, in terms of activity, ability of reactions to run to completion in reasonable times, and linearity of standard curves. We find that addition of BSA to the reaction mixtures improves the quality of the data obtained. No such result has been observed with additions either of dithiothreitol or dAMP.
3. We designed templates and primer different from those used by Sherman and Fyfe *(7)*. The Sherman–Fyfe procedure allows flexibility in the choice of radiolabeled counternucleotide, and our choice of templates was based upon the availability of a source of [^{3}H]dTTP of high quality, high specific activity, and moderate price.
4. A comment about nucleotide extraction procedures is in order. Nucleotides can be extracted from tissue or cell samples by strong acid (perchloric or trichloroacetic) or by alcohol–water mixtures, typically, 60% methanol. Acid extraction requires a neutralization step and removal of the acid. For perchloric acid extraction, neutralization with KOH also removes the perchlorate, as the insoluble potassium salt. For trichloroacetic acid extraction, the TCA is removed by solvent extraction with a mixture of tri-*N*-octylamine in Freon. Both the procedures are widely used, but carry the risk of partial degradation of dNTPs in the extract. Accordingly, most early investigators working with whole cells turned to 60% methanol. Our laboratory, however, found that 60% methanol treatment of intact cells extracts some active enzymes, including nucleases and nucleotide kinases, which interfere with the enzymatic dNTP assay *(12)*. Accordingly, we and others *(13)* treated the methanol extract with heat, by immersion in a boiling water bath. This turns out to be optimal for mitochondrial dNTP extraction as well. Typically, we extract a mitochondrial sample with 2 ml of 60% aqueous methanol, leaving the mixture at –20°C for up to 1 h. Then the mixture is immersed in boiling water for 3 min.

After centrifugation the supernatant is taken to dryness under vacuum in a Speed-Vac^{TM}. The residue is dissolved in 70 µl of water and is then ready for analysis. The optimal extraction time varies with the source of tissue used, from 30 min for mitochondria from animal tissues to 1 h for cultured mammalian cell mitochondria to 2 h for mitochondria from yeast cultures.

5. For every analysis we run a standard curve, with 5-µl aliquots of standard dNTP solutions at 0.4, 0.2, 0.1, 0.05, 0.02, and 0.01 µM, representing a range from 0.05 to 2.0 pmol. Several precautions must be taken to ensure linear standard curves. First, it must be established that polymerase reactions run to completion, so that a plot of cpm incorporated vs time of incubation reaches a plateau. For the amount of enzyme we use, a 45-min incubation suffices, but this must be checked routinely, particularly when a new batch of DNA polymerase is being used. If a polymerase preparation is contaminated with a nuclease, a plateau may never be reached, as the radioactive product begins to be digested while its synthesis is continuing.
6. When working with cultured cells, we can estimate the mitochondrial dNTP pools in terms of pmol per 10^8 cells or some similar unit of mass per cell. This is more difficult with tissue samples, so we report pools here in terms of pmol per mg mitochondrial protein. For studies on mutagenesis it is desirable to estimate the molar concentration of each dNTP within the fluid volume of the mitochondrion. We have not measured this directly, but we find reasonable agreement among values in the literature. Three laboratories (cited in Ref. *(11)*) reported values of 0.67, 0.82, and 1.04 µl aqueous volume per mg mitochondrial protein for rat heart mitochondria. We have used the midrange value, 0.82. Using this value, with pools measured as described in several rat tissues, we have found remarkable asymmetry in the mitochondrial dNTP pools, particularly in heart and skeletal muscles *(11)*. Typical data are shown in **Fig. 22.1**. Note that, whereas dGTP is the

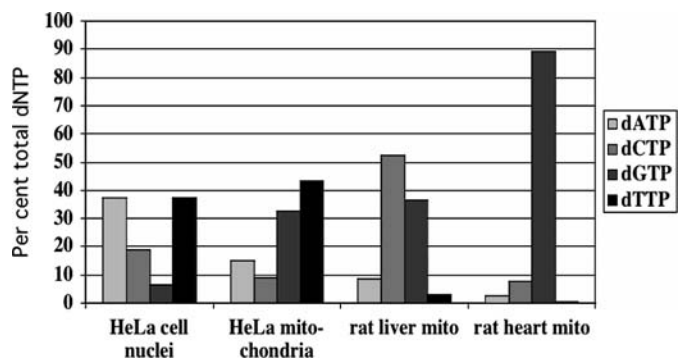


Fig. 22.1. Mitochondrial dNTP pools. Data are replotted from Refs. *(9)*, *(11)*, and *(14)*.

least abundant nucleotide in whole-cell extracts or extracts of rapidly prepared nuclei *(14)*, dGTP is greatly overrepresented in mitochondria. In the rat heart mitochondrial preparation depicted in **Fig. 22.1**, we estimate the concentration of dGTP as 140 μM. Although this value is well below corresponding values for mitochondrial ribonucleoside triphosphates, which are in the millimolar range (at least for ATP), it is still startlingly high, and we do not yet know the biological significance of this asymmetry.

7. Finally, we discuss two areas in which we have found mitochondrial dNTP levels to influence mutagenesis. The natural pool asymmetry mentioned above evidently helps to determine the spontaneous mutation rate, because in a collaborative study *(11)* we found that DNA replication reactions run in vitro, at dNTP concentrations corresponding to the determined rat heart or skeletal muscle mitochondrial values, the replication error rate was several-fold higher than that seen with an equimolar dNTP mixture. Moreover, the dGTP excess specifically forced AT→GC transitions, known to be the most prominent mitochondrial mutations in humans.

   Another precaution that should be taken when dissecting an animal for mitochondrial dNTP extraction and analysis is the rapid removal of each organ and its transfer into cold buffer. Ferraro et al. *(13)* have pointed out that mitochondria rapidly go anaerobic after death, and the resultant decline in ATP/ADP ratio within the mitochondrion could cause dNTP breakdown. Although it is difficult to see how this would affect the four dNTPs differentially so as to generate the observed asymmetry, our laboratory is modifying its procedures so that ATP and ADP levels are routinely monitored in our mitochondrial extracts.

   The other example in which our studies have demonstrated a link between mitochondrial dNTP abnormalities and mutagenesis involves the disease mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). In this condition the mitochondrial genome accumulates both point mutations and deletions. Nishino et al. *(15)* showed that MNGIE results from a deficiency of the nuclear-encoded enzyme thymidine phosphorylase. They speculated that the resultant accumulation of thymidine in the plasma of affected individuals creates a mutagenic dNTP pool imbalance within the mitochondrion. We found this to be the case in a model study, in which HeLa cells were cultured in the presence of thymidine *(9)*. The mitochondrial dTTP pool expanded, as expected, but the dCTP pool dropped to nearly unmeasurable levels; this was not seen in corresponding whole-cell extracts. DNA polymerase stalling during replication is known to cause

deletions, through inappropriate strand pairing after fraying at the primer-template junction. By use of both Southern blotting and PCR analyses, Song et al. *(9)* were able to show that this condition, when applied over a period of several months, caused several long deletions to appear in mitochondrial DNA.

References

1. Randerath, K., and Randerath, E. (1967) Thin-layer separation methods for nucleic acid derivatives. *Meth. Enzymol.* **XIIA**, 323–347.
2. Garrett, C., and Santi, D. V. (1979) A rapid and sensitive high pressure liquid chromatography assay for deoxyribonucleoside triphosphates in cell extracts. *Anal. Biochem.* **99**, 268–273.
3. Shewach, D. S. (1992) Quantitation of deoxyribonucleoside 5'-triphosphates by a sequential boronate and anion-exchange high-pressure liquid chromatographic procedure. *Anal. Biochem.* **206**, 178–182.
4. Decosterd, L. A., Cottin, E., Chen, X., Lejeune, F., Mirimanoff, R. O., Biollaz, J., and Coucke, P. A. (1999) Simultaneous determination of deoxyribonucleoside triphosphates in the presence of ribonucleoside triphosphates in human carcinoma cells by high-performance liquid chromatography. *Anal. Biochem.* **270**, 59–68.
5. Lindberg, U., and Skoog, L. (1970) A method for the determination of dATP and dTTP in picomole amounts. *Anal. Biochem.* **34**, 152–160.
6. Bestwick, R. K., Moffett, G. L., and Mathews, C. K. (1982) Selective expansion of mitochondrial nucleoside triphosphate pools in antimetabolite-treated HeLa cells. *J. Biol. Chem.* **257**, 9300–9305.
7. Sherman, P. A., and Fyfe, J. A. (1989) Enzymatic assay for deoxyribonucleoside triphosphates using synthetic oligonucleotides as template primers. *Anal. Biochem.* **180**, 222–226.
8. Schnaitman, C., and Greenawalt, J. W. (1968) Enzymatic properties of the inner and outer membranes of rat liver mitochondria. *J. Cell Biol.* **38**, 158–175.
9. Song, S., Wheeler, L. J., and Mathews, C. K. (2003) Deoxyribonucleotide pool imbalance stimulates deletions in HeLa cell mitochondrial DNA. *J. Biol. Chem.* **278**, 43893–43896.
10. Glick, B. S., and Pon, L. A. (1995) Isolation of highly purified mitochondria from *Saccharomyces cerevisiae*. *Methods in Enzymol.* **260**, 213–223.
11. Song, L., Pursell, Z., Copeland, W. C., Longley, M. J., Kunkel, T. A., and Mathews, C. K. (2005) DNA precursor asymmetries in mammalian tissue mitochondria and possible contribution to mutagenesis through reduced replication fidelity. *Proc. Natl. Acad. Sci. USA.* **102**, 4990–4995.
12. North, T. W., Bestwick, R. K., and Mathews, C. K. (1980) Detection of activities that interfere with the enzymatic assay of deoxyribonucleoside 5'-triphosphates. *J. Biol. Chem.* **253**, 6640–6645.
13. Ferraro, P., Nicolosi, L., Bernardi, P., Reichard, P., and Bianchi, V. (2006) Mitochondrial deoxynucleotide pool sizes in mouse liver and evidence for a transport mechanism for thymidine monophosphate. *Proc. Natl. Acad. Sci. USA.* **103**, 18586–18591.
14. Leeds, J. M., Slabaugh, M. B., and Mathews, C. K. (1985) DNA precursor pools and ribonucleotide reductase activity: Distribution between nucleus and cytoplasm of mammalian cells. *Molec. Cell. Biol.* **5**, 3443–3450.
15. Nishino, I., Spinazzola, A., and Hirano, M. (1999) Thymidine phosphorylase gene mutations in MNGIE, a human mitochondrial disorder. *Science.* **283**, 689–692.

Chapter 23

Establishment of Human Cell Lines Lacking Mitochondrial DNA

Kazunari Hashiguchi and Qiu-Mei Zhang-Akiyama

Abstract

Mitochondria have their own genome, and mitochondrial DNA (mtDNA) encodes 2 ribosomal RNAs, 22 transfer RNAs, and 13 polypeptides that function in oxidative phosphorylation (OXPHOS). mtDNA mutations lead to dysfunction of OXPHOS, resulting in cell death and/or compromised cellular activity. Cell lines lacking mtDNA (termed ρ^0 cells) are very effective tools for studying the consequences of mtDNA mutations. ρ^0cell lines have been used widely to investigate relationships between mtDNA mutation, mitochondrial function, and a variety of cellular processes. In this chapter, we summarize the yeast and animal ρ^0 cell lines that have been studied. We provide simple protocols for the generation of human ρ^0 cells by exposure to ethidium bromide and PCR verification of their ρ^0 status.

Key words: Mitochondria, mitochondrial DNA (mtDNA), ρ^0 cells, oxidative phosphorylation (OXPHOS), uridine, pyruvate, ethidium bromide, PCR.

1. Introduction

Since mitochondrial DNA (mtDNA) encodes genes related to oxidative phosphorylation (OXPHOS), the stability of mtDNA is crucial for the survival of cells in vivo (*1–4)*. However, cells depleted of mtDNA (ρ^0 cells) are viable in culture, provided appropriate conditions are met *(5–7)*. These ρ^0 cells are important tools for investigating the pathogenesis of specific mtDNA mutations, and for developing a better understanding of interactions between nuclear and mitochondrial genomes in mitochondrial disease *(8)*. The ρ^0 cells also have been used to study mtDNA replication and repair processes, since many of the proteins involved in mtDNA transactions continue to localize to mitochondria even in the absence of

Jeffrey A. Stuart (ed.), *Mitochondrial DNA, Methods and Protocols, vol. 554*

DOI 10.1007/978-1-59745-521-3_23 Springerprotocols.com

mtDNA *(9–11)*. Cybrid cells have been produced by fusing ρ^0 cells with enucleated cells *(6)*, and using this technique, mitochondrial disorders have been investigated in human *(12, 13)* and mouse cells *(14–16)*.

As summarized in **Table 23.1**, ρ^0 cell lines have now been established from various tissues and species. Human cells lacking mtDNA (ρ^0 cells) were originally obtained from the human cell line 143B.TK$^-$ *(6)* by chronic exposure to a DNA intercalating dye ethidium bromide (EtBr). This same approach has been used to establish an avian ρ^0cell line *(17)*. In mouse cells, EtBr is not effective in producing ρ^0 cells for unknown reasons. Inoue and co-workers, however, successfully isolated ρ^0 mouse cell lines by exposure to the antitumor bis-intercalating agent ditercalinium (DC) *(14)*.

Table 23.1
Some examples of ρ^0 cell lines

Species	Cell line	Cell type	Treatment	References
Yeast			EtBr	Goldring *(23)*
Avian	LSCC-H32	Fibroblast	EtBr	Desjardins *(17)*
Rat	NRK52E	Kidney epithelial	ERI	Kukat *(19)*
Mouse	C2	Myoblast	DC	Inoue *(14)*
	MIN6	Pancreatic β cell	DC	Inoue *(14)*
	B82cap	Fibroblast	DC	Inoue *(22)*
	NIH3T3	Fibroblast	DC	Inoue *(22)*
	LMTK$^-$	Fibroblast	ERI	Kukat *(19)*
Human	143B206	Osteosarcoma	EtBr	King *(6)*
	HeLa	Cervical carcinoma	EtBr	Attardi *(24)*
	A549	Lung carcinoma		Bodnar *(25)*
	U937	Promonocytic leukemia	EtBr	Gamen *(20)*
	64/5	Neuroblastoma	EtBr	Miller *(21)*
	HL60	Promyelocytic leukemia	EtBr	Herst *(26)*
	143BTK$^-$	Osteosarcoma	ERI	Kukat *(19)*

EtBr: ethidium bromide; DC: ditercalinium; ERI: *Eco* RI expression.

The mechanism by which EtBr renders cells ρ^0 involves the binding to DNA. When present at low doses (0.1–2 μg/ml) in culture medium, mtDNA replication is prevented, but at these same low doses, nuclear DNA replication is not prevented. Thus, in replicating cells, daughter cells are populated with nuclear

DNA, but not mtDNA, leading to the eventual disappearance of mtDNA over many rounds of replication. Interestingly, cells lacking mtDNA retain some aspects of normal mitochondrial structure, because most of the >1,000 different mitochondrial proteins are encoded in the nuclear genome. ρ^0 mitochondria maintain an electrochemical potential across the inner membrane by a mechanism coupled to ATP hydrolysis *(18)*, thus making them net consumers of ATP in cells.

Theoretically, if a cell contains 10^4 copies of mtDNA, the number of copies of mtDNA will become less than 1 after 14 rounds of cell division ($10^4/2^{14} = 0.61$). This suggests that continuous cultivation of cells in the presence of EtBr for more than 14 cycles of cell division is typically required to obtain ρ^0 cells.

Another approach to generate ρ^0 cells without exposure to chemical treatment has been developed recently by Kukat and coworkers *(19)*. These authors targeted restriction endonuclease *Eco*RI to mitochondria in human, mouse, and rat cell lines. Since human, mouse, and rat mtDNA have three, three, and seven *Eco*RI-sensitive sites, respectively (accession number of human, mouse, and rat mtDNA reference sequence; AC 000021, NC 005089, and AC 000022), it is thought that *Eco*RI cleaves mtDNA, producing several short linear segments that are not effectively replicated. The comparison of the human ρ^0 line generated using the restriction approach (143B.TK$^-$K7) with 143B.TK$^-\rho^0$ cells generated by EtBr treatment revealed very similar proliferation rates, glucose consumption rates, lactate production rates, and mitochondrial morphology and reticular structure.

Here, we describe a method for establishing human ρ^0 cells and propagating these cell lines in culture. A convenient PCR analysis for verifying the ρ^0 state is also described. Modifications to the culture medium required to support the viability of ρ^0 cells are provided and explained. While the method is outlined for a human cell line, it is broadly applicable, with suitable modifications (*see* **Table 23.1**) for use with other cells.

2. Materials

2.1. Cell Culture

1. Human cultured cell lines (HeLa, 143B.TK$^-$, and so on) can be obtained from ATCC (http://www.atcc.org).
2. Culture medium: Dulbecco's Modified Eagle's Medium (high glucose, cat# 10938-025, Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml sodium pyruvate (Invitrogen), and 50 μg/ml uridine (Sigma). For culture of 143B.TK$^-$ cells, 100 μg/ml bromodeoxyuridine (BrdU, Sigma) is also required (*see* **Note 1**). A stock solution

of 10 mg/ml uridine and 10 mg/ml BrdU is dissolved in distilled water, sterilized by filtration, and stored at –20°C. BrdU is light-sensitive, so this should be shielded from light.

3. EtBr stock solution: 10 mg/ml; prepared in distilled water, filter-sterilized, and stored at 4°C in dark (*see* **Note 2**). Before use, dilute this stock solution with sterilized water to 5 μg/ml.
4. Sterilized PBS (Ca, Mg-free).
5. Trypsin–EDTA solution (Invitrogen).
6. CO_2 incubator.
7. Centrifuge with swinging rotor.
8. Centrifuge with 1.5-mL minifuge tube rotor.

2.2. DNA Extraction, PCR, and Polyacrylamide Gel Electrophoresis

1. PCR primer sequences for amplification of mtDNA encoded tRNA-Leu gene and the nuclear encoded polγ gene (as positive control) are as follows (*see* **Note 3**).
   For mtDNA encoded tRNA-Leu gene:
   5′-GATGGCAGAGCCCGGTAATCGC-3′
   5′-TAAGCATTAGGAATGCCATTGCG-3′
   For nuclear polγ gene:
   5′-AGCGACGGGCAGCGGCGGCGGCA-3′
   5′-CCCTCCGAGGATAGCACTTGCGGC-3′
2. Cell scraper (can be obtained from BD Falcon, Corning, and Greiner Bio-one).
3. Total DNA extraction kit. Any commercial DNA extraction kit for use with cultured cells is acceptable.
4. Conventional recombinant Taq polymerase is useful as PCR enzyme (Promega, Takara, Roche, NEB, Stratagene). High-fidelity enzyme is not required. PCR buffer and dNTPs are generally supplemented with PCR enzyme.
5. Radioactive [γ-^{32}P]ATP and [α-^{32}P]dCTP (3,000 Ci/mmol) can be purchased from ICN (*see* **Note 4**).
6. Molecular weight marker (50 Base-Pair Ladder, GE Healthcare).
7. T4 polynucleotide kinase (New England BioLabs).
8. Electrophoresis buffer: 1X TBE buffer (89 mM Tris–borate and 2 mM EDTA). Store at room temperature.
9. 8% Polyacrylamide gel prepared from 30% acrylamide/bis-acrylamide (29:1) stock solution and contains 1X TBE.
10. 10% Ammonium persulfate (APS) solution prepared in distilled water. Store aliquot at –20°C, and should be used in 6 months.
11. *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED) can be purchased from Bio-Rad.
12. Appropriate electrophoresis system for polyacrylamide gel.

13. Phosphorimager (e.g., Storm 860; Molecular Dynamics).
14. Transilluminator (if not using phosphorimager).
15. UV–Vis spectrophotometer for qualitative analysis of isolated DNA purity.

3. Methods

A high concentration of glucose, as well as addition of uridine and pyruvate to the culture medium *(6)* is required for the viability of cells lacking mtDNA. The requirement for glucose is due to the complete reliance of ρ^0 cells on glucose fermentation for ATP synthesis. The requirement for uridine is due to a deficiency of pyrimidine biosynthesis in ρ^0 cells. Addition of uridine and pyruvate into the culture medium and at least 1 month of culture in the presence of EtBr or DC are commonly used for generating ρ^0 vertebrate cell lines. Once mtDNA is completely lost, there is no further requirement for EtBr in the culture medium. The method described below for human cells can be applied with minor modifications to a wide variety of eukaryotic cells (*see* **Note 5**).

3.1. Isolation of a Human ρ^0 Cell Line

1. Maintain human cell lines in culture medium and passage by trypsinization twice a week or as required. Do not allow cells to reach confluency.
2. Twenty-four hours after plating cells to a 100-mm dish ($\sim 1 \times 10^5$ cells), add 50 ng/ml EtBr to culture medium.
3. Approximately 1 week after the addition of EtBr, dead cells will begin to peel off from the dish. Remove these cells by washing the plate once with PBS, then add fresh medium supplemented with 50 ng/ml EtBr.
4. Monitor the pH of the culture medium closely, and change medium as required (*see* **Note 6**), continuing to supplement with 50 ng/ml EtBr.
5. Approximately 1 month following the initial addition of EtBr, colonies will be visible (these might be ρ^0 cells). Isolate single colonies by penicillin cup, and transfer cell suspension to 24-well.
6. Continue to grow cells, changing medium every 2 days or sooner if medium begins to appear yellow.
7. Expand cells to isolate total DNA (*see* **Section 3.2** below).
8. Isolated cell lines can be stored in liquid nitrogen using a general cell freezing medium.

3.2. Total DNA Extraction and PCR

1. Propagate candidate ρ^0 cell lines generated above in 100-mm culture dishes to near confluency (this will be approx. $5 \times 10^6 \sim 1 \times 10^7$ cells).
2. Remove the culture medium by aspiration and wash cell surface with 5 ml of PBS.
3. Add fresh 1 ml of PBS and scrape cells by cell scraper (*see* **Note 7**).
4. Transfer cell suspension to a minifuge tube.
5. Centrifuge at 300 × g for 3 min.
6. Aspirate supernatant. At this point, the cell pellet can be stored at –80°C until use.
7. Extract total DNA from the cell pellet using any commercial kit, according to the instructions provided.
8. Measure DNA concentration and purity using a spectro photometer.
9. Prepare 50 µl reactions in PCR tubes according to the manufacturer of your PCR enzyme. Generally add 1 × PCR buffer, $MgCl_2$ (if not included in PCR buffer). Include 200 µM dNTPs and 10 µCi [α-^{32}P]dCTP (3,000 Ci/mmol) to the reaction (*see* **Note 4**). Add 0.1 µM each primer, 20 ng of total DNA, and 0.5 U Taq DNA polymerase.
10. Incubate reaction mixtures at 95°C for 3 min followed by 30 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min in a PCR machine. The reaction is completed by a final incubation at 72°C for 5 min, and the reaction products are stored at 4°C until use (*see* **Note 8**).

3.3. Polyacrylamide Gel Electrophoresis

1. Before electrophoresis, mix 50 pmol of molecular weight marker with 50 pmol of [γ-^{32}P]ATP and 20 units of T4 polynucleotide kinase in 1 × T4 polynucleotide kinase buffer and incubate at 37°C for 30 min. To inactivate kinase, incubate reaction at 65°C for 20 min. This reaction can be loaded directly onto gel and should be stored at –20°C.
2. Clean the glass plates, comb, and spacers. It is better to wipe with ethanol before use.
3. Assemble the glass plates.
4. Mix 3.2 ml of 30% acrylamide stock with 7.6 ml of distilled water, 1.2 ml of 10 × TBE, and 10 µl of TEMED (*see* **Note 9**).
5. Add 200 µl of 10% APS to the gel mixture, and immediately pour into gel plates and set the comb into the top of the gel (*see* **Note 10**).
6. Allow at least 60 min for gel polymerization (*see* **Note 11**).
7. Set the gel plate into the electrophoresis apparatus and pour the 1 × TBE running buffer.

8. Mix PCR product with appropriate gel loading dye and load sample onto the gel.
9. Run the gel at 1–8 V/cm constant voltage. Stop gel running at appropriate point.
10. When radioactive materials have been used in PCR, visualize products using a phosphorimager. If not using radioactive materials, soak the gel in 1:20,000 diluted 10 mg/ml EtBr in 1 × TBE and visualize using a transilluminator.

4. Notes

1. Normally, Dulbecco's Modified Eagle Medium contains pyruvate. You may choose pyruvate pre-mixed medium. Important materials in medium are high concentrations of glucose (4,500 mg/l), pyruvate, L-glutamine, and uridine. The reason for the addition of BrdU is that this cell line is deficient in thymidine kinase and is resistant to BrdU.
2. Filtration of lower than 10 mg/ml EtBr causes significant reduction of EtBr concentration, because of binding to the filter.
3. The sizes of PCR products from mitochondrial tRNA-Leu and nuclear polymerase γ genes are 139 and 92 bp, respectively.
4. This protocol is a highly sensitive detection method because of the use of radioactive materials. If use of radioactivity is a complication in your lab, this can be omitted, and conventional PCR without radioactive materials can be done.
5. Gamen et al. *(20)* used 5 ng/ml EtBr for isolation of human U937 ρ^0 cells. Miller et al. *(21)* used 5 μg/ml EtBr for isolation of human 64/5 ρ^0 cells. Compared with EtBr concentration used for 143B206 cells (50 ng/ml), it is obvious that the optimal concentration of EtBr differs in different cell lines. For isolation of murine ρ^0 cell lines, concentrations of DC used include 1.5 μg/ml for C2, B82cap, and NIH3T3 cell lines and 56 ng/ml for MIN6 cell line *(14, 22)*. For the isolation of the avian LSCC-32H ρ^0cell line, a final concentration of 400 ng/ml EtBr is used *(17)*. Thus, when generating ρ^0cells using a unique cell line, optimization of EtBr/DC concentration is required. For generation of ρ^0cells in the budding yeast *Saccharomyces cerevisiae*, the parental line is cultivated for 40 generations in the presence of 20 μg/ml EtBr in YPD (2% Bacto-pepton, 1% Bacto-yeast extract, and 2% glucose) medium. Cells are plated onto YPD plate, and single colonies that appeared are replicated onto both fresh

YPD and YPG [*2*% Bacto-pepton, *1*% Bacto-yeast extract, and *3*% (v/v) glycerol] plates. Since yeast ρ^0 line cannot use glycerol as a carbon source, the ρ^0 state of candidates that cannot replicate on YPG can be used as a preliminary check of mtDNA status that is verified by PCR.

6. Due to their reliance on glucose fermentation, ρ^0 cells produce large amounts of lactic acid that can rapidly acidify the culture medium and interfere with cell growth and viability. Therefore, close attention to media pH is necessary.
7. This step can be replaced by trypsinization.
8. These reaction times are not critical. Obey your instruction manual attached to PCR enzyme.
9. The total volume of gel is 12 ml. You may change this volume according to your gel size. Acrylamide before polymerization is neurotoxic. You need to wear gloves when you handle with unpolymerized acrylamide.
10. After addition of APS, polymerization is started. Any bubble in the gel has to be omitted.
11. Before use ensure that the gel is completely polymerized. With lower room temperature, acrylamide becomes difficult to polymerize. Keep around 25°C as room temperature.

References

1. Gillham, N.W. (1994) *Organelle Genes and Genomes*. Oxford University Press, New York, NY.
2. Hashiguchi, K., Bohr, V.A. and de Souza-Pinto, N.C. (2004) Oxidative stress and mitochondrial DNA repair: implications for NRTIs induced DNA damage. *Mitochondrion*, **4**, 215–222.
3. Kang, D. and Hamasaki, N. (2002) Maintenance of mitochondrial DNA integrity: repair and degradation. *Curr Genet*, **41**, 311–322.
4. Shadel, G.S. and Clayton, D.A. (1997) Mitochondrial DNA maintenance in vertebrates. *Annu Rev Biochem*, **66**, 409–435.
5. Desjardins, P., Frost, E. and Morais, R. (1985) Ethidium bromide-induced loss of mitochondrial DNA from primary chicken embryo fibroblasts. *Mol Cell Biol*, **5**, 1163–1169.
6. King, M.P. and Attardi, G. (1989) Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. *Science*, **246**, 500–503.
7. Morais, R., Gregoire, M., Jeannotte, L. and Gravel, D. (1980) Chick embryo cells rendered respiration-deficient by chloramphenicol and ethidium bromide are auxotrophic for pyrimidines. *Biochem Biophys Res Commun*, **94**, 71–77.
8. Chandel, N.S. and Schumacker, P.T. (1999) Cells depleted of mitochondrial DNA (rho0) yield insight into physiological mechanisms. *FEBS Lett*, **454**, 173–176.
9. Hashiguchi, K., Stuart, J.A., de Souza-Pinto, N.C. and Bohr, V.A. (2004) The C-terminal alphaO helix of human Ogg1 is essential for 8-oxoguanine DNA glycosylase activity: the mitochondrial beta-Ogg1 lacks this domain and does not have glycosylase activity. *Nucleic Acids Res*, **32**, 5596–5608.
10. Stuart, J.A., Hashiguchi, K., Wilson, D.M., 3rd, Copeland, W.C., Souza-Pinto, N.C. and Bohr, V.A. (2004) DNA base excision repair activities and pathway function in mitochondrial and cellular lysates from cells lacking mitochondrial DNA. *Nucleic Acids Res*, **32**, 2181–2192.
11. Stuart, J.A., Mayard, S., Hashiguchi, K., Souza-Pinto, N.C. and Bohr, V.A. (2005) Localization of mitochondrial DNA base

excision repair to an inner membrane-associated particulate fraction. *Nucleic Acids Res*, **33**, 3722–3732.

12. Hayashi, J., Ohta, S., Kagawa, Y., Kondo, H., Kaneda, H., Yonekawa, H., Takai, D. and Miyabayashi, S. (1994) Nuclear but not mitochondrial genome involvement in human age-related mitochondrial dysfunction. Functional integrity of mitochondrial DNA from aged subjects. *J Biol Chem*, **269**, 6878–6883.
13. Hayashi, J., Ohta, S., Kikuchi, A., Takemitsu, M., Goto, Y. and Nonaka, I. (1991) Introduction of disease-related mitochondrial DNA deletions into HeLa cells lacking mitochondrial DNA results in mitochondrial dysfunction. *Proc Natl Acad Sci U S A*, **88**, 10614–10618.
14. Inoue, K., Ito, S., Takai, D., Soejima, A., Shisa, H., LePecq, J.B., Segal-Bendirdjian, E., Kagawa, Y. and Hayashi, J.I. (1997) Isolation of mitochondrial DNA-less mouse cell lines and their application for trapping mouse synaptosomal mitochondrial DNA with deletion mutations. *J Biol Chem*, **272**, 15510–15515.
15. Inoue, K., Nakada, K., Ogura, A., Isobe, K., Goto, Y., Nonaka, I. and Hayashi, J.I. (2000) Generation of mice with mitochondrial dysfunction by introducing mouse mtDNA carrying a deletion into zygotes. *Nat Genet*, **26**, 176–181.
16. Nakada, K., Inoue, K., Ono, T., Isobe, K., Ogura, A., Goto, Y.I., Nonaka, I. and Hayashi, J.I. (2001) Inter-mitochondrial complementation: Mitochondria-specific system preventing mice from expression of disease phenotypes by mutant mtDNA. *Nat Med*, **7**, 934–940.
17. Desjardins, P., de Muys, J.M. and Morais, R. (1986) An established avian fibroblast cell line without mitochondrial DNA. *Somat Cell Mol Genet*, **12**, 133–139.
18. Porteous, W.K., James, A.M., Sheard, P.W., Porteous, C.M., Packer, M.A., Hyslop, S.J., Melton, J.V., Pang, C.Y., Wei, Y.H. and Murphy, M.P. (1998) Bioenergetic consequences of accumulating the common 4977-bp mitochondrial DNA deletion. *Eur J Biochem*, **257**, 192–201.
19. Kukat, A., Kukat, C., Brocher, J., Schafer, I., Krohne, G., Trounce, I.A., Villani, G. and Seibel, P. (2008) Generation of rho0 cells utilizing a mitochondrially targeted restriction endonuclease and comparative analyses. *Nucleic Acids Res*, **36**, e44.
20. Gamen, S., Anel, A., Montoya, J., Marzo, I., Pineiro, A. and Naval, J. (1995) mtDNA-depleted U937 cells are sensitive to TNF and Fas-mediated cytotoxicity. *FEBS Lett*, **376**, 15–18.
21. Miller, S.W., Trimmer, P.A., Parker, W.D., Jr. and Davis, R.E. (1996) Creation and characterization of mitochondrial DNA-depleted cell lines with "neuronal-like" properties. *J Neurochem*, **67**, 1897–1907.
22. Inoue, K., Takai, D., Hosaka, H., Ito, S., Shitara, H., Isobe, K., LePecq, J.B., Segal-Bendirdjian, E. and Hayashi, J. (1997) Isolation and characterization of mitochondrial DNA-less lines from various mammalian cell lines by application of an anticancer drug, ditercalinium. *Biochem Biophys Res Commun*, **239**, 257–260.
23. Goldring, E.S., Grossman, L.I., Krupnick, D., Cryer, D.R. and Marmur, J. (1970) The petite mutation in yeast. Loss of mitochondrial deoxyribonucleic acid during induction of petites with ethidium bromide. *J Mol Biol*, **52**, 323–335.
24. Attardi, G., King, M.P., Chomyn, A. and Loguercio-Polosa, P. (1991) Novel genetic and molecular approaches to the study of mitochondrial biogenesis and mitochondrial diseases in human cells, in *Progress in Neuropathology*, vol. 7 (Sato T. and DiMauro, S., ed.), Raven Press, New York, pp. 75–92.
25. Bodnar, A.G., Cooper, J.M., Holt, I.J., Leonard, J.V. and Schapira, A.H. (1993) Nuclear complementation restores mtDNA levels in cultured cells from a patient with mtDNA depletion. *Am J Hum Genet*, **53**, 663–669.
26. Herst, P.M., Tan, A.S., Scarlett, D.J. and Berridge, M.V. (2004) Cell surface oxygen consumption by mitochondrial gene knockout cells. *Biochim Biophys Acta*, **1656**, 79–87.

INDEX

D

E

F

G

H

I

L

M

N

O

P

R

S

T

U

V

W

Y