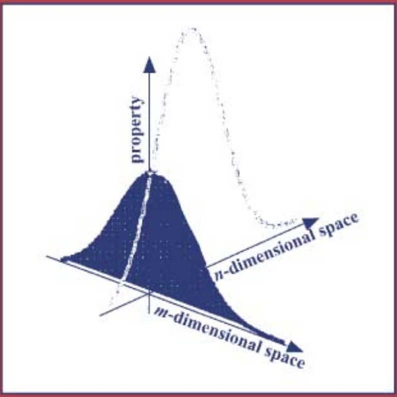
Methods in Molecular Biology™

VOLUME 182

In Vitro Mutagenesis Protocols

SECOND EDITION

Edited by Jeff Braman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In Vitro Mutagenesis Protocols

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Humana Press **Totowa, New Jersey**

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999 Riverview Drive, Suite 208
Totowa, New Jersey 07512
humanapress.com

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Cover illustration: Fig. 2. from Chapter 22, "Evolutionary Molecular Engineering by Random Elongation Mutagenesis," by Tomoaki Matsuura, Tetsuya Yomo, and Itaru Urabe.

Cover design by Patricia F. Cleary.

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Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1

Library of Congress Cataloging in Publication Data

Main entry under title: Methods in molecular biology™.
In vitro mutagenesis protocols/edited by Jeff Braman.—2nd ed.
p.cm.—(Methods in molecular biology; v. 182)
Includes bibliographical references and index.
ISBN 0-89603-910-2 (alk. paper)
1. Mutagenesis—Methodology. I. Braman, Jeff. II. Methods in molecular biology (Totowa, N.J.); v. 182.

QH465.A1 15 2001
660.6'5—dc21 2001039227

Dedication

To Barbara, Ryan, Emily, Rebecca, Michael, Colin, and Connor

Jeff Braman, PhD

Preface

In vitro mutagenesis is a major tool used by molecular biologists to make connections between nucleotide sequence and sequence function. In the post-genome era, in vitro mutagenesis is being used to establish the function of components of the proteome. There has never been a more exciting and critical time for molecular biologists to master the use of efficient and reliable in vitro mutagenesis protocols.

Anyone skilled in the use of tools will tell you that a well-equipped toolbox is essential for solving the myriad problems encountered in the practice of their art. Likewise, molecular biologists require an arsenal of reliable tools appropriate to solve complex problems they encounter. *In Vitro Mutagenesis Protocols* is intended to represent such a toolbox. Chapter authors were chosen because their protocols (tools) have been published in reputable, peer-reviewed journals. Their chapters focus on improvements to conventional site-directed mutagenesis, including a chapter on chemical site-directed mutagenesis, PCR-based mutagenesis and modifications thereto allowing high throughput mutagenesis experiments, and mutagenesis based on gene disruption (both in vitro and *in situ* based). Last, but certainly not least, a section of chapters is devoted to the subject of accelerated protein evolution relying on in vitro evolution, gene shuffling, and random mutagenesis.

I trust that these protocols will be successful in your hands and allow you to quickly reach the point of analyzing the results for inclusion in the discussion section of your own peer reviewed journal article. I am indebted to Humana Press representatives Craig Adams and Professor John Walker for guiding me through the process of editing this book and to the many good scientists from Massachusetts to California who took the time to help me with my own research.

Jeff Braman, PhD

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1

Rapid and Reliable Site-Directed Mutagenesis Using Kunkel's Approach

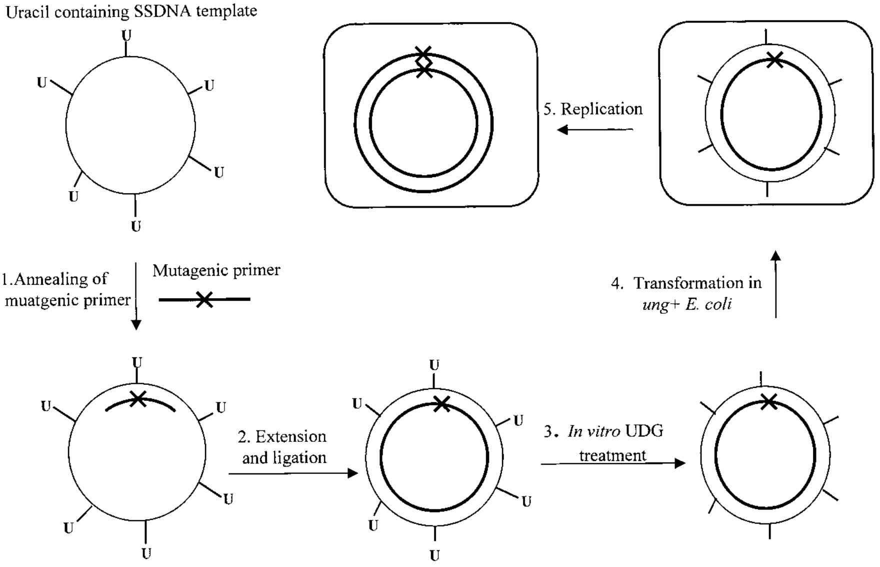
Priya Handa, Swapna Thanedar, and Umesh Varshney

1. Introduction

Site-specific mutagenesis is a powerful tool in molecular biology research. A number of techniques are available today for carrying out site-directed mutagenesis (SDM). Common among them is the oligonucleotide-directed mutagenesis ***(1)***. Three widely used procedures, which are based on this principle, are the polymerase chian reaction (PCR)-based approach ***(2)***, and Kunkel's ***(3)*** and Eckstein's ***(4)*** methods. Kunkel's method, which takes advantage of a strong biological selection, although inexpensive, has a drawback, in that its efficiency of selecting against the wild-type parent strand from the heteroduplex is not efficient. In addition, the enzymes used in this procedure are contaminated with uracil DNA glycosylase (UDG), which may also contribute to the overall low efficiency of mutagenesis.

A number of modifications have been conceived over the years to improve the efficiency of the originally proposed Kunkel method ***(5)***. Following is the strategy that the authors have adopted to increase the efficiency of selecting for the mutant strand. This protocol employs a modified T7 DNA polymerase (Sequenase, Amersham Pharmacia Biotech) for extension of the mutagenic primer ***(6)***. Because Sequenase is highly processive, it ensures complete extension of the template in less than one-half hour compared to the prolonged periods (2–16 h) reported earlier ***(7)*** with the other DNA polymerases which are time consuming and prone to inefficient extension, because of potential secondary structures that could form in the template at lower incubation temperatures. Furthermore, in the authors' protocol ***(8)***, subsequent to the extension and ligation step, a step of in vitro UDG treatment has been introduced to effectively eliminate the parent strand, and thereby increase the efficacy of

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ


Uracil containing SSDNA template
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5. Replication
1.Annealing of
muatgenic primer
Mutagenic primer
4. Transformation in
ung+ E. coli
2. Extension
and ligation
3. In vitro UDG
treatment

Fig. 1. Schematic representation of the various steps used in the SDM protocol (*see* also **Note 7**). (Reproduced with permission from **ref. *8***.)

selecting the desired mutant strand (**Fig. 1**). Although the use of UDG has earlier been reported (Boehringer Mannheim), this protocol differs, in that it utilizes T7 DNA polymerase (Sequenase, Amersham Pharmacia Biotech) for primer extension, and works well, even with phagemid vectors. In addition, the authors' protocol is simplified, step-by-step, convenient, quick, and cost-effective: all reactions are carried out in a single tube. Time taken for the whole procedure, from annealing the template to the mutagenic primer to the plating of the final reaction products on the selective medium, is less than 3 h. This method gives high efficiency of mutagenesis, so that screening of a large number of transformants is circumvented.

2. Materials

1. *Escherichia coli* strains used for the various steps were RZ1032 and TG1. The genotypes of the two strains are RZ1032: *dut1 ung1*; TG1: F'*tra*D36*lacI*$^q\Delta$ *(lacZ)M15proA*$^+$*B*$^+$*/supE*Δ(*hsdM-mcrB*)5($r_K^-m_K^-$McrB$^-$) *thi*Δ*(lac-proAB)*.
2. The gene, or portion of the gene of interest, was cloned into a phagemid vector (the authors used pTZ19R, Amersham Pharmacia Biotech) to obtain single stranded uracil containing DNA from *E. coli* RZ1032 (or any other *ung*$^-$*dut*$^-$ strain of *E. coli*).
3. 10× T4 polynucleotide kinase buffer: 700 m*M* Tris-HCl (pH 7.6), 100 m*M* $MgCl_2$, and 5 m*M* dithiothreitol (DTT).
4. 5× annealing buffer: 200 m*M* Tris-HCl (pH 7.5), 100 m*M* $MgCl_2$, and 250 m*M* NaCl.

5. 10× T4 DNA ligase buffer: 660 m*M* Tris-HCl (pH 7.5), 10 m*M* DTT, 50 mM $MgCl_2$, and 10 m*M* adenosine triphosphate (ATP).
6. 10 m*M* ATP.
7. 2.5 m*M* deoxynucleoside triphosphate (dNTP) mix: 2.5 m*M* dATP, 2.5 m*M* deoxycytodine triphosphate, 2.5 m*M* deoxyguanosine triphosphate, and 2.5 m*M* deoxythymidine triphosphate.
8. 2× YT broth (per L): 16 g tryptone, 10 g yeast extract, and 5 g NaCl. Add deionized H_2O to make up the volume to 1 L. Autoclave.
9. 2× YT agar (per L): 16 g tryptone, 10 g yeast extract, 5 g NaCl, and 15 g agar. Add deionized H_2O to make up the volume to 1 L. Autoclave. Pour into sterile Petri dishes. Store the plates at 4°C.
10. 2× YT ampicillin agar (per L): 1 L 2× YT agar. Autoclave. Cool to 55°C. Add 10 mL 10 mg/mL filter-sterilized ampicillin. Pour into Petri dishes. Store the plates at 4°C.
11. *E. coli* competent cells (competent cells prepared by $CaCl_2$ method, **ref.** ***7***).

3. Methods

3.1. Design of the Mutagenic Oligomer

The mutagenic primers must be designed according to the number and kind of mutations desired in the gene of interest. The following guidelines can be followed when designing oligomers for mutagenesis.

1. Mutagenic oligomers, 20–25 bases in length, were used.
2. The mismatch(es) required for generating the mutation should be placed in the center of the oligomer. The terminal nucleotides of the oligomer should preferably be G or C.
3. The mutagenic oligomers should be purified and desalted (the authors purify these from urea polyacrylamide gels, and subsequently desalt them by passing through a gel filtration column).
4. The mutagenic oligomer should be 5'-phosphorylated, before the mutagenesis reaction (*see* **Subheading 3.3.**).

3.2. Preparation of Single-Stranded Uracil-Substituted Template

Single-stranded uracil containing phagemid DNA, containing the insert of interest, was prepared from *E. coli* RZ1032, using established procedures ***(7)***.

3.3. Phosphorylation of the Mutagenic Oligomer

1. To 1 µL mutagenic DNA oligomer (20 pmol), add 1 µL 10× T4 polynucleotide kinase buffer, 1 µL 10 m*M* ATP, 6.5 µL H_2O, and 0.5 µL T4 polynucleotide kinase (5 U, New England Biolabs) (*see* **Note 1**) in a 1.5-mL microcentrifuge tube.
2. Incubate the reaction at 37°C for 30 min, followed by heat-inactivation of T4 polynucleotide kinase at 65°C for 10 min.

3.4. Mutagenesis

3.4.1. Step 1: Annealing the Mutagenic Primer to the Single-Stranded Template

1. Mix 1 µL single-stranded DNA template (~1 µg), 10 µL phosphorylated mutagenic oligomer (from **Subheading 3.3.**), and 2 µL 5× annealing buffer (final volume, 13 µL).
2. Incubate the microcentrifuge tube at 65°C for 5 min, and allow to cool slowly to room temperature (25–27°C) over 30 min.
3. Give a brief spin, and remove a 3-µL aliquot (A-1) for analysis on agarose gel.
4. Process the remaining 10 µL for extension and ligation reaction.

3.4.2. Step 2: Extension and Ligation Reaction

The reaction mixture from the annealing reaction is extended using Sequenase (Amersham), and the newly synthesized strands are ligated using T4 DNA ligase.

1. To 10 µL annealed reaction mixture (*see* **Subheading 3.4.1.**), add 3 µL 2.5 m*M* dNTP mix, 3 µL 10× T4 DNA ligase buffer, 0.5 µL Sequenase (6 U, Amersham) (*see* **Notes 1–3**), 1 µL T4 DNA ligase (1 Weiss unit, BM), and 12.5 µL H_2O (total reaction vol, 30 µL).
2. Incubate first at 4°C for 5 min then at room temperature for 5 min, (for stable initiation of DNA synthesis), then at 37°C for 30 min (for efficient extension), and finally at 70°C for 15 min, for the heat-inactivation of the enzyme.
3. Give a quick spin, mix, and remove another aliquot of 5 µL (A-2) from this reaction, for analysis on agarose gel, and process the remainder for in vitro UDG treatment.

3.4.3. Step 3: In Vitro UDG Reaction

In this step, the heteroduplex (*see* **Subheading 3.4.2.**) is subjected to in vitro UDG treatment, to facilitate removal of uracils and generation of the apyrimidinic sites. Subsequent nicking of the apyrimidinic sites in the wild-type template strand increases the efficiency of selecting the newly synthesized mutated strand.

1. Add 1 µL *E. coli* UDG (~100 ng) to the remaining 25 µL vol of the extended heteroduplex, and incubate at 37°C for 30 min (because the salts in the reaction are inhibitory to UDG, an excess of UDG has been used).
2. Remove another aliquot of 5 µL (A-3) for analysis on agarose gel.

3.4.4. Step 4: Transformation into UDG Proficient Strain of E. coli and Screening for Mutants

1. Transform 1- and 5-µL aliquots of the UDG treated reaction mixture into TG1 (or any other strain of *E. coli*, which is wild-type for *ung*) (*see* **Note 4**). The remainder of the reaction is stored at –20°C.
2. Transformants are screened directly by nucleotide sequencing of the miniplasmid preparations (*see* **Notes 2** and **5**).

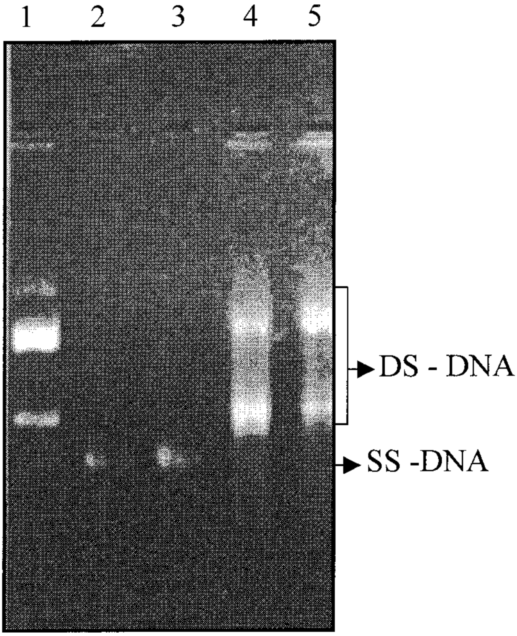

Fig. 2. Electrophoresis of the reaction aliquots to monitor the success of the various steps (*see* **Notes 6** and **7**) of the protocol on a 1% agarose gel. (Lane 1: control plasmid DNA sample; lane 2: single-stranded template DNA; lane 3: aliquot [A-1] from the annealing reaction; lane 4: aliquot [A-2] from the extension and ligation reaction; lane 5: aliquot [A-3] after UDG treatment. Single- and double-stranded DNA are indicated as ss- and ds-DNA, respectively.)

4. Notes

1. Various enzymes used by the authors did not contain any detectable UDG activity. However, many of the commercially available enzymes could contain detectable UDG activity and result in lower efficiency of Kunkel's method as encountered by some investigators. In such cases, the authors advise that approx 10 ng UDG inhibitor protein, Ugi ***(9)***, be added to the phosphorylation reaction. Because Ugi is heat stable, supplementation with Ugi in subsequent steps is unnecessary. The concentration of Ugi used will not interfere with the UDG step of the protocol, because an excess of the latter is provided.
2. By combining the use of Sequenase (a highly processive DNA polymerase) for extension, and the in vitro UDG treatment step (to facilitate efficient removal of the wild-type DNA template) in the Kunkel's method, the authors have achieved >80% efficiency of mutagenesis. Such a high efficiency of mutagenesis allows the mutations to be identified directly by nucleotide sequencing of a small number of the transformants.

3. None of the mutants obtained showed any undesired changes in the DNA sequence. Thus, the lack of the 3'→5' exonuclease activity of this enzyme does not appear to be a serious concern. In any case, DNA fragments should normally be sequenced to their entirety, to ensure that no inadvertent mutations have occurred.
4. The authors also attempted to carry out second-strand synthesis in vitro prior to transformation, but this additional step did not enhance the efficiency of the method any further. Therefore, in the final protocol, this step has been eliminated.
5. This procedure has been used successfully, even for the mutagenesis of tRNA genes, which are highly structured in single-stranded DNA.
6. Agarose gel profile of aliquots A-1 to A-3 can be used as a guide to assess the efficiency of each step (**Fig. 2**).
7. Although not shown in **Fig. 1**, subsequent to UDG treatment (*see* **Subheading 3.4.3.**), the Tris base buffer will also result in nicking at apyrimidinic sites. Presumably, this nicking results in preferential selection of the in vitro synthesized strand as template during in vivo replication, which, in turn, results in increased efficiency of mutagenesis.

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2

Site-Directed Mutagenesis Using Altered β-Lactamase Specificity

Christine A. Andrews and Scott A. Lesley

1. Introduction

Site-directed mutagenesis (SDM) is a powerful tool for the study of gene expression/regulation and protein structure and function. Hutchinson et al. ***(1)*** developed a general method for the introduction of specific changes in DNA sequence, which involves hybridization of a synthetic oligonucleotide (ON) containing the desired mutation to a single-stranded DNA (ssDNA) target template. Following hybridization, the oligonucleotide is extended with a DNA polymerase to create a double-stranded structure. The heteroduplex DNA is then transformed into an *Escherichia coli,* in which where both wild type and mutant strands are replicated. In the absence of any selection this method is very inefficient, often resulting in only a few percent of mutants obtained. Various strategies of selection have since been developed, which can increase mutagenesis efficiencies well above the theoretical yield of 50%. The methods of Kunkel ***(2)***, Eckstein ***(3)***, and Deng ***(4,5)*** employ negative selection against the wild-type DNA strand, in which the parental DNA is selectively degraded, either by growth in an alternate host strain, or by digestion with a nuclease or restriction enzyme. The methods of Lewis and Thompson ***(6)*** and Bonsack ***(7)*** utilize antibiotic resistance to positively select for the mutant DNA strand. This chapter describes a method for the positive selection of mutant strand DNA, which relies on the altered activity of the enzyme β-lactamase against extended spectrum cephalosporins ***(8)***.

Various amino acid substitutions in the active site of TEM-1 β-lactamase, the enzyme responsible for resistance to ampicillin (AMP), have been reported ***(9–17)***. These mutations alter the substrate specificity of the enzyme and result in increased hydrolytic activity of the enzyme against extended spectrum β-lactam

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ

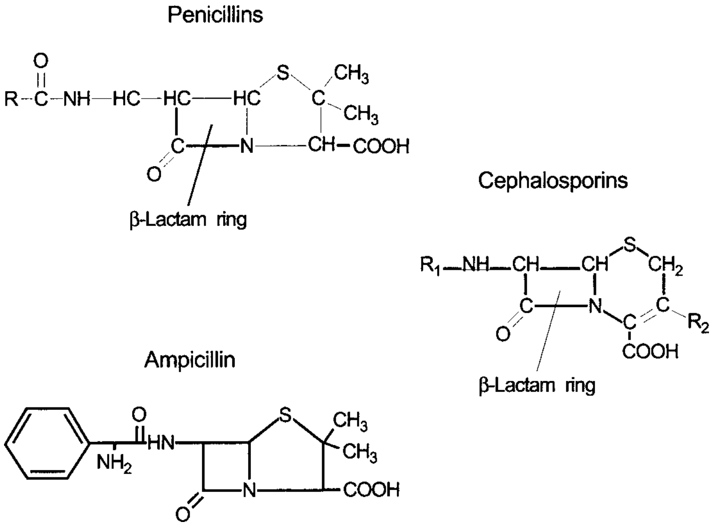
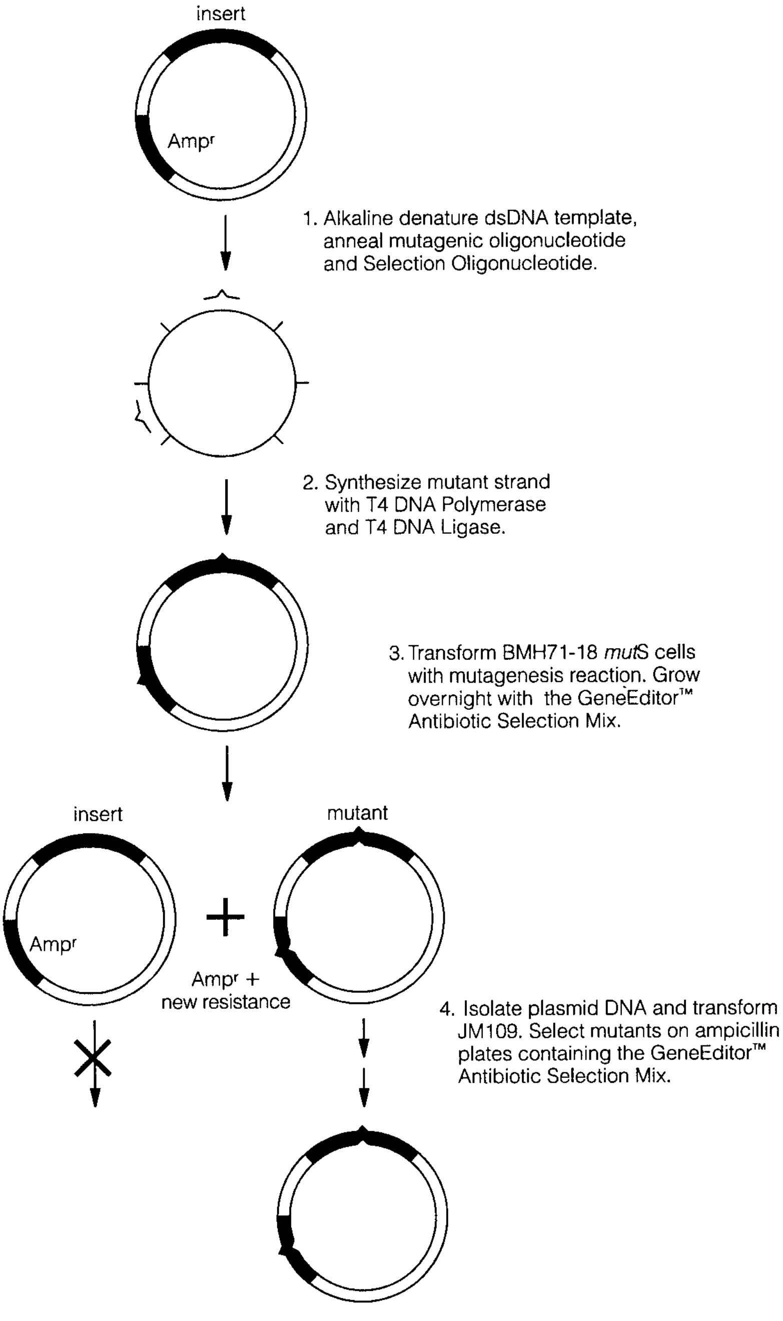


Fig. 1. Structure of β-lactam antibiotics.

antibiotics and cephalosporins (**Fig. 1**). This increased activity results in increased resistance specific to cells expressing the mutant enzyme. The triple mutant, G238S:E240K:R241G, displays increased resistance to cefotaxime ***(9,10)***, and ceftriaxone (unpublished result), and is the basis for the selection strategy used in the GeneEditor™ Mutagenesis System. Residues 238, 240, and 241 are adjacent in the β-lactamase sequence, but are numbered according to the system of Ambler ***(18)***. The numbering system for amino acid residues starts at the N-terminus of the longest form of the *TEM-1* gene from *Bacillus lichenformis*, and takes into account the postulated gaps necessary for optimal sequence alignment of the various forms of the *TEM-1* gene.

Figure 2 is a schematic outline of the GeneEditor procedure. Double-stranded (ds) plasmid DNA is first alkaline denatured. Subsequently, two synthetic ONs are simultaneously annealed to the template. The first ON is the selection ON, which encodes the residue changes in the V-lactamase gene that result in increased resistance to the extended spectrum antibiotics. **Table 1** shows the sequence of the selection ON, compared to the wild-type sequence in the V-lactamase gene. The second ON is the mutagenic ON, and codes for the desired sequence changes in the target DNA. This mutagenic ON hybridizes to the same DNA strand as the selection ON. Synthesis and ligation of the mutant strand by T4 DNA polymerase and T4 DNA ligase creates a heteroduplex, effectively linking the conferred antibiotic resistance with the desired



1. Alkaline denature dsDNA template, anneal mutagenic oligonucleotide and Selection Oligonucleotide.
2. Synthesize mutant strand with T4 DNA Polymerase and T4 DNA Ligase.
3. Transform BMH71-18 *mut*S cells with mutagenesis reaction. Grow overnight with the GeneEditor™ Antibiotic Selection Mix.
4. Isolate plasmid DNA and transform JM109. Select mutants on ampicillin plates containing the GeneEditor™ Antibiotic Selection Mix.

Fig. 2. Schematic diagram of the GeneEditor in vitro SDM procedure.

Table 1
Sequence Alignment of Cephalosporin Selection Oligonucleotide with Native β-Lactamase Sequence

Native												
	D	K	S	G	A	**G**	**E**	**R**	G	S	R	G
	GAT	AAA	TCT	GGA	GCC	**GGT**	**GAG**	**CGT**	GGG	TCT	CGC	GGT
Mutant												
	GAT	AAA	TCT	GGA	GCC	**TCC**	**AAG**	**GGT**	GGG	TCT	CGC	GGT
	D	K	S	G	A	**S**	**K**	**G**	G	S	R	G

The sequence of the selection oligonucleotide is shown relative to the native β-lactamase sequence. The oligonucleotide hybridizes to residues 233–245 (using the numbering sequence of Ambler et al. ***[18]***). Substitutions are in boldface. Incorporation of the selection oligonucleotide results in the formation of a *Sty*I site within the β-lactamase gene.

mutation in the target gene. The DNA is then transformed into a repair-deficient *E. coli mutS* host, such as BMH71-18 ***(19)***, followed by clonal segregation in a second host. Linkage of the antibiotic resistance to the desired mutation results in a high efficiency of mutagenesis. More than one mutagenic ON may be annealed along with the selection ON, to create several linked mutations on the same plasmid. The authors have effectively coupled seven separate mutations with the altered substrate specificity, in a single mutagenesis reaction, with 30% efficiency.

The GeneEditor protocol can be used with any plasmid vector containing the *TEM-1* β-lactamase sequence (commonly designated the "amp" gene), without the need for subcloning into a specialized vector. A Basic Local Alignment Search Tool (BLAST) search of the vector database at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/) indicates that the *TEM-1* sequence is present in over 90% of the commonly used cloning vectors. Guidelines for the design of mutagenic ONs are discussed in the Notes section.

2. Materials

1. 2 *M* NaOH, 2 m*M* ethylenediamine tetraacetic acid (EDTA).
2. 2 *M* Ammonium Acetate pH 4.6.
3. 70 and 100% ethanol.
4. TE Buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
5. Oligonucleotides, 5' phosphorylated (*see* **Table 1** and **Note 1**).
6. 10X Annealing buffer: 200 m*M* Tris-HCl, pH 7.5, 100 m*M* $MgCl_2$, 500 m*M* NaCl.
7. 10X Synthesis buffer: 100 m*M* Tris-HCl, pH 7.5, 5 m*M* deoxyribonucleoside triphosphate (dNTPs), 10 m*M* adenosine triphosphate, 20 mm dithiothreitol.
8. T4 DNA Polymerase (10 U/µL).

9. T4 DNA Ligase (5 U/μL).
10. Competent cells from *E. coli* strains BMH71-18*mutS* and JM109 (Promega, Madison, WI).
11. Luria-Bertani (LB) media: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl.
12. SOC media (100 mL): 2 g tryptone, 0.5 g yeast extract, 1 mL of 1 *M* NaCl, 0.25 mL of 1 *M* KCl, 1 mL of 2 *M* Mg^{2+} stock (1 *M* $MgCl_2 \cdot 6H_2O$/1 *M* $MgSO_4 \cdot 7H_2O$), 1 mL of 2 *M* glucose.
13. Antibiotic selection mix: 5 mg/mL ampicillin/25 μg/mL cefotaxime/25 μg/mL ceftriaxone (Sigma, St. Louis, MO)/100 m*M* potassium phosphate, pH 6.0 (*see* **Note 2**).
14. Chloroform:isoamyl alcohol (24:1).
15. TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1).
16. Miniprep resuspension buffer: 25 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA, pH 8.0, 50 m*M* glucose.
17. Miniprep lysis buffer: 0.2 *M* NaOH, 1% sodium dodecyl sulfate. Prepare fresh.
18. Neutralization solution: 3.5 *M* potassium acetate, pH 4.8.
19. Ampicillin solution, 100 mg/mL in deionized H_2O, filter-sterilized.
20. LB agar (1.5%).

3. Methods

3.1. Step 1: Denaturation of dsDNA Templates

1. Set up the following alkaline denaturation reaction: 1.0 pmol (approx 2 μg for a 3-kb plasmid) dsDNA template; 2 μL of 2 *M* NaOH, 2 m*M* EDTA; sterile, deionized H_2O to a final volume of 20 μL. This generates enough DNA for 10 mutagenesis reactions. To ensure good recovery, do not denature less than 1.0 pmol dsDNA. In general, ng dsDNA = pmol dsDNA × 0.66 × *N*, where *N* = length of dsDNA in bases.
2. Incubate at room temperature for 5 min.
3. Add 2 μL of 2 *M* ammonium acetate, pH 4.6, and 75 μL of 100% ethanol.
4. Incubate at –70°C for 30 min.
5. Precipitate the DNA by centrifugation at top speed in a microcentrifuge for 15 min at 4°C.
6. Decant supernatant, and wash the pellet with 200 μL of 70% ethanol.
7. Centrifuge again as in **step 5**. Dry the pellet under vacuum.
8. Resuspend the pellet in 100 μL TE buffer pH 8.0. Analyze a 10-μL sample of the denatured DNA on an agarose gel, to verify that no significant loss occurred, before proceeding to the annealing reaction. Include nondenatured DNA of known concentration in a neighboring well, to help quantify DNA losses, and to ensure that the DNA has been denatured. Denatured, ssDNA will generally run faster than nondenatured dsDNA, and will appear more smeared. The denatured DNA may be stored at –20°C for up to several months, with no loss in mutagenesis efficiency.

3.2. Step 2: Annealing Reaction and Synthesis of Mutant Strand

1. Prepare the annealing reaction as outlined here: 0.10 pmol (200 ng) 10 μL denatured template DNA; 0.25 pmol 1 μL selection ON, phosphorylated (*see* **Note 1**);

1.25 pmol mutagenic ON, phosphorylated (*see* **Note 1**); 2 µL 10x annealing buffer; and sterile, deionized water to 20 µL.

2. Heat the annealing reaction to 75°C for 5 min, then allow to cool slowly to room temperature. Slow cooling minimizes nonspecific annealing of the ONs. Cooling the reactions at a rate of approx 1.5°C/min is recommended (*see* **Note 3**).
3. Once the annealing reactions have cooled to room temperature, spin briefly in a microcentrifuge to collect the contents at the bottom of the tube. Add the following components in the order listed (*see* **Note 4**): 5 µL sterile, deionized H_2O; 3 µL of 10x synthesis buffer; 5–10 U T4 DNA polymerase; and 1–3 U T4 DNA ligase.
4. Incubate the reaction at 37°C for 90 min. Incubation times longer than 90 min are not recommended, because template degradation can occur as dNTP levels are depleted.

3.3. Step 3: Transformation into BMH71-18mutS Competent Cells

1. Prechill sterile 17 × 100 mm polypropylene culture tubes on ice (the use of microcentrifuge tubes reduces the transformation efficiency twofold because of inefficient heat-shock treatment).
2. Thaw competent BMH71-*18mutS* cells on ice. Add 1.5 µL mutagenesis reaction to 100 µL competent cells, and mix gently.
3. Incubate cells on ice for 10 min.
4. Heat-shock the cells for 45–50 s in a water bath exactly at 42°C.
5. Immediately place the tubes on ice for 2 min.
6. Add 900 µL room-temperature LB media, without antibiotic, to each transformation reaction, and incubate for 60 min at 37°C, with shaking.
7. Prepare overnight cultures by adding 4 mL LB media to each reaction (5 mL total), then add 100 µL antibiotic selection mix to each 5 mL culture (*see* **Note 5**).
8. Incubate overnight (16–20 h) at 37°C, with vigorous shaking (*see* **Note 6**).

3.4. Step 4: Plasmid DNA Miniprep Procedure

1. Place 3 mL overnight culture into an appropriate tube, and centrifuge at 12,000*g* for 5 min.
2. Remove the media by aspiration or decantation.
3. Resuspend the pellet by vortexing in 100 µL resuspension buffer.
4. Add 200 µL lysis buffer. Mix by inversion. Incubate on ice 5 min.
5. Add 200 µL neutralization solution. Mix by inversion, and incubate on ice for 5 min.
6. Centrifuge at 12,000*g* for 5 min.
7. Transfer the supernatant to a fresh tube, avoiding the white precipitate.
8. Add 1 vol of TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1). Vortex for 1 min then centrifuge at 12,000*g* for 2 min.
9. Transfer the upper aqueous phase to a fresh tube, and add 1 vol of chloroform:isoamyl alcohol (24:1). Vortex for 1 min, and centrifuge as in **step 8**.
10. Transfer the upper aqueous phase to a fresh tube, and add 2.5 vol of ice-cold 100% ethanol. Mix, and incubate on dry ice for 30 min.
11. Centrifuge at 12,000*g* for 15 min. Wash the pellet with cold 70% ethanol, and dry the pellet under vacuum.

12. Dissolve the pellet in 50 µL sterile, deionized H_2O.
13. Quantitate the DNA by taking a A_{260}/A_{280} absorbance reading (*see* **Note 7**).

3.5. Step 5: Transformation into JM109 and Clonal Segregation

1. Before beginning the transformation procedure, prepare plates by pouring molten LB agar, containing 7.5 mL/L antibiotic selection mix and 1 mL/L 100 mg/mL ampicillin solution. Alternatively, plates may be prepared by evenly spreading 100 µL the antibiotic selection mix onto 20–25 mL LB agar plates containing 100 µg/mL AMP (*see* **Note 8**).
2. Prechill sterile 17 × 100 mm polypropylene culture tubes on ice (the use of microcentrifuge tubes reduces the transformation efficiency twofold because of inefficient heat-shock treatment).
3. Thaw competent JM109 cells on ice.
4. Add 5–10 ng BMH miniprep DNA (or a dilution of the miniprep DNA) to 100 µL competent cells, and mix gently.
5. Incubate cells on ice for 30 min.
6. Heat-shock the cells for 45–50 s in a water bath exactly at 42°C.
7. Immediately place the tubes on ice for 2 min.
8. Add 900 µL of room temperature SOC media, without antibiotic, to each transformation reaction, and incubate for 60 min at 37°C, with shaking.
9. Plate 100 µL transformation reaction onto each of two plates prepared in **step 1** above. Incubate the plates at 37°C for 12–14 h. If cells are highly competent, it may be necessary to plate less than 100 µL, in order to obtain isolated colonies.
10. The GeneEditor mutagenesis system generally produces 60–90% mutants; therefore, colonies may be screened by direct sequencing. Engineering a restriction site into the mutagenic ON, if possible, may be useful to aid in screening. Assuming that greater than 60% mutants are obtained, screening five colonies will give greater than 95% chance of finding the desired mutation. Continued growth of the mutants is not necessary in the presence of the cephalosporin antibiotic selection mix, because the triple mutation in β-lactamase is stable. The authors do, however, recommend further outgrowth of the mutants in AMP, for maintainance of the plasmid DNA.
11. **Notes 9–12** contain suggestions for troubleshooting the system, some of which have been observed during the development of the GeneEditor system.

4. Notes

1. The mutagenic ON and the selection ON must be complementary to the same strand of DNA, in order to achieve coupling of the antibiotic resistance to the desired mutation. **Table 1** shows the sequence of the selection ON for the coding strand of the β-lactamase gene. This ON, or its complement, may be used, depending on the orientation of the cloned insert to be mutagenized. If the orientation of the cloned insert is not known, two separate mutagenesis reactions may be prepared, using each selection ON. Only one reaction will generate the target mutation.

The length and base composition of the mutagenic ON will depend on the nature of the desired mutation and the sequence of the template DNA. For single-base-pair substitutions, insertions, or deletions, a mutagenic oligonucleotide of about 20 bases is sufficient if the region of mismatch is located near the center. Larger mutations, particularly, large insertions or deletions, may require an ON having larger regions of complementarity on either side of the mismatched region. We have successfully used an ON of 90 bp to create a 50-bp insertion. This would allow for 20 perfectly matched bases on either side of the region of mismatch. However, the use of particularly large mutagenic ONs may decrease overall mutagenesis efficiency, because of formation of secondary structures within the ON.

To stabilize annealing between the ON and template DNA, and promote extension by T4 polymerase, the 3' end of the ON should end with a G or a C nucleotide. A significant increase in the number of mutants is observed when ONs are phosphorylated. The authors recommend 5' phosphorylation of both the selection ON, as well as any mutagenic ON used with this system.

2. Preparation and storage of the antibiotic selection mix:
   a. All three antibiotics are light-sensitive, and should be stored in the dark (i.e., foil-wrapped), both as a solution and in powder form.
   b. Powders should be stored at 4°C, and solutions at –20°C.
   c. All three antibiotics are members of the penicillin family of antibiotics, and as such have the potential to cause an allergic reaction in individuals who are sensitive to penicillin. The powders should be handled in a hood.
   d. The antibiotic selection mix should be filter-sterilized with a 0.22-μ filter, prior to use.
   e. The antibiotic selection mix is sensitive to freeze–thaw cycling. The authors recommend that the solution be aliquoted into single-use volumes of 1–2 mL and stored at –20°C.
3. Annealing conditions will vary for each mutagenic ON, and may need to be determined empirically. In general, longer ONs and G–C-rich ONs may require higher annealing temperatures; shorter ONs or A–T-rich ONs may require lower annealing temperatures. A slow cooling of the annealing reaction has been observed to give a higher overall mutagenic efficiency. The amount of ON used in the annealing reaction has been optimized for the selection ON, as well as for a number of mutagenic ONs. A 12.5:1 ON:template molar ratio for the mutagenic ON, and a 2.5:1 ON:template molar ratio for the selection ON are recommended for a typical reaction.
4. Add the components exactly in the order listed. Addition of the polymerase in the absence of dNTPs (in the synthesis buffer) can induce exonuclease activity associated with the polymerase, and result in degradation of the template.
5. Thaw the antibiotic selection mix thoroughly, and mix well, before use. Aliquot the thawed material into 1–2-mL amounts prior to refreezing, to avoid freeze–thaw cycles. Do not add greater than 100 μL antibiotic selection mix to the 5 mL overnight culture. Unlike AMP, the antibiotics in this mix can inhibit growth of resistant cells, when provided in excess of the recommended levels, especially

with low-copy number plasmids. Some low-copy-number plasmids may require a decrease in antibiotic concentration. This must be determined empirically.

6. BMH71-*18mutS* cells grow very slowly, and therefore require longer incubation times than most *E. coli* strains. The overnight culture may take longer than 16–20 h to reach density. Optimal growth rate can be attained by maximizing aeration with vigorous shaking and slanting the tubes to increase surface area. BMH71-*18mutS* cells tend to aggregate when grown in the presence of the antibiotic selection mix and often settle at the bottom of the culture tube.
7. Limit the amount of DNA used in the second transformation reaction to a maximum of 10 ng, to avoid cotransformation of cells with both wild-type and mutant plasmids. The authors therefore recommend that the DNA miniprep be quantitated by measurement of A_{260}.
8. Pouring plates, containing the antibiotic selection mix, reduce edging effects that are often seen when plates are spread with the antibiotic mix. Uneven spreading may result in the growth of wild-type cells in areas with low antibiotic concentration, and, alternatively, in inhibition of growth of the mutant cells in areas of high antibiotic concentration. Poured plates should be prepared ahead of time and used within 1 wk when stored at 4°C.
9. *Problem:* No growth in the BMH*mutS* overnight culture. *Possible causes* and s*uggestions:* β-Lactamase expression may be too low to overcome the effects of the antibiotics. This has been seen when using low-copy-number plasmids. Decrease the amount of antibiotic in the overnight culture to 50 µL instead of 100 µL. DNA used in the transformation is derived from an *hsd* modification minus strain, which is restricted by BMH*mutS.* Use DNA grown in a modification (+) K12 strain. Do not use strains such as HB101,MN522, or BL21 (*E. coli* B strain). Low competency of BMH*mutS* cells. Use only high-efficiency competent cells. Check competency by plating only on AMP.
10. *Problem:* No JM109 colonies. *Possible causes and suggestions:* Excessive amount of cephalosporin antibiotic selection mix used: Try using 50 µL instead of 100 µL on selective plates. Low competency of JM109 cells: Use only high-competency cells. Check competency by plating only on AMP.
11. *Problem:* JM109 antibiotic-resistant colonies, but low mutation frequency. *Possible causes and suggestions:* Co-transformation of JM109 cells with both wild-type and mutant plasmids. Do not use more than 10 ng DNA in the transformation reaction. Quantitate DNA in the miniprep by measuring the A_{260} and dilute the DNA, if necessary.
12. *Problem:* JM109 antibiotic resistant colonies, but no mutations. *Possible causes and suggestions:* Mutagenic ON is not annealed to the same strand as the selection ON. Recheck the orientation of the cloned insert. Repeat the mutagenesis using the complementary selection ON. Inadequate annealing of mutagenic ON to template DNA. Secondary structure in cloned insert or mutageneic ON. Prepare template as ssDNA, and/or redesign mutageneic ON. Antibiotic selection mix is no longer active: Check for activity by plating JM109 cells transformed with an Amp^r plasmid on plates spread with 100 µL selection mix. If the selection

mix is active, no colonies should be obtained. Remake fresh antibiotic selection mix, if necessary.

Acknowledgments

The authors would like to thank Isobel McIvor and Neal Cosby for assistance with the graphics work, and also thank Martin K. Lewis, Kris Zimmerman, and Jackie Kinney for critical reading of the manuscript.

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3

Site-Directed Mutagenesis Facilitated by *Dpn*I Selection on Hemimethylated DNA

Fusheng Li and James I. Mullins

1. Introduction

Oligonucleotide-based, site-directed mutagenesis (SDM) of cloned DNA has become a fundamental tool of modern molecular biology, used to introduce insertions, deletions, and substitutions into DNA. Current techniques available for performing in vitro mutagenesis fall into three categories:

1. Those employing single-stranded DNA (ssDNA) as template ***(1,2)***, originally using phage M13 vector to produce the ssDNA. Several methods were developed to reduce the background of nonmutated parental DNA, including in vivo incorporation of uracil in the parental strand ***(3,4)***, and in vitro incorporation of 5-methyl cytidine or thiophosphates in the synthesized strand ***(5,6)***. Selection against the parental strand was accomplished during intracellular DNA repair, or by differential restriction endonuclease sensitivity in vitro, respectively.
2. Use of double-stranded DNA (dsDNA) as template. A widely used method called unique site elimination (USE) ***(7)*** allows introduction of specific mutations into a target gene or region cloned into a ds plasmid with a unique restriction site.
3. Polymerase chain reaction (PCR)-based methods, in which site-specific mutants are created by introducing mismatches into oligonucleotides used to prime in vitro amplification ***(8–11)***. Such methods often include splicing by overlap extension, which involves a two-step PCR procedure, and subsequent cloning.

Although each of the mutagenesis methods described above has proved successful in some cases, certain limitations remain for each: A phagemid or phage vector must be used to produce a ssDNA template. In some cases, no suitable restriction sites are available for subcloning, and insertion of large fragments in M13 vectors results in instability. The USE approach requires a second mutagenic oligonucleotide to eliminate a unique restriction site. PCR-based

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ

mutagenesis suffers from the low fidelity of *Taq* DNA polymerase, and the expense of multiple primers.

The authors have overcome each of these limitations by using a dsDNA template combined with *Dpn*I digestion ***(12)***. *Dpn*I was first used for SDM with dsDNA templates by Weiner et al. ***(13)***. In their protocol, and in others ***(14,15)***, the mutation-containing DNA was amplified by PCR, and *Dpn*I was used to destroy the parental DNA, as well as hemimethylated DNA. In the authors' protocol, DpnI is used in a different way. Briefly, a mutagenic oligo is first annealed to the dsDNA. Subsequent DNA synthesis using T4 DNA polymerase and ligation with T4 DNA ligase results in the formation of two kinds of molecules. One is the fully methylated parental dsDNA; the other contains the newly synthesized hemi-methylated dsDNA. In high-salt buffers, *Dpn*I can selectively destroy fully methylated dsDNA, and leave the hemimethylated dsDNA intact ***(16)***. The resulting *Dpn*I-treated reaction is then transformed into a mismatch repair-defective strain of *Escherichia coli* (BMH71-18*mutS*) ***(7)***. Mutated plasmids are then detected by direct sequencing or restriction-site analysis (**Fig. 1**). Reasonably high mutation efficiencies can be achieved by this simple protocol (35–50%). To gain higher mutation efficiency, the authors introduced two optional modifications to the protocol.

Use of uracil-containing dsDNA templates is easily achieved by passage through *E. coli* strain CJ236 (ung^-, dut^-) ***(4)***. As a dsDNA replication template, uracil-containing DNA is indistinguishable from thymidine-containing DNA ***(4)***. However, when hemimethylated dsDNA is transformed into ung^+ *E. coli*, the uracil-containing strand is selectively destroyed by *N*-glycosylase. Only the newly synthesized mutated strand can survive this selection pressure, thus giving rise to a high mutation efficiency (>80%).

Use of a second "marker" oligo, a procedure similar to the USE approach, can be introduced to help screen for mutated clones ***(7)***. However, the authors' approach differs from USE, in that the marker oligo is not necessary to eliminate a unique site in the DNA template, but rather to simply change a restriction site, and selection for the mutated plasmid is imposed by *Dpn*I, rather than the unique site. Thus, the restriction site changed by marker oligo serves just as a marker to screen clones that survive transformation and colony formation. Because of the high coupling effect of the two oligos, the desired mutation clone can be screened by a simple restriction site analysis (**Fig. 2**). This modifications can also consistently achieve >80% mutagenesis efficiency.

2. Materials

2.1. E. coli *Strains*

1. CJ236: *dut, ung, thi, relA*; pCJ105(Cm^r) ***(17)***.
2. BMH71-18*mutS*: thi, supE, (lac-proAB), [mutS::Tn10] [F'proAB, $lacI^qZ$(M15] (*see* **Note 1**) ***(18)***.

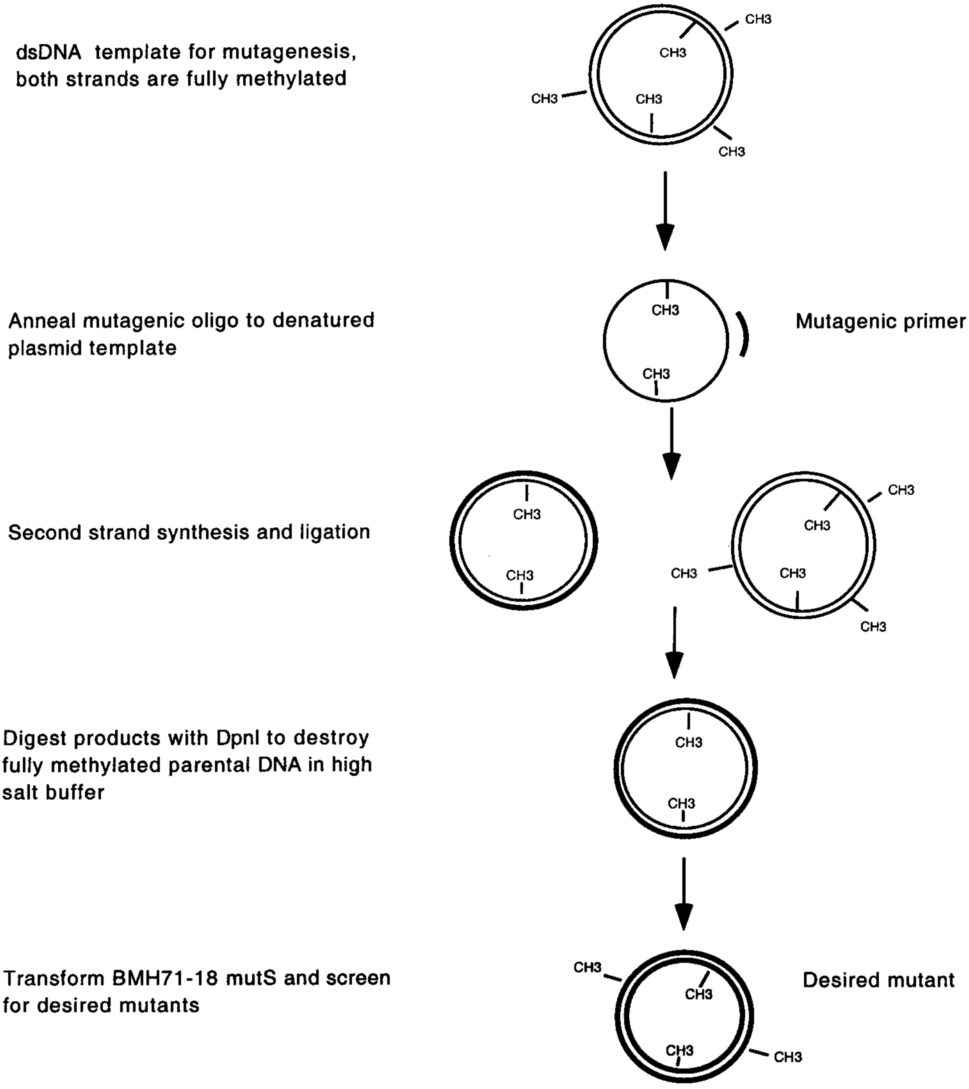
dsDNA template for mutagenesis, both strands are fully methylated
CH3
CH3
CH3
CH3
CH3
Anneal mutagenic oligo to denatured plasmid template
CH3
CH3
Mutagenic primer
Second strand synthesis and ligation
CH3
CH3
CH3
CH3
CH3
CH3
CH3
Digest products with DpnI to destroy fully methylated parental DNA in high salt buffer
CH3
CH3
Transform BMH71-18 mutS and screen for desired mutants
CH3
CH3
CH3
CH3
Desired mutant

Fig. 1. Schematic representation of mutagenesis protocol.

2.2. Buffers

1. 10X Annealing buffer: 200 m*M* Tris-HCl, pH 7.5, 100 m*M* $MgCl_2$, and 500 m*M* NaCl.
2. 10X Synthesis buffer: 100 m*M* Tris-HCl, pH 7.5, 5 m*M* of each deoxyribonucleoside triphosphate, 10 m*M* adenosine triphosphate (ATP), and 20 m*M* dithiothreitol (DTT).

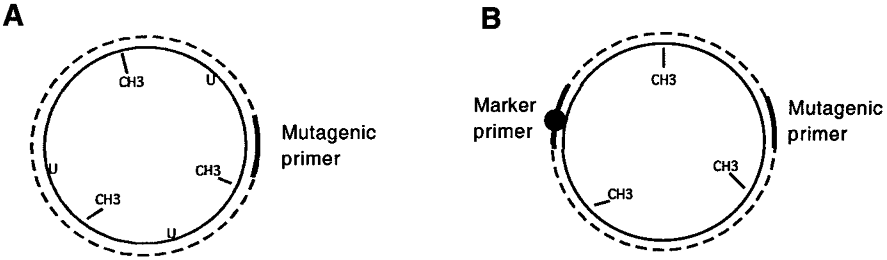

Fig. 2. Alternative ways to improve mutation efficiency. **(A)** Uracil-containing dsDNA as templates. **(B)** Coupling of marker primer to change a restriction site.

3. 10X *Dpn*I buffer: 1.2 *M* NaCl.
4. 10X Kinase buffer: 500 m*M* Tris-HCl, pH 7.5, 100 m*M* MgCl2, 50 m*M* DTT, 10 m*M* ATP.
5. 2 *M* NaOH.
6. 3 *M* NaOAc (pH 4.8).
7. Isopropyl-β-D-thio-galactopyranoside (IPTG), 20 m*M* stock solution in sterile, distilled H_2O. Store at 4°C. Use 10 µL/10-cm plate.
8. 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). 20 mg/mL stock solution in dimethylformamide. Store at –20°C. Use 40 µL/10-cm plate.
9. Ampicillin, 100 mg/mL (1000X) stock solution in H_2O. Filter-sterilize, and store at 4°C for no more than 1 mo.
10. Tetracycline, 5 mg/mL (100X) stock solution in ethanol. Keep in dark at –20°C.
11. Luria-Bertani (LB) medium, 10 g/L Bacto-tryptone, 5 g/L Bacto-yeast extract, 10 g/L NaCl. Adjust pH to 7.0 with 5 *N* NaOH. Autoclave to sterilize.
12. LB agar plates, add agar (15 g/L) to LB medium and autoclave.
13. Transformation and storage solution for chemical transformation (TSS): 85% LB medium, 10% polyethylene glycol 8000, 5% dimethylsulfoxide, 50 m*M* $MgCl_2$ (pH 6.5). Autoclave or filter sterilize. Store at 4°C for up to 2 wk.
14. Uridine, 0.26 mg/mL stock solution in distilled H_2O. Store at –20°C.
15. Glucose–Tris–EDTA (GTE) solution: 50 m*M* glucose, 25 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA.
16. TE buffer: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, pH 8.0.
17. NaOH-sodium dodecyl sulfate (SDS) solution: 0.2 *M* NaOH, 1% (w/v) SDS.
18. Potassium acetate solution, pH 4.8: 29.5 mL glacial acetic acid, add H_2O to ~90 mL, KOH pellets to pH 4.8, then H_2O to 100 mL. Store at room temperature (do not autoclave).

2.3. Plasmid and Enzymes

1. pUC19M (Transformer™ Site-Directed Mutagenesis Kit, Clontech, Palo Alto, CA) contains a mutation that interrupts the coding sequence of the *lacZ* gene by

converting the UGG tryptophan codon to the amber stop condon, UAG, which results in white colonies on LB agar plates containing X-gal and IPTG. The mutation primer (5'-GAG TGC ACC ATG GGC GGT GTG AAA T-3') can revert the stop codon in the *lacZ* gene to result in the production of blue colonies on X-gal/IPTG plates.
2. T4 DNA polymerase (New England Biolabs [NEB], Beverly, MA).
3. T4 DNA ligase (NEB).
4. T4 polynucleotide kinase (NEB).
5. *Dpn*I (NEB).

3. Methods

3.1. Competent Cell Preparation

1. Streak the cell stock on an LB plate containing 1.5% agar and 50 µg/mL tetracycline (for BMH 71-18*mutS*) (*see* **Notes 2** and **3**). Incubate at 37°C overnight.
2. Pick a single, well-separated colony, and inoculate into a sterile tube containing 3 mL LB broth, plus 50 µg/mL (BMH71-18*mutS*). Incubate at 37°C overnight, with shaking at 220 rpm.
3. Transfer 1 mL saturated overnight culture of *E. coli* cells to a fresh, sterile 500-mL flask containing 100 mL LB medium (no antibiotics). Incubate the cells at 37°C, with shaking at 220 rpm, until OD_{600} reaches 0.5 (usually takes 2.5–3 h). Check the OD_{600} frequently, to avoid overgrowth.
4. Chill the flask on ice for 15–20 min, then collect cells by centrifugation at 1200*g* for 5 min at 4°C.
5. Resuspend the cells in 10 mL ice-cold TSS solution. These transformation-competent cells can be used immediately to achieve the highest transformation efficiency, or may be stored at –70°C for up to several months (*see* **Note 4**).

3.2. Plasmid Template Preparation

1. Inoculate 5 mL sterile LB medium containing 100 µg/mL ampicillin with a single bacterial colony, and grow to saturation at 37°C with rotation (Add 5 µg/mL uridine to the LB medium, if CJ236 strain is cultured).
2. Microcentrifuge 1.5 mL of cells for 20 s. Resuspend cell pellet in 100 µL GTE solution, and let sit 5 min at room temperature.
3. Add 200 µL NaOH/SDS solution, mix, and place on ice for 5 min.
4. Add 150 µL potassium acetate solution, vortex 2 s, and place on ice for 5 min.
5. Microcentrifuge for 3 min and transfer 0.4 mL supernatant to a new tube. Add 0.8 mL 95% ethanol and mix, let sit 2 min at room temperature.
6. Microcentrifuge for 3 min at room temperature, wash pellet with 1 mL 70% ethanol, and dry under vacuum.
7. Resuspend pellet in 30 µL TE buffer.
8. Add 1 µL 10 mg/mL RNase solution, to destroy RNA (*see* **Note 5**).

3.3. Phosphorylation of Oligonucleotide Primers

1. Combine 2.0 µL 10X kinase buffer, 1.0 µL T4 polynucleotide kinase (10 U/µL) and 1 µg primer. Adjust the volume to 20 µL with ddH_2O, and mix well.

2. Incubate at 37°C for 60 min.
3. Stop the reaction by heating at 65°C for 10 min.
4. Use 2.0 µL phosphorylated primer solution in each mutagenesis reaction.

3.4. Mutagenic Strand Extension

1. Add 100 ng of each phosphorylated mutagenesis primer (*see* **Notes 6–9**), 100 ng plasmid DNA, and 2 µL 10X Annealing buffer to a 0.5-mL tube (*see* **Note 10**). Adjust with H_2O to a total volume of 20 µL. Mix well, and briefly centrifuge.
2. Incubate at 100°C for 3 min to completely denature the plasmid (*see* **Note 11**).
3. Chill immediately in an ice water bath (0°C) for 5 min (*see* **Note 12**).
4. To the primer/plasmid annealing reaction, add 3 µL of 10X synthesis buffer, 1 µL T4 DNA polymerase (3 U), 1 µL T4 DNA ligase (5 U), and 5 µL ddH_2O (final volume 30 µL).
5. Incubate at 37°C for 1–2 h.
6. Stop the reaction by heating at 70°C for 5 min, to inactivate the enzyme.
7. Let the tube cool to room temperature.

3.5. DpnI Digestion and Transformation

1. Add 3 µL 1.2 *M* NaCl to the 30 µL reaction, and mix well (*see* **Note 13**).
2. Add 1–2 U *Dpn*I to the reaction, and mix well. This is usually done by diluting 1 µL *Dpn*I (20 U) in 10–20 µL H_2O, and adding 1 mL (1–2 U) into the reaction (*see* **Note 13**).
3. Incubate at 37°C for 1 h.
4. Thaw 100 µL transformation-competent cells on ice and immediately add 5 µL of the *Dpn*I-cleaved DNA reaction.
5. Incubate on ice for 30 min.
6. Transfer to 42°C for 1 min.
7. Immediately add 1 mL LB medium (with no antibiotic) to the tube.
8. Incubate at 37°C for 60 min with shaking at ~220 rpm.
9. Add 40 µL 20 mg/mL X-gal solution, 10 µL 20 m*M* IPTG solution. Mix well, and spread evenly onto a room temperature LB plate containing the antibiotic for selection of transformants.
10. Incubate the plates at 37°C overnight.

3.6. Characterization of Mutant Plasmids

1. The efficiency of mutagenesis is estimated by dividing the number of blue colonies by the total number of blue and white colonies (*see* **Note 14**).
2. In mutagenesis experiments, plasmid DNA should be isolated, to characterize the mutation by DNA sequencing.

4. Notes

1. The *E.coli* strain, BMH71-18*mutS*, or other mismatch repair-deficient strain, should be used for transformation, to achieve higher mutation efficiency.
2. The DNA mismatch repair-deficiency mutation (*mutS*) in BMH71-18 was generated by insertion of the transposon, Tn*10* ***(18)***. To maintain selective pressure on

the transposon, tetracycline (25–50 µg/mL) should be included in the medium. Tn*10* carries the gene conferring resistance to tetracycline [tet^r].

3. Since the pilus encoding F' episome pCJ105 in *E. Coli* strain, CJ236, is not necessary in this protocol, this strain can be grown in the absence of chloramphenicol.
4. Competent cells should be stored at –70°C for long-term use. Good transformation frequency can be maintained for 2–3 mo.
5. Other plasmid extraction methods can be used. The purity of the plasmid DNA is important for successful mutagenesis. DNA should have an A_{260}/A_{280} ratio of 1.8–2.0. If the ratio <1.7, protein contamination is indicated, and further purification is required.
6. When the two-oligo protocol is used, some additional considerations are needed. The marker and mutagenic primer must anneal to the same strand of the plasmid. The restriction site in the marker oligo should be easy to analyze. Although the distance between the marker primer and the mutagenic primer is not critical, it is desirable that two primers can be evenly spaced in the plasmid, which will allow the DNA polymerase to extend both primers for an equivalent distance. In addition, the annealing temperature of the mutagenic primer should be no less than that of the marker primer.
7. The authors suggest using a marker primer in every experiment. Apart from use as a screening tool, it serves as an internal control to determine the mutagenesis efficiency.
8. Design of mutagenic primers: Over 10 nucleotides of uninterrupted matched sequences, flanking each side of the mismatch site, should give sufficient annealing stability, with the mismatch base placed in the center of the primer sequence. If the GC content is less than 50%, the lengths of the primer arms should be extended. For optimum primer annealing, the oligonucleotides should start and end with a G or C. If a large deletion is desired, the mutagenic primer should have at least 15 bp of matching sequences flanking both sides of the segment to be deleted.
9. Multiple mutagenic primers can be annealed to the same plasmid, to simultaneously create mutations at several sites.
10. The ratio of mutagenic primer molecules to DNA template should be ~100–200. The amount of DNA template and oligo given above (*see* **Subheading 3.4., step 1**) presume that DNA templates are ~3 kb and that oligos are 25 bp long. Appropriate adjustment should be made, based on the plasmid and oligo length.
11. Instead of heat, alkali denaturation can be used to denature a large plasmids (>7–8 kb). Mix 1–2 µg of plasmid in a 16-µL vol with 4 µL 2 *M* NaOH. Incubate at room temperature for 10 min and then neutralize with 4 µL 3 *M* NaOAc (pH 4.8). Add 70 µL ethanol and cool to –70°C. Centrifuge for 10 min to pellet the DNA. Carefully wash the pellet with 70% ethanol, and dry. Resuspend DNA in H_2O at a concentration of ≥10 ng/µL, and use immediately, or store at 4°C for up to several days. For annealing, the plasmid and primers are mixed and incubated at 37°C for 10 min, then at room temperature for another 10 min.
12. After denaturation, the reaction should be placed in an ice-water bath as soon as possible. Slower cooling seems to result in lower mutation efficiency.

13. *Dpn*I digestion is the key step in this protocol. The mutation efficiency is determined chiefly by the efficiency of *Dpn*I digestion. Important factors include salt concentration, *Dpn*I concentration, and digestion time.
    a. Salt concentration determines the *Dpn*I specificity on hemimethylated DNA. In a lower-salt-concentration buffer (NaCl <100 m*M*), *Dpn*I can cleave both fully methylated DNA and hemimethylated DNA. In a higher-salt-concentration buffer (150–200 m*M* NaCl), *Dpn*I only digests fully methylated DNA, and can leave the hemimethylated DNA intact ***(16)***.
    b. Addition of high-concentration DpnI should be avoided (such as directly adding over 1 µL undiluted DpnI to a reaction). The authors suspect that a nonspecific activity, similar to star activity (20) may appear. They authors have observed that prolonged digestion time can also result in nonspecific activity.
14. Troubleshooting:
    a. No colonies or very few colonies are produced. Possible explanations include: Too much *Dpn*I, too prolonged a digestion time, or too low a salt concentration. Take care to add *Dpn*I buffer before *Dpn*I and DNA are in contact; Competent cell transformation efficiency is too low: Use freshly made competent cells with a high transformation efficiency (>10^7 transformants/µg plasmid DNA).
    b. Many colonies but the mutation efficiency is low. The plasmid template might not have been fully denatured. Denature plasmid in 100°C for >3 min, or use the alkaline method to assure denaturation; *Dpn*I's activity is too low: Use a new batch of *Dpn*I.

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4

Multiple Site-Directed Mutagenesis In Vitro

Yang-Gyun Kim and Stefan Maas

1. Introduction

Polymerase chain reaction (PCR)-based site-directed mutagenesis (SDM) methods are widely used in molecular biology to selectively alter gene sequences ***(1)***. However, common protocols allow for the introduction of only one ***(1–3)*** or two ***(4)*** independent point mutations at a time, followed by the time-consuming phenotypic selection in bacteria cells and isolation of plasmid DNA, to identify the correctly targeted clones. Here is described an improved protocol for mutagenesis that allows introduction of multiple independent mutations with high efficiency, involving only one cloning step ***(5)***. The authors' procedure is based on the QuikChange™ SDM method ***(2)***, which combines PCR mutagenesis using *Pfu* DNA-polymerase and mutagenic primers, with the subsequent elimination of the template DNA by *Dpn*I digestion ***(2,3,6)***. The high fidelity of *Pfu* DNA polymerase minimizes undesirable mutations on newly amplified DNA during PCR and the selectivity of *Dpn*I for fully or hemimethylated 5'-G^{m6}ATC-3' sequences quantitatively degrades the parental DNA, resulting in high mutagenesis efficiencies ***(2,3,6)***.

As outlined in **Fig. 1**, we use repeated cycles of PCR-mediated SDM forgoing the time-consuming in vivo selection between mutagenesis steps. Instead, in vitro Dam-methylation and DNA ligation are performed, and thus multiple independent point mutations can be introduced in a much shorter period of time ***(5)***. Only one cloning step with DNA transformation and plasmid isolation is required after the final round of in vitro mutagenesis. Below is described the multiple mutagenesis procedure which can be applied to any DNA sequence for the introduction of multiple exchanges, insertions or deletions.

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ

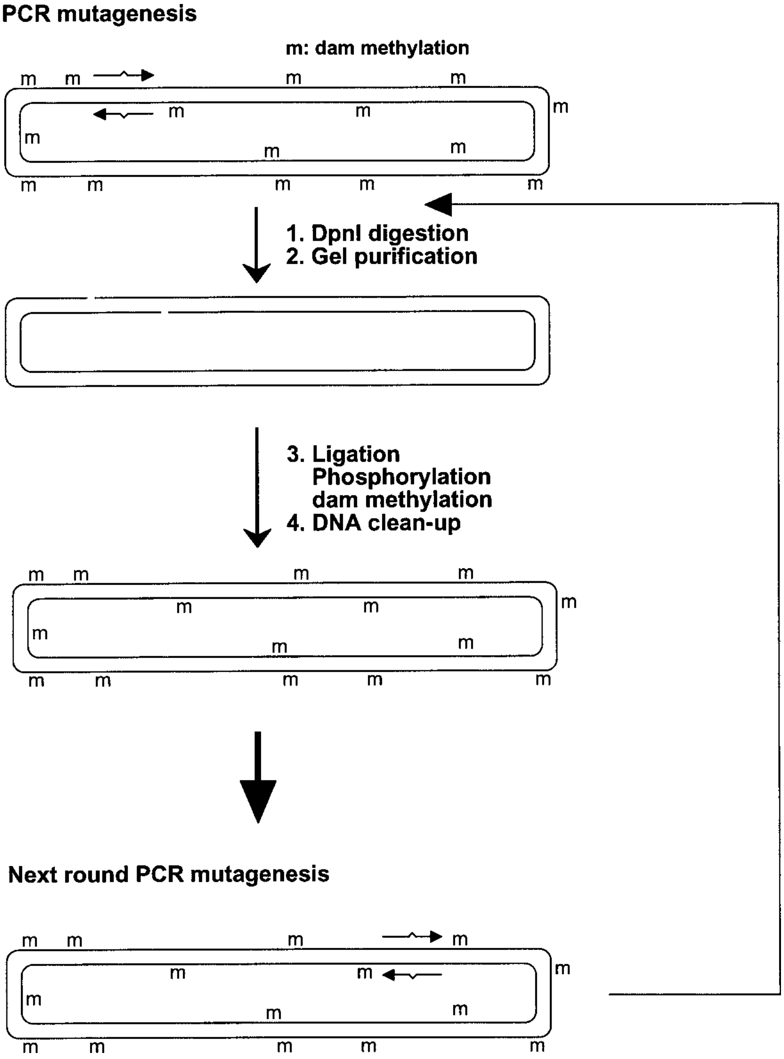
PCR mutagenesis
m: dam methylation
m
1. DpnI digestion
2. Gel purification
3. Ligation
Phosphorylation
dam methylation
4. DNA clean-up
Next round PCR mutagenesis

Fig. 1. Outline of the multiple-site mutagenesis protocol. Circular plasmid DNA with Dam-methylated sites (m) is used as template for the first round of PCR-mediated mutagenesis. The mutagenic primers are indicated by arrows. Following **step 1** and **2**, the majority of remaining DNA molecules is represented by unmethylated, nicked duplexes. The subsequent **steps 3** and **4** of methylation and ligation prepare the DNA for the next round of mutagenesis with a new set of mutagenic primers. The cycle is repeated until all mutations have been introduced. DNA resulting from either **step 1** or **2** (*see* **Notes 13** and **14**) is used for transfection into *E. coli* cells.

2. Materials

1. DNA plasmid template containing the cloned gene to be mutated prepared using standard protocols ***(7)***. 50 ng/μL stock solution in TE buffer. The DNA concentration is determined by spectrophotometry (1 OD_{260} = 50 μg/mL dsDNA).
2. Sets of oligonucleotide primers: 100 ng/μL stock solution in TE buffer (10 m*M* Tris-HCl and 1 m*M* EDTA, pH 8.0) (*see* **Note 1**).
3. Native *Pfu* DNA polymerase (Stratagene, La Jolla, CA): 2.5 U/μL.
4. 10X native *Pfu* DNA polymerase buffer: 200 m*M* Tris-HCl, 20 m*M* $MgCl_2$, 100 m*M* KCl, 60 m*M* Triton®X-100 and 100 μg/mL nuclease-free bovine serum albumin, pH 8.0 (Stratagene).
5. Deoxyribonucleoside triphosphate (dNTP) stock: 10 m*M* of each of deoxyadenosine triphosphate (dATP), deoxycytodine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate in TE buffer (10 m*M* Tris-HCl and 1 m*M* EDTA, pH 8.0).
6. *Dpn*I restriction enzyme (New England Biolabs [NEB], Beverly, MA): 10 U/μL.
7. Reagents for agarose gel electrophoresis.
8. TAE electrophoresis buffer (50X stock): 2 *M* Tris-acetate and 50 m*M* EDTA (pH 8.3).
9. Ethidium bromide solution: 10 mg/mL stock in sterile H_2O, store at 4°C in the dark.
10. 10X gel-loading dye: 50% sucrose, 1 mg/mL bromophenol blue, 2 mg/mL xylene cyanole.
11. DNA size markers: 1-kb ladder containing DNA fragments differing by 1 kb (Life Technologies, Gaithersburg, MD).
12. T4 polynucleotide kinase (NEB): 10 U/μL.
13. T4 DNA ligase (NEB): 400 U/μL.
14. *dam* methyltransferase (NEB): 10 U/μL.
15. 80 μ*M* S-adenosylmethionine (NEB).
16. 10X T4 DNA ligase buffer: 500 m*M* Tris-HCl, 100 m*M* $MgCl_2$, 100 m*M* dithiothreitol, 10 m*M* ATP and 250 μg/mL bovine serum albumin, pH 7.5 (NEB).
17. Qiaex II® gel extraction kit (Qiagen, Valencia, CA).
18. Qiaquick® PCR purification kit (Qiagen).
19. Competent cells: Frozen competent cells of *E. coli* strain, Epicurian Coli SURE (Stratagene), prepared according to Hanahan ***(8)***. Store in 200-μL aliquots at –80°C.
20. Luria-Bertani (LB) agar plate containing 100 μg/mL ampicillin.
21. 5-Bromo-4-chloro-3-indoly-β-D-galactoside stock: 200 mg/mL in *N,N*-dimethylformamide.

3. Methods

The multiple mutagenesis protocol consists of three basic steps:

1. PCR amplification, with one mutagenic primer set introducing the first mutation and digestion of template DNA with *Dpn*I.
2. DNA purification and in vitro methylation/ligation.
3. The final step of PCR-mediated mutagenesis and *Dpn*I digestion, followed by *E. coli* transformation, selection, and DNA isolation.

According to the desired number of independent mutations to be introduced, **steps 1** and **2** are repeated, using different sets of mutagenesis primers (*see also* **Note 12**).

3.1. Preparation of First-Round PCR Product

1. Design a set of mutagenic primers to introduce the desired change into the target DNA template (*see* **Note 1**). The primers are phosphorylated at their 5' ends, using T4 polynucleotide kinase ***(7)*** prior to use. Alternatively, the primers may be purchased already as 5'-phosphorylated derivatives.
2. Prepare the PCR reaction for the first round of mutagenesis using the primers described in **step 1**: 100 ng template DNA, 150 ng (15 pmol) of each primer, 200 m*M* each dNTPs and 2.5 U native *Pfu* DNA polymerase in 1X native *Pfu* DNA polymerase buffer (*see* **Note 2**), and with a total volume of 50 µL.
3. Add 25 µL of mineral oil to overlay the reaction (*see* **Note 3**).
4. Perform PCR reaction: Initial denaturation, 94°C, 2 min, followed by 15 cycles with each 30 s denaturation at 95°C, 1 min annealing at 55°C, and 2 min/kb extension (*see* **Note 4**) at 68°C, using the GeneAmp PCR thermocycler system 9600 (PE Biosystems, Foster City, CA) or an equivalent apparatus (*see* **Note 5**).
5. *Dpn*I digestion: Add 10 U *Dpn*I restriction enzyme into the 50 µL PCR reaction. Incubate reaction tube for 1 h at 37°C (*see* **Note 6**).

3.2. In Vitro Dam Methylation and Ligation

1. Run *Dpn*I-digested PCR reaction on 1.2% agarose gel at ~80 V in 1X TAE buffer, to separate undigested full-length DNA plasmid from digested DNA (*see* **Note 7**).
2. After staining with ethidium bromide and visualizing the DNA bands (*see* **Note 8**), excise the full-length DNA from the agarose gel, and purify DNA by the Qiaex II gel extraction kit (*see* **Note 9**).
3. Elute DNA with 55 µL dH_2O (*see* **Note 10**).
4. For the in vitro methylation/ligation reaction add 400 U T4 DNA ligase, 10 U T4 polynucleotide kinase and 10 U *E. coli* Dam methylase in 1X T4 DNA ligase buffer supplemented with 400 n*M* S-adenosylmethionine (*see* **Note 11**). The final volume is approx 60 µL.
5. Incubate the reaction for 1 h at 37°C.
6. Purify DNA by using Qiaquick PCR purification kit. Elute DNA with 55 µL dH_2O (*see* **Note 10**).

3.3. Preparation of Successive Rounds of PCR

1. Use 20 µL eluted DNA from **step 6** in **Subheading 3.2.** for the PCR reaction with the next set of mutagenic primers. Combine 150 ng (15 pmol) of each primer, 200 µ*M* of each dNTP, and 2.5 U native *Pfu* DNA polymerase in 1X native *Pfu* DNA polymerase buffer (*see* **Note 2**), in a total volume of 50 µL. Perform the amplification cycles according to the PCR conditions described in **Subheading 3.1., step 4** (*see* **Note 12**).
2. Digest with *Dpn*I (*see* **Subheading 3.1., step 5**).

3. If additional mutations are to be introduced (*see* **Note 12**), repeat protocol starting with **Subheading 3.2.**, for the next round of in vitro Dam methylation/ligation. Otherwise, proceed to **Subheading 3.4.** After the final PCR amplification, the mutagenized DNA need not be separated from the reaction mixture.

3.4. Transformation of E. coli *with Mutated DNA*

1. Use 1–2 µL eluted DNA from **step 2** of **Subheading 3.3.** for transfection into competent *E. coli* cells, using standard protocols ***(7),*** and streak out on selective LB agar plates containing an appropriate antibiotic (*see* **Notes 13** and **14**).
2. The transformation should yield ~0.1–10 × 10^2 colonies. Typically, >90% of the colonies should contain the multimutagenized DNA sequence (*see also* **Note 13**). Isolate plasmid DNA from bacterial clones, and sequence to confirm that the desired mutations have been introduced.

4. Notes

1. Both of the mutagenic primers must contain the desired mutation, and anneal to the same sequence on opposite strands of the plasmid. They may be designed to contain a point mutation, insertion, or deletion, as desired. The primers can be 25–45 bases in length, and should have at least 10–15 bases of complementarity on both sites of the mutation. This is especially important when multiple base mismatches are present. The melting temperature (T_m) of the primers should be ~10°C above the extension temperature of 68°C. The authors use the following formula for estimating the T_m of primers:

$$T_m \cong (2°C) \times (A + T) + (4°C) \times (G + C)$$

   Optimally, the primers should terminate in one or more C or G bases, for improved annealing properties. The consideration of the length and GC content of primers, in general, follows the instruction manual for the QuikChange™ SDM system (Stratagene). Purification of the mutagenic primers is not required.
2. Thermostable DNA polymerase: Although the authors use native *Pfu* DNA polymerase, other high-fidelity, thermostable DNA polymerases (such as *PfuTurbo* or Vent DNA polymerase) can be used for PCR mutagenesis, instead. *PfuTurbo* DNA polymerase would be especially suitable for amplification of long DNA templates.
3. Mineral oil is added to avoid evaporation during cycling. The use of mineral oil is advised because of the low reaction volume (50 µL) and long extension times, even though the PCR thermocycler used has a heated coverlid.
4. Extension time is calculated as 2 min/kb, based on the length of the DNA template. The mutagenesis procedure works best with target DNAs of 3–5 kb in size. DNAs over 6 kb tend to lower DNA yield during PCR mutagenesis. To overcome inefficient PCR mutagenesis when a long DNA template is used, longer extension times (up to 3 min/kb) are recommended (*see also* **Note 11**).
5. The listed PCR conditions are optimal for the PCR thermocycler indicated, with 0.2 mL thin-wall PCR tubes. Other types of thermocyclers may be used, but cycle parameters need to be adjusted.

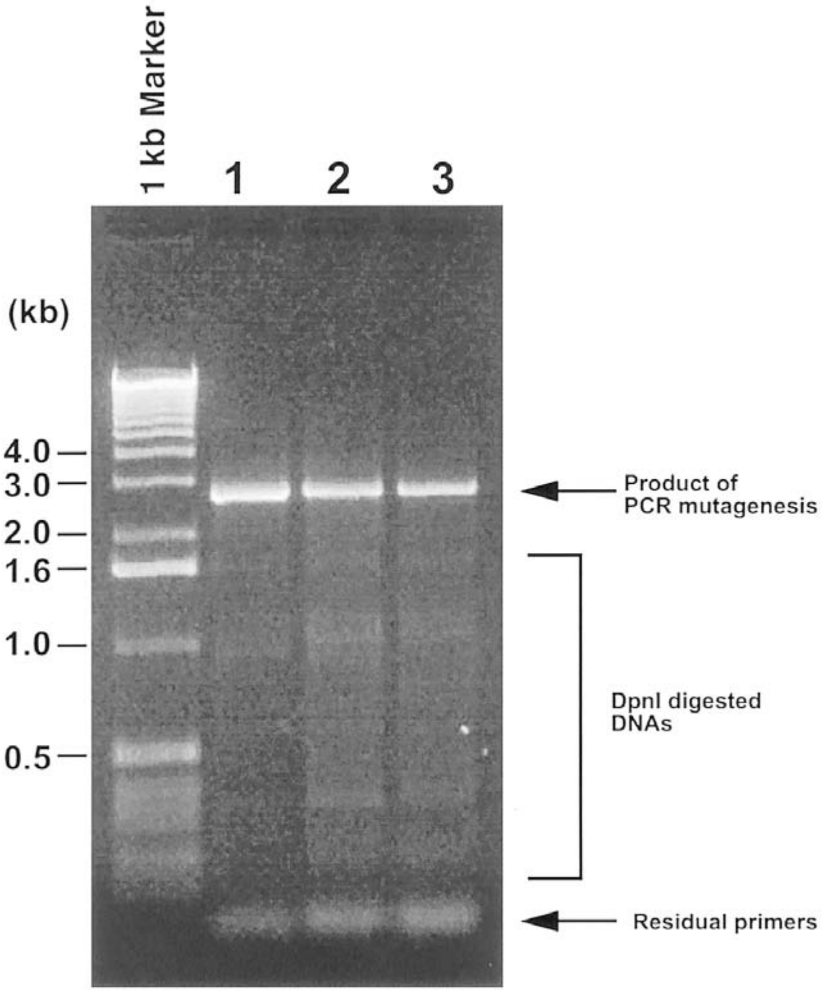
1 kb Marker
1
2
3
(kb)
4.0
3.0
2.0
1.6
1.0
0.5
Product of PCR mutagenesis
DpnI digested DNAs
Residual primers

Fig. 2. Agarose gel electrophoresis of *Dpn*I-digested PCR products after each step of multiple-site mutagenesis. 7 µL of the 50 µL DpnI-treated PCRs were loaded onto a 1.5% agarose gel after one (lane 1), two (lane 2), and three rounds (lane 3) of mutagenesis. This step corresponds to zero, one, or two cycles of intermittent in vitro methylation/ligation, respectively.

6. Avoid mineral oil: After completion of the PCR amplification, the reaction mixture may be transferred first into a new tube, to minimize residue mineral oil.
7. For control purposes, 10% of the PCR reaction from individual rounds of mutagenesis may be run out on an agarose gel, to monitor the yield of full-length, *Dpn*I-resistant PCR product during multiple mutagenesis (*see* **Fig. 2**). When an

amplification reaction yields insufficient DNA amounts (*see* **Note 14**), the product should be increased by scaling up the reaction, rather than increasing the number of PCR cycles (*see also* **Note 12**).

8. The necessary time for gel electrophoreses depends on the template lengths involved, but, for DNAs of 3–8 kb, a gel-running time of ~1 h suffices. For prompt detection and recovery of the mutagenized, *Dpn*I-resistant DNA from the gel, ethidium bromide can be included in the agarose gel.
9. Gel extraction: Various companies manufacture kits for DNA extraction from agarose gels, and these may all work equally well. In this lab, the Qiaex gel extraction kit was used with success.
10. Elution volume after Qiaex gel extraction or Qiaquick DNA purification kit: the final elution volume after the last spin will be ~50 µL when 55 µL dH_2O are used for the elution step.
11. Ligation, phosphorylation, and Dam methylation: The unit definitions of enzymes follow those of their manufacturers. Therefore, the unit numbers may be different if enzymes from other suppliers are used. T4 polynucletide kinase is active in the presence of 1 m*M* ATP contained in the T4 DNA ligase buffer, and Dam methylation is also efficient in T4 DNA ligase buffer supplemented with 400 n*M* S-adenosylmethionine.
12. Amount of DNA for the next round of PCR: As shown in **Fig. 2**, the yields of newly synthesized DNAs tend to decrease slightly with each cycle of mutagenesis. The reason for this may be the limited ligation and phosphorylation efficiency in vitro. To compensate for the diminished DNA yield, increase the volume of DNA template used for the subsequent round of PCR, starting after three cycles of mutagenesis.

    If the number of point mutations is large, then the authors recommend including a transformation and selection step after 4–6 rounds of in vitro methylation/ligation, to preserve high mutagenesis efficiency, especially when large DNA templates (>5 kb) are involved. This in vivo methylation/ligation procedure works efficiently, as follows:
    1. Transfect DNA from **step 2** of **Subheading 3.3.** into *E .coli*, according to the method of Hannahan ***(8)***.
    2. After plating and growing the bacteria at 37°C overnight, collect *E. coli* cells directly from the plate.
    3. Isolate plasmid DNA.
    4. Proceed to the next round of PCR mutagenesis.

    For the authors, this method proved to be highly efficient.
13. Options for DNA transfection into *E. coli*: The DNA for the final transfection does not need to be purified prior to use, but the authors observed that gel purification of *Dpn*I-digested DNA (as obtained after **step 3**, **Subheading 3.2.**) can improve the efficiency of mutagenesis by several percent.
14. Competent cells: The transformation efficiency of the competent bacteria cells needs to be high, for a successful experiment. With a control plasmid DNA, the transformation efficiency should measure at least 10^6 transformants/µg DNA, to

give several hundred colonies, when 1–2 µL DNA from a PCR mutagenesis reaction is used. Lack of colonies after transformation may result from low competence of *E. coli* cells or low yield in the PCR. In a successful mutagenesis experiment, intact-size DNA should be visible (>100 ng) when 7–10 µL *Dpn*I-digested reaction is loaded on an agarose gel (*see also* **Note 7**).

Acknowledgments

The authors are grateful to Dr. Alexander Rich for his support and for critically reading the manuscript. Research in the authors' laboratory was supported by a long-term fellowship of the European Molecular Biology Organization to Stefan Maas, and by grants to A. Rich from National Institutes of Health and the National Cancer Research Foundation.

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5

Two-Stage Polymerase Chain Reaction Protocol Allowing Introduction of Multiple Mutations, Deletions, and Insertions, Using QuikChange™ Site-Directed Mutagenesis

Wenyan Wang and Bruce A. Malcolm

1. Introduction

The invention of polymerase chain reaction (PCR) ***(1,2)*** provided a powerful tool to modify DNA sequences in genetic engineering. With numerous mutagenesis methods available, such as traditional sequential PCR ***(3)***, "megaprimer PCR"***(4–7)***, marker-coupled PCR ***(8)***, and so on, introducing changes to DNA sequences has become less tedious and more efficient. Recently, marketed site-directed mutagenesis (SDM) kits, such as Transformer™ Site-Directed Mutagenesis Kit (Clontech, San Francisco, CA), and Altered Site® II in vitro Site-Directed Mutagenesis Systems (Promega, Madison, WI), even eliminate the necessity of subcloning the amplified fragment.

One of the most broadly applicable protocols currently available is the QuikChange™ Site-Directed Mutagenesis System (QCM) developed by Stratagene (La Jolla, CA). This method allows the rapid introduction of point mutations into sequences of interest, using a pair of complementary mutagenesis primers to amplify the entire plasmid in a single PCR reaction. Destruction of the parental template plasmid by *Dpn*I digestion, followed by direct transformation into *Escherichia coli* cells, allows introduction of the desired mutation with high efficiency (70–90%), in as little as 24 h.

However, this approach is basically limited to primer pairs of 25–45 bases (with annealing/melting temperature [T_m] values, between the primer and the template, no lower than 78°C, as indicated in the accompanying

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ

literature, and confirmed in the authors' studies). When longer primers are attempted, the mutagenesis efficiency is drastically decreased, largely because of the more favorable primer dimer formation (100% complementary), compared with the primer–template annealing (i.e., because of the multiple mismatches). When multiple mutations, spanning longer sequences (e.g., five amino acid residues) are desired, two or more primer pairs and rounds of mutagenesis are needed, leading to higher cost and longer completion time.

A simple modification, "two stage PCR mutagenesis protocol,"***(9)*** is described, to address the problem. By performing two single-primer PCR reactions preceding the standard QCM procedure, the primer dimer formation is abolished in the first stage, and the DNA molecule, with the mutations generated in this stage, can serve as a PCR template in the following QCM step. The newly generated hybrid plasmids (i.e., with one wild-type and one newly synthesized mutant strand) allow the subsequent annealing of the mutagenesis primers to the template strands to be more efficient, because the primers can now hybridize to a perfectly matched, newly synthesized template strand. This method retains the simplicity and mutation efficiency of the original protocol. It not only overcomes the limitation of the primer length, but also provides a convenient and efficient approach to gene modifications, previously not possible with the standard QCM protocol, such as multiple and cassette mutagenesis, insertions, and deletions, irrespective of primer length and target DNA sequence (*see* **Notes 1** and **2**).

2. Materials

1. Complementary oligonucleotide primer pairs should be designed so that each hybridizing stretch flanking the area of mutation (insertion or deletion) has a T_m value ≥66°C, based on the following formula (*see* **Note 3** and **Table 1**):

$$T_m\ (^\circ\text{C}) = (\text{numbers of G or C}) \times 4 + (\text{number of A or T}) \times 2$$

2. All the primers should be purified by polyacrylamide gel electrophoresis (PAGE) (*see* **Note 4**). The primers are stored in TE (10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA) at –20°C at 100 µ*M* (storage) and 2 µ*M* (working stock) concentrations. The working stock of 2 µ*M* primers can be thawed and frozen several times. *Dpn*I restriction enzyme, *turbopfu* DNA polymerase, and XL-1-Blue *E. coli* competent cells can be purchased from Stratagene.

3. Method

The procedure consists of two stages. In stage 1, two extension reactions are performed in separate tubes, one containing the forward primer and the other containing the reverse primer. Subsequently, the two reactions are mixed, and the standard QCM procedure is carried out on the mixture.

Table 1
Oligonucleotide Primers Used in the Mutagenesis Experiments

Primer	Pairs	Primer sequences
P1[a]	B01F	GGCGCGGTATTATCC**T**G**A**GTTGACGCCGGGCAAG
	B02R	CTTGCCCGGCGTCAAC**T**C**A**GGATAATACCGCGCC
P2[a]	B03F	GTTCTGCTATGTGGCGCGGTATTATCC**T**G**A**GTTGACGCCGGGCAAGAGCAACTCGGTCGC
	B04R	GCGACCGAGTTGCTCTTGCCCGGCGTCAAC**T**C**A**GGATAATACCGCGCCACATAGCAGAAC
P3[a]	B05F	GTTCTGCTATGTGGCGCGGTA**GC**AT**GGTAG**G**C**TGACG**A**GGGGCAAGAGCAACTCGGTCGC
	B06R	GCGACCGAGTTGCTCTTGCCCC**T**CGTCA**G**C**CTACC**AT**GC**TACCGCGCCACATAGCAGAAC
P4[b]	B07F	GTTCTGCTATGTGGCGCGGTA(**TTATCCCGTGTTGACGCCGGGCAAGAGCAAC**)TCGGTCGCCGCATACACTATTC
	B08R	GAATAGTGTATGCGGCGACCGA(**GTTGCTCTTGCCCGGCGTCAACACGGGATAA**)TACCGCGCCACATAGCAGAAC
P5[c]	B09F	GTTCTGCTATGTGGCGCGGTA**GCCTAACCAAACGACGAGCGTGACACCACGG**TTACCCGTGTTGACGCCGGGC
	B10R	GCCCGGCGTCAACACGGGTAA**CCGTGGTGTCACGCTCGTCGTTTGGTTAGGC**TACCGCGCCACATAGCAGAAC

[a] Bases that are bold and underlined are designed mutations in the *bla* sequence.
[b] Bases that are marked in the brackets are the designed deletions in the *bla* sequence.
[c] Bases that are bold and underlined are the expected insertions in the *bla* sequence.

3.1. Stage 1

1. In two 0.5-mL thin-wall PCR tubes, assemble the pair of reactions, as follows: 50–200 ng template plasmid, 10 pmol one primer (forward or reverse), 0.2 m*M*/ each deoxyribonucleoside triphosphate mix, 5 µL 10X *Pfu Turbo* polymerase reaction buffer, add deionized water to total volume of 49 µL.
2. A hot start (preheat reactions to 95°C for 3 min) is recommended; use a thermocycler with a hot lid (94°C), or cover the reaction mix with mineral oil, to prevent evaporation during all PCR steps.
3. Add 2.5 U of *Pfu Turbo* DNA polymerase (1 µL), and mix well. 1–5 polymerization cycles are conducted: 95°C for 30 s, 55°C for 1 min, and 68°C for the length of time decided by the following equation:

   Extension time in minutes = 2 × length of the plasmid in kb

4. Reactions can be held at 4°C until ready for proceeding with Stage 2.

3.2. Stage 2

1. Following the completion of the single primer reactions, 50 µL of each reaction pair is mixed in one 0.5-mL thin-wall PCR tube, and subjected to 16 cycles of QCM reactions, as follows: 95°C for 30 s, 55°C for 1 min, and 68°C for the length of time decided above.
2. Reactions can be held at 4°C, until ready for *Dpn*I digestion.
3. Add 10 U *Dpn*I (per the QCM protocol), mix well, and incubate at 37°C for another hour, prior to transformation or agarose gel electrophoresis (**Fig. 1**).

4. Notes

1. This technique can also be combined with other PCR strategies, such as multiple round PCR mutagenesis without transformation ***(14)***, an approach to accelerate the introduction of multiple mutations, insertions, and deletions. The main requirement for the successful application of this strategy is that sufficient PCR product be generated in each mutagenesis reaction to be visualized and purified by agarose gel electrophoresis, thereby obviating the need for transformation and amplification prior to the subsequent round(s) of mutagenesis. By using the two-stage mutagenesis protocol, maximum PCR product is ensured.
2. Insertion of large DNA fragments (i.e., the megaprimer approach ***[4]***) can also be accomplished using the two-stage mutagenesis protocol. By carefully designing amplification primers to generate an appropriate fragment (the megaprimer) with ends suitable for insertion mutagenesis, and to optimize its concentration in the subsequent PCR reaction ***(15)***, entire genes may be directly inserted anywhere in a target plasmid (e.g., behind promoters, leader sequences, epitope tags, proteins, and so on). By using either asymmetric PCR (two separate amplification reactions, each with an excess of one of the primers) ***(16–19)***, or a strand-separating gel ***(20)***, single megaprimers may be generated for the two-stage mutagenesis protocol.

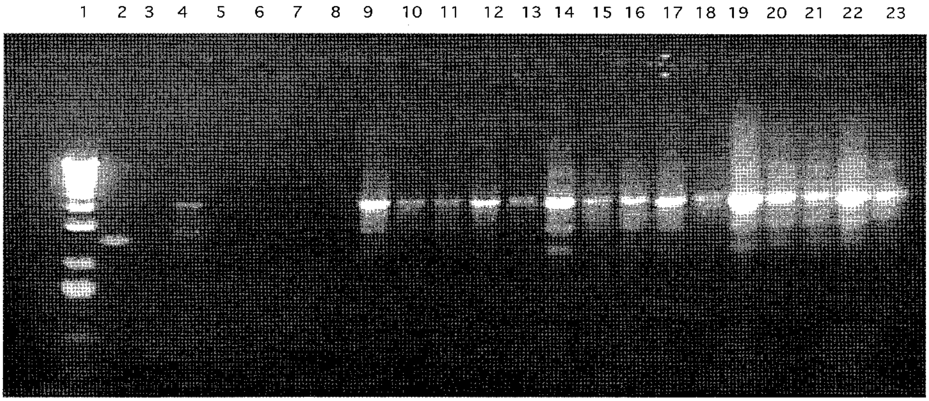

Fig. 1. Agarose gel electrophoresis of mutagenesis reactions. Lane 1, 1-kb DNA ladder standard (Gibco-Lifetech); lane 2, template plasmid PUC4K (Amersham-Pharmacia Biotech); lane 3, template plasmid PUC4K after *Dpn*I digestion; lanes 4–8, mutagenesis reactions using standard QCM procedure with primer pairs P1, P2, P3, P4, and P5, respectively; mutagenesis reactions using the modified protocol incorporating single primer extension reactions prior to QCM procedure: lanes 9–13, with one cycle of extension (primer pairs P1, P2, P3, P4, and P5, respectively); lanes 14–18, with three cycles of extension (primer pairs P1, P2, P3, P4, and P5, respectively); and lanes 19–23, with 10 cycles of extension (primer pairs P1, P2, P3, P4, and P5, respectively).

3. The general rules concerning primer design still apply. Ideally, the hybridizing sequences flanking the mutations in the primers should have similar T_m values (±4°C), and not be too GC-rich (i.e., %GC <70%). Strong hairpin structures (i.e., self-complementary sequences within the primer or primed region) are most problematic. As intramolecular interactions, they will tend to self-anneal, independent of the concentration, effectively lowering the concentration available for the desired priming of the template, even in the single-primer stage reaction. In this case, it is important to design the primers, and conduct the annealing reaction, to favor the thermodynamic equilibrium process, rather than the kinetic one. This problem can usually be fixed by appropriate silent codon changes, adding more primer (because the higher concentration promotes intermolecular annealing), and/or slowing down the ramping time (e.g., ~–0.5°C/min) from the denaturation to the annealing temperature (thus favoring the extended base pairing between the template and the primer more than the less stable self complementation. The touch down PCR approach ***(12,13)*** (an algorithm that gradually lowers the annealing temperature with each cycle from a few degrees above the predicted T_m) can also be applied to the first-stage PCR reaction, to minimize hairpin formation, and ensure more annealing between the primer and template. If the self-annealing DNA sequence cannot be modified (i.e., it is part of a required DNA sequence),

the primer length should be carefully designed so that the annealing temperature between the template and the primer is higher than that of the stem-loop structure within the template and/or the primer.

4. Unlike the primer titration strategy ***(10)*** and the use of partially overlapping primers to optimize priming, this current approach is universal irrespective of primer length and target sequences. However, the purity of the primers does affect the mutagenesis efficiency. Primers should be purified by PAGE, because of its high resolution and capacity. The purification step is relatively costly when it is done by commercial companies. Using the denaturing PAGE method described in **ref. *11***, one can easily perform the task oneself.

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6

Efficient and Accurate Site-Directed Mutagenesis of Large Plasmids

Susan A. Nadin-Davis

1. Introduction

Since the development of the polymerase chain reaction (PCR) technique ***(1)***, its potential use as a tool for site-directed mutagenesis (SDM) has been extensively explored, as illustrated by several chapters in this volume. In particular, the relative ease with which DNA fragments of 2–3 kb can be generated facilitated the development of the highly efficient procedure known as "recombination PCR" (RPCR), the principles of which were first described by Jones and Howard ***(2)*** and were later refined ***(3,4)***. Briefly, in this technique, the entire plasmid, containing the cloned gene of interest, is amplified as two overlapping fragments, each generated using a primer pair comprising a nonmutagenic primer directed to vector sequence and a mutagenic primer targeting the mutation site. Following separate amplification of the plasmid in two halves, using both primer sets, the complete plasmid is regenerated by recombination in vivo following cotransformation of the two fragments into competent recA$^-$ *Escherichia coli* cells. Since both DNA strands carry the desired mutation as directed by the mutagenic primers, the recovery of mutant clones should, in theory, approach 100%, although, in practice, mutant yields, ranging from 50 to 100% have been reported ***(3)***.

The advantage of this procedure is its rapidity, because traditional subcloning of fragments is avoided. One disadvantage is the requirement for synthesis of four different primers for the first mutagenesis reaction, although, with careful primer design, the nonmutagenic primers targeting the vector sequence can be used for subsequent multiple mutagenesis reactions, thereby reducing the number of primers needed to two/mutation. Another limitation that impacts any PCR-based strategy for SDM is the accuracy of the amplifica-

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ

tion reactions themselves. *Thermus aquaticus (Taq)* DNA polymerase, the first thermostable enzyme widely employed for PCR, was reported to lack a proofreading exonuclease activity and to exhibit a base substitution error rate of 1/9000, a value significantly higher than that observed for other DNA polymerases ***(5)***. During PCR, misincorporation rates by *Taq* polymerase were initially estimated at 10^{-4}/cycle ***(1)***, although it was later reported ***(6)*** that this value can be reduced to 10^{-5}/cycle by decreasing the concentrations of key components (deoxyribonucleoside triphosphate [dNTPs] and magnesium chloride) and limiting extension times. Notwithstanding these improvements, it has been proposed that the enzyme's error rate ultimately limits the length of fragments that can be efficiently generated, because of nucleotide misincorporation and the consequent immediate termination of extension of elongating DNA chains ***(7)***. Thus, the inherent properties of *Taq* DNA polymerase limit the size of fragments that can be generated, and consequently the size of plasmid amenable to SDM by RPCR. Although a plasmid of 7.1 kb was reported to be successfully mutated by RPCR ***(8)***, larger plasmids would pose an increasingly difficult challenge. Moreover, as the size of the plasmid increases, the risk of incorporating additional inadvertant mutations increases.

The discovery of additional thermostable DNA polymerases with proofreading capabilities, and hence higher fidelity, e.g., *Vent* DNA polymerase ***(9)***, promised to help resolve this dilemma. However, by themselves, these enzymes did not prove as successful for PCR as hoped, perhaps because they degrade the PCR primers as a result of their 3'-exonuclease activity ***(7)***. In 1994, Barnes ***(7)*** made the significant observation that the combination of *Taq* DNA polymerase with other thermostable proofreading polymerases, in the appropriate ratio, allows both efficient and accurate DNA synthesis, and facilitates the amplification of DNA fragments up to 35 kb in size, a phenomenon that came to be designated as "long distance PCR." Clearly, the application of long-distance PCR principles could facilitate the application of RPCR to SDM of plasmids of sizes >10 kb. This might be required, for example, when studying large genes, or when the gene of interest must be cloned into a vector that is itself of substantial size. The latter situation exists for transfer vectors, which are used to transfer genes into viral genomes via homologous recombination, as employed extensively in the generation of recombinant baculoviruses that direct heterologous protein expression in insect cells ***(10)***.

This chapter describes the successful application of RPCR and long-distance PCR principles to the SDM of a plasmid of 11.4 kb. This plasmid, pAcMP-RG, as illustrated in **Fig. 1**, comprises the pAcMP3 baculovirus transfer vector into which a rabies virus G gene, encoding the viral surface glycoprotein, has been cloned as a *Bam*HI expression cassette ***(11)***. A procedure for the mutagenesis of two sites, S1 and S2, of the glycoprotein gene is described.

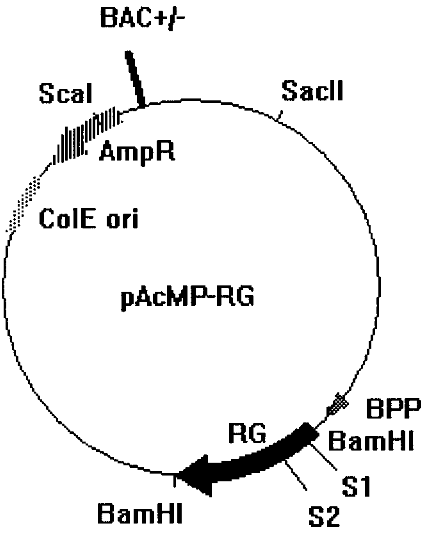

Fig. 1. Map of pAcMP-RG plasmid showing the *Bam*HI insert containing the rabies glycoprotein (RG) expression cassette under the control of the basic protein promoter (BPP), the position of the two mutagenesis sites (S1,2) within the rabies G gene, the two restriction endonuclease sites (*Sca*I and *Sst*II [or *Sac*II]) used for plasmid template linearization, and the position of the sequence targeted by the $BAC^{+/-}$ nonmutagenic primers within the pUC portion of the vector.

2. Materials

1. The plasmid, pAcMP-RG, was constructed at this laboratory, and stocks suitable for use as a template in the SDM procedure were prepared by cesium chloride gradient centrifugation (***12)*** (*see* **Note 1**).
2. Restriction endonucleases (*Sst*II and *Bam*HI) were obtained from Life Technologies (Gaithersburg, MD), or in the case of *Sca*I, from New England Biolabs (Mississauga, ON).
3. Primers (**Table 1**) were synthesized in-house by an Applied Biosystems 391 DNA synthesiser (Foster City, CA), and processed using an oligo prep OP120 instrument (Savant, Farmingdale, NY). Lyophilized, deprotected primers were dissolved in sterile H_2O and diluted to a working concentration of 10 μ*M* before use.
4. The Expand™ long template PCR kit was from Roche Molecular Biochemicals (Laval, PQ); the Expand enzyme is a proprietary formulation containing both *Taq* and *Pwo* DNA polymerases.
5. Dilute stocks of 100 m*M* dNTPs (Amersham Pharmacia Biotech) to a working dilution in 2 m*M* Tris-HCl, pH 7.5, containing all four dNTPs, each at 10 m*M.*
6. PCR was performed using a 9600 thermal cycler (Perkin-Elmer/Cetus).
7. Phenol (ultra-pure) (Life Technologies).
8. MAX Efficiency DH5α™ competent *E. coli* cells (Life Technologies).

Table 1
Primers Used for SDM

Function	Name	Sequence 5'–3'
Nonmutagenic	BAC^{+}	GCGCGTTTCGGTGATGACGGTGAA
	BAC^{-}	GTCAGAGGTTTTCACCGTCATCAC
Mutagenic	S1^{+}	GATGCACCAACTTGACTGGTTTCTCCTACTTGGA
	S1^{-}	TCCAAGTAGGAGAAACCAGTCAAGTTGGTGCATC
	S2^{+}	GAGAGCATCCAAAGGGAACAAGACTTGCGGATTTGTT
	S2^{-}	AACAAATCCGCAAGTCTTGTTCCCTTTGGATGCTCTC

Mutagenic bases are underlined.

9. Luria-Bertani (LB) medium (per L): 10 g tryptone (Difco, Becton Dickinson, Sparks, MD), 5 g yeast extract (Difco), 10 g NaCl, adjusted to pH 7.5 with 5 *N* NaOH. Autoclave.
10. LB-ampicillin agar plates (per L): 10 g tryptone, 5 g yeast extract (Difco), 10 g NaCl, adjusted to pH 7.5 with 5 *N* NaOH, and 15 g agar (Difco). Autoclave; cool to 50°C. Add 1 mL filter-sterilized 100 µg/mL ampicillin (Sigma). Pour into Petri dishes (25 mL/100 mm plate). Allow to solidify, then store plates (up to 1 mo) at 4°C.
11. SOC medium (per L): 20 g tryptone, 5 g yeast extract, 10 mL 1 *M* NaCl (10 m*M* final concentration), 0.25 mL 1 *M* KCl (2.5 m*M* final concentration). Autoclave, then cool. Add 20 mL of filter-sterilized 1 *M* $MgCl_2$ and 20 mL filter-sterilized 1 *M* glucose, before use.
12. 0.1X TE buffer: 1 m*M* Tris-HCl, pH 7.5, 0.1 m*M* EDTA.

3. Methods

3.1. Design of Primers for SDM

The procedure requires the design of two sets of primers; a pair of complementary nonmutagenic primers that target a portion of the plasmid vector, and a pair of complementary mutagenic primers for each mutagenesis reaction to be applied to the gene of interest. To maximize the cost-effectiveness of the procedure, the nonmutagenic primers should be designed to target a nucleotide sequence commonly encountered in a wide range of vector constructs. The two nonmutagenic primers, BAC$^{+/-}$ (*see* **Note 2**), employed in these studies target both strands of sequence collectively spanning bases 3–36 inclusive of the pUC18 vector ***(13)***. This portion of the pUC series of vectors has been employed in the construction of many other plasmids in current use, including the pAcMP3 transfer vector.

Design of the mutagenic primers will be dictated by the nature of the sequence to be mutated. The four mutagenic primers employed in the protocol reported here (*see* **Note 3**) were designed so as to introduce the following coding changes to the mature rabies glycoprotein (505 residues): S1, serine (TCT) to threonine (ACT) at amino acid residue 39; S2, serine·lysine·alanine (AGCAAGGCT) to asparagine·lysine·threonine (AACAAGACT), which introduces an extra glycosylation site at amino acids 204–206.

3.2. Preparation of Plasmid Template for PCR

The plasmid to be targeted for SDM must be digested, in separate reactions, by two restriction endonucleases, each targeting outside the respective amplicon sequences (*see* **Note 4**). In these studies, the restriction endonucleases, *Sca*I and *Sst*II (*Sac*II is an isoschizomer of this latter enzyme), were employed.

1. Digest aliquots of the plasmid template DNA in two separate reactions. In each case, mix 2 μg plasmid DNA, 1 μL bovine serum albumin (1 mg/mL), 1 μL 10X reaction buffer (supplied by the manufacturer with each enzyme), and 20 U enzyme, in a final volume of 10 μL.
2. Incubate digests at 37°C for 3 h.
3. Verify completion of digestions by analysis of a 1-μL aliquot of each digest by agarose gel electrophoresis (*see* **Note 5**).
4. Inactivate the restriction endonucleases by heating the digests at 65°C for 10 min and adding 30 μL 0.1X TE buffer: The final plasmid concentration is 50 ng/μL (*see* **Note 6**).

3.3. Amplification of Plasmid Templates

1. Perform PCR by following the general instructions provided with the Expand long template PCR kit. The various combinations of plasmid template and primer pairs employed for each of the two SDM reactions is provided in **Table 2**; note that each SDM requires two amplifications employing different template and primer combinations.
2. For each amplification, prepare two master mixes as follows:
   a. Master mix 1: Combine 5 μL 10X PCR buffer #1 (supplied in kit and provides a final $MgCl_2$ concentration of 1.75 m*M*), 1.75 μL 10 m*M* dNTPs (to a final concentration of 350 μ*M*) and 1.5 μL each of the two 10 μ*M* primer stocks (final concentration of 300 n*M*) in a volume of 25 μL.
   b. Master mix 2: Combine 1 μL DNA template (50 ng) and 0.75 μL Expand enzyme in sterile H_2O, to a final volume of 25 μL .
3. Combine master mixes 1 and 2 (50 μL final reaction volume) in thin-walled PCR tubes.
4. Place tubes in a 9600 thermal cycler for thermal cycling. The program utilized was: 93°C, 2 min, followed by 10 cycles of 93°C, 10 s, 65°C, 30 s, 68°C, 4 min, followed by an additional 15 cycles using similar parameters, except that the 4-min 68°C extension step was extended by 20 s/cycle, with a final additional extension period at 68°C for 5 min prior to a 4°C soak file (*see* **Note 7**).
5. Confirm the success of the amplification by analysis of aliquots (2–5 μL) of the PCR products by agarose gel electrophoresis.

Table 2
Summary of Use of Template and Primer Pairs for SDM of pAcMP-RG by RPCR

Site targeted	Template digestion	Primer pair	PCR product size (kb)
S1	*Sca*I	$BAC^+/S1^-$	5.0
S1	*Sst*II	$BAC^-/S1^+$	6.4
S2	*Sca*I	$BAC^+/S2^-$	5.6
S2	*Sst*II	$BAC^-/S2^+$	5.8

3.4. Purification of PCR Products (see *Note 8*)

1. Bring each remaining PCR product to a final volume of 100 µL by addition of sterile H_2O and transfer to a 1.5-mL microcentrifuge tube.
2. Extract once by mixing with 100 µL phenol, then separate the phenolic and aqueous phases by centrifugation (12,000*g* for 3 min) in a microcentrifuge.
3. Recover the upper aqueous phase, transfer to a fresh tube, and repeat the extraction, using a 1:1 mix of phenol:chloroform.
4. Recover the final aqueous phase to a fresh tube, and precipitate the DNA by the addition of 10 µL 3 *M* sodium acetate and 250 µL 95% ethanol. Invert several times, and place on dry ice for 30 min.
5. Spin the tube at 12,000*g* in a microcentrifuge for 15 min, remove the liquid, and rinse the pellet in 250 µL 70% ethanol. Dry the pellet.
6. Dissolve the DNA in 10 µL 0.1X TE buffer.

3.5. PCR Product Transformation into E. coli

1. For each SDM reaction, perform five transformations, each using a 50-µL aliquot of MAX Efficiency DH5α competent cells in a 1.5-mL microcentrifuge tube. The transformation procedure supplied with the cells was followed, with minor modifications, as detailed below (*see* **steps 2–7**).
2. Make the following additions to the five cell aliquots: to separate tubes (1 and 2), add 1 µL of one of the two paired PCR products, to tube 3, add 1 µL of both PCR products together, to tube 4, add 2 µL (20 pg) pUC 19 vector (supplied with the cells) as a positive control; and to tube 5, add 2 µL sterile H_2O (negative control).
3. Maintain the tubes on ice for 30 min.
4. Heat tubes at 42°C for 45 s, then replace them on ice for 2 min.
5. Add 0.45 mL SOC medium to each transformation, and gently agitate the cells at 37°C for 1 h.
6. For transformations 1–3 and 5, plate out 250 µL cells in duplicate onto LB-ampicillin agar plates. Plate out 100 µL 1/10 dilution for the pUC control transformation (tube 4).
7. Incubate agar plates overnight at 37°C.
8. Count the numbers of transformants on all agar plates. The presence of severalfold higher numbers of colonies on the plates receiving transformation 3 (both

PCR products), compared to those transformed with single PCR products (1 and 2) is indicative of successful homologous recombination (*see* **Note 9**).

3.6. Characterization of Plasmids from Recombinant Clones

1. Pick colonies (usually 6–12) at random from the LB-ampicillin agar plates, bearing cells transformed with both PCR products (3), and regrow clones in 2.5 mL liquid LB medium containing 100 µg/mL ampicillin, at 37°C overnight.
2. Transfer each culture to a 1.5-mL microcentrifuge tube (by sequential centrifugation of 2 × 1.25-mL aliquots), and use the cell pellet for plasmid DNA preparation (*see* **Note 10**).
3. Digest a 2–5-µL aliquot of the plasmid DNA, to confirm that its size and organization is similar to that of the parental vector. In these studies, *Bam*HI, which removes the rabies G gene insert from the pAcMP3 vector, was employed. Perform digestions as described in **Subheading 3.2.**
4. Select several independent plasmids with the correct size for nucleotide sequencing across the SDM site (*see* **Notes 11** and **12**). As for all SDM procedures, the incorporation of the desired mutation into the recovered plasmids should be confirmed by sequence determination.

4. Notes

1. Although plasmid DNA, prepared by cesium chloride gradient centrifugation, is an excellent source of starting material for these procedures, a number of alternative methods for plasmid preparation, including many commercial protocols, could be employed successfully. The main provision is that the plasmid can be cleaved efficiently by restriction endonucleases, and that it can then be subsequently amplified by PCR (i.e., the plasmid preparation should not contain PCR inhibitors).
2. The nonmutagenic primers, $BAC^{+/-}$, were designed to target pUC plasmid sequence, which is incorporated into a large number of different cloning vectors; thus, these primers can be widely employed for SDM by this protocol. They have a sequence overlap of 14 bases which, based on previous reports ***(2)***, should efficiently support homologous recombination. However, consideration must be given to the orientation of this pUC sequence within each vector, so as to determine the correct pairing of these primers with the mutagenic primers, thus ensuring successful PCR. For example, in the pAcMP-RG vector, amplification required the pairing of the BAC^{+} primer with mutagenic primers $S1^{-}$ and $S2^{-}$; SDM of this same rabies G gene, when cloned into the pAcYM1 plasmid, required pairing of the BAC^{-} primer with these same mutagenic primers ***(11)***. This is a consequence of the insertion of the promoter, which drives expression of the gene insert into these two transfer vectors, in opposite orientation with respect to the pUC sequence.
3. The primers employed here are long: the S1 primers contain 34 bases, with the single mutagenic base positioned 20 or 15 bases from the 3' end. These provide a sequence overlap, at the site of mutagenesis, of 34 bases, thus facilitating highly

efficient homologous recombination. S2 primers were slightly longer (37 bases), but incorporated two base substitutions within a six-base region. The positioning of the mutagenic base(s) well away from the 3' end ensures their protection from any proofreading activities of the DNA polymerase, a factor of greater concern than when using Taq DNA polymerase alone. However, it is possible to successfully utilize shorter primers in this procedure. A primer, S1$^+$ (a), composed of the 24 bases of the 5' end of primer S1$^+$, was tested, together with S1$^-$, for its ability to introduce the S1 mutation. Whilst absolute numbers of transformants were lower using the S1$^+$ (a) primer, the same mutation rate (100% of those sequenced) was obtained in the clones generated, regardless of which coding sense primer was used ***(11)***, which indicates that a mutagenic base at position 10 from the 3' end of the primer is efficient for this SDM protocol. The effect on successful SDM of further reductions in the size of the mutagenic primers, and the position of the mutagenic base with respect to the 3' end, was not examined, but these factors will certainly limit the extent to which shortened primer sequences can be employed before reduced efficiency of SDM is observed.

4. Cleavage of the plasmid is required to prevent copying of the complete circular vector by extension from each primer, a process that might severely compromise the efficiency of SDM, and to minimize the presence of circular nonmutated plasmid in the *E. coli* transformation process.The choice of enzymes will depend on the restriction map of the vector being used and the relative positions of the mutagenesis site and sequence target of the nonmutagenic primers. The enzyme should, of course, not cut sequence within the amplicon targeted by a specific primer pair, and, since amplification of the entire plasmid in two separate PCRs is needed, digestion of the vector by two separate enzymes is unavoidable. The information provided in **Table 2**, together with the vector map illustrated in **Fig. 1**, indicate how these considerations were met in this particular series of experiments.
5. Since circular plasmid DNA transforms cells very efficiently, complete digestion of target plasmid should be ensured. Otherwise, the small amount of intact plasmid that is carried through the procedure into the transformation will result in a high transformant background and low SDM efficiency. If digestion is incomplete, add additional enzyme (5–10 U), and continue incubation at 37°C for 1–2 h.
6. Removal of restriction endonuclease activity is not strictly necessary prior to initiating PCR, and it is recognized that not all restriction endonucleases are fully inactivated by 10 min heating at 65°C. The heat treatment, as well as the addition of EDTA to the reaction, helps to prevent any nonspecific nuclease digestion of the DNA template before amplification. During PCR, any residual nuclease activity will be quickly eliminated, upon the initiation of the thermocycling program.
7. These cycling parameters worked well for the primers employed in these studies. Obviously, adjustments may need to be made, especially to the annealing temperature, for primers of different lengths and base composition.
8. Contrary to a previous report on RPCR ***(3)***, purification of the PCR products prior to transformation into *E. coli* was found to be essential to permit transformant recovery. Initially, purification was done, as decribed here, by standard phenol:chloroform

extraction, followed by ethanol precipitation. However, more rapid commercial methods of PCR purification can be utilized; e.g., a Wizard PCR purification system (available from Promega, Madison, WI) has been successfully used.

9. The following tips may help in trouble-shooting problems encountered at this stage. Based on the use of MAX Efficiency DH5α competent cells, which provide a high transformation efficiency (up to 1 × 10^9/µg plasmid DNA), plates receiving transformations 1 and 2 typically yield <20 colonies/plate; transformation 3 yields >100 colonies/plate. The positive-control transformation (4) should generate several hundred colonies/plating; the negative-control transformation (5) should yield no colonies. If transformations 1, 2, and 3 yield few or no colonies, check the transformation efficiency of the cells achieved with transformation 4. If this value is much lower than expected, repeat the transformation with a new batch of cells; if this efficiency is close to expected check the yield of the PCR products (by agarose gel electrophoresis of 1-µL aliquots), to ensure that they were recovered during purification. If all plates, including no. 5, yield large numbers of colonies, this indicates that the selection medium is not functioning properly, and freshly prepared LB-ampicillin agar plates should be used. If similar numbers of colonies are obtained from transformations 1 and/or 2 and 3, one or both of the PCR products may contain trace amounts of intact plasmid. Either repeat the entire procedure using redigested plasmid as PCR template, or, alternatively, try digesting each of the PCR products with an appropriate restriction endonuclease (one which does not cleave the PCR product itself but does cleave the parental vector), repurify the PCR products, and repeat the transformations.
10. A Wizard Plus DNA purification system (Promega) was employed for plasmid DNA recovery into a final volume of 50 µL 0.1X TE buffer. Many alternative methods for small-scale plasmid DNA preparation are also available ***(12)***.
11. In these studies, manual cycle sequencing was performed using a fmol DNA sequencing system (Promega) employing ^{32}P-labeled primers directed to portions of the rabies virus G gene sequence ***(14)***. Many alternative manual sequencing strategies are available ***(12)***, and automated sequencing services are provided by many service laboratories.
12. Extensive study of the accuracy of this SDM technique (*see* **ref. *11***) reported that an overall mutation efficiency of 94.2% (49/52 clones examined) was achieved. Moreover, out of 16,200 bases sequenced, only one base substitution was observed: a C-to-T change in one clone close to the mutagenesis site. Based on these results, one could predict that plasmids significantly larger than 11.4 kb could be targeted by the SDM protocol described here. This high level of sequence fidelity is dependent on the nature of the thermostable DNA polymerase cocktail employed for PCR. Mixtures of such enzymes, other than the Expand system employed here, could yield higher or lower rates of spurious mutations.

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7

Combining Site-Specific Chemical Modification with Site-Directed Mutagenesis

Versatile Strategy to Move Beyond Structural Limitations of 20 Natural Amino Acids Side Chains in Protein Engineering

Grace DeSantis and J. Bryan Jones

1. Introduction

The invention of site-directed amino acid (AA) mutagenesis ***(1)*** has revolutionized the way in which enzyme mechanism and specificity can be probed, while simultaneously providing a route for engineering novel enzyme properties. Prior to this very precise method of AA replacement, protein chemists utilized chemical modification and chemical mutagenesis techniques to alter protein and enzyme properties ***(2–4)***.

However, chemical modification is often fraught with difficulties, because of the generation of heterogeneous product mixtures ***(5)***. By contrast, DNA based site-directed mutagenesis (SDM) and random mutagenesis techniques are limited to exchange of one AA for one of the 19 other natural AAs. Eukaryotes and, to a lesser extent, prokaryotes overcome this limitation by further expanding the structural and functional repertoire of the 20 common AAs via posttranslational modifications ***(6)***. The incorporation of unusual moieties into enzymes and proteins can provide unprecedented mechanistic insights, yield proteins with novel properties, and facilitate the study of structure–activity relationships of natural posttranslationally modified peptides and proteins.

This limitation of DNA directed mutagenesis has begun to be addressed. For example, the chemical synthesis of peptides containing unnatural AAs or modified AAs followed by the ligation of these peptides either chemically ***(7)*** or enzymatically ***(8,9)***, provides a route to proteins containing modified or unnatural AA side chains. However, since protein folding occurs concurrently

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ

with translation, and may be mediated by posttranslational events, proper function and folding may be precluded by this patchwork approach ***(10)***. Furthermore, the chemical synthesis of long stretches of peptides, especially those containing unusual AAs is not cost-effective on a large scale. An elegant in vitro transcription and translation approach to unnatural AA incorporation employs a tRNA, which is modified to contain an unnatural engineered anticodon, and which is chemically charged with an unnatural AA ***(11)***. Unnatural AAs have also been incorporated biosynthetically, by supplementing the growth medium of an expression system of an organism that is auxotrophic for the natural AA to be replaced with an unnatural analog ***(12,13)***. Recently, native chemical ligation has emerged as a promising strategy to ligate peptides, while incorporating additional diversity beyond the common 20 AAs ***(14,15)***.

1.2. Strategy and Theory

To address the 20 AA limitation of SDM and the lack of specificity of the chemical modification technique the authors have developed and implemented the chemical modification of mutant (CMM) proteins strategy. This technique can be undertaken successfully in any laboratory, without specialized equipment or expertise. It is an experimentally simple, robust, and inexpensive method by which unnatural AA side chains, or modifications thereof, can be incorporated specifically and cleanly into proteins.

As a representative template to develop the CMM methodology, the authors have employed the alkaline serine protease, subtilisin *Bacillus lentus* (SBL). However, the method is by no means limited to this protein. The strategy involves the introduction of one cysteine residue at a key active site position via SDM which is then thioalkylated with an alkyl methanethiosulfonate (MTS) reagent (CH_3SO_2S-R) to give a CMM enzyme, as illustrated in **Fig. 1**. MTS reagents react specifically and quantitatively with sulfhydryls ***(16–19)***. SBL is a particularly well-suited template for this approach, since it does not contain any natural cysteine residues. However, any protein that does not have a catalytically critical cysteine residue can be effectively modified using this strategy. Cysteine residues engaged in disulfide linkages, and those buried within the protein core, are protected from reaction with MTS reagents. Furthermore, if there are any exposed cysteines in the wild-type (WT) protein, one simply needs to mutate them to serine, this being a conservative mutation that is not expected to significantly alter activity, in most cases; then the uniquely reactive cysteine (s) for CMM derivatization can be introduced at the desired location(s).

The combined SDM chemical modification approach has emerged as a powerful and versatile technique for the creation of new enzyme active-site environments ***(20,21)*** for mechanistic studies ***(22,23)***, and to stabilize subtilisin against oxidation ***(24)***. The authors have reported on the utility of the CMM

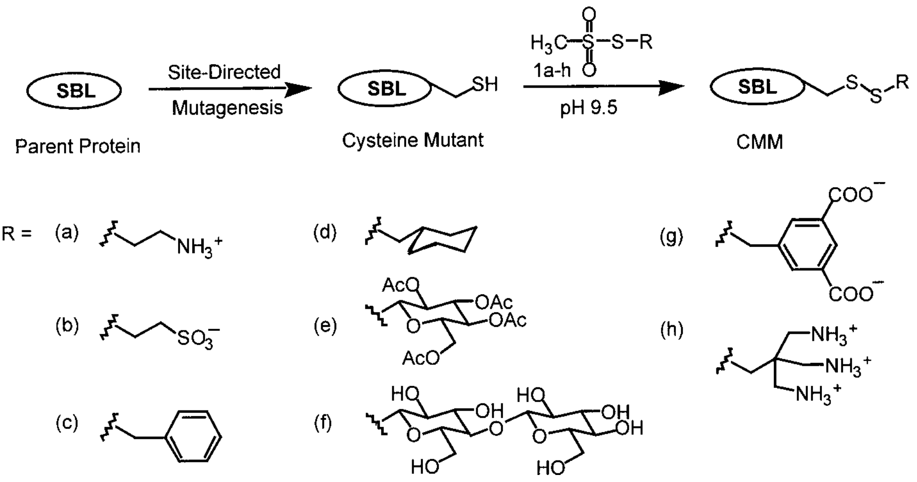
SBL
Parent Protein
Site-Directed
Mutagenesis
SBL
SH
Cysteine Mutant
$H_3C-\overset{O}{\underset{O}{S}}-S-R$
1a-h
pH 9.5
SBL
S
S
R
CMM
R =
(a)
NH_3^+
(b)
SO_3^-
(c)
(d)
(e)
AcO
OAc
OAc
O
AcO
(f)
HO
OH
O
O
HO
HO
OH
OH
O
HO
(g)
COO^-
COO^-
(h)
NH_3^+
NH_3^+
NH_3^+

Fig. 1. The CMM approach: A unique cysteine residue is introduced into a target protein by SDM. The introduced thiol side chain is then modified by reaction with a methanethiosulfonate (MTS) (CH_3SO_2S-R, **a–h**) reagent. This permits the introduction of an essentially infinite variety of –R groups, cleanly and quantitatively. Some representative examples are shown.

methodology to modulate activity ***(25,26)***, specificity ***(27)***, and pH activity profiles ***(28)*** of SBL in a predictable, monotonic fashion. The scope of this approach was recently demonstrated by its applicability to the difficult problem of generating well-characterized homogeneous neoglycoproteins ***(29)***. The amenability of this approach to generating a combinatorial library of CMMs in a controlled fashion ***(30)***, and toward the practical implementation of these CMMs as biocatalysts in preparative reactions, has also been demonstrated ***(31,32)***.

2. Materials

1. A protein that contains only one reactive cysteine residue. We use SBL as a representative example.
2. Column packed with Sephadex G-25 desalting matrix (cat. no. 17-0034-02, Pharmacia Biotech, Piscataway, NJ).
3. Column packed with SP Sepharose FF cation exchange matrix (cat. no. 17-0729-01, Pharmacia Biotech).
4. Running buffer A for Sephadex column: 20 m*M* sodium acetate, 5 m*M* $CaCl_2$, pH 5.2, filtered through 0.2-μ*M* membrane.
5. Dialysis exchange buffer: 20 m*M* N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 2 m*M* $CaCl_2$, pH 7.8.

6. Running buffer B for Sepharose column: 20 m*M* HEPES, 2 m*M* $CaCl_2$, pH 7.8, filtered through 0.2-µm membrane.
7. Elution buffer B for Sepharose column: 20 m*M* HEPES, 2 m*M* $CaCl_2$, 200 m*M* NaCl, 20 m*M* dithiothreitol (DTT) (Sigma, St. Louis, MO), pH 7.8, filtered through 0.2-µ*M* membrane.
8. Dialysis membrane. SPECTRA/POR®, MWCO 12,000-14,000 (Spectrum Medical, Los Angeles, CA).
9. Amicon ultrafiltration cell with a YM 10 filter (*see* **Note 1**) or Centricon® Centrifugal Filter Devices with a YM 10 filter (Millipore, Bedford, MA).
10. 2-(N-morpholino)ethanesulfonic acid (MES) storage buffer: 10 m*M* MES, 2 m*M* $CaCl_2$, pH 5.8 (*see* **Note 2**).
11. Bio-Rad (Bradford) protein dye concentrate and bovine serum albumin protein standard (Hercules, CA).
12. Several MTS reagents are commercially available from Toronto Reseach (North York, ON, Canada). For example, methyl MTS, 2- (aminocarbonyl)ethyl MTS, benzl MTS, N- (β-D-glucopyranosyl-N'-[2-methanethiosulfonyl)ethyl)urea. A fresh 0.2 *M* aqueous solution is recommended. If the MTS reagent is not water-soluble, a water-miscible organic solvent, such as ethanol, methanol, dimethyl sulfoxide, or dimethylformamide, may be used.
13. For the preparation of MTS-R reagents: Mesyl chloride, sodium sulfide and various alkylhalides, absolute ethanol, anhydrous DMF, diethyl ether, brine, Aldrich Chemical Co. (Milwaukee, WI), Merck silica gel 60 (230-400 mesh). Equipment: hot plate and oil bath or heating mantel, round bottom flask and water jacketed condenser, mortar and pestle, rotary evaporator, vacuum pump equipped with manifold, glass column for flash chromatography.
14. 2-(N-cylcohexylamino)ethanesulfonic acid (CHES) modification buffer: 140 m*M* CHES, 2 m*M* $CaCl_2$, pH 9.5.
15. MES quench buffer: 5 m*M* MES, 2 m*M* $CaCl_2$, pH 6.5.
16. Disposable PD-10 Sephadex G-25 desalting columns (cat. no. 17-0851-01, Pharmacia Biotech).
17. End-over-end rotator or shaker.
18. Pharmacia PhastSystem, Native Buffer Strips, and Gradient PhastGel (8–25%, or other appropriate range) (Pharmacia Biotech). Any other polyacrylamide gel electrophoresis (PAGE) apparatus available may be used.
19. Ellman's reagent (5,5'-dithio-*bis* (2-nitro-benzoic acid) 2.5×10^{-4} *M* in phosphate buffer 0.25 *M*, pH 6.9, stored at 4 °C (Sigma).
20. Fast protein liquid chromatography (FPLC) (Bio-Rad, Biologic System), FPLC column packed with source 15 reverse-phase chromotography matrix (cat. no. 17-0727-20 from Pharmacia).
21. FPLC Running buffer: 5% acetonitrile, 0.01% trifluoroacetic acid (TFA), filtered through 0.2-µm membrane.
22. FPLC Elution buffer: 80% acetonitrile, 0.01% TFA, filtered through 0.2-µm membrane.

3. Method

3.1. Site-Directed Mutagenesis

Several efficient and rapid methods for the generation and expression of site-directed mutants have been developed and are well-described ***(1)***. In addition, numerous commercial mutagenesis and expression kits are available.

3.2. Protein Purification (33)

1. Purify the crude protein concentrate of SBL, which contains polyethyleneglycol (50%) as a stabilizer, by passing it through a Sephadex G-25 desalting matrix with running buffer A, to remove small mol-wt contaminants.
2. Pool the fractions from the desalting column and dialyze (SPECTRA/POR, MWCO 12,000–14,000, dialysis membrane) twice against 2 L dialysis buffer for 3 h, at 4°C.
3. Apply dialyzed enzyme sample to a strong cation exchange column (SP Sepharose FF) in running buffer B. Elute the enzyme with a one-step gradient of 0–200 m*M* NaCl elution buffer B.
4. Dialyze the enzyme 3× for 3 h against 2 L Storage buffer, at 4°C.
5. Further concentrate the enzyme to about 25 mg/mL, using an Amicon ultrafiltration cell with a YM 10 filtration membrane.
6. Aliquot enzyme in 0.5 or 1.0 mL vol, flash-freeze in N_2 *(l)*, and store at less than –20°C.

3.3. Determination of Protein Concentration

Determine the concentration of SBL, using the Bio-Rad dye reagent kit, according to the manufacturer's directions. Use serial dilutions of bovine serum albumin as a protein standard.

3.4. Synthesis of Representative MTS Reagents

3.4.1. Preparation of Sodium MTS (16,17)

1. To a solution of $Na_2S\cdot 9H_2O$ (142.2 g, 0.592 mol) in H_2O (150 mL), contained in a 250-mL roundbottomed flask equipped with a water jacketed condenser, and heated to 80°C, add mesyl chloride (46.6 mL, 0.602 mol) dropwise via syringe.
2. After the addition, heat the reaction mixture under reflux for 15 h. The reaction mixture turns from pale yellow to yellow, and some yellow precipitate is formed.
3. Cool the reaction mixture to room temperature, then evaporate the H_2O on a rotary evaporator.
4. Grind the solid residue with a mortar and pestle, then further dry the powder under vacuum (1 torr) at 50°C.
5. Triturate the powder in four portions with EtOH (700 mL).
6. Concentrate the EtOH filtrate, then cool it with an ice bath, to obtain a precipitate, which is collected by vacuum filtration. Further concentrate the filtrate to obtain a second crop of precipitate. After repeated concentration and filtration (4×), the final volume of the filtrate is approx 10 mL.

7. Dissolve the combined precipitates in absolute EtOH at room temperature, and filter to remove trace amounts of Na chloride and Na sulfide. Concentrate the filtrate, and cool, then collect the precipitate by vacuum filtration. Repeat the concentration, cooling, and filtration process 3×, to give white, flaky crystals, which are dried further at 1 torr overnight, (24.51 g, 31%) IR (KBr): 3004, 2916, 1420, 1326, 1203, 1095, 980, 772/cm. ^{1}H NMR (200 MHz, D_2O): δ 3.23 (s). ^{13}C NMR (50 MHz, D_2O, with dimethylsulfoxide d_6 as an internal standard): δ 39.72 ppm (*see* **Note 3**).

3.4.2. Preparation of Cyclohexylmethyl MTS **(25)**

1. Heat a stirred reaction mixture of bromomethylcyclohexane (1.56 g, 0.00881 mol), sodium MTS (1.18 g, 0.00881 mol) and dry dimethyl formamide (DMF) (6 mL) at 50°C for 24 h.
2. After cooling the reaction mixture to room temperature, transfer the mixture to a separatory funnel, add H_2O and extract with ether (3 × 30 mL). Wash the ether extract with brine, dry over anhydrous magnesium sulfate, filter, then concentrate.
3. Subject the residue to flash column chromatography ***(33)*** on silica gel with EtOAc-hexanes (1:4),to afford a colorless oil (1.5033 g, 82%). IR ($CDCl_3$ cast): 3030 (w), 3012 (w), 2926 (st), 2853 (st), 1446 (m), 1410 (m), 1320 (st), 1134 (st), 955 (st), 746/cm (st); ^{1}H NMR (200 MHz, $CDCl_3$): δ 3.32 (s, 3H, C**H**$_3SO_2S$), 3.07 (d, J = 6.9 Hz, 2H, SC**H**$_2$CH), 1.95–1.55 (m, 6H), 1.40–0.90 (m, 5H); ^{13}C NMR (50 MHz, $CDCl_3$): δ 50.42, 43.30, 37.83, 32.43, 26.02, 25.82.

3.4.3. Preparation of Isopropyl MTS **(25)**

1. To a solution of diisopropyl disulfide (3.0 g, 0.02 mol) in aqueous acetone (75 mL, 50:50), add a solution of silver nitrate (4.25 g, 0.025 mol) and sodium MTS (2.53 g, 0.025 mol) in the same solvent mixture (75 mL). Heat the reaction mixture under reflux for 9 h.
2. After cooling the reaction mixture to room temperature, filter off the solid, then concentrate the filtrate, to remove most of the acetone.
3. Extract the aqueous residue with ether (3 × 30 mL). Wash the etheral extracts with brine, dry the organic phase over anhydrous magnesium sulfate, filter, then concentrate.
4. Subject the residue to flash chromatography ***(34)*** on silica gel with ethyl acetate (EtO-Ac)-hexanes (1:4), to obtain the product as a colorless oil (2.012 g, 65%). IR (film): 2974 (m), 2928 (m), 2871 (w), 1462 (m), 1320 (st), 1134 (st), 1057 (m), 956 (st), 692/cm (st); ^{1}H NMR (200 MHz, $CDCl_3$): δ 3.73 (heptet, J = 6.9 Hz, 1H, SC**H**Me$_2$), 3.34 (s, 3H, C**H**$_3SO_2S$), 1.49 (d, J = 6.9 Hz, 6H, SCH**Me**$_2$); ^{13}C NMR (50 MHz, $CDCl_3$): δ 51.43, 43.15, 23.81.

3.5. Site-Specific Chemical Modification (25–27)

1. In a 5-mL polypropylene tube, which has been coated with polyethelene glycol 10,000, then dried, mix: 1.2 mL mutant SBL (~25 mg/mL) in storage buffer, 1.2 mL CHES modification buffer, and 100 µL 0.2 *M* solution of MTS reagent. Gently agitate the mixture at 20°C, using an end-over-end rotator. Run blank reactions containing 100 µL solvent, instead of the reagent solution in parallel.

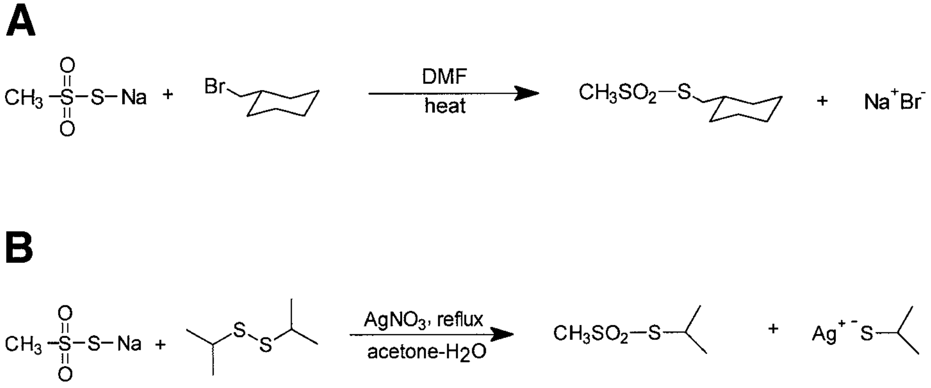

Fig. 2. Synthetic methods for preparation of alkyl MTS. **(A)** Substitution method: for primary –R groups. **(B)** Disulfide exchange method: for secondary –R groups.

2. Monitor reaction progress by determination of residual free thiol content, using Ellman's reagent. At regular time intervals, withdraw 10 µL of the reaction mixture, then test for residual free thiol content by mixing with 10 µL Ellman's reagent (2.5 × 10^{-4} *M*). The absence of yellow by visual inspection indicates complete reaction, usually within 20 min. At this point add a further 100 mL MTS solution, and allow reaction to proceed for a further 30 min (*see* **Note 4**).
3. After establishing the absence of no further change in free thiol concentration, apply the 2.5-mL reaction mixture to a disposable PD-10 Sephadex G-25 *M* desalting column, which was pre-equilibrated with MES quench buffer. After letting the mixture flow into the column, elute the CMM with MES quench buffer (3.5 mL), then dialyze 3× for 3 h against MES storage buffer (3 × 1 L) at 4°C (*see* **Note 4**).

3.6. Polyacrylamide Gel Electrophoresis

Analyze the modified enzymes, together with the parent cysteine mutant and the WT enzyme, by nondenaturing gradient (8–25%) PAGE. For SBL, run samples towards the cathode on the Pharmacia Phast-System, using pH 4.2 buffer strips. Develop gels with Coomassie brilliant blue. Each CMM should appear as one single band (*see* **Note 5**).

3.7. Electrospray Mass Spectrometry

Prior to electrospray mass spectometry analysis, purify the CMMs by FPLC (Bio-Rad, Biologic System) on a Source 15 RPC matrix with FPLC running buffer, and elute with FPLC elution buffer in a one-step gradient. Electrospray mass spectra may be recorded on a PE SCIEX API III Biomolecular Mass Analyzer (*see* **Note 6**).

3.8. Regeneration of Unmodified Enzyme by Treatment with β-Mercaptoethanol or DTT

To a solution of CMM (2.0 mg) in 250 μL CHES-buffer (70 m*M* CHES, 5 m*M* MES, 2 m*M* $CaCl_2$, pH 9.5), add 10 μL solution of β-mercaptoethanol or DTT (1 *M* in 95% EtOH or H_2O, respectively). This procedure should restore the full activity and the original mass of the unmodified cysteine mutant parent.

3.9. Free Thiol Titration to Assess Completion of Reaction

Dilute the analyte CMM with 0.25 *M* phosphate buffer, pH 8.0, to a volume of 800 μL in a 1-mL cuvet. Determine the absorbance of the protein solution at 412 nm, and use as a blank. Then add 200 μL Ellman's reagent (2.5×10^{-4} *M* in 0.25 *M* phosphate buffer, pH 6.9, ε_{412} = 13600/M/cm). Allow the mixture to equilibrate for 15 min before reading the new absorbance at 412 nm.

4. Notes

1. Store the YM10 membrane at 4°C in 10% EtOH and wash with dH_2O before use.
2. SBL and its CMMs are dialyzed against buffer containing $CaCl_2$, to preserve the bound and structurally critical Ca^{2+}. However, the CMM should be dialyzed against any appropriate buffer (low salt and concentration), in which the particular protein is stable, then stored in small aliquots.
3. Secondary and tertiary halides (even iodides) did not undergo substitution reactions, because of the steric congestion at the substitution site. Instead, silver nitrate assisted reaction of dialkyl disulfide with sodium methanesulfinate is applied to prepare secondary alkyl MTS. With tertiary compounds, such as di-*tert*-butyl disulfide, no product was isolated, probably because of the cleavage of the S-C bond, resulting in the formation of the more stable tertiary carbocation.
4. The modification of cysteine residues in poorly accessible protein cavities, or on the interior, can be accomplished by unfolding or partially unfolding the protein, followed by refolding. However, some proteins will not refold readily. The authors accomplished modification of the M222C mutant of SBL, which is in a partially buried pocket, by modifying this mutant in the presence of 1.0 *M* guanidinium chloride. Extensive dialysis was then used to remove the denaturant. For very precious or expensive MTS-R reagents, one may use as little as 5 mM MTS reagent.

   In addition, when using very base labile MTS modifying reagents, e.g., **1e**, the modification can be performed at neutral pH. In both cases, care should be taken to ensure that reaction is complete.

   The base labile nature of acetate-protected sugar MTS reagent **1e**, and its facile deprotection by the esterase activity of subtilisin itself, was exploited to effect the *in situ* deprotection, thus generating a neoglycoconjugate of SBL ***(29)***.
5. Run gel of crude and purified samples in parallel, for comparison. This will show if any dimer is present. Surface exposed cysteines, such as for S156C-SBL, may dimerize during concentration, and may require the addition of small amounts

(less than 1 m*M*) of DTT to prevent this. Alternatively, lyophilization may be used in lieu of freezing, because this does not seem to promote dimerization as quickly. Under native nondenaturing conditions, protein separation occurs on the basis of charge and mass, therefore, introduction of a charged –R group will alter the electrophoretic mobility of the resultant protein.

6. Matrix-assisted laser desorption time-of-flight mass spectrometry may be used instead of electrospray mass spectometry. The former is less sensitive to interfering ion contaminants. However, the latter is preferable, since it yields a better indication of the ratio of component protein modification products ***(35)***. In the absence of an FPLC system for sample preparation, extensive dialysis against dH_2O may be used.
7. The CMM approach is readily amenable to combinatorial microtiter (96-well) plate format, by simply reducing the modification scale ***(30)***.

Acknowledgments

The authors would like to thank Per Berglund, Ben Davis, Michael Dickman, Marvin Gold, David Hodgson, Richard Lloyd, Kanjai Khumtaveeporn, Kazutsugu Matsumoto, Erika Plettner, Rafael Sala, Andreas Seger , Michele Stabile, Xiao Shang, and Astrid Ullman, of the University of Toronto and Rick Bott, Tom Graycar, Colin Mitchinson, Christian Peach, and Karl Sanford of Genencor for their seminal contributions to developing this area of research. The authors are grateful to Genencor, the Natural Sciences and Engineering Research Council of Canada (NSERC), and the University of Toronto for generous financial support. The authors also thank Genencor for providing WT and mutant SBL.

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8

Site-Directed Mutagenesis Mediated by a Single Polymerase Chain Reaction Product

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1. Introduction

Site-directed mutagenesis (SDM) by deletion of a unique restriction site, introduced by Deng and Nickoloff ***(1)***, allows site-specific mutagenesis of a plasmid DNA without any subcloning steps. This procedure uses two mutagenic primers: one carries the desired mutation; the second, acting as a selection primer, carries a mutation in a unique, nonessential restriction site in the target plasmid. The method relies on the simultaneous annealing of two primers (mutagenic and selection primers) to one strand of the denatured double-stranded plasmid DNA. After DNA elongation and ligation, plasmids lacking the selection-restriction site are expected to encode the desired second site mutation. This strategy is efficient on a substantial number of templates; however, the authors have found that, in the case of some plasmids, the recovery of the desired mutation is much lower, yielding the desired mutant products at a frequency of less than 10%. The reason for this low efficiency may stem from the nucleotide sequence of the target DNA, which may acquire stable secondary structures, such as stem-loops. These nucleotide structures may interfere with the annealing of the mutagenic primers, and could result in low yields of the mutant plasmids.

In order to circumvent this limitation, the authors developed a mutagenesis method based on polymerase chain reaction (PCR)-fragment directed DNA synthesis. The procedure relies on the use of selection and mutagenic primers to amplify a nucleotide region lying between the annealed primers, thus generating the desired mutant DNA fragment, which is then used as a primer for the subsequent production of heteroduplex DNA, which is subsequently introduced into a *mutS Escherichia coli* strain. Next, plasmid DNA preparation from the

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ

culture of transformed bacterial cultures is treated with the appropriate restriction enzyme; plasmids carrying mutations at the unique site remain circular upon restriction enzyme digestion; nonmutant plasmids are linearized. When the mixture of circular and linearized DNA is subsequently introduced into *E. coli*, the circular mutant plasmids are efficiently recovered. Since the amplified DNA fragment contains both the desired and the selection mutations, this method results in a high efficiency of SDM. A mutagenesis efficiency of more than 80% is routinely obtained by using this strategy ***(2)***.

2. Materials

All chemicals should be molecular-biology-grade. All solutions should be made by using DNAse-free double-distilled or deionized H_2O.

2.1. PCR Amplification

1. 10X reaction buffer: 200 m*M* Tris-HCl, pH 8.0, 100 m*M* KCl, 60 m*M* ammonium sulfate, 20 m*M* $MgCl_2$, 1% Triton X-100, 100 mg/mL bovine serum albumin.
2. *Pfu* DNA polymerase (Stratagene).
3. 2 m*M* of each deoxyribonucleoside triphosphate (dNTP) (Roche Molecular Biochemicals).
4. Minute PCR Prep DNA Purification Kit (Chimerx).
5. QIAprep Spin Miniprep Kit (Qiagen).
6. 10X phosphorylation buffer: 500 m*M* Tris-HCl, pH 7.5, 100 m*M* $MgCl_2$, 50 m*M* dithiothreitol (DTT), and 10 m*M* adenosine triphosphate (ATP).
7. T4 polynucleotide kinase (Stratagene).

2.2. Mutagenesis of Double-Stranded DNA

1. 10X Annealing buffer: 200 m*M* Tris-HCl, pH 7.5, 100 m*M* $MgCl_2$ and 500 m*M* NaCl. 10X DNA synthesis buffer: 100 m*M* Tris-HCl, pH 7.5, 5 m*M* dNTPs, 10 m*M* ATP, and 20 m*M* DTT.
2. T4 DNA polymerase (3 U/µL, New England Biolabs [NEB]).
3. T4 DNA ligase (5 U/µL, Roche Molecular Biochemicals).
4. BMH71-18 and DH5α *E. coli* strains (Clontech).
5. Electroporation apparatus with a pulse controller (Bio-Rad).
6. Chilled electroporation cuvets, 0.2-cm electrode gap (Bio-Rad).
7. SOC medium: 0.5% (w/v) yeast extract, 2% (w/v) tryptone, 10 m*M* NaCl, 2.5 m*M* KCl, 10 m*M* $MgCl_2$, 20 m*M* $MgSO_4$, 20 m*M* glucose.
8. Luria-Bertani (LB) medium with appropriate antibiotics.
9. Appropriate restriction enzymes and buffers.

3. Methods

3.1. Production of Mutant Fragment by PCR

1. Primer design and synthesis: primer 1 includes the desired mutation (mutagenic primer); primer 2 carries a base substitution in any unique, nonessential restric-

tion site in the target plasmid (selection primer). Flanking sequences at both ends of the mutant site should contain at least 10 bases that are homologous to the template DNA (*see* **Note 1**).
2. Assemble the reaction in a thermal cycle tube as follows: 3 fmol template DNA; primers, 30 pM each (*see* **Note 2**); 10 µL 10X reaction buffer; 10 µL 2 m*M* dNTPs; 5 U *pfu* DNA polymerase; add H_2O to final volume of 100 µL.
3. Overlay the reaction with 50 µL light mineral oil.
4. PCR amplify under the following conditions: one cycle of 95°C for 5 min; 25 cycles of 94°C for 1.5 min; 50°C for 2 min; 72°C for 4 min; one cycle of 72°C for 10 min (*see* **Note 3**).
5. Analyze 10 µL PCR reaction products by conventional agarose gel electrophoresis. This provides information concerning the size of the amplified fragment.
6. Purify the PCR product by using Minute PCR Prep DNA Purification Kit.

3.2. Phosphorylation of PCR Products

PCR fragment is phosphorylated by incubating 3–10 pmol primers, 1X phosphorylation buffer, and 5 U of T4 polynucleotide kinase in a volume of 10 µL for 1 h at 37°C.

3.3. DNA Elongation, Digestion, Ligation, and Electroporation

1. 10 µL solution containing the phosphorylated fragments (*see* **Note 4**), 50 ng template plasmid, and 1X annealing buffer, is heated to 100°C for 3 min and immediately quenched in ice for 10 min. Fragment–directed DNA synthesis is performed by adding 1.5 µL 10X DNA polymerase buffer, 2.5 µL H_2O, 0.5 µL DNA polymeraseI and 0.5 µL T4 DNA ligase. The reaction is incubated in ice for 5 min, at room temperature for 5 min, and at 37°C for 120 min. The reaction is stopped by heating for 10 min at 68°C.
2. The elongated DNAs are digested by an appropriate restriction enzyme in a 10 µL incubation volume (*see* **Note 5**).
3. 1 µL digested mixture is used to transform *E. coli* BMH71-18 *mut* strain by electroporation. Set electroporation apparatus at 2.5 kV, 25 µF, and pulse controller at 200 Ω; apply pulse. Remove cuvet, immediately add 1 mL SOC medium, and transfer to a culture tube. Incubate 45 min, with moderate shaking, at 37°C ***(3)***, add 4 mL LB medium containing the appropriate antibiotics, and continue the cultures' incubation overnight at 37°C (*see* **Note 5**).
4. Plasmid DNAs are prepared from cultures by using QIAprep Spin Miniprep Kit (Qiagen). 0.5 µg DNA is digested with the appropriate enzyme (*see* **Note 6**). The digested DNA is electroporated into DH5α, as described above. The transformed cells are plated on LB plates containing the appropriate antibiotics. The mutant plasmids are subjected to further identification by enzyme digestion and sequencing.

4. Notes

1. The primers should end at 3' with two nucleotides capable of forming GC pairs. Minimize the extent of intra- and interprimer homology. This rule is particularly important for the primer's 3' terminal nucleotides.

2. The authors found that the optimal amount of primers depends on the size of the fragment to be amplified. In fact, when 146 and 1010 bp fragments were amplified, the optimal concentrations of primers were 1 µ*M* and 15–30 pM, respectively.
3. The authors use *Pfu* DNA polymerase, because it is endowed with a 3' to 5' exonuclease proofreading activity that enables the polymerase to correct nucleotide-misincorporation errors ***(4)***. As *Pfu* DNA polymerase has a low processivity, the extension time is increased.
4. The authors found that the optimal fragment/template ratio was dependent on the size of the amplified fragment. In the case of a 200–300 bp fragment, a 50-fold molar excess, beyond the wild-type template concentration, was optimal; in the case of a 1000-bp fragment, a 10-fold molar excess worked better ***(2)***.
5. The appropriate restriction enzyme is the one that recognizes the unique restriction site. If adjustment of the required buffer components is difficult, the phosphorylated fragment should be ethanol precipitated before the digestion.
6. To determine the number of primary transformants, aliquots of the solution is plated onto LB agar plates containing the appropriate antibiotics and cultured overnight at 37°C.
7. The second digestion step is important, because it may affect the mutation efficiency. The authors usually perform the digestion in a 20-µL volume with 10 U enzyme for 3–4 h.

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9

Megaprimer Method for Polymerase Chain Reaction-Mediated Generation of Specific Mutations in DNA

Jesper Brøns-Poulsen, Jane Nøhr, and Leif Kongskov Larsen

1. Introduction

During the past decade, a number of methods using polymerase chain reaction (PCR) for the generation of specific mutations in any nucleotide sequence have been described, these methods include such drawbacks as the possibility of generating undesired secondary mutations, because of the low fidelity of some of the thermostable DNA polymerases, the need for four or more specific oligonucleotide primers, and the use of sophisticated, individually optimized protocols. Finally, the selection of correctly mutated clones may also prove to be laborious ***(1–3)***. In contrast, the megaprimer principle described here is cost-efficient, fast, reliable, and convenient.

The principle of the megaprimer method for PCR site-directed mutagenesis is shown in **Fig. 1**. The first round of PCR generates a fragment with the desired mutation introduced by one of the primers. The resulting product is electrophoresed on an appropriate agarose gel, and the fragment of interest is purified. Subsequently, the fragment is used as a primer for a second round of PCR. The final fragment generated from the second reaction is purified, and optionally restricted for cloning into the appropriate vector. By choosing a universal set of primers for the distal regions containing restriction sites compatible with the vector of choice, it is possible to use only one new primer for each mutant clone, which is both a cost- and a time-efficient feature of this method.

Protocols described in this chapter are highly flexible, and in the authors' hands, have been successfully applied on several different templates with both low and high G+C content, amplifying megaprimers ranging from 71 to 800 bp, and final products between 400 and 2500 bp. In principle, several different

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
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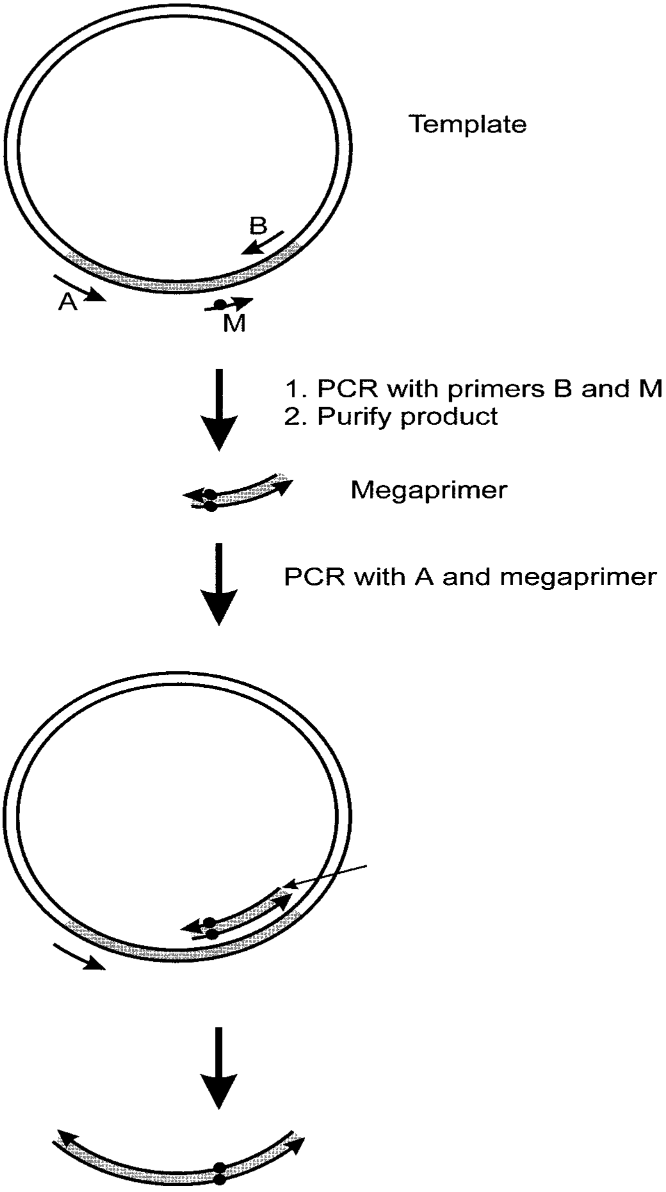
Template
B
A
M
1. PCR with primers B and M
2. Purify product
Megaprimer
PCR with A and megaprimer

Fig. 1. Megaprimer method. A megaprimer is formed by making a PCR with a flanking primer B and a mutation primer M from a template of choice. The megaprimer is purified on a gel, and a new PCR is set up with the megaprimer and a flanking primer A, using the same template as in the first reaction. The mutated product is purified, cloned into a vector of choice, and subsequently characterized.

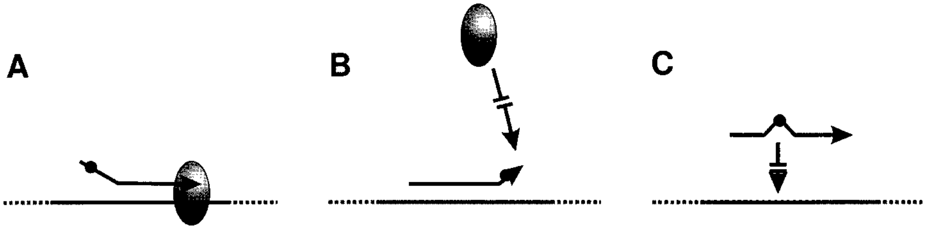

Fig. 2. Primer design for mutagenesis. **(A)** When placing the mutation in the 5'-end of the M primer, the first round of PCR (the synthesis of the megaprimer) is expected to be very efficient, but the second round of PCR (the synthesis of the final mutated fragment) may be troublesome. **(B)** A mutation placed in the 3'-end of the M primer can seriously inhibit the yield in the synthesis of the megaprimer. **(C)** A mutation placed in the central region of the primer may have a profound effect on annealing and, subsequently, the yield of the first round of PCR.

types of mutations could be introduced using this method. The authors have successfully applied the procedure for introducing mutations spanning, from single bp substitutions to 24 bp deletions and substitutions.

2. Materials

1. DNA template containing the sequence of interest (*see* **Note 1**).
2. Oligonucleotide primers A, B, and M (**Fig. 2**).
3. Reaction buffer for PCR (usually supplied with the polymerase, *see* **Note 2**).
4. Deoxyribonucleotide triphosphate stock solution (dNTP), 10 m*M* each.
5. Thermostable DNA polymerase (*see* **Note 3**).
6. Thermocycler.
7. Equipment for agarose gel electrophoresis of PCR products, DNA size marker.
8. Long-wave UV-transilluminator.
9. Reagents for purification of template and PCR products (*see* **Note 4**).
10. Linearized plasmid vector for cloning of the mutated fragment.
11. Reagents and equipment for ligation and for transformation of *Escherichia coli*.

3. Methods

3.1. Primer Design

When designing primers, the size of the final product (amplified with the megaprimer and primer B) must differ considerably from the size of the megaprimer. This ensures proper separation upon electrophoresis of the two fragments, after the second PCR.

Preferably, the mutation(s) in the M primer should reside in the central part of the primer. If placed near the 3' end, the mutations may interfere with polymerase activity in the first round of PCR, and, when placed in the 5' end, the mutations may interfere with polymerase activity in the second round of PCR. The mutations usually have the greatest impact on melting temperature when placed in the middle of the primer (**Fig. 2**), and the length of the primer should be adjusted accordingly, to secure an efficient annealing. Otherwise, the result may be a low yield in the first round of PCR.

Furthermore, for convenience and ease when cloning the final product, primers A and B may contain adequate restriction sites compatible with restriction sites in the cloning site of the chosen vector.

For all three primers, A, B, and M, standard rules for primer design should be applied: The annealing temperature should preferably lie between 50 and 60°C, the G+C content should be approx 50%, and the two 3'-terminal bases should be GC, CG, CC, or GG, if possible. A number of problems, such as self-annealing, may arise, and can be avoided using one of numerous computer programs for primer design ***(4)***.

3.2. Step1 : PCR 1, Synthesizing Megaprimer

1. DNA fragments are PCR-amplified with a proofreading thermostable DNA polymerase, such as *Pfu* or *Pwo*, in a total reaction volume of 100 µL. A standard reaction, using plasmid DNA as template, typically contains: 1X PCR buffer (containing magnesium, typically supplied with the enzyme), 0.2 µg template, 100 pmol of both downstream and upstream primers, 200 µ*M* of each of the four dNTPs, and, finally, 2.5 U DNA polymerase. PCR conditions for synthesis of mutant megaprimers ranges from 95°C for 1-5 min, followed by 10–25 cycles of 94°C for 20-45 s, 50–60°C for 30–120 s, and 72°C for 30–90 s. The reactions are terminated with an extension at 72°C for 10 min and cooled to 4°C (*see* **Note 6**).
2. Run the product obtained from the PCR on an appropriate agarose gel.
3. Visualize the product using a long-wave UV-transilluminator cut out the band containing the megaprimer, and purify the DNA (*see* **Note 4**).

3.3. Step 2: PCR 2, Synthesizing Product for Cloning

After purification (*see* **Note 4**), the megaprimer, together with primer B, serves to amplify the final product (*see* **Fig. 1**). The crucial parameter in this reaction is primer concentration ***(5,6)*** (*see* **Note 7**).

1. Set up a reaction for the second PCR, typically consisting of 1X PCR buffer (supplied with the enzyme), 0.2 µg template, 4 pmol of both megaprimer and distal (up- or downstream) primers, 200 µ*M* of each of the four dNTPs, and 2.5 U DNA polymerase in a final volume of 100 µL. PCR conditions for synthesis of the final mutated product ranges from 95°C for 1–5 min, followed by 10–25 cycles of 94°C for 20–45 s, 50–60°C for 120 s, and 72°C for 30–90 s. The reactions are terminated with an extension at 72°C for 10 min and cooled to 4°C
2. Run the product on an appropriate agarose gel, visualize using a long-wave UV-transilluminator, and cut the final, mutated fragment out of the gel.

3. Purify the fragment (*see* **Note 4**).
4. The purified fragment is ready for cloning into a plasmid vector.

4. Notes

1. This will usually be, e.g., a cDNA or a promoter sequence inserted in a plasmid, through cloning via adequate restriction sites.
2. Cations, such as Mg^{2+}, Mn^{2+}, and NH_4^+, and their concentration are of paramount importance for polymerase performance, probably because of shielding of enzyme and coordination of nucleotides. Various water-activity-lowering molecules, such as glycerol, Triton™ X-100, or other detergents, may improve the yield of PCR product. Additives such as spermine and spermidine reduce nonspecific reactions between the polymerase and DNA. Other additives that may improve the PCR are dimethylsulfoxide, betaine, and bovine serum albumin. Determining the optimal composition of the PCR reaction may be neccessary, empirically, if problems arise in achieving a good specificity and yield.
3. The performance of different polymerases is dependent on the composition of the template, and should be determined empirically, if encountering problems with yield. Concentration of nucleotides may also influence fidelity and performance of the polymerase. If secondary mutations pose a problem, lowering the initial concentration of dNTP may improve polymerase performance and yield of specific product. Furthermore, a relationship between increased annealing efficiency and decreased polymerase fidelity upon increased Mg^{2+} concentration, is a frequent observation. Finally, the choice of commercially available thermocycler may influence the yield of PCR product. This is, in the authors' opinion, mainly the result of differences in ramp time between instruments.
4. Various commercially available kits perform very well. In these laboratories, QuantumPrep™ (Bio-Rad, Hercules, CA), GenElute™ (Sigma, St. Louis, MO), Qiagen™ (Qiagen, Hilden, Germany) for preparation of plasmid template, and QiaEx™ and QiaExII™ for purification of PCR products from agarose gels, were successfully used. However, if problems with described PCR procedures are experienced, and supposedly relate to DNA quality, a standard phenol/chloroform (note that these compounds are labeled hazardous and toxic) extraction of the DNA may be beneficial.
5. If multiple products are formed during the PCR (as determined on the gel), elevating the annealing temperature often abolishes this problem.
6. The PCR reaction is optimized with the usual parameters influencing success of a PCR: longer denaturation for higher G+C content in products, longer elongation for longer products, and higher annealing temperature with longer primers (*see* **Note 5**). Furthermore, problems with repeatedly occurring, specific secondary mutations may arise. In these cases, adjustment of some of the parameters mentioned in **Notes 2** and **3** may be advantageous.
7. Although the reasons for this observation remain elusive, it probably relates to the secondary and tertiary structures formed, when single-stranded DNA of considerable size reaches critical concentrations. Others ***(6)*** have found that

megaprimer concentrations higher than 0.01 µ*M* inhibit the reaction. In contrast, the authors' experiences show that a megaprimer concentration between 0.02 and 0.04 µ*M* is advantageous, compared to concentrations of 0.01 µ*M* and below. Furthermore, an annealing step for as long as 15 min has been used ***(7)***; in the authors' protocol, an annealing time of 30–120 s is sufficient for amplification of the fragment of interest, regardless of size. With certain megaprimers, a complete inhibition of the reaction may be observed. In this case, the concentration of the megaprimer should be decreased to an absolute minimum. Note that both primers should be equal in concentration, to avoid asymmetric amplification, resulting in single-stranded product.

Acknowledgments

Jane Nøhr was supported by grant no. 9502914 from the Danish Research Councils Committee on Biotechnology.

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10

Generation of Epitope-Tagged Proteins by Inverse Polymerase Chain Reaction Mutagenesis

Lucio Gama and Gerda E. Breitwieser

1. Introduction

Generation of fusion proteins is a routine procedure in an increasing number of laboratories worldwide. Generally, the cDNA sequence of the protein under study is subcloned in-frame into a vector containing the sequence of a well-established epitope. This procedure, although simple and widespread, presents some important limitations: The vector generally contains a single, unidirectional, multiple-cloning site that allows the epitope to be incorporated into only the N- or C-terminus of the protein; it requires the use of unique restriction endonucleases that have no sites within the inserted cDNA sequence; when working with different expression systems, it is often necessary to acquire different, and potentially expensive, plasmid vectors; and it is a multistep procedure involving polymerase chain reaction (PCR), digestion with restriction enzymes, subcloning, and bacterial transformation and selection.

To circumvent these problems, the authors have extended the inverse polymerase chain reaction mutagenesis (IPCRM) technique to permit the insertion of short peptide sequences into any position in a target protein. The original method was developed for the generation of point mutations or deletions in sequences already cloned into a specific vector ***(1–4)***. It begins with PCR amplification of the entire plasmid, using a pair of prephosphorylated, adjacent, nonoverlapping oligonucleotides. The PCR product is then ligated, treated with *Dpn*I to digest the methylated, contaminating parental plasmid, and subsequently introduced into competent cells through transformation.

To extend the general IPCRM technique for the generation of fusion proteins, a pair of primers, each containing roughly half of the epitope sequence,

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ

is used. No restriction enzyme site is required, and the additional nucleotides defining the epitope can be incorporated anywhere within the parental plasmid, without subcloning into a new vector. The whole procedure (from PCR to selection) takes less than 48 h, and is highly efficient (on average, 70% of selected colonies are positive for the insertion and in-frame) ***(5)***.

2. Materials

1. T4 polynucleotide kinase and 10X reaction buffer (500 m*M* Tris-HCl, pH 7.5, 100 m*M* $MgCl_2$, 100 m*M* 2-mercaptoethanol) (Amersham Pharmacia Biotech, Piscataway, NJ).
2. 10X deoxyadenosine triphosphate (dATP) (10 m*M*).
3. *Pfu* DNA polymerase and 10X reaction buffer (200 m*M* Tris-HCl, pH 8.8, 100 m*M* KCl, 100 m*M* $[NH_4]_2SO_4$, 20 m*M* Mg_2SO_4, 1% Triton X-100, 1 mg/mL nuclease-free bovine serum albumin) (Stratagene, La Jolla, CA).
4. Deoxyribonucleoside triphosphate (dNTPs) stock (4 mM).
5. Geneclean® kit (BIO 101, Vista, CA).
6. 10 m*M* Tris-HCl, pH 8.5, for elution.
7. T4 DNA Ligase and 10X reaction buffer (500 m*M* Tris-HCl, pH 7.5, 100 m*M* $MgCl_2$, 100 m*M* dithiothreitol (DTT), 10 m*M* ATP, 250 µg/mL bovine serum albumin) (New England BioLabs [NEB], Beverly, MA).
8. *Dpn*I (NEB).
9. One Shot™ TOP10 Competent Cells (Invitrogen, Carlsbad, CA).
10. Suitable restriction enzyme for diagnostic digests.

3. Method

3.1. Designing the Primers

To illustrate the procedure (**Fig. 1**), the FLAG, epitope sequence (DYKDDDDK) will be fused in-frame at the C-terminus of calcium-sensing receptor (CaR) cDNA, which was already subcloned into pcDNA3.1 (+) vector, from Invitrogen (*see* **Note 1**).

1. The upstream primer consists of 20 nucleotides complementary to the end of the CaR cDNA (minus the stop codon), plus 12 in-frame nucleotides corresponding to the first half of the FLAG DNA sequence (5'-**GTCCTTGTAGTC**TGA ATTCACTACGT TTTCTG-3') (*see* **Note 2**).
2. The downstream primer contains the other half of the epitope sequence, plus 20 nucleotides complementary to the vector (5'-**GATGACGACAAGTAA**TCGA GTCTAGAGGGCCCGTT-3').
3. To aid in selection of correct clones, generating a novel restriction enzyme site in one of the primers is useful (but not always possible), by incorporating a silent mutation (*see* **Note 3**). In this specific case, an *Xho*I site was incorporated within the downstream primer (5'-…AGT*CTCGAG*GGC…-3').

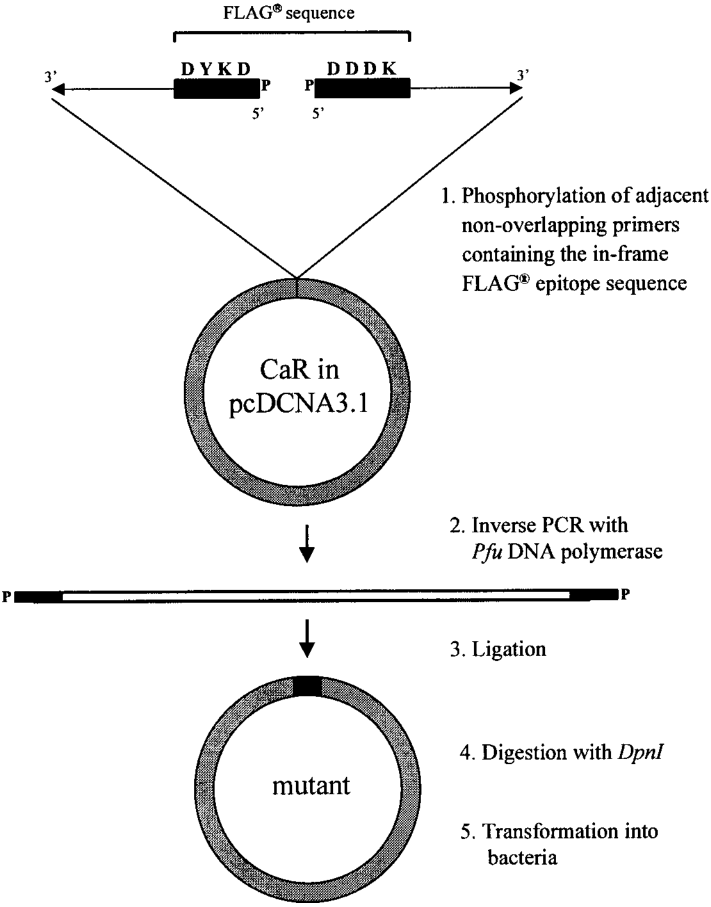
FLAG® sequence
DYKD
DDDK
3'
3'
P
P
5'
5'
1. Phosphorylation of adjacent non-overlapping primers containing the in-frame FLAG® epitope sequence
CaR in pcDCNA3.1
2. Inverse PCR with *Pfu* DNA polymerase
P
P
3. Ligation
mutant
4. Digestion with *DpnI*
5. Transformation into bacteria

Fig. 1. Overview of modified IPCRM protocol.

3.2. Primer Phosphorylation

1. Both primers (50 pmol each) were combined with 1 µL 10X ATP (10 m*M*), 1 µL 10X kinase reaction buffer and 3 U T4 polynucleotide kinase (Amersham Pharmacia Biotech) to a final volume of 10 µL (*see* **Note 4**).
2. After 30 min at 37°C, the reaction was stopped by heating to 65°C for 5 min.

3.3. PCR

The PCR product can be >10 kb (amplification of entire plasmid plus insert); therefore, the use of *Pfu* DNA polymerase is essential, not only because of its high fidelity, but also because it generates blunt-ended PCR products, eliminating the need for end polishing.

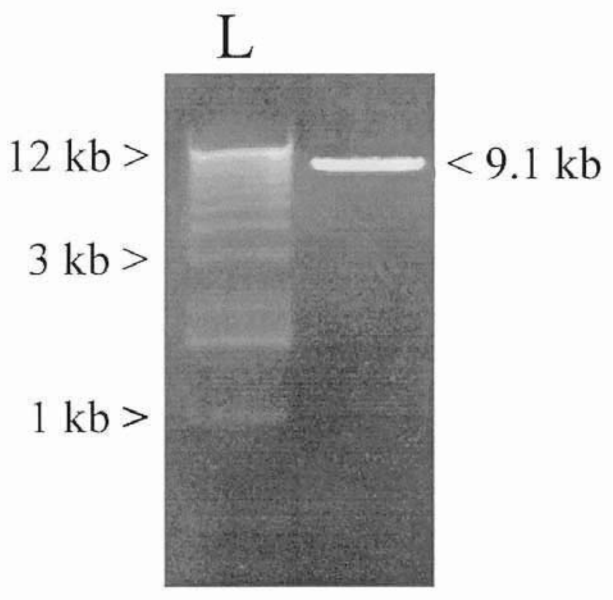

Fig. 2. 1% Agarose gel of 10 μL PCR product. L, ladder.

1. The PCR mixture (50 μL) contained 8 μL 4 m*M* dNTP (final concentration of 160 μ*M* for each NTP), 2.5 U *Pfu* polymerase, 10–20 ng template plasmid, 5 μL 10X *Pfu* reaction buffer (Stratagene), and 5 μL phosphorylated primer mix, which was prepared in the previous step (25 pmol of each primer).
2. Following an initial 94°C denaturing step (4 min), the reaction was cycled 16× through 94°C for 30 s, 60°C for 30 s, and 72°C for 20 min (2.5 min/kb template).
3. The reaction was terminated by a final extension step at 72°C for 25 min.
4. Amplification is confirmed by running a 5-μL aliquot of the reaction mixture on a 1% agarose gel. The product should be easily seen and unique, i.e., a single band (**Fig. 2**). If the mixture contains multiple products, *see* **Note 5**.

3.4. Ligation

1. The PCR product is purified by Geneclean (Bio101), resuspended in 8 μL 10 mM Tris-HCl, pH 8.5 (*see* **Note 6**), and mixed with 1 μL 10X ligation buffer, and 6 U T4 DNA ligase (NEB).
2. The ligation reaction is incubated for 2 h at room temperature, followed by heat-inactivation of the ligase for 10 min at 65°C.

3.5. **Dpn*I*** *Digestion*

To eliminate wild-type template DNA, 8 μL H_2O and 2 μL *Dpn*I (NEB) (40 U) are added to the sample, and incubated for 4 h at 37°C (*see* **Note 7**).

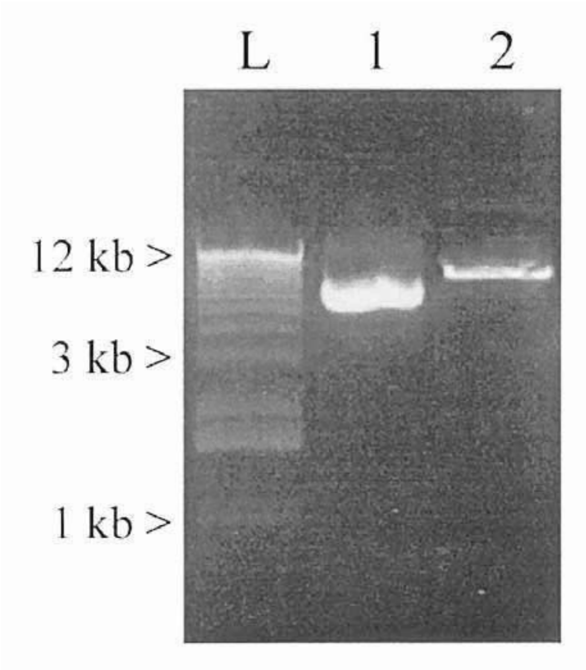

Fig. 3. 1% Agarose gel of DNA samples after digestion with the diagnostic restriction enzyme. The *Xho*I site is not present in the original plasmid, but is present in the product. L, ladder; 1, wild-type vector (uncut form); 2, positive clone (one band at 9.1 kb).

3.6. Transformation

1. 1 µL *Dpn*I digest is used to transform 50 µL One Shot TOP10 Competent Cells (Invitrogen), according to the manufacturer's protocol (30 min on ice, 45 s at 42°C to heat-shock the bacteria, 2 min recovery on ice, dilution to 200 µL with SOC media (20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 2.5 m*M* KCl, 10 m*M* $MgCl_2$, 20 m*M* glucose), 1 h incubation at 37°C).
2. The entire transformation reaction is plated onto an Luria-Bertani–kanamycin (10 µg/mL) plate and incubated for 37°C overnight.

3.7. Selection

1. Colonies are expanded overnight in 1.5 mL Luria-Bertani containing 10 µg/mL kanamycin. Minipreps were performed, and correct clones were selected after restriction digests were run with the appropriate diagnostic restriction enzyme, in this case *Xho*I (**Fig. 3**). Clones selected by restriction digests can be sequenced directly. However, other procedures for confirming clones, such as the transfection method described below, may help minimize sequencing costs.
2. To confirm that the FLAG epitope sequence was correct and in-frame, selected clones were transfected into HEK-293 cells previously plated in 8-well Lab-Tek® Chamber Slides (Nalge Nunc, Rochester, NY). After 24 h, the cells were fixed

with acetone and analyzed by immunofluorescence with anti-FLAG antibody (Sigma, St. Louis, MO).

3. Positive clones were confirmed by automated sequencing (Model 377 DNA Sequencer; PE Biosystems, Foster City, CA) in the region encompassing the epitope tag.
4. Constructs chosen for use in further studies were sequenced over the entire coding region, to confirm the absence of PCR-induced errors.

4. Notes

1. The method described here was also used to create additional recombinant proteins by inserting other peptides, including c-Myc protein (EQKLISEEDL), an enterokinase site (DDDDK) or His_6 (HHHHHH) into various expressed proteins. The inserted peptides varied from 5 to 10 amino acids, and were introduced into different regions of the target protein.
2. Primers for epitopes with repetitive sequences like His_6 should be designed to prevent overlapping of complementary nucleotides. The entire DNA sequence for the epitope must be incorporated into only one of the two primers.
3. Incorporating silent mutations (unique restriction sites) within the primers is facilitated by using a number of computer programs, such as Primer Generator ***(6)***.
4. An excess of primers during the phosphorylation step, or an excess of PCR product during the ligation step, can lead to high levels of nonligated plasmids during bacterial transformation, considerably decreasing the efficiency of the method. Better results are achieved when ligating 0.5 µg or less of the PCR product.
5. Occasionally, despite attempts to optimize the annealing temperature, the PCR amplification can yield multiple products. One solution to this problem is to run 10–20 µL PCR product on an agarose gel, and to extract the correct band from the gel with Geneclean.
6. Traces of ethanol can contaminate the PCR product after the Geneclean process and negatively affect the ligation reaction. The glassmilk must be thoroughly dry before adding the elution buffer.
7. The efficiency of the *Dpn*I digestion may vary, depending on the amount and quality of the DNA vector used as a template for the PCR reaction. Supercoiled forms of DNA can be more resistant to enzyme digestion, and can lead to an increased number of colonies containing the wild-type plasmid. Incubating the *Dpn*I digestion overnight will minimize this problem.

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Site-Directed Mutagenesis by Polymerase Chain Reaction

Albert Jeltsch and Thomas Lanio

1. Introduction

Site-directed mutagenesis (SDM) is a powerful tool for analyze protein structure and function, protein folding, and enzyme mechanism ***(1)***. Several protocols for SDM by polymerase chain reaction (PCR) are published ***(2–8)***. Here, two protocols, which turned out to be robust and efficient in the authors' hands, are described in detail. Both methods comprise two steps: In the first step the desired mutation is introduced by a PCR primer used to amplify one part of the target gene. In a second step, this PCR product is then used as a megaprimer to amplify the full gene containing the mutation. Both methods deviate in how the mutated gene is introduced back into its cloning vector: In method 1, the whole vector plasmid is amplified in a PCR reaction, method 2 relies on restriction enzyme cleavage of the gene and vector, followed by ligation. In both methods, together with the mutation, a restriction enzyme marker site is introduced into the target gene by silent mutations, to allow fast and convenient screening for the presence of the mutation ***(4,7,9)***. Because random mutagenesis and directed evolution are being widely used for protein engineering ***(10)***, a method for random mutagenesis by PCR is described, using spiked oligonucleotides, which contain a mixture of all four nucleotides, to a certain degree ***(11)***. This technique allows randomization of a small part of a gene, at a level that can be chosen at will, and is a convenient alternative to cassette mutagenesis methods described previously ***(12,13)***. The protocol described here allows the construction of large mutant libraries (10^4–10^5 clones). Because wild-type (WT) alleles are efficiently excluded from transformants, screening for the presence of the mutational primer is not necessary.

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
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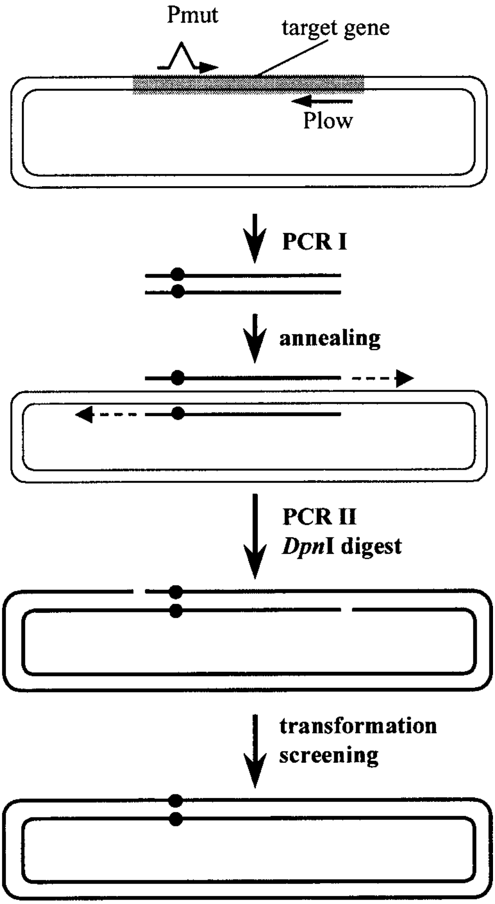

Fig. 1. Schematic drawing of the site-directed PCR mutagenesis method 1. The method involves a complete amplification of the vector plasmid. It does not require any restriction enzyme cleavage and ligation steps.

1.1. Site-Directed PCR Mutagenesis (Method 1)

Method 1 is a modified version of the PCR mutagenesis protocol described by Kirsch and Joly ***(8)***, and consists of two PCR reactions (**Fig. 1**). In the first PCR reaction (PCR I), the mutation, together with a marker restriction site, is introduced by a PCR primer into a first PCR product. This first product is employed as a megaprimer in a second PCR reaction (PCR II) using the circular WT plasmid as template to amplify a linear DNA fragment with large, complementary, single-stranded ends, whose size corresponds to the length of the megaprimer (*see* **Note 1**). These ends can hybridize to form stable circular

DNA molecules, which contain the mutation and a nick in each DNA strand. The product mixture of PCR II also contains the original template of the PCR reaction, which can be removed by a *Dpn*I digest. This restriction enzyme only cleaves DNA containing GATC sites whose adenine residues are methylated, as purified from dam$^+$ *Escherichia coli* strains ***(14–16)***. Since only the original template, but not the PCR products, contain such modified G^{m}ATC sites, the PCR products are not cleaved by *Dpn*I. Finally, the DNA is precipitated and directly used to transform competent *E. coli* cells, without further cloning steps. This method is also well-suited to transfer variants of the target gene containing certain mutations from one vector construct to other vectors containing the target gene, in order to change the expression system.

1.2. Site-Directed PCR Mutagenesis (Method 2)

PCR mutagenesis is performed using a megaprimer protocol modified from Ito et al. ***(4)***. The method employs three PCR primers and three PCR reactions (**Fig. 2**): Pup binds to the coding strand upstream of the target gene, Plow1 binds to the lower strand downstream of the target gene, and removes the cloning site 3' to the gene, and Plow2 binds downstream to Plow1, but leaves the 3' cloning site intact. The desired mutation is introduced into the gene (together with a marker site) by an additional upper primer (Pmut) in a PCR reaction, together with Plow2 (PCR I) (*see* **Note 2**). The PCR product, which consists of a part of the target gene and carries the 3' cloning site is hybridized to a PCR product, produced using Pup and Plow1 (PCR II), which contains the 5' cloning site, but no 3' cloning site. After three PCR fill-in steps, a PCR, using Pup and Plow2 is carried out (PCR III). After this procedure, only mutated products carry the 3' cloning site, and can be cloned into the large fragment of the cloning vector cleaved with the cloning restriction enzyme. Usually, 30–50% of the obtained clones contain the marker site and the desired mutation.

1.3. PCR Mutagenesis Using Spiked Oligonucleotides (Method 3)

The method comprises two steps (**Fig. 3**) ***(11)***: In step one, PCR mutagenesis is carried out following method 2, except that the mutagenic PCR primer does not contain a defined mutation, but is degenerated to a certain degree, i.e., certain positions are doted with a small amount of a mixture of all four nucleotides (*see* **Note 3**). After this step, a library is obtained, which contains a large number of different mutations introduced by the mutational primer. However, the pool still contains considerable amounts (depending on the success of the mutagenesis procedure, 5–60%) of genes that are WT, because the mutational primer is not introduced. These genes in principal can be identified by restriction analysis, because the mutational primer also introduces a characteristic restriction site. However, it is impossible with respect to time, and costs to

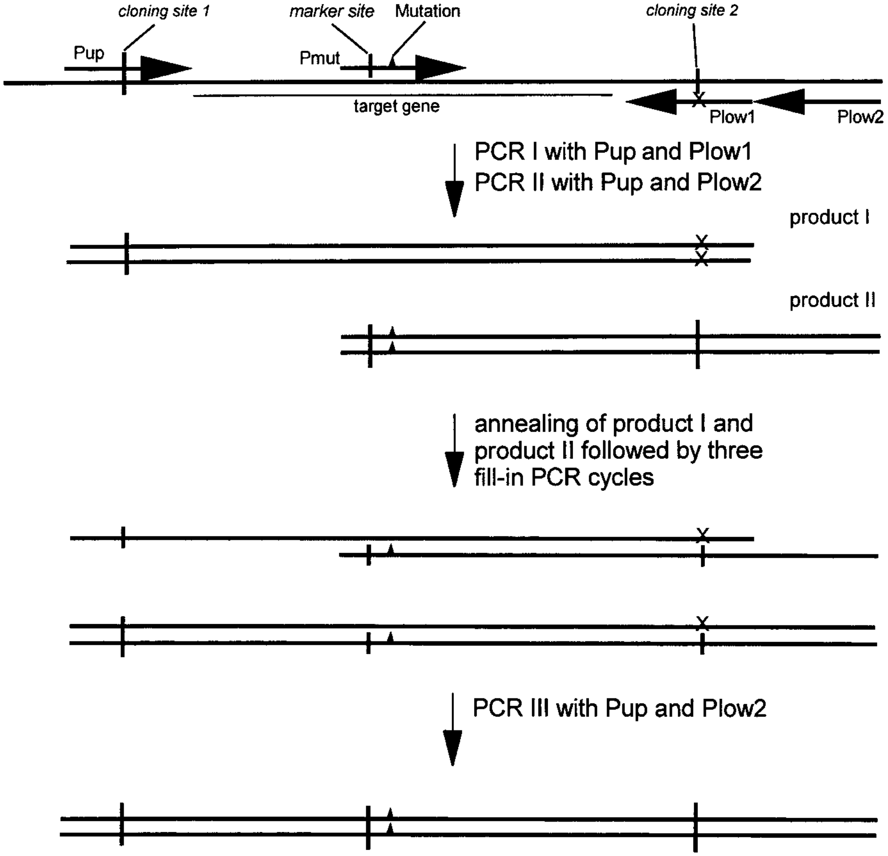
cloning site 1
marker site
Mutation
cloning site 2
Pup
Pmut
target gene
Plow1
Plow2
PCR I with Pup and Plow1
PCR II with Pup and Plow2
product I
product II
annealing of product I and product II followed by three fill-in PCR cycles
PCR III with Pup and Plow2

Fig. 2. Schematic drawing of the site-directed PCR mutagenesis method 2. Following three PCR reactions, only products containing the desired mutations also contain the restriction sites required for cloning.

screen more than some hundreds of clones, with respect to the presence of the marker site. The protocol described here overcomes this limitation and allows the construction of large mutant libraries (10^4–10^5 clones), because the WT allele is efficiently excluded from transformants, and, consequently, screening for the presence of the mutation is not necessary. To construct a large library of mutated genes without WT background, a second step (step two) is carried out. In this step, the marker site is employed as cloning site. After step one, only one marker positive clone must be isolated, and the plasmid of this clone digested with the marker restriction enzyme and the second cloning restriction enzyme to generate a new large fragment, which already carries the part of the

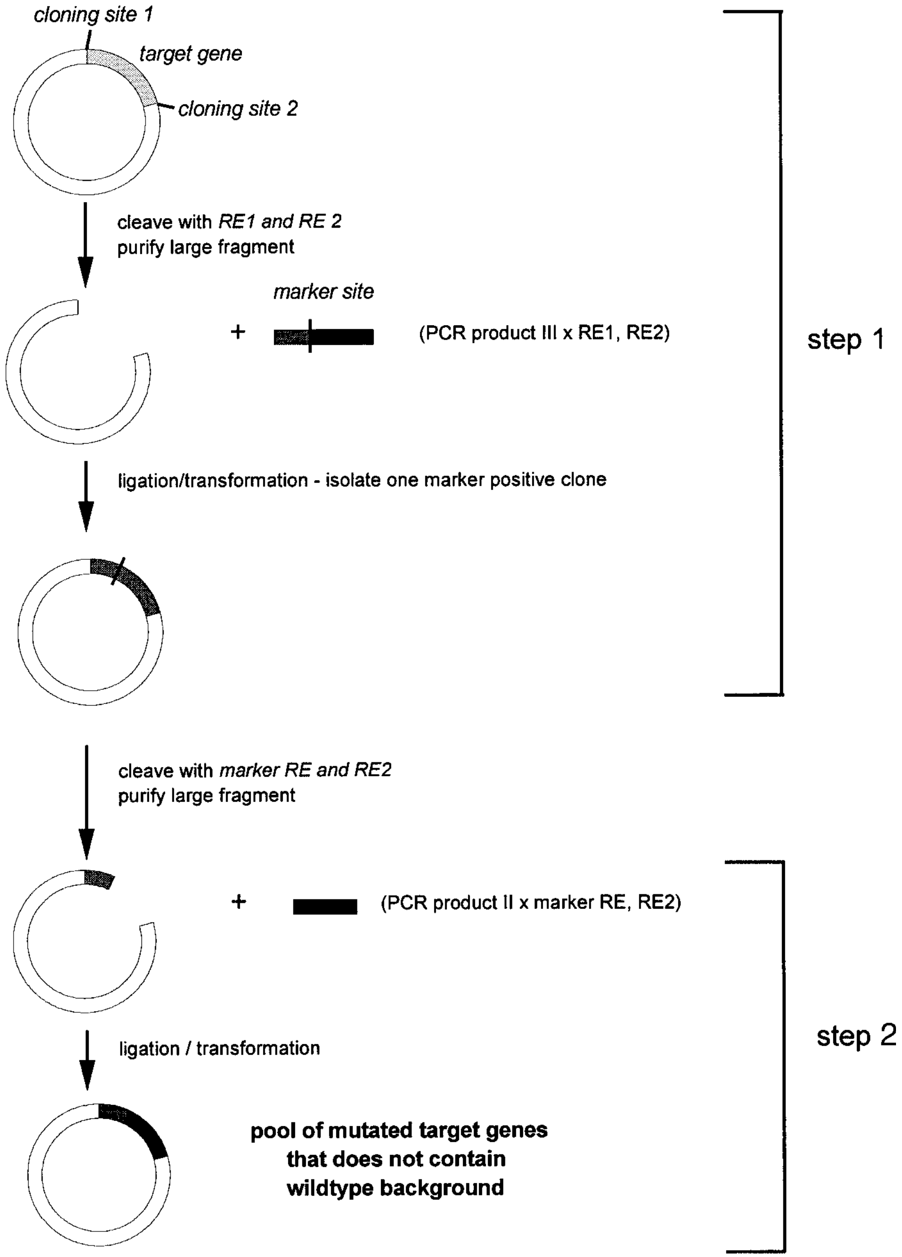
cloning site 1
target gene
cloning site 2
cleave with RE1 and RE 2
purify large fragment
marker site
+
(PCR product III x RE1, RE2)
step 1
ligation/transformation - isolate one marker positive clone
cleave with marker RE and RE2
purify large fragment
+
(PCR product II x marker RE, RE2)
step 2
ligation / transformation
pool of mutated target genes
that does not contain
wildtype background

Fig. 3. Schematic drawing of the PCR mutagenesis method that almost completely excludes the WT allele from the pool of transformants.

target gene upstream to the marker site. The PCR product obtained in PCR II of step one is also cleaved with the marker restriction, enzyme and the second cloning restriction enzyme and ligated into the large fragment. The ligation product is used to transform *E. coli* cells. This procedure leads to a zero WT background in the obtained clones because only PCR products containing the desired mutations can carry a marker site ***(11,17)***. Any PCR products amplified from contaminating WT genes irrespective to the origin of the contamination cannot be cloned. In practice, step two needs only to be carried out, if the WT background obtained after step one is too high for the experimental purpose. Under such circumstances, instead of repeating step one (and probably repeating the same mistakes), this protocol provides an easy tool to eliminate any WT background.

2. Materials

1. PCR cycler.
2. Electroporator and appropriate cuvets for electroporation.
3. Competent *E.coli* cells to be used in electroporation.
4. Equipment for agarose gel electrophoresis and acrylamide gel electrophoresis.
5. Gel extraction kit, e.g., silica beads (Merck, Darmstadt).
6. PCR purification kit, e.g., Qiaquick PCR purification kit (Qiagen, Hilden).
7. PCR primer (stock solutions 2–10 µ*M*).
8. Deoxyribonucleoside triphosphate (dNTP) stock solution (each 2 m*M*).
9. DNA polymerase with proofreading activity, e.g., *Pfu* polymerase (Stratagene).
10. Polymerase 10X buffer (as supplied with the polymerase).
11. Restriction enzymes (marker enzyme, cloning enzymes, *Dpn*I, depending on the method used).
12. 10X restriction enzyme buffers.
13. DNA ligase.
14. 10X DNA ligase buffer (as supplied with the ligase).
15. Template plasmid containing the gene of interest.

3. Methods

3.1. Method 1

1. PCR I: Mix 2 ng/µL template with PRC primers pmut (*see* **Note 4**) and plow (each 400 n*M*), dNTPs (200 µ*M*) and 0.02 U/µL *Pfu*-DNA Polymerase (Stratagene) in 1× *Pfu* reaction buffer (*see* **Note 5**). PCR conditions are 1× 150 s at 95°C, 20× (60 s at annealing temperature, 90 s at 72°C, 30 s at 95°C) and 1× (60 s at 45°C, 230 s at 72°C). The actual annealing temperature can vary between 40 and 55°C, and depends on the length and number of mismatches of mutagenesis primer.
2. Purify the product of PCR I, using a PCR purification kit. Elute with 50 µL.
3. PCR II: Mix 20 µL purified product of PCR I with circular WT plasmid (2 ng/µL), dNTPs (200 µ*M*), and *Pfu* polymerase (0.02 U/µL) in *Pfu* reaction buffer. PCR

conditions are: 1× 90 s at 95°C, 20× (60 s at 50°C, 900 s at 68°C, 30 s at 95°C) and 1× 60 s at 50°C, 900 s at 68°C.

4. Optional: Purify the product mixture, using a PCR purification kit. Elute with 50 µL.
5. *Dpn*I digest: Incubate 44 µL purified product of the second PCR reaction with 0.25 U/µL *Dpn*I (NEB) in *Dpn*I reaction buffer for 3 h at 37°C.
6. Precipitate the DNA with ethanol, and transform competent *E. coli* cells by electrotransformation.
7. Screen the colonies obtained for the presence of the marker site (*see* **Notes 6** and **7**).
8. The mutation, as well as the absence of additional mutations, must be verified by DNA sequencing.

3.2. Method 2

1. Cleave 30 µg vector plasmid with both cloning restriction enzymes.
2. Separate the cleavage products on an agarose gel, and purify the vector fragment from the gel, using silica beads (Merck, Darmstadt), according to the instructions of the supplier (*see* **Note 8**).
3. PCR I: 50 ng linearized template plasmid carrying the target gene, were mixed with Pup and Plow1 (400 n*M*), 200 µ*M* dNTPs, and 2 U *Pfu* DNA polymerase in a total volume of 50 µL. PCR conditions are 1× 120 s at 95°C, 15× (30 s at 95°C, 60 s at annealing temperature, 60 s at 72°C). Typical yields of PCR I are 100–500 ng product I.
4. PCR II is carried out as PCR I, using Pmut and Plow2. Typically, yields are 100–500 ng product II.
5. PCR III: Mix 10-50 ng of product I and product II, 200 µ*M* dNTPs, 400 n*M* Pup and Plow2 and 2 U *Pfu* DNA polymerase. PCR conditions are 3× (60 s at 95°C, 60 s at 60°C, 120 s at 72°C), 15× (30 s at 95°C, 60 s at annealing temperature, 60 s at 72°C). During the first three cycles of denaturation and reannealing primer binding is disfavored, and the heteroduplexes are filled in by the *Pfu* polymerase. The resulting products were amplified in the following cycles. Typical yields of this reaction were between 500 ng and 1 µg DNA.
6. Digest the purified full-length PCR products with the cloning restriction enzyme, purify the DNA, using a PCR purification kit to remove the restriction enzymes and short cleavage fragments; ligate into the large vector fragment, using 1 U T4-ligase at 16°C for 16 h.
7. Precipitate the DNA with ethanol, and transform competent *E. coli* cells by electrotransformation.
8. Screen the colonies obtained for the presence of the marker site (*see* **Notes 6** and **7**).
9. The mutation, as well as the absence of additional mutations, must be verified by DNA sequencing.

3.3. Method 3

1. Step 1 is performed as described in method 2.
2. Cleave 30 µg plasmid of one marker positive clone obtained in step 1 with the marker restriction enzyme and cloning restriction enzyme.

3. Separate the cleavage products on an agarose gel, and purify the vector fragment from the gel using silica beads (Merck, Darmstadt), according to the instructions of the supplier (*see* **Note 8**).
4. Digest PCR product II from step 1 with the marker restriction enzyme and cloning restriction enzyme, and purify the cleaved PCR product, using a PCR purification kit.
5. Ligate 100–1000 ng cleaved PCR product into 100 ng of the vector fragment, using 1 U T4 DNA ligase at 16°C for 16 h.
6. Transform *E. coli* cells with the ligation mixture, using an electroporator.
7. All transformants should contain the desired mutations.

4. Notes

1. This method works best if the megaprimer has a length between 100 and 400 bp.
2. The marker yield of this method decreases with the length of the product of PCR I. Sizes larger than 600–800 bp should be avoided.
3. The probability (P) to find X mutations within a stretch of N randomized nucleotides depends on the fraction (f) of wrong nucleotides at each position by:

$$P(x) = \{N!/[x!(N-x)!]\}\ (f)^x\ (1-f)^{N-x}$$

4. The mutagenic primer should contain as few mismatches as possible. The length of the primer must be increased, if many mismatches are necessary. Mismatches should be located at least 6 bp from both ends of the primer.
5. A polymerase with proofreading activity should be employed to minimize the number of mutations outside of the target region.
6. Screening is carried out by restriction enzyme digests with the marker enzyme, using a DNA miniprep as template. Alternatively, a template can be generated by PCR: Pick one clone with a toothpick, and suspend the cells in 30 µL H_2O. Heat to 95°C for 10 min, and use 1 µL as template for a PCR reaction. If screening is done out by PCR, a negative control without template always must be carried out, to exclude any contamination of one of the reagent. The obtained PCR product could also serve as template for the following sequencing reaction.
7. Given a certain fraction (f) of positive clones in the pool of transformants, the probability (P) to obtain at least one positive clone depends on the number (N) of clones screened by:

$$P = 1 - (1 - f)^N$$

   This means that screening of 5, 10, 15, 20, 25, and 30 will identify at least one positive clones with a probability of 95%, if f is 0.45, 0.26, 0.18, 0.14, 0.11, or 0.10, respectively. Therefore, it is not reasonable to screen more than 20 clones from one transformation, because the chance of finding a positive clone, after 20 clones were negative, is very low.
8. An alternative protocol, to produce the large vector fragment, does not invoke gel purification: Cleave 30 µg plasmid of one marker-positive clone obtained in step 1 with the marker restriction enzyme and cloning restriction enzyme. If pos-

sible, inactivate the restriction enzymes by heating to 65°C for an appropriate period of time (depending on the restriction enzyme used). Alternatively, precipitate the cleaved DNA with ethanol, to remove the restriction enzyme. Incubate the DNA with 0.5 U shrimps alkaline phosphatase for 1 h in shrimps alkaline phosphatase buffer. Heat to 65°C for 30 min to inactivate the shrimps alkaline phosphatase.

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12

Generation of Multiple Site-Specific Mutations by Polymerase Chain Reaction

Amom Ruhikanta Meetei and M. R. S. Rao

1. Introduction

Generating mutant proteins has become a routine strategy for molecular and structural biologists, to understand the structure–function relationship of a given protein of interest. In this, site-directed mutagenesis has proven to be a valuable technique. After the advent of polymerase chain reaction (PCR), PCR-mediated mutagenesis has been the method of choice to change from one amino acid to another, which has replaced the classic Kunkel's method ***(1)***.

Several procedures have been described in the literature ***(2–13)*** to achieve the desired mutation using the PCR approach. Although, in most cases, the change is only with respect to one amino acid, often one encounters a situation in which more than one amino acid needs to be changed. For example, multiple mutations are required in the same gene to examine whether a second mutation modulates the effect of the primary mutation.

Another example wherein multiple mutations are required is when one is dealing with zinc (Zn) finger proteins, in which multiple residues of cysteine and histidine are involved in coordination with Zn. This becomes much more relevant when there are many more cysteine and histidine residues in the protein than what is necessary for making tetrahedral coordination with Zn. Recently, the authors encountered such a problem in solving the Zn finger domains of the spermatidal protein, TP2, which appears transiently during mammalian spermiogenesis ***(14–18)***.

There are at least three procedures described in the literature ***(19–21)*** for generating the multiple site-specific mutations. The first method is termed the "MM-SSP" method, described by Dwivedi et al. ***(19)***, which involves stepwise creation of the mutations, using the previous single-mutant clone generated as

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ

the template. Here, the first mutant gene created by the PCR method must be cloned, the sequence confirmed, then used as the template for the second cycle of mutation.

In the second method, described by Tu and Sun ***(20)***, a set of mutagenic primers have been used in the same PCR reaction to create a combination of mutations in a given gene. This method involves generation of single-stranded DNA template containing deoxyuridine monophosphate and subsequent screening of mutant clones (essentially extension of Kunkel's method). Moreover, the efficiency of mutagenesis in this method is also not very high.

More recently, Bi and Stambrook ***(21)*** developed a technique called combined chain reaction for generating single and multiple mutations. Although this method proved to be very efficient for generating single and double mutations, it needs special considerations, i.e., the annealing efficiency of the mutant primers should not be less than that of side primers, and the DNA polymerase should not possess 5' to 3' exonuclease activity. They also did not check the efficiency of their method for generating three or more mutations in the given gene.

These three methods require DNA ligase (thermostable ligase in the third method) and 5'phosphorylation of the mutagenic primers. The authors recently developed an efficient method for generating multiple site-specific mutations, which was necessitated by our attempts to identify the Zn-coordinating amino acid residues of TP2 ***(22)***, which involves a series of PCR reactions with a new mutant primer provided at each round of PCR, without the necessity of cloning the PCR product after each reaction. The authors have successfully employed this method to delineate novel Zn finger modules in TP2 ***(23)***. Described below is the procedure, which should be useful for generating multiple site-specific mutants in any given gene. The strategy of the present method is summarized in **Fig. 1**.

2. Materials

1. Restriction enzymes and Deoxynucleoside triphosphate (dNTP) were from New England Biolabs.
2. dNTP mix: 10 m*M* each of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate.
3. *Pfu* polymerase (2 U/µL) and 10X *Pfu* polymerase buffer were from Stratagene.
4. Oligonucleotides or mutagenic primers (5 µ*M*) were either from Life Technologies or Bangalore Genei.
5. Acrylamide and agarose were from Life Technologies.
6. All fine chemicals were from Sigma, St. Louis, MO.
7. DNA purification kit was from Qiagen.
8. Plasmid pET-22b (Novagen).
9. Plasmid pTrc99A (Amersham-Pharmacia Biotech).
10. 1X Tris-EDTA (TE): 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0.

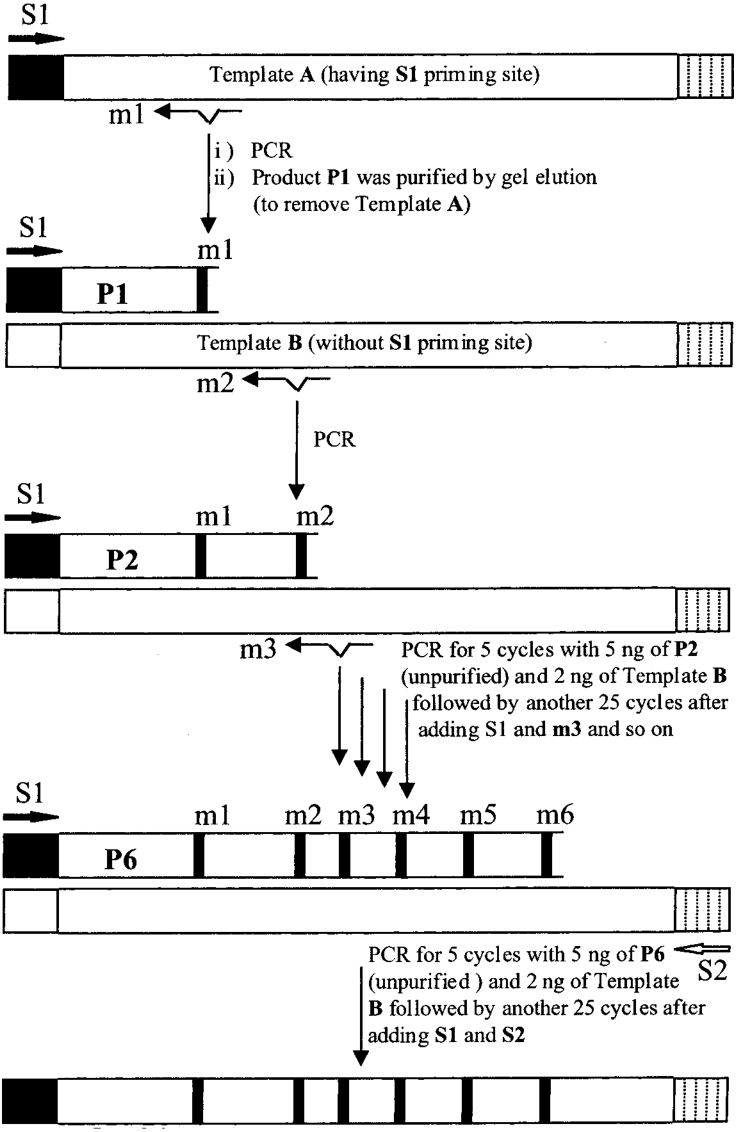
S1
Template **A** (having **S1** priming site)
m1
i) PCR
ii) Product **P1** was purified by gel elution
(to remove Template **A**)
S1
m1
P1
Template **B** (without **S1** priming site)
m2
PCR
S1
m1
m2
P2
m3
PCR for 5 cycles with 5 ng of **P2**
(unpurified) and 2 ng of Template **B**
followed by another 25 cycles after
adding S1 and **m3** and so on
S1
m1
m2
m3
m4
m5
m6
P6
PCR for 5 cycles with 5 ng of **P6**
(unpurified) and 2 ng of Template
B followed by another 25 cycles after
adding **S1** and **S2**
S2

Fig. 1. A schematic representation of the strategy for generating multiple site-specific mutations. S1, side primer; P1, P2...P6, mutagenic PCR products; m1, m2...m6, mutagenic primers; and S2, 3' end specific primer. (Reproduced with permission from **ref.** ***22***.)

11. 1X Tris-acetate-EDTA (TAE): 40 m*M* Tris-acetate, 1 m*M* EDTA.
12. Ethidium bromide stock: 10 mg/mL.
13. 3 *M* Sodium acetate, pH 5.2.

14. Butanol.
15. Ethanol, 95% (ice-cold).

3. Methods

3.1. Generation of PCR Templates A and B

The gene of interest should be cloned into two different vector backgrounds so that the primer S1 sequence is present only in template A, but not in template B.

1. Template A: TP2 cDNA was subcloned into *Nde*I and *Hin*dIII sites of plasmid, pET-22b (*see* **Note 1**).
2. Template B: TP2 cDNA was subcloned into *Nco*I and *Hin*dIII sites of plasmid, pTrc99A.

3.2. Designing Oligonucleotide Primers

The following consideration should be made in designing side and mutagenic primers:

1. Side primer S1 should anneal only to template A. This can be simply achieved by selecting vector (pET22b) DNA sequence from template A, which is at least 30 nucleotides away from the multiple cloning site (MCS) and the cDNA insert. In the authors' study, S1 was designed to anneal at the 5' region of the vector, with respect to the start codon of TP2 cDNA.
2. All the mutagenic primers should be designed in such a way that the mismatched portions of mutagenic primers should be in the middle of the primer, with approx 12–15 bases of exact complementary sequence on either side. The orientation of all the mutagenic primers should be toward side primer S1 (*see* **Note 2**).
3. Side primer S2 should anneal to the 3' end of TP2 cDNA. S2 should also have stop codon in-frame with TP2 cDNA, with appropriate restriction enzyme site (*Hin*dIII, in this study), for further subcloning of the PCR product after mutagenesis. S2 can also be designed to anneal to the vector (pTrc99A) sequence, which is downstream to stop codon of the TP2 cDNA. In this case, no stop codon and restriction enzyme site need to be included in the primer sequence.

3.3. First Cycle PCR

1. Prepare the PCR reaction mix, containing 2 ng template A, with 8 pmol each primers S1 and mL, 10 nmol dNTP and 1 U *Pfu* polymerase (*see* **Note 3**), in a total volume of 50 μL.
2. Carry out the PCR reaction with cycling conditions of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, for a total of 25 cycles, followed by a further extension at 72°C for 3 min.

3.4. Removal of Template A by Gel Elution

1. Separate the PCR product, P1 (after the first PCR reaction), from the template A, on a 4–8% (depending on the size of the PCR product) polyacrylamide gel electrophoresis (PAGE) in 1X TAE buffer, using a minigel PAGE setup (*see* **Note 4**).
2. Stain the DNA with 1X TAE containing ethidium bromide (0.5 ng/mL) for 30 min and excise out the desired band (by viewing on a UV transilluminator).
3. Crush the gel pieces by passing through 1-mL syringe (without the needle). Add 3–5 vol of 1X TE, and keep for shaking for 16 h at 37°C.
4. Recover the eluted DNA by butanol extraction, followed by ethanol precipitation (product P1). Estimate the concentration of DNA (product P1) after dissolving in 20 µL 1X TE.

3.5. Second PCR Cycle

1. The PCR reaction should contain 5 ng purified PCR product P1 (*see* **Note 5**) and 2 ng template B, 8 pmol each of primer S1 and second mutagenic primer m2, 10 nmol dNTPs, and 1 U *Pfu* DNA polymerase, in a total volume of 50 µL.
2. Set up the PCR reaction with the same cycling conditions as in the first-cycle PCR.
3. Check the PCR product, P2, by running a 1% agarose gel, and aliquot it, if necessary.

3.6. Third and Subsequent PCR Cycles

1. Carry out a 5-cycle PCR reaction with 5 ng unpurified PCR product P2, 2 ng template B, and other components, without any primers added (*see* **Note 6**). Keep the cycling condition the same as with that of the first PCR cycle. This step allows the extension of the 3' end of P2 along the template, enriching the template with two mutations. A few template molecules, extended from P1 (having only one mutation), will be outnumbered by template with two mutations.
2. After 5 cycles, carefully add 8 pmol each of primers S1 and mutant primer m3, and allow the reaction to proceed for another 25 cycles, giving rise to PCR product P3 with three mutations.
3. Repeat these steps with subsequent mutagenic primers m4, m5, m6, and so on, to obtain products P4, P5, P6, and so on, containing four, five, and six mutations, respectively (*see* **Notes 7–9**).
4. In the last step, carry out the PCR reaction, using extreme primers S1 and S2 to obtain the full length PCR product, which can be used for further cloning in the desired vector.

4. Notes

1. If the cloned gene of interest is more than 1 kb, it is preferable to perform the mutagenesis procedures only in the region where mutagensis is desired, by subcloning the smaller fragment into a suitable plasmid vector, and recloning back, after generating the desired mutation by the above method. This is necessary to avoid unwanted surrounding mutation generated during PCR reaction, and to overcome the problem of low efficiency of long template PCR with high-fidelity polymerases.

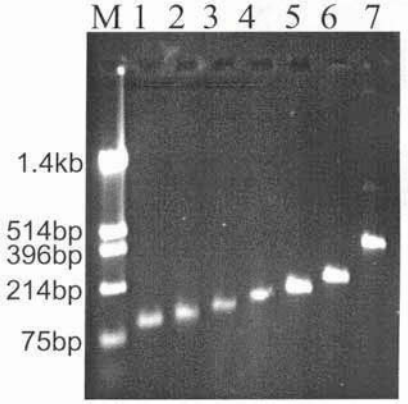

Fig. 2. Generation of multiple site-specific mutations in a single PCR product Ethidium bromide stained agarose gel pattern of PCR products generated after each step of mutagenesis. M, molecular size marker. Lane 1–7, mutant products after the first and subsequent PCR reactions. (Reproduced with permission from **ref.** ***22***.)

2. It is important to choose the orientation of the mutagenic primer toward the side, which will generate smaller products in the subsequent mutant products.
3. Because this protocol involves a series of PCR reactions, the authors recommend use of a DNA polymerase with high fidelity, such as *Pfu* (Stratagene), *Pfx* (Life Technologies), and *Vent* (New England Biolabs).
4. Although elution of DNA from agarose gel is much easier and faster, the authors have found that the removal of template A from the PCR product P1, by elution from a 4–8% PAGE, always gives much less background, compared to that of elution from agarose gel.
5. The authors consistently obtained the best result of amplification of specific product with a minimum amount of template (5 ng) for the subsequent round of PCR reaction.
6. The initial steps of five PCR cycles, without the primers, is crucial for achieving maximum efficiency of the present method.
7. In most cases, except in the first step of PCR, purification of DNA by gel elution is not necessary; however, if there are persistent problems in amplifying the next mutant product in any of the subsequent steps even after varying the annealing temperature and Mg^{2+} concentration, it is preferable to include another gel elution step prior to the next PCR reaction.
8. In each PCR reaction step, except in the last reaction wherein the full-length product is required to be cloned into a suitable expression vector, one tube of 50 µL PCR reaction is more than sufficient.
9. The frequency of clones containing all of the desired mutations vary with the efficiency of priming by the mutagenic primer in each round of PCR. Since each PCR is independent of others, the annealing condition can be optimized with

respect to each specific primer, to further improve the efficiency of the present method. The authors have successfully incorporated six site-specific mutations in spermatidal protein TP2 by this method ***(22)***. A representative example of the PCR products obtained after each round of PCR mutagenesis of TP2 is given in **Fig. 2**.

Acknowledgments

This work was financially supported by the Department of Atomic Energy, Government of India.

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13

Phenotypic Expression of Polymerase Chain Reaction-Generated Random Mutations in a Foreign Gene After its Introduction into an *Acinetobacter* Chromosome by Natural Transformation

David M. Young, Ruben G. Kok, and L. Nicholas Ornston

1. Introduction

Acinetobacter strain ADP1 (also known as strain BD413) is unusual among bacteria in the frequency with which it incorporates DNA into its chromosome (chr) by natural transformation. Since there is no requirement for uptake sequences to be present in the donor DNA, strain ADP1 can be transformed by DNA from any source. Unlike many other naturally transformable bacteria, *Acinetobacter* strain ADP1 is competent throughout most of its growth cycle, in virtually any medium that supports growth (1). Recombinant colonies can be either selected or screened on simple growth media within a day after transformation. Manipulated DNA can be substituted for its wild-type counterpart in the chr, and the function of the substituted DNA can be assessed at the level of phenotype. As described here, natural transformation allows chromosomal recovery of DNA that has gained random nucleotide substitutions during polymerase chain reaction (PCR) amplification. Observed changes in phenotype can be correlated to changes in nucleotide sequence, and thus permit a general survey of how changes in structure influence function in the gene product. Alternatively, nucleotide substitution can be used to alter the substrate specificity or kinetics of a gene product.

PCR mutagenesis, coupled to natural transformation, was initially used on resident genes on the *Acinetobacter* strain ADP1 chr ***(2,3)***. Amino acid substitutions, causing either null or conditional mutations in genes encoding various enzymes and transcriptional activators, were easily identified. Given the nature

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ

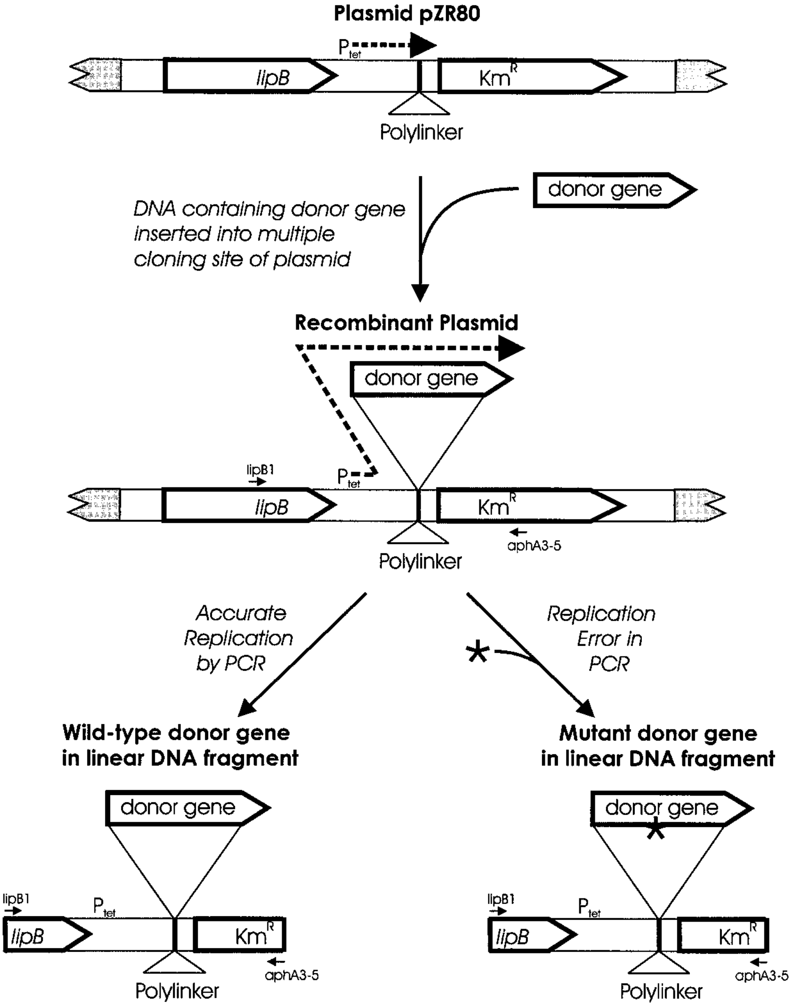
Plasmid pZR80
P_tet
lipB
Km^R
Polylinker
DNA containing donor gene inserted into multiple cloning site of plasmid
donor gene
Recombinant Plasmid
donor gene
lipB1
P_tet
lipB
Km^R
aphA3-5
Polylinker
Accurate Replication by PCR
Replication Error in PCR
*
Wild-type donor gene in linear DNA fragment
Mutant donor gene in linear DNA fragment
donor gene
lipB1
P_tet
lipB
Km^R
aphA3-5
Polylinker
donor gene
lipB1
P_tet
lipB
Km^R
aphA3-5
Polylinker

Fig. 1. Preparation of DNA fragments containing a foreign donor gene. A recombinant plasmid is formed by introduction of the donor gene into the polylinker downstream of the tetracycline promoter (P_{tet}) of pZR80. PCR amplification with primers lipB1 and aphA3-5 produces a donor DNA fragment containing either wild-type *(left)* or mutated *(right)* DNA.

of strain ADP1's natural transformation system, in principle, it should be possible to apply *Acinetobacter* genetics to any gene, provided it can be introduced stably into the cell.

The authors have developed a vector–recipient system that allows the cloning and selectable insertion of foreign genes into a docking site in the

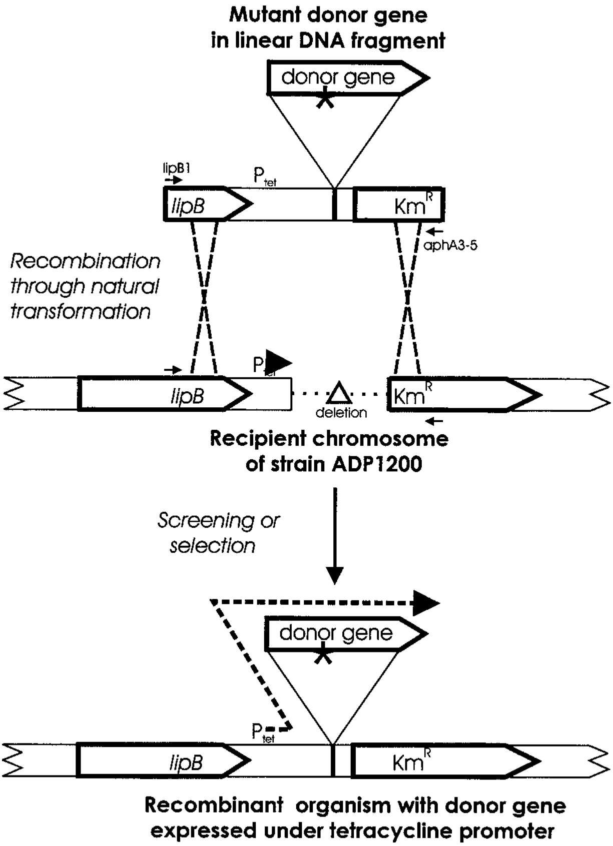
Mutant donor gene
in linear DNA fragment
donor gene
lipB1
P tet
lipB
Km R
aphA3-5
Recombination
through natural
transformation
P
lipB
deletion
Km R
Recipient chromosome
of strain ADP1200
Screening or
selection
donor gene
P tet
lipB
Km R
Recombinant organism with donor gene
expressed under tetracycline promoter

Fig. 2. Using natural transformation to prepare *Acinetobacter* recombinants that express the donor gene. The amplified PCR fragment containing the donor gene is mixed with the recipient *Acinetobacter* strain, ADP1200, which contains only a portion of the KM-resistance gene. Selection for KM-resistant recombinants produces organisms in which the donor (either mutant, as shown here, or wild-type) is expressed from the tetracycline promoter (P_{tet}). The phenotypes of recombinant colonies can be identified by either screening or selection.

Acinetobacter chr (*see* **Note 1**). Donor genes can be studied using all of the unique genetic manipulations facilitated by natural transformation. As depicted in **Figs. 1** and **2**, the system utilizes a vector that allows cloning of a foreign gene into a polylinker, where it is expressed from a constitutive tetracycline promoter. The polylinker is flanked by a kanamycin (KM) resistance gene and portions of the *lipAB* operon from strain ADP1, allowing the cloned gene to be stably inserted into the *Acinetobacter* chromosome by homologous recombination. The advantage of this system is that, once fragments containing PCR-generated mutations are produced, they can be recovered as individual clones, without resorting to cloning the fragment into a plasmid vector.

This chapter details the cloning vector, the *Acinetobacter* recipient strain, and details the PCR mutagenesis, transformation, and selection procedures. The authors previously ***(4)*** used this system to mutagenize a gene from *Pseudomonas putida*. The high G+C content of *Pseudomonas* DNA was not a barrier to expression from the A+T-rich *Acinetobacter* chromosome, so the system appears to be generally applicable.

2. Materials

2.1. Cloning Foreign Gene into pZR80

1. Bacterial strains: *Escherichia coli* DH5α (pZR80), competent *E. coli* DH5α (Life Technologies, Rockville, MD).
2. KM: Prepare a 25-mg/ml stock of KM sulfate, dissolved in deionized H_2O, and filter-sterilize with a 0.2-µm filter. Store at –20°C.
3. Luria-Bertani (LB) broth: In 1 L H_2O, dissolve 10 g Bacto-tryptone, 5 g Bacto yeast extract, 10 g NaCl; adjust pH to 7.2 with NaOH, and autoclave.
4. LB + KM agar plates: add 18 g agar to 1 L LB broth, autoclave, allow to media to cool to 55–60°C and add 1 mL 25 mg/mL KM.
5. Restriction endonucleases: available from several different suppliers. Store at –20°C.
6. T4 DNA ligase: available from several different suppliers. Store at –20°C.
7. 30% Glycerol (w/v): Add 30 g glycerol to flask, fill to 100 mL mark with deionized H_2O, and mix. Autoclave.

2.2. PCR Mutagenesis

1. pZR80 + foreign gene construct: diluted to approx 3 ng/µL with H_2O.
2. Deoxyribonucleoside triphosphate (dNTP) Mix: deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxycytidine triphosphate (dCTP), and deoxyguanosine triphosphate (dGTP). 2 m*M* for each in sterile water. Store a –20°C, and keep on ice after thawing.
3. PCR Primers (10 pmol/µL): lipB1 (5'-TGCAGGGCTGTTCGGCTCAG-3'), aphA3-5 (5'-GGCAATGTCATACCACTTGTCCG-3').
4. Sterile deionized H_2O.
5. *Taq* DNA polymerase (Boehringer Mannheim, Germany, or another supplier).
6. 10X *Taq* buffer: Use standard 10X buffer, containing 15 m*M* $MgCl_2$, supplied with *Taq* polymerase.
7. Mineral oil.
8. 6X loading dye: To 50 mL deionized water add 0.25 g bromphenol blue, 0.25 g xylene cyanol, 40.0 g sucrose, 5.0 mL 20% sodium dodecyl sulfate (SDS), and 20.0 mL 0.5 *M* EDTA (pH 8.0). Dissolve, and add deionized H_2O to 100 mL mark.
9. 50X TAE: To 600 mL deionized water add 242.0 g Tris-base, 57.1 mL glacial acetic acid, and 100.0 mL 0.5 *M* EDTA; dissolve, and adjust volume to 1000 mL with deionized H_2O. Dilute 1/50 with deionized H_2O to make 1X stock.

10. 1% agarose in 1X TAE: Add 1 g agarose to 100 mL 1X TAE, and dissolve by heating on hotplate or in microwave.

2.3. Transformation

1. Bacterial strain: *Acinetobacter* strain ADP1200.
2. Mineral media: When supplemented with an appropriate carbon source, most mineral media will support the growth of *Acinetobacter* strain ADP1 and its derivatives. One such mineral medium ***(5)*** is 11 m*M* KH_2PO_4, 95 m*M* Na_2PO_4, 0.81 m*M* $MgSO_4$, 37 m*M* NH_4Cl, 0.068 m*M* $CaCl_2$, and 1.8 µ*M* $FeSO_4$. Dissolve in distilled water and autoclave. For plates, add 18 g agar/L, prior to autoclaving. Add desired carbon source after autoclaving.
3. 1 *M* succinate stock: Dissolve 60 g succinic acid in 250 mL distilled H_2O. Bring to pH 7.0 using NaOH crystals (approx 40 g). Bring total volume to 500 mL, and autoclave.
4. 10 m*M* succinate mineral media: Prepare mineral media as described. After autoclaving, add 10 mL sterile 1 *M* succinate stock/L, and mix.
5. 10 m*M* succinate mineral media plates: Prepare mineral media + 1.8% agar as described. After autoclaving, add 10 mL sterile 1 *M* succinate stock/L, and mix.
6. 10 m*M* succinate mineral media plates with 25 µg/mL KM (succinate + KM): Prepare 10 m*M* succinate plates as described. After autoclaving, allow solution to cool to 55–60°C, and then add 1 mL sterile KM (25 mg/mL) stock, and mix.
7. Lysis buffer: 1.75 g NaCl, 0.88 g Na citrate·$2H_2O$, 0.1 g SDS in 200 mL H_2O. Autoclave.
8. 30% (w/v) glycerol: *See* **Subheading 2.1.**

2.4. Preparation of DNA Sequence Template

1. dNTP Mix: *See* **Subheading 2.2.**
2. PCR Primers (10 pmol/µL): lipB1 (5'-TGCAGGGCTGTTCGGCTCAG-3'), aphA3-5 (5'-GGCAATGTCATACCACTTGTCCG-3').
3. Sterile deionized H_2O.
4. *Taq* DNA polymerase: *See* **Subheading 2.2.**
5. 10X *Taq* buffer: *See* **Subheading 2.2.**
6. Geneclean II kit (Bio101, Vista, CA).

3. Methods

3.1. Cloning Foreign Gene into pZR80

The system requires that the donor gene be cloned into the polylinker of pZR80, so that the gene and its ribosomal binding site are downstream of the tetracycline promoter (P_{TET}). The cloning strategy used for each gene will vary, depending on the restriction sites flanking the gene, and their compatibility with those in the polylinker. Therefore, rather than describing a detailed procedure for cloning, an overview is given and common protocols are suggested, which can be refered to once a strategy has been developed for the donor gene.

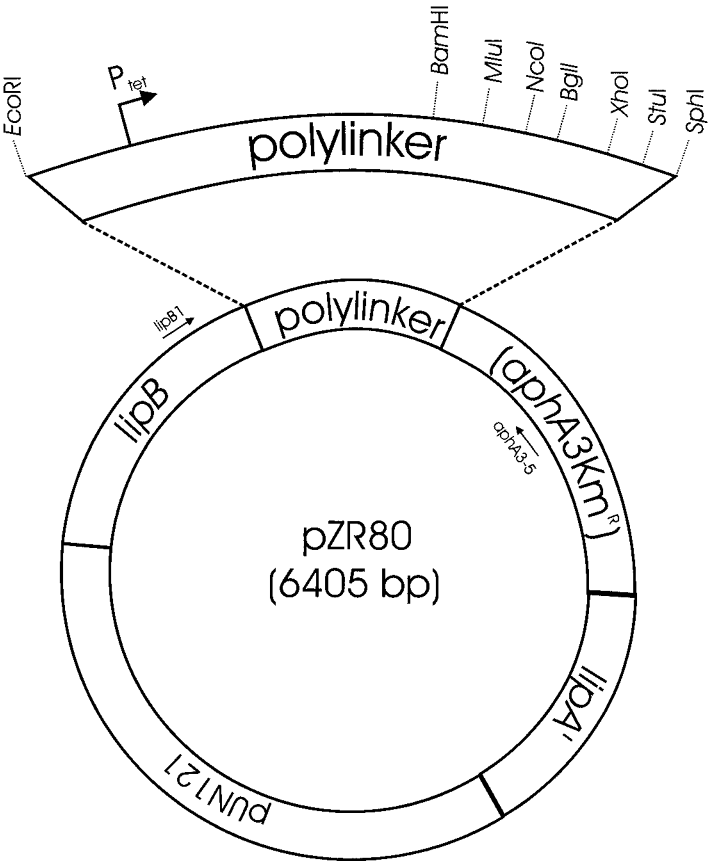
EcoRI
Ptet
BamHI
MluI
NcoI
BglI
XhoI
StuI
SphI
polylinker
polylinker
lipB1
lipB
(aphA3Km^R)
aphA3-5
pZR80
(6405 bp)
lipA'
pUNU121

Fig. 3. Essential features of pZR80. The backbone of the plasmid, including the origin of replication, is derived from the ColE1 plasmid pUNU121 ***(2)***. *lip* DNA from the *Acinetobacter* chromosome flanks the KM-resistance gene, *aphA3*, and the polylinker containing the tetracycline promoter (P_{tet}). Positions of the lipB1 and aphA3-5 primer annealing sites are indicated by small arrows.

Figure 3 depicts the specialized cloning vector, pZR80, and the unique restriction sites of its polylinker. Details concerning the construction of pZR80 can be found in the paper that originally described the system ***(2)***, and its complete nucleotide sequence can be accessed via the National Center for Biotechnology Information's GenBank database, under the accession number AY003885. A good approach for cloning genes into pZR80 is to PCR-amplify the gene (*see* **Note 2**), using primers possessing "tails" containing restriction sites that are compatible with those located in the polylinker of pZR80 (*see* **Note 3**). If possible, design the strategy so that the gene is ligated into the vector by directional cloning, allowing it to be expressed from P_{tet} upstream. If none of the restriction sites in the polylinker are of use for a particular gene, the polylinker contains a site for *Stu*I, a blunt cutting enzyme. This allows one to PCR-amplify their gene, and ligate it into the vector by blunt-ended ligation (*see* **Note 4**). Numerous protocols for the design of primers ***(6,7)*** and the cloning PCR products ***(8–10)*** are available in the literature.

Following ligation of the gene into pZR80, the ligation reaction can be transformed into competent *E. coli* cells (*see* **Note 5**), and transformants can be selected as follows:

1. Select transformants on LB + KM plates at 37°C (*see* **Note 6**).
2. When colonies arise on the selection plates, streak-purify clones on LB + KM at 37°C.
3. Prepare 5-mL LB + KM broth cultures of the clones, using single colonies from the streak-purification plates as inoculum. Incubate at 37°C, with shaking, overnight. Perform plasmid purification on 2–3 mL of each (*see* **Note 7**), and analyze the clones by restriction digestion and agarose gel electrophoresis, to identify those containing the insert in the proper orientation (*see* **Note 8**).
4. Once the correct construct has been identified, prepare frozen stocks of the *E. coli* strain harboring it. In a 2.0-mL CryoTube (Nalge Nunc, Denmark), mix 0.9 mL overnight culture (grown in LB + KM) with 0.9 mL sterile 30% glycerol, and freeze at –80°C.

3.2. PCR Mutagenesis

Once a gene has been cloned in pZR80, the construct is used as a template for mutagenic PCR amplification. A single pair of primers is used to amplify any gene that has been cloned in the vector. The PCR reaction amplifies the inserted gene, as well as flanking DNA, which shares sequence identity with the recipient's chr at an engineered docking site. Therefore, the PCR product can be transformed directly into the recipient, where it is inserted into the chr by homologous recombination. This alleviates the need to ligate the product into a plasmid vector. Transformants that undergo the recombination event are recovered by selecting for KM resistance.

There are many procedures for increasing the rate of misincorporation by *Taq* polymerase. However, even under standard conditions, the enzyme exhibits a relatively high rate of error (approx 10^{-4}/base) ***(11)*** (*see* **Note 9**). The authors have found that using standard PCR amplification reaction conditions, followed by transformation into *Acinetobacter* recipient strains, yields high numbers of unique mutants, the majority containing single substitutions in the gene of interest (*see* **Note 10**).

Table 1
PCR Reaction Mixture

Reagent	Stock concentration	Volume (μL)
pZR80 + gene	3 ng/μL	5
Standard *Taq* buffer	10X (15 mM $MgCl_2$)	5
dNTPs	2 m*M* for each dNTP	5
Primer lipB1	10 pmol/μL	1
Primer aphA3-5	10 pmol/μL	1
Sterile H_2O		32
Taq Polymerase	5 U/μL	1

1. Prepare a dilution of the construct so that the final concentration is approx 3 ng/μL. For most plasmid preparations, this requires about a 1/100 dilution (*see* **Note 11**).
2. Set up 50-μL PCR reactions in 0.6-mL tubes, as in **Table 1**.
   After adding the *Taq*, mix the sample, overlay with mineral oil, and place in the thermocycler (*see* **Note 12**).
3. Depending on the G+C content and length of the gene under investigation, the cycle used for amplification will vary. However the following cycle generally works well when using the primers lipB1 and aphA3-5: step 1; 95°C, 45 s; step 2; 58°C, 30 s; step 3; 72°C, 1 min/1000 bp target sequence. Repeat steps 1–3 for 30 cycles, followed by a final extension step at 72°C for 9 min.
4. When the reactions are complete confirm that the products are correct by removing 6 μL from each reaction and mixing them with 1 μL 6X loading dye. Run the samples on a 1% agarose gel by electrophoresis in 1X TAE buffer. Run DNA size standards on the gel, and confirm that the size of the product is correct. Using the primers lipB1 and aphA3-5, the size of the product should equal the size of the inserted DNA plus 1300 bp flanking DNA.
5. The authors recommend performing 5–10 individual PCR reactions, combining the products in a single tube, and using this mixture as the donor DNA for the transformation procedure. Using one PCR reaction as donor DNA increases the chance that a single mutation, arising early during PCR amplification, will be enriched, and therefore be the primary mutation in the transformant population. Combining many separate reaction products (the authors routinely combine 10) increases the randomness of the mutation spectrum.

3.3. Transformation

This subheading describes the general procedure for transforming *Acinetobacter* strain ADP1200 with mutagenic PCR products and selection of recombinants, based on repair of the truncated *Km*^{r} gene located at its chr docking site (**Fig. 2**). For cell culturing, the authors use mineral media with 10 m*M* succinate as a carbon source and the cells are grown at 30°C. However, the recipient strain is capable of growth on a wide range of carbon sources and media types, and grows well at temperatures up to 37°C. Therefore, growth conditions can be tailored to a particular selection scheme.

The authors recommend that multiple transformation cultures be performed for each mutagenesis experiment. Preparing multiple transformations reduces the risk that recombinant populations can be swept by cells that take up mutations that offer a selective advantage, even under the theoretically nonselective transformation culture conditions.

1. Start a 5-mL culture of *Acinetobacter* strain ADP1200 in 10 m*M* succinate mineral media and incubate overnight at 30°C, with shaking (*see* **Note 13**).
2. Transfer 20 µL culture to a sterile 14-mL polypropylene tube, add 480 µL fresh succinate mineral media, and incubate at 30°C, with shaking, for 1 h (*see* **Note 14**).
3. Add 0.5–1.0 µg PCR amplified DNA, and incubate at 30°C, with shaking, for an additional 3–5 h (*see* **Note 15**).
4. Transfer 400 µL culture to a sterile Eppendorf tube, and pellet by centrifugation. Remove the supernatant, and resuspend in 400 µL mineral media, with no carbon source added. Prepare a dilution series of the cells, and plate aliquots onto succinate + KM selection plates (*see* **Note 16**).
5. To obtain a viable cell count, plate dilutions of the cell suspension on succinate plates that do not contain KM. The transformation frequency can be calculated as the number of Km^r colonies per viable cell (*see* **Note 17**).
6. Incubate the plates at 30°C until colonies appear (usually overnight). Only cells that have been transformed by the PCR product should arise on the succinate + KM plates. If the gene under investigation can be screened for, or selected, the necessary reagents or treatments can be added directly to the succinate + KM selection plates. Alternatively, colonies can be analyzed following streak purification, alleviating any background effects (*see* **Note 18**).
7. Pick individual colonies from the selection plates and streak-purify on succinate + KM plates, incubate at 30°C.
8. Once mutant strains have been identified, they can be frozen in 30% glycerol, as described in **Subheading 3.1.** In addition, DNA from the cells can be stored as a crude lysate (*see* **Note 19**).

3.4. Preparation of DNA Sequencing Template

If DNA sequence analysis is to be performed on the mutant DNA, sequence template can be amplified from each strain, using primers lipB1 and aphA3-5.

1. In an Eppendorf tube, resuspend a single colony in 50 µL distilled H_2O and place in a boiling-water bath for 5 min. Centrifuge at high speed for 2 min, and use 5 µL supernatant as template for the PCR reaction.
2. Set up PCR reactions as described in **Subheading 3.2.** PCR-amplify, using the conditions found to give optimal amplification in **Subheading 3.2.** (*see* **Note 20**).
3. Check the 5 µL PCR products on an agarose gel, as described in **Subheading 3.2.**

4. Remove unincorporated PCR primers from the product, using the Geneclean kit (Bio101, Vista, CA), as described by the supplier (*see* **Note 21**). Elute the purified product in 35 µL sterile H_2O.
6. For automated fluorescent sequencing reactions, the optimum template concentration is ~10 ng/200 bp PCR fragment length. Using the above procedure, one can expect to generate enough template for 3–4 sequence reactions, assuming the PCR reaction yields an average amount of product.

4. Notes

1. Bacterial strains required for this system are available by request from the authors. Send requests to: L. Nicholas Ornston, Yale University, Department of Molecular, Cellular, and Developmental Biology, Kline Biology Tower, Room 752, P.O. Box 208103, New Haven, CT 06520-8103.
2. Use a proofreading polymerase, such as *Pfu* DNA polymerase (Stratagene, La Jolla, CA), to reduce the risk of mutation during the initial cloning. In addition, unlike other thermostable polymerases, which commonly have “extendase” activity, proofreading polymerases do not produce products with 3'-terminal adenosine residues. Therefore, the products can be cloned by blunt-end ligation, without additional manipulation. Be sure to perform sequence analysis on the final clone, to ensure that mutations were not introduced during cloning.
3. When digesting PCR products with restriction enzymes that create overhangs, gel-purify or extract the product with phenol/chloroform, prior to digestion. These treatments remove the polymerase, and prevent it from filling in the overhangs created by digestion.
4. When digesting the vector with a single enzyme, it is useful to dephosphorylate the linearized vector, before performing the ligation with the insert DNA. This prevents recircularization, and increases the ligation efficiency. However, when cloning a PCR product by blunt-ended ligation, be sure that either the PCR primers or the PCR product have been phosphorylated to provide the 5'-phosphates required for the formation of phosphodiester bonds during ligation.
5. The authors routinely use subcloning efficiency *E. coli* DH5α cells purchased from a commercial supplier (Life Technologies, Rockville , MD); however, competent cells prepared by any method can be used. The authors use *E. coli* DH5α, other *recA*$^-$ *E. coli* strains can be used.
6. Although the vector encodes both ampicillin (AMP) and KM resistance markers, only the *Km*^{r} marker is required for selection. Unlike KM, however, AMP should not be used alone, even for routine maintenance of clones, because the lack of selection for the *Km*^{r} gene may result in instability of the polylinker region of the construct, particularly if the foreign gene product is somewhat toxic to the cell.
7. Because the newly constructed plasmid is to be used as template in a PCR reaction, the authors suggest using one of the many commercially available plasmid-purification kits, and resuspending the DNA in H_2O, rather than in TE buffer. Such kits tend to yield a cleaner preparation, and therefore better PCR template.

8. If the inserted gene provides a selectable or screenable phenotype (e.g., antibiotic resistance, production of pigmented colonies, and so on), constructs may be screened/selected, while harbored in the *E. coli* strains. This is particularly useful in situations in which forced directional cloning is not possible. Since expression of the gene should rely on it being positioned correctly, relative to P_{TET}, clones with the correct construct can be initially identified via phenotype.
9. The authors have found that the error rate of *Taq* polymerase varies, depending on the manufacturer. For the procedure described here, Boehringer Mannheim *Taq* was used, and achieved good results. In addition, the length of the PCR product and the amplification conditions all influence error rate. Therefore, the authors suggest doing initial trial experiments to determine the conditions that yield the best results for a particular gene.
10. In general, *Taq* polymerase errors are heavily biased toward A:T→G:C transition mutations. Mutagenic PCR protocols usually call for increasing the dCTP and dTTP concentrations to 1.0 m*M*, increasing the $MgCl_2$ concentration to 7 m*M*, and adding 0.5 m*M* $MnCl_2$ ***(12)***. Although these reaction conditions do decrease sequence bias, they can also increase the overall mutation frequency and give rise to mutant populations in which the majority of the mutants contain more than one substitution. Certain types of studies (e.g., enzyme structure–function studies) require that mutants contain a single substitution that can subsequently be correlated to a given phenotype. Therefore, the investigator must optimize the PCR reaction conditions as required.
11. Although usually not necessary, linearizing the plasmid template often results in increased PCR amplification. Therefore, if a unique restriction site within the vector sequence exists, the authors suggest linearizing the plasmid, prior to using it as template. Care must be taken to ensure that the restriction enzyme does not cut either the gene insert or the vector sequence that lies between the lipB1 and aphA3-5 primer annealing sites. Refer to the pZR80 sequence from GenBank (accession no. AY003885) to determine unique sites.
12. If the thermocycler is equipped with a heated lid, it is not necessary to overlay with oil. If oil is added, transfer the reaction product to a fresh tube following PCR amplification, however, there is no need to extract with phenol/chloroform prior to transformation.
13. *Acinetobacter* strains grow well with many media types, including LB. However, using a defined media may produce more consistent results during the screening and selection procedures, by reducing effects such as catabolite repression, and other physiological effects that are hard to anticipate for cells grown in complex media.
14. *Acinetobacter* strain ADP1, and its derivatives, become competent at the beginning of log phase, and remain competent well into stationary phase ***(1)***. However, best competence is achieved shortly after transferring starved cells to new media.
15. Keeping the incubation time for the transformation culture to a minimum reduces the number of siblings in the final population.
16. Plating dilutions of 10^{-1}–10^{-3} usually will yield plates with manageable numbers of colonies.

17. Use the same type of medium and growth conditions used in the selection plates (i.e., LB or mineral at 30°C) for the viable counts.
18. Genes inserted into the chr are normally very stable, therefore, KM is generally not required in growth media once the gene is inserted. However, determining whether a particular gene is stable is important because some gene products produce toxic effects in the cell, which may lead to deletions.
19. Preparing a crude lysate is an quick and efficient way to store DNA in a readily usable form. Grow an overnight culture of the strain, and transfer 1 mL to an Eppendorf tube. Pellet the cells by centrifugation, and remove the supernatant. Resuspend the pellet in 0.5 mL lysis buffer ***(13)***, and incubate in a 60°C water bath for 30–60 min, vortexing periodically. Lysates can be stored frozen for at least 1 yr. Use 1–5 µL lysate as donor DNA, to transform *Acinetobacter* strains, as described above in **Subheading 3.3**.
20. Use a thermocycler program that yields a single product when amplifying sequence template. Nonspecific bands in the template will cause poor sequence results.
21. Although the authors use the Geneclean procedure, any convenient method, including gel purification or spin columns, can be used to remove unincorporated primers.

Acknowledgments

This research was supported by grant MCB-9603980 from the National Science Foundation and grant DAAG55-98-10232 from the U.S. Army Research Office. This is publication 25 from the Biological Transformation Center in the Yale Institute for Biospheric Studies.

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Polymerase Chain Reaction-Mediated Mutagenesis in Sequences Resistant to Homogeneous Amplification

Ross N. Nazar, P. D. Abeyrathne, and Robert V. A. Intine

1. Introduction

For many biologists, the polymerase chain reaction (PCR) has become an indispensable tool serving many diverse applications, from simple DNA amplification to complex diagnostics. In basic research, a very important application has been the use of PCR in the introduction of site-specific mutations into target DNA ***(1,2)***. A simple and commonly used example of this approach is the two-step "megaprimer" method (**Fig. 1**), in which the mutant oligomer is first incorporated into DNA in one direction, then this DNA itself is used as a megaprimer to complete the mutant sequence in the other direction ***(3)***. Over the years, such an approach has been used in many laboratories with considerable success. In the authors' experiments, this approach has been used for over a decade to introduce many changes into the ribosomal genes of yeast cells ***(4–6)***. Although some modifications were made some modifications in the amplification conditions ***(7)***, to improve the efficiency of the megaprimer method, the basic strategy, as originally described, has proven to be effective and reliable in most instances.

As with many applications of the PCR, in specific instances, artifacts may arise that prevent a successful mutation. The most common result from recombination between complementary sequence elements during DNA amplification, particularly with DNA sequences that contain repeated elements, or, as single strands, are likely to form extended hairpins. Once formed, such recombinants are rapidly amplified, together with the normal DNA template, ultimately giving rise to a heterogeneous collection of products, which frequently

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ

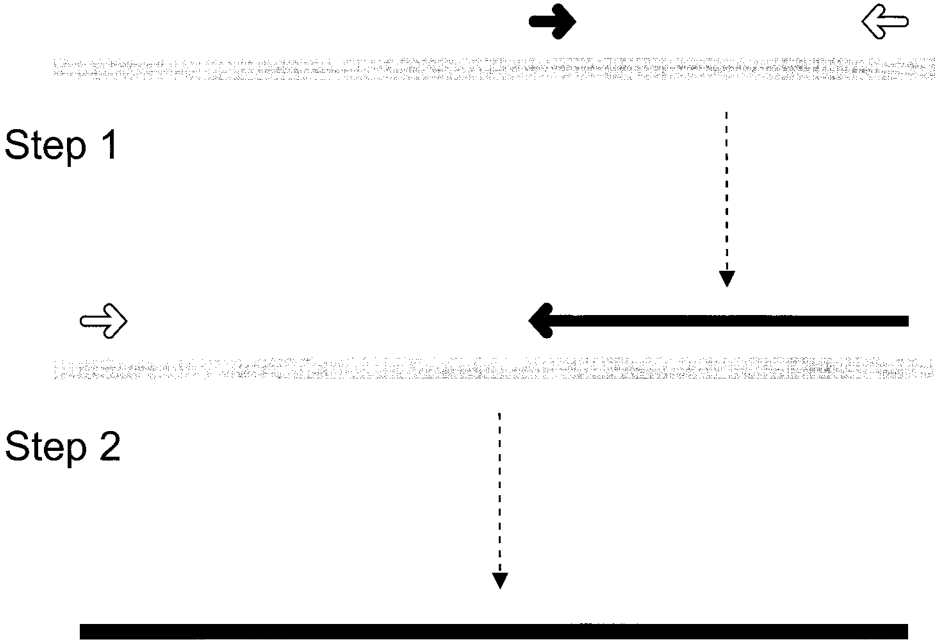

Fig.1. Overview of the two-step megaprimer method for PCR-mediated mutagenesis.

present extensive streaks after fractionation by gel electrophoresis. With rDNA spacers that form extensively paired structures, this artifact is fairly common, and often frustrates the introduction of key mutations.

To overcome or minimize this type of artifact, the authors initially examined a range of reaction conditions that are recommended in standard protocols, including changes in the annealing temperature, annealing time, amount of primer, amount of template, magnesium chloride concentration, and cycle number ***(8)***. Although some improvements were observed in specific instances, many attempts to introduce deletions or nucleotide changes continued to fail. Specialized approaches, such as "hot start" PCR ***(9)*** and "touchdown" PCR ***(10)***, also were attempted, with the same unsatisfactory results. "Overlap extension" was also examined ***(11)***, a method which, like the megaprimer approach, has been designed to embed mutations in a DNA sequence by PCR amplification. This method was shown ***(12)*** to be more efficient for generating large-deletion mutations (>200 bp). However, as found with the standard megaprimer strategy, the first amplifications of the overlapping fragments usually were successful, but the extension steps again resulted in smeary mixtures of heterogeneous products ***(8)***. Once again, alternate pairing between comple-

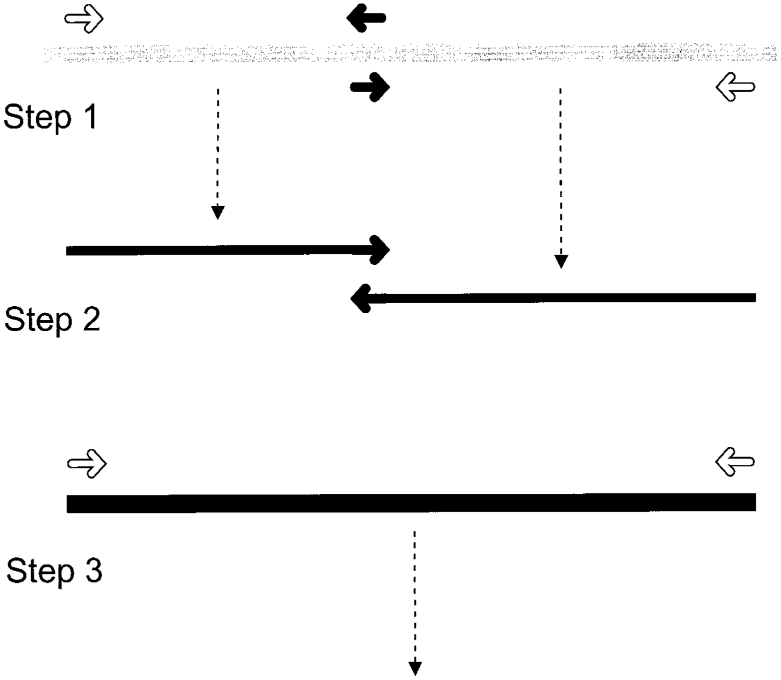

Fig.2. Overview of the SSOE method for PCR-mediated mutagenesis.

mentary strands continued to result in heterogeneous initiations of DNA replication and, ultimately, highly recombined populations of replicated molecules that appeared as broad streaks after gel electrophoresis.

The authors' first successful resolution of this problem ***(8)*** made use of a modified overlap extension strategy (**Fig. 2**), which we termed "single-stranded overlap extension" (SSOE). Since the product heterogeneity was probably the result of recombination between complementary sequences in the opposite strands, the authors proceeded to eliminate these competing factors, by using only one pair of overlapping single strands in the final amplification step. Strand separation methods, previously developed for DNA sequencing by chemical degradation ***(13)***, were adapted for the separation and gel purification of strands from products of the first PCR amplification. The isolated complementary strands were then annealed, extended by *Taq* DNA polymerase, and subsequently used as a template in the final PCR amplification step. This approach was successfully applied to many difficult mutations, yielding very satisfactory results, usually with a single major mutant product.

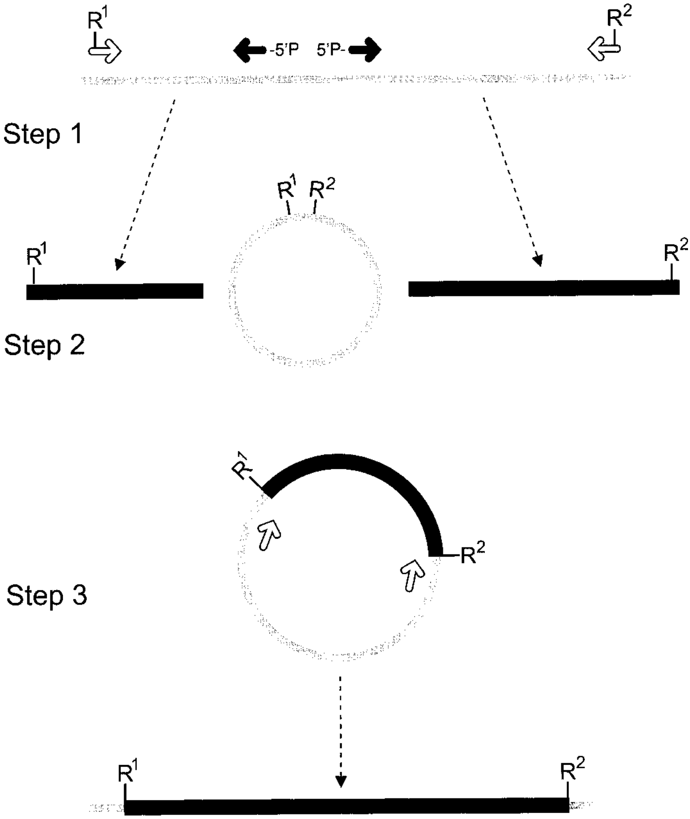

Fig. 3. Overview of the PEP method for PCR-mediated mutagenesis.

The SSOE approach was almost always successful, but the need for strand separation, as in chemical DNA sequencing, was labor-intensive, which was a major disadvantage to this procedure. To avoid this, the authors have developed a simplified alternate approach that eliminates the need for strand separation ***(14)***. As illustrated in **Fig. 3**, with this alternate strategy, termed "plasmid-enhanced PCR-mediated" (PEP) mutagenesis, the mutant DNA is amplified as two halves of the final sequence, and simply joined by blunt ligation. For a deletion, the targeted sequence is simply omitted, and, for base substitutions or insertions, the mutant sequence is incorporated into one of the primers. The efficiency and orientation of the ligation process is enhanced or controlled by sequence-specific overlapping interactions with the plasmid, using compatible restriction sites. Blunt ligation is used, because, in most instances, a restriction site in the central junction would require a change in the gene sequence. Instead, the plasmid-compatible restriction sites provide for an

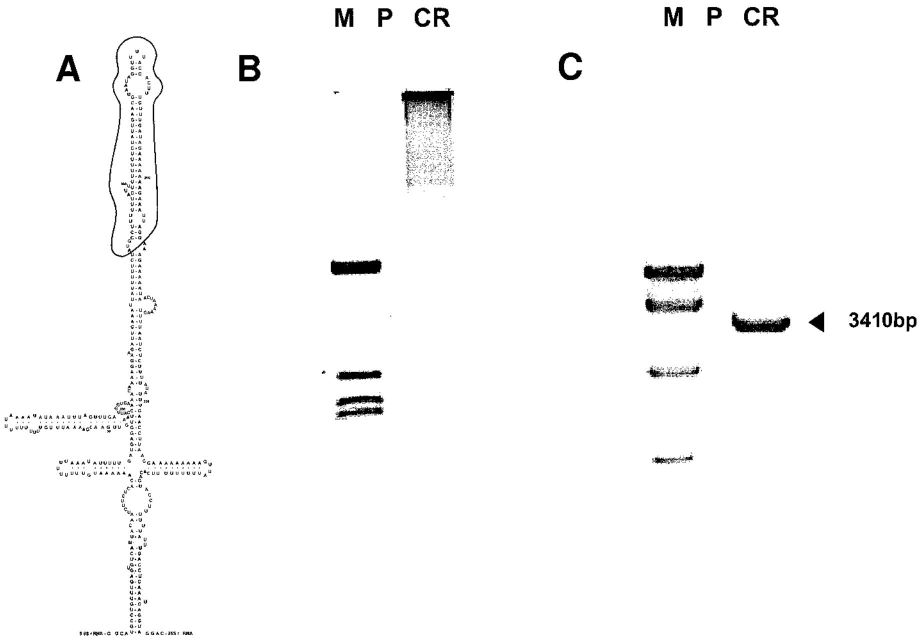

Fig. 4. Introduction of a large stem deletion in the ITS2 region of *S. pombe* preribosomal RNA. **(A)** Structure of the central extended hairpin in the ITS2 region. The lightly shaded region indicates the targeted deletion; the darkly shaded sequences indiacate the 3' and 5' ends of the mature 5.8S and 25S rRNAs. **(B)** Unsuccessful attempt to introduce the mutation using a standard megaprimer strategy. **(C)** Successful introduction of the mutation, using the PEP strategy.

efficient assembly of fragments without additional sequence changes. These sites also can be usefully applied in further subcloning. As noted in **Fig. 3** (solid arrows), to ensure ligation at the blunt junction, the internal primers are 5'- phosphorylated before use. As also noted (*see* **Subheading 3.3.3., step 3**), the ligated mixture actually can be used as a template for a direct amplification of the mutant sequence, using only plasmid-specific primers to provide mutated DNA (*see* **Subheading 3.3.3., step 3**), without a need for DNA cloning. As illustrated with the example shown in **Fig. 4**, with this approach, the authors routinely are able to resolve problems with difficult templates in a reliable manner.

2. Materials

1. 10X Polynucleotide kinase (PNK) reaction buffer: 100 m*M* $MgCl_2$, 100 m*M* 2-mercaptoethanol, 0.5 *M* Tris-HCl, pH 7.6.

2. 10X PCR buffer: 0.5 *M* potassium chloride, 15 m*M* $MgCl_2$, 1% (v/v) Triton X-100, 100 m*M* Tris-HCl, pH 9.0.
3. 5X ligation reaction buffer: 66 m*M* $MgCl_2$, 100 m*M* dithiotreitol, 660 m*M* adenosine triphosphate (ATP), 660 μ*M* Tris-HCl, pH 7.6.
4. 10X TBE buffer: 55 g boric acid, 9.3 g ethylenediamine tetraacetic acid (EDTA), 108 g Tris-base/L. The pH of this buffer is 8.3.
5. All reactions are conducted in disposable 1.5-mL microcentrifuge tubes or 0.5-mL PCR tubes.
6. All modifying enzymes and restriction endonucleases are commercially available. Significant differences in activity between enzymes from different suppliers were not observed.
7. 20% polyacrylamide gel (PAG): 19 g acrylamide, 1 g bis-acrylamide/L TBE buffer.
8. 5% PAG: 47.5 g acrylamide, 2.5 g bis-acrylamide/L TBE buffer.
9. 2% agarose gel: 2 g agarose/100 mL TBE buffer.

3. Methods

3.1. Mutagenic and Selection Primer Design

Both mutagenic and normal sequence primers are required. All are designed to anneal efficiently to the templates, and to prime selectively in complementary pairs.

1. For sequence deletions, all the primers are fully complementary to the template DNA. Primers, which range in length from 14 to 18 nucleotides, are usually effective and cost-efficient.
2. For base substitutions, at least one of the phosphorylated primers contains a mismatched portion in the middle of the oligonucleotide, with approx 9–12 bases of correct sequence on either side.
3. If possible, primer pairs should be similar in GC content. Although not essential, in general, they also should be GC-rich, and terminate in one or more G or C bases.
4. The melting temperature should be determined for each primer, and used to choose the annealing temperature during PCR amplification. This can be calculated manually using published predictive formulae, such as those reported by (Breslauer et al. ***[15]***). Commercial software or utilities, available on the internet (e.g., http://www.sci.lib.uci.edu/HSG/RefCalculators3.htm), also are very convenient for this purpose.
5. The two nonphosphorylated primers used in **Step 1** (*see* **Fig. 3**) either can include or should preceed appropriate restiction sites.
6. The selective primers used in **Step 3** (*see* **Subheading 3.3.1.** and **Fig. 3**) can be universal sequencing primers, which precede the multiple cloning site in the plasmid. These can be used in all experiments, and provide for multiple cloning sites in the final PCR product. The two nonphosphorylated template-specific primers used in **Step 1** also can be used, if additional cloning sites are not required in subsequent experiments.

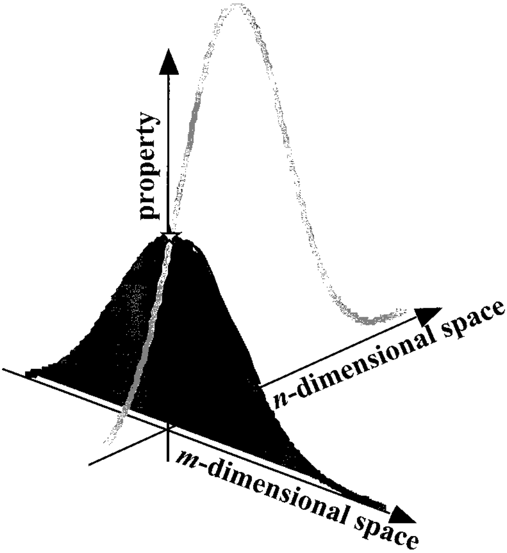

Fig. 2. Sketch of the landscape of a property of an enzyme in the *m* and *n*-dimensional sequence space. Landscape in the *m*-dimensional space is illustrated by plotting the values of the property of all mutants with *m* amino acid residues as obtained by substitution random mutagenesis on the axis for *m*-dimensional space. Landscape in the *n*-dimensional space illustrate all the elongation mutants prepared by adding a peptide tail with *n* amino acid residues to the C-terminal end of the enzyme with *m* amino acid residues, which was located at the highest peak of the *m*-dimensional landscape. The *m*- and *n*-dimensional sequence spaces are degenerated to the corresponding axes.

Basically, the concept of REM encompasses the theory of the landscape in a sequence space. For the protein with a length *m* amino-acid residues, all possible 20^m sequences are located in the *m*-dimensional sequence space. Population of the mutant libraries generated from a protein with *m* amino acid residues via substitution mutagenesis, including DNA shuffling, StEP, and RPR, then, will always be located in the same dimensional sequence space, because there will be no change in the length of the target protein. Consequently, serial cycles of consecutive substitution mutation and selection are restricted to pursue for the best sequence of a property in the *m*-dimensional sequence space. On the contrary, since elongation mutagenesis extends the peptide by the terminal attachment of certain length of a random peptide, e.g., *n*, the length of *m* is expanded to $m + n$ (**Fig. 2**). Therefore, acquiring a longer length of peptide from the original one would mean an expansion of dimension, and thus a search for better properties in higher dimensional sequence space.

3.3.2. Step 2: Fragment Ligation

The PCR amplified fragments and plasmid DNA generally should be digested with two appropriate restriction endonucleases, and recombinant constructs should be joined with DNA ligase using conditions specified by the enzyme manufacturers. Essentially, any cloning plasmid can be used. In most studies, the authors have used pTZR19, a widely used phagemid vector (PL-Pharmacia, Peapack, NJ). Depending on the restriction enzyme used, to permit efficient digestions, the PCR amplified fragments from **Step 1** (*see* **Subheading 3.1., step 1**) may first have to be purified by reverse phase chromatography or gel electrophoresis, to permit satisfactory digestion. The authors commonly fractionate the fragments on a 5% PAG (*see* **Note 5**).

1. Mix together 4–5 mg linearized plasmid DNA and a fourfold molar excess of the two digested PCR-amplified fragments, and precipitate the mixed DNAs with 2.5 vol ethanol containing 2% potassium acetate (*see* **Note 6**).
2. Collect the precipitate by centrifugation, discard the liquid phase, and dry the pellet with a gentle air stream or under vacuum (*see* **Note 3**).
3. Dissolve the DNA pellet in 8 μL H_2O (*see* **Note 7**).
4. Add 2 μL 5X ligation reaction buffer, mix by vortex, and spin briefly.
5. Heat at 70°C for 5 min and cool rapidly on ice.
6. Add 1 μL ATP solution (10 m*M*) and 1 μL T4 DNA ligase enzyme (1 U/μL), mix by vortex, and spin briefly.
7. Incubate at room temperature for 4 h.

The efficiency of ligation can be assessed by an electrophoretic comparison of the pre- and postligation mixes, using a 2% agarose gel. A 0.5–1-μL aliquot is sufficient for this purpose.

3.3.3. Step 3: PCR Amplification of Mutant DNA

The mutant DNA sequence is selectively amplified, using plasmid-specific primers and the same PCR amplification protocol described in **Subheading 3.3.1.**

1. Dilute 1 μL ligated DNA mixture prepared in **Subheading 3.3.2.** with 19 μL H_2O.
2. Add 5 μL 10X PCR buffer and the other reagents described in **Subheading 3.3.1.** Use the plasmid-specific primers, in this case.
3. With a temperature cycler, amplify the mutated DNA sequence, using 30 reaction cycles, as described in **Subheading 3.3.1.**

Again, the quality and yield of the product can be confirmed by an electrophoretic separation, using a 2% agarose or 5% PAG and appropriate chain-length markers. Frequently, only one band is evident, as illustrated in **Fig. 4C**. Large deletions can be confirmed by chain-length measurement; smaller changes should be confirmed directly by DNA sequencing ***(17)***. For cloning, the PCR-amplified fragment can be digested and ligated, as described in **Subheading 3.3.2.** Clones also may be isolated directly by using the remaining ligation mixture, prepared in **Subheading 3.3.2.**, to transform appropriate host cells, followed with selection based on antibiotic resistance or DNA hybridization ***(18)***.

4. Notes

1. To avoid nonspecific nuclease degradation, the H_2O used to dissolve DNA, or to prepare enzyme buffers, should be glass-distilled or deionized and boiled. Also, use disposable plasticware or washed glassware that has been heated to a high temperature (>150°C).
2. Commercial liquified or crystalline phenol can be used directly, if the solution is colorless. A water-saturated solution is prepared by adding sufficient H_2O or buffer (>10% w/w) to permit some separation of phases. The solution can be stored at 4°C, but should be discarded if pink or yellow color is present. Since phenol is corrosive, and can cause severe burns, gloves and safety glasses should be worn.
3. If an air stream is used, the air should be filtered to avoid contamination. If vacuum is used, a centrifugal concentrator, such as the CentriVap (Labconco, Kansas City, MO) is convenient, and avoids possible disruptions and loss of the pellet.
4. The details for electophoretic separations of products are not given, because these procedures are those commonly used in all molecular genetics laboratories, and the apparatus may be of commercial or lab design. The basic formulae are listed in **Subheading 2.**; reagent-grade acrylamide, agarose, and buffer ingredients are suitable.
5. With some restriction enzymes, poor digestion is often observed using PCR-amplified DNA, a problem necessitating that the fragments be purified before digestion. The method of purification is not critical, and various commercial chromatography kits or other aids are in wide use and entirely suitable. Although more labor-intensive, the authors prefer to use gel electrophoresis, because it simultaneously provides a measure of fragment quality and yield. The authors elute the DNA by homogenization, but electroelution is entirely suitable as well.
6. The absolute concentration of DNA is important for efficient ligation, and should not be reduced significantly.
7. Depending on how exhaustively it is dried, precipitated DNA can be difficult to dissolve. Sufficient time and mixing should be applied, to make sure the DNA is fully dissolved.

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Polymerase Chain Reaction-Based Signature-Tagged Mutagenesis

Dario E. Lehoux and Roger C. Levesque

1. Introduction

The study of bacterial pathogenicity in vitro has identified many signals, at the molecular and cellular levels that affect expression of virulence and other factors in causing disease. Because these pathways are not necessarily reproduced in vitro, their implication and their regulation in pathogenesis in vivo remains circumstantial. Genomics-based technologies can now be used to study pathogenesis in vivo ***(1)***. Signature-tagged mutagenesis (STM) ***(2)*** is an elegant method, based on negative selection, to identify mutations in a gene, which is essential during the infection process. In STM, transposon (Tn) mutants are generated, and each unique cell clone is tagged with a specific DNA sequence ***(2)***. Compared to traditional pathogenicity assays, STM minimizes the number of animals to be utilized, and eliminates false-positive and false-negative results. The strategy of STM depends on tagged Tn mutants, defective in virulence, which cannot be maintained in vivo. Attenuated mutants are selected and retested to confirm attenuation; disrupted genes are cloned via the Tn marker, and the inactivated gene confirmed by DNA sequencing. Modifications of STM, allowing rapid and easy identification of attenuated mutants, have recently been described and called "polymerase chain reaction (PCR)-based STM" ***(3)***.

STM is divided into two steps: the construction of a library of tagged mutants by Tn mutagenesis, and the in vivo screening step. A crucial step in STM depends on a high frequency of random Tn insertions into the chromosome (chr), which is not always possible; insertion into an essential gene gives a lethal phenotype (defined here as a gene essential for growth in vitro). Also, the bacterial genetic tools of Tns are not easily amenable to genetic analysis in

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ

Table 1
DNA Sequences of ONs Synthesized for STM *(3)*

Tag number	Nucleotide sequence
1	5'-GTACCGCGCTTAA**ACGTTCA**G-3'
2	GTACCGCGCTTAA**ATAGCCT**G
3	GTACCGCGCTTAA**AAGTCTC**G
4	GTACCGCGCTTAA**TAACGTG**G
5	GTACCGCGCTTAA**ACTGGTA**G
6	GTACCGCGCTTAA**GCATGTT**G
7	GTACCGCGCTTAA**TGTAACC**G
8	GTACCGCGCTTAA**AATCTCG**G
9	GTACCGCGCTTAA**TAGGCAA**G
10	GTACCGCGCTTAA**CAATCGT**G
11	GTACCGCGCTTAA**TCAAGAC**G
12	GTACCGCGCTTAA**CTAGTAG**G

Each 21-mer has a melting temperature of 64°C and permits PCRs in one step when primer combinations are used for screening. The consensus 5' ends, comprising the first 13 nucleotides, have higher ΔGs for optimizing PCRs. The variable 3' ends, indicated in bold, define tag specificity, and allow amplification of specific DNA fragments. Each tag is used as a primer in PCR, with a primer synthesized within the KM-resistant gene of mini-Tn*5* Km2. The set of 12 21-mers, representing the complementary DNA strand in each tag, are not represented, and can be deduced from the sequences presented.

some species of bacteria. The PCR-based STM scheme involves designing pairs (12 in this case, but 24, 48, and 96 could be utilized) of 21-mers (**Table 1**) synthesized as complementary DNA strands for cloning into the mini-Tn*5* plasmid vector. The sets of 12 tags are repeatedly used to construct 12 libraries (**Fig. 1**), and used for specific DNA amplifications as signature tags easily detectable by PCR (**Fig. 2**). Tagged products from arrayed bacterial clones can be compared as DNA products of a specific length separated by agarose gel electrophoresis.

Twelve pairs of 21-mers were designed as tags, following three basic rules: Similar melting temperature of 64°C, to simplify tag comparisons by using one step of PCR reactions; invariable 5' ends with higher ΔG than at the 3' end, to optimize PCR amplification reactions (10); a variable 3' end, for an optimized yield of specific amplification product from each tag. The 21-mers are double-stranded, and are cloned into a miniTn (mini-Tn*5*), which is used to mutagenize, and, hence, tag bacteria. A series of suicide plasmids carrying mini-Tn*5*s, each with a specific tag, were transferred into the targeted bacteria, giving 12 libraries of mutants; 96 groups of 12 mutants are pooled, and arrayed into 96-well plates. The 12 mutants from the same pool are grown separately

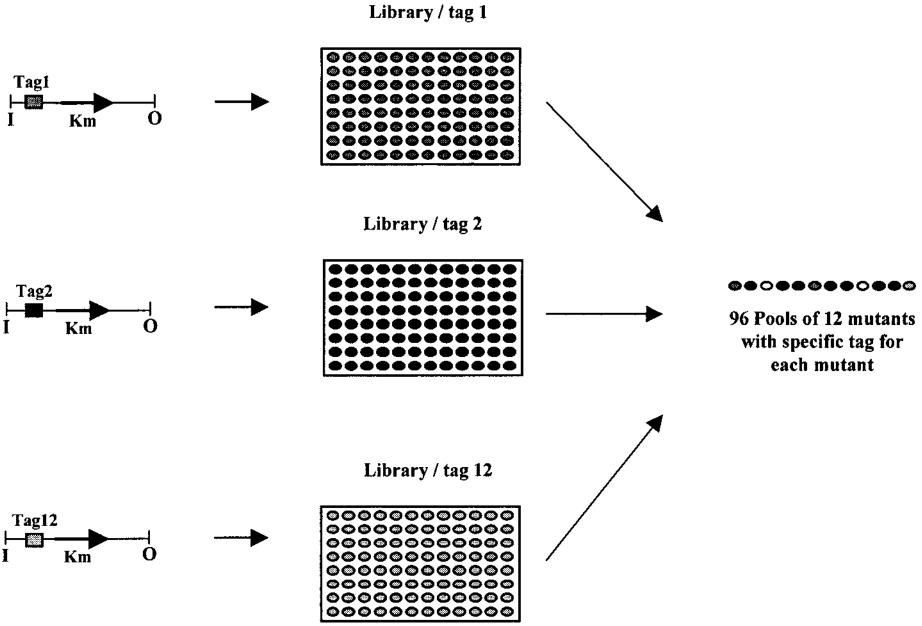

Fig. 1. Construction of 12 libraries of *P. aeruginosa* mutants tagged with mini-Tn*5 Km2*. Double-stranded DNA tags were cloned into the pUTmini-Tn*5 Km2* plasmid (*see* **Methods**). KM-resistant exconjugants were arrayed as libraries of 96 clones. In a defined library, each mutant has the same tag, but is inserted at different locations in the bacterial chr. One mutant from each library is picked to form 96 pools of 12 mutants with a unique tag for each. The differences between tags are represented by the gray scale colors. O and I represent the 19-bp inverted repeats at each extremity of the mini-Tn*5*.

overnight at 37°C. Aliquots of these cultures were pooled and a sample was removed for PCR analysis (the in vitro pool). A second sample from the same pool is used for the in vivo passage. After this passage, bacteria recovered from the animal organ (the in vivo pool) and the in vitro pool are used as templates in 12 distinct PCR reactions. Amplicons obtained with the in vitro and in vivo pools are compared. Mutants whose tags have not been detected after the in vivo passage are in vivo attenuated. This simple STM method can be adapted to any bacterial system, and used for genome scanning in various growth conditions.

2. Materials

2.1. Tags Annealing

10X Medium salt buffer: 10 m*M* Tris-HCl, pH 7.5, 10 m*M* $MgCl_2$, 50 m*M* NaCl, 1 m*M* dithiothreitol.

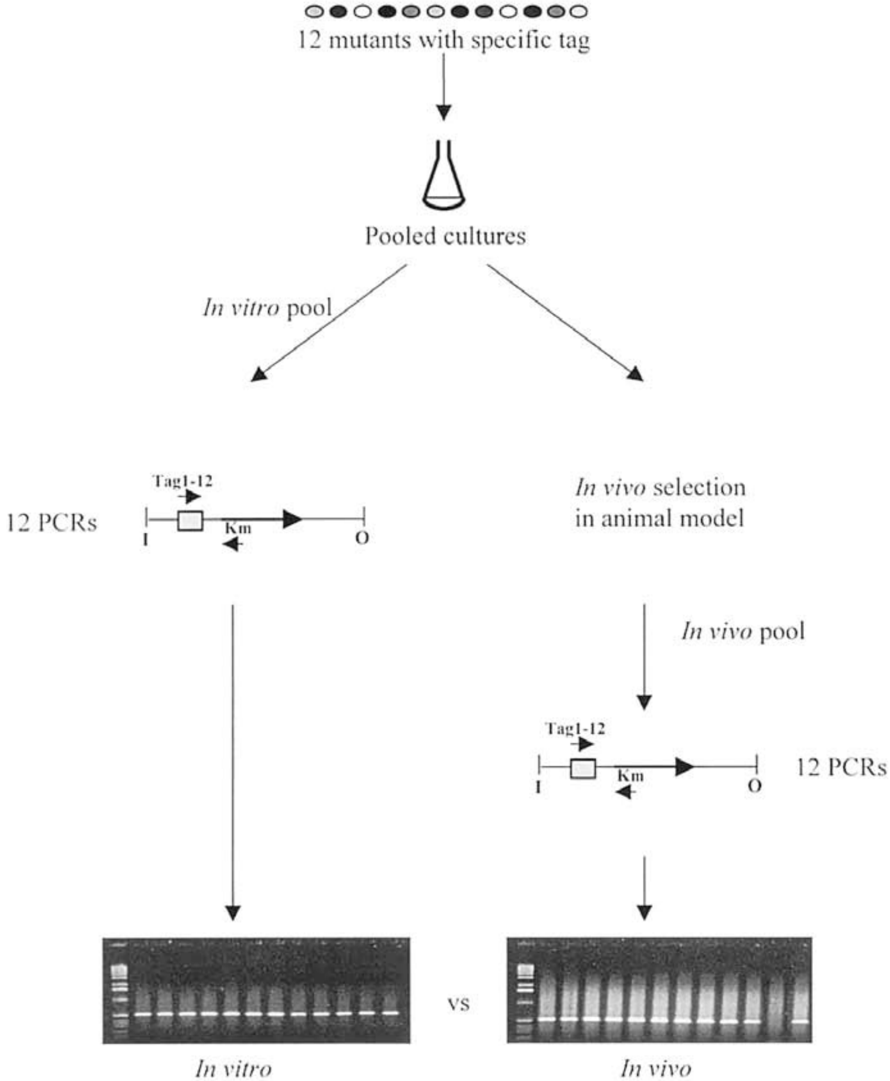
12 mutants with specific tag
Pooled cultures
In vitro pool
12 PCRs
Tag1-12
Km
I
O
In vivo selection in animal model
In vivo pool
Tag1-12
Km
I
O
12 PCRs
vs
In vitro
In vivo

Fig. 2. STM scheme for comparisons between the in vitro and in vivo negative selection step. Mutants from the same pool were grown as separate cultures. An aliquot was kept as the in vitro pool, and a second aliquot was used for injection into an animal model for in vivo selection. After this passage, bacteria were recovered from animal organs, and constitute the in vivo pool. The in vitro and in vivo pools of bacteria were used to prepare DNA templates in 12 PCRs, using the 21-mers 1–12 in **Table 1** and the KM primer. PCR products were analyzed by agarose gel electrophoresis. Lanes 1–12: PCR products obtained with the primers 1–12. In this example, the mutant with Tag11 was not recovered after the in vivo selection.

2.2. Plasmid Preparation

1. pUT mini-Tn5 Km2 plasmid (4).
2. *Kpn*I (New England Biolabs [NEB], Mississauga, ON, Canada).
3. 10X NEB #1 buffer: 10 m*M* Bis-Tris propane-HCl, 10 m*M* $MgCl_2$, 1 m*M* dithiothreitol, pH 7.0.
4. 10X bovine serum albumin (1 mg/mL) (NEB).
5. T4 DNA polymerase (Gibco-BRL, Burlington, ON, Canada).
6. Deoxyribonucleoside triphosphates (dNTPs) (deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate, and deoxythymidine triphosphate from Amersham Pharmacia Biotech, Baie d’Urfé, PQc, Canada).

2.3. Plasmid and DNA Tag Ligation and Electroporation

1. T4 DNA ligase 10X buffer (NEB): 50 m*M* Tris-HCl, pH 7.5, 10 m*M* $MgCl_2$, 10 m*M* dithiothreitol, 1 m*M* adenosine triphosphate, 25 µg/mL bovine serum albumin.
2. T4 DNA ligase (NEB).
3. Micropure-EZ pure (Millipore, Montréal, PQ, Canada).
4. Microcon 30 (Millipore).
5. Microcon PCR (Millipore).
6. Electrocompetent *Escherichia coli* S17-1λ*pir* (washed in glycerol 10%).
7. 2-mm electroporation gap cuvets (BTX, distributed by VWR Can Lab, Mississauga, ON, Canada).
8. SOC medium (Fisher, Montréal, PQ, Canada): Formula per liter: 20 g Bacto-tryptone, 5 g Bacto-yeast extract, 0.5 g sodium chloride, 2.4 g magnesium sulfate anhydrous, 0.186 g potassium chloride, 20 mL filter-sterilized 20% glucose.
9. Tryptic soy broth (TSB: Fisher): Formula per liter: 17 g Bacto-tryptone, 3 g Bacto-soytone, 2.5 g Bacto-dextrose, 5 g sodium chloride, 2.5 g dipotassium phosphate.
10. Ampicillin (AMP) (Sigma, St. Louis, MO).
11. Kanamycin (KM) (Sigma).
12. TE PCR: 10 m*M* Tris-HCl, pH 7.4, ethylenediamine tetraacetic acid (EDTA) 0.1 m*M*.
13. PCR reaction premix: 10X *Taq* polymerase (Gibco-BRL) reaction buffer, without Mg^{2+}: 200 m*M* Tris HCl, pH 8.4, 500 m*M* KCl, 50 m*M* $MgCl_2$, 1.25 m*M* dNTPs, 10 pmols oligonucleotide (ON) tag (**Table 1**), 10 pmols pUTKanaR1 (5'-GCGGCCTCGAGCAAGACGTTT-'3), *Taq* polymerase (Gibco-BRL).
14. Mineral oil.
15. Agarose.
16. 1X Tris-borate EDTA buffer: 5X concentrated stock solution per liter: 54 g Tris-base, 27.5 g boric acid, 20 mL EDTA, pH 8.0.
17. 0.5 µg/ml ethidium bromide solution.

2.4. Tn Mutagenesis

1. Nylon membrane.
2. Brain–heart infusion agar (BHIA) (Fisher).
3. BHI (without agar).
4. Sterile 1X phosphate-buffered saline (PBS): 137 m*M* NaCl, 3 m*M* KCl, 10 m*M* Na_2HPO_4, 1.3 m*M* KH_2PO_4, pH 7.4.
5. 2 mL 96-well plates (Qiagen).

2.5. In Vivo Screening of Tagged Mutants

1. Typtic soy broth.
2. Kanamycin.
3. Brain–heart infusion.
4. 96-well microtiter plates.
5. Brain–heart infusion agar.
6. 1X PBS.
7. TE PCR buffer.
8. PCR reaction premix.
9. Mineral oil.
10. Agarose.
11. 1X Tris-borate-EDTA buffer.
12. 0.5 µg/mL ethidium bromide solution.
13. Animals.
14. Dissection kit.
15. Potter homogenizer.

2.6. Cloning and Analysis of Disrupted Genes from Attenuated Mutants

1. Qiagen genomic Tips (Qiagen, Mississauga, ON, Canada).
2. Selected endonuclease.
3. pTZ18R (Amersham Pharmacia Biotech).
4. 10X T4 DNA ligase buffer.
5. T4 DNA ligase (NEB).
6. Microcon PCR (Millipore).
7. Electrocompetent *E. coli* DH5α (washed in 10% glycerol).
8. 2-mm electroporation gap cuvets.
9. SOC media (Fisher).
10. X-gal (Sigma).
11. Isopropyl thiogalastose (Roche Diagnostics, Laval, PQ, Canada).
12. Ampicillin.
13. Qiagen midipreparation kit.
14. DNA sequencing service (Nucleic acids analysis and synthesis units, Laval University, http://www.rsvs.ulaval.ca).
15. PC computer.

3. Methods

3.1. Construction of Tagged Plasmids

3.1.1. Tags Annealing

1. A collection of 12 defined 21-mers ONs should be synthesized, along with their complementary DNA strands, as tags (**Table 1**) (Nucleic acids analysis and synthesis units, Laval University, http://www.rsvs.ulaval.ca).
2. Annealing reactions contained 50 pmol of both complementary ONs in 100 µL medium salt buffer.
3. This ON mixture is heated 5 min at 95°C, left to cool slowly at room temperature in the block heater, and kept on ice (*see* **Note 1**).

3.1.2. Plasmid Preparation

1. 20 µg pUT mini-Tn*5* plasmid are digested with 20 U *Kpn*I in 40 µL 1X NEB #1 buffer containing 1X bovine serum albumin (*see* **Note 2**).
2. Incubate 2 h at 37°C.
3. Inactivate 20 min at 65°C.
4. Extremities are blunted with T_4 DNA polymerase by adding 4 nmols of each dNTPs and 5 U T4 DNA polymerase.
5. Purify blunted plasmid from endonuclease and T4 DNA polymerase reactions with Micropure-EZ and Microcon 30 systems in a single step, as described by the manufacturer's protocol.

3.1.3. Plasmid and DNA Tag Ligation and Electroporation

1. 0.04 pmol plasmid are ligated to 1 pmol of double-stranded DNA tags in a final volume of 10 µL T4 DNA ligase 1X buffer containing 400 U T4 DNA ligase (*see* **Note 3**).
2. Ligated products are purified using Microcon PCR (Millipore), as described by the manufacturer's instructions, and resuspended in 5 µL H_2O.
3. All of the 5 µL containing ligated products, are transferred into *E. coli* S17-λ*pir* ***(5)*** (*see* **Note 4**) by electroporation, using a Bio-Rad apparatus at 2.5 kV, 200 Ω, 25 µF in a 2-mm electroporation gap cuvet. After electroporation, 0.8 mL SOC are added to cells, which are transferred in culture tubes, to be incubated for 1 h at 37°C.
4. Transformed bacteria containing tagged plasmids are selected on TSB supplemented with 50 µg/mL AMP and 50 µg/mL KM by plating 100 µL transformed cells.
5. Single colonies are selected, purified, and screened by colony PCR (*see* **Note 5**) in 50 µL reaction volumes containing: 10 µL boiled bacterial colonies in 100 µL TE PCR; 5 µL 10X *Taq* polymerase (Gibco-BRL) reaction buffer; 1.5 m*M* $MgCl_2$; 200 µ*M* of each dNTPs; 10 pmol one of the ON tag ***(3)*** used to construct the DNA tags as a 5' primer and 10 pmol the pUTKanaR1 (5'-GCGGCCTCGAGC AAGACGTTT-'3) as the 3' primer in the KM-resistance gene; 2,5 U *Taq* polymerase (Gibco-BRL). Thermal cycling conditions were (touchdown PCR): a hot

start for 7 min at 95°C, 2 cycles at 95°C for 1 min, 70 to 60°C for 1 min, and at 72°C for 1 min, then followed by 10 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min in a DNA Thermal Cycler (Perkin-Elmer/Cetus). 10 µL of DNA amplified products were analyzed by electrophoresis in a 1% agarose gel, 1X Tris-borate EDTA buffer, and stained for 10 min in 0.5 µg/mL ethidium bromide solution ***(6)***. The amplicons should be ~500 bp (**Fig. 2**).

3.2. Construction of Libraries of Tagged Mutants

3.2.1. Tn Mutagenesis

1. *E. coli* S17-λ*pir* containing the pUTtag plasmids, is used as a donor for conjugal transfer into the recipient strain. The donor:recipient ratio should be established to obtain the maximum exconjugants by preliminary experiments. For *Pseudomonas aeruginosa*, the authors used 1 donor:10 recipient cells. Cells are mixed and spotted as a 50-µL drop on a nylon membrane placed on a nonselective BHIA plate. Plates are incubated at 30°C for 8 h (*see* **Note 6**).
2. Filters are washed with 10 mL PBS to recover bacteria.
3. Five 100-µL aliquots of the PBS solution, containing exconjugants, are plated on five BHIA plates, supplemented with the appropriate antibiotic to select for the strain. KM is used to select exconjugants with the mini-Tn*5* inserted into their chr (*see* **Note 7**). Plates are incubated overnight at 37°C.
4. Selected colonies are picked on BHIA, supplemented with AMP, to exclude bacterial colonies having the suicide donor plasmid, pUT mini-Tn*5* Km2, inserted into the chr by homologous recombination.
5. KM-resistant and AMP-sensitive exconjugants are arrayed as libraries of 96 clones in 2-mL 96-well plate in 1.5 mL BHI supplemented with KM and appropriate antibiotic. The 2-mL 96-well plates are incubated 18–22 h at 37°C (*see* **Note 8**).
6. As an STM working scheme, one mutant from each library is picked to form 96 pools of 12 unique tagged mutants (**Fig. 1**) contained in the 2-mL 96-well plates.

3.3. In Vivo Screening of Tagged Mutants

1. The 12 mutants from the same pool are grown separately overnight at 37°C in 200 µL TSB containing KM in 96-well micotiter plates.
2. Aliquots of these cultures are pooled.
3. A first sample is diluted from 10^{-1} to 10^{-4}, and plated on BHIA supplemented with KM.
4. After overnight incubation at 37°C, 10^4 colonies are recovered in 5 mL PBS, and a sample of 1 mL is removed for PCR, and called the in vitro pool.
5. The 1 mL in vitro pool sample is spun down, and the cell pellet is resuspended in 1 mL TE PCR buffer.
6. The in vitro pool is boiled for 10 min, spun down, and 10 µL supernatant are used in PCR analysis, as described above.
7. A second sample from the pooled cultures is used to inoculate animals.
8. After the appropriate in vivo incubation time, animals are sacrificed, and bacteria are recovered from the targeted organs (*see* **Note 9**).

9. Tissues are recovered by dissection and homogenized with a Potter homogenizer in 10 mL sterile PBS, pH 7.0, contained in a 50-mL Falcon tube (*see* **Note 10**).
10. 100 µL homogenized tissues are plated on BHIA supplemented with KM. After the in vivo selection, 10^4 colonies, recovered from a single plate, are pooled in 5 mL PBS. From the 5 mL, 1 mL is spun down and resuspended in 1 mL TE PCR (the in vivo pool).
11. The in vivo pool is boiled for 10 min, spun down, and 10 µL supernatant is used in PCR analysis, *see* **Subheading 3.1.3., step 5.** 10 µL PCR are used for 1% agarose gel electrophoresis separation.
12. PCR amplification products of tags present in the in vivo pool are compared with amplified products of tags present in the in vitro pool (**Fig. 2**).
13. Mutants that give PCR amplicon from in vitro pool, and not from in vivo pool, are purified and kept for further analysis (*see* **Note 11**).

3.4. Cloning and Analysis of Disrupted Genes from Attenuated Mutants

1. Chr DNA from attenuated mutants is prepared using the Qiagen genomic DNA extraction kit, as described in the manufacturer's protocol.
2. Chr DNA (1 µg) is digested with endonuclease, giving a large range of fragment sizes (*see* **Note 12**).
3. Digested chr DNAs are cloned into pTZ18R (Amersham Pharmacia Biotech) predigested with the corresponding endonuclease. Ligation reactions are done as follows: 1 µg digested chr DNA is mixed with 50 ng digested pTZ18r in 20 µL 1X T_4 DNA ligase buffer with 40 U T4 DNA ligase.
4. Incubate overnight at 16°C.
5. Ligated products are purified using Microcon PCR (Millipore), as described by the manufacturer's instructions, and resuspended in 5 µL H_2O.
6. The 5-µL recombinant plasmid solution is used for electroporation in *E. coli* DH5α, as described in **Subheading 3.1.3., step 3**.
7. All the electroporation cells are spun down and resuspended in 100 µL BHI, to be plated on a selective plate. Recombinant bacteria are selected as white vs blue colonies on X-gal/isopropyl thiogalastose-containing plates (0.005% and 0.1 m*M*, respectively) with AMP (100 mg/mL) and KM (50 µg/mL) (*see* **Note 13**).
8. Clones are kept and purified for plasmid analysis.
9. Plasmid DNAs are prepared with Qiagen midipreparation kit, as described by the manufacturer.
10. These plasmids are sequenced using the complementary primer of the corresponding tagged mutant. Automated sequencing (ABI 373) is done as suggested by the manufacturer.
11. DNA sequences obtained are assembled and subjected to database searches, using BLAST included in the GCG Wisconsin package (version 10.0). Complete open reading frames of disrupted genes and similarity searches with complete genomes, can be performed at the National Center for Biotechnology Information, using the microbial genome sequences (http://www.ncbi.nlm.nih.gov).

4. Notes

1. The annealing ON mixture should be made before each ligation.
2. Sometimes, unconcentrated plasmid preparation may contain endonuclease inhibitors, and using large volumes of DNA preparation in digestion reaction can inhibit enzyme activity. In this case, it is possible to digest several small quantities of DNA preparation, and, after purification (*see* **Subheading 3.1.2., step 5**), pool all digested plasmid preparations.
3. Using freshly made annealing ON mixture raises the efficiency of ligation, since tags might be degraded.
4. For replication and maintenance of the recombinant plasmid, it might be useful to use the well-known *E. coli* DH5αλ*pir* strain. However, it will be necessary to transfer plasmids in the S17-1λ*pir* strain, to transfer DNA by conjugation.
5. Screening several colonies may be necessary to find the good recombinant. Pooling several colonies is possible, to reduce the number of PCRs ***(7)***, which ensures that the good recombinant is among the selected colonies in few PCRs. To bypass the necessity of doing plasmid preparations, PCRs can be done on bacterial cell lysates. One or several colonies are resuspended in 100 µL TE PCR buffer, boiled 10 min, and span down. 10 µL supernatant are used for the PCR template. After the pool PCR, the specific clone containing tagged plasmid should be identify within the pool.
6. Temperature and incubation time should be determined by preliminary experiments.
7. Using the good KM concentration is very important, to eliminate background related to the inoculum effect. Minimal inhibitory concentration can be determine, to evaluate the effective KM concentration.
8. In a defined library, each mutant has the same tag, but is assumed to be inserted at a different location in the bacterial chr. Southern blot hybridization is necessary to confirm the random integration of the mini-Tn*5* ***(6)***.
9. Parameters concerning animal model should be particularly well-defined. The inoculum size necessary to cause infection determines the complexity of mutants pooled. In fact, each mutant in a defined input pool must be in a sufficient cell number to initiate infection. The inoculum size must not be too high, resulting in the growth of mutants that would otherwise have not been detected ***(8)***. Other important parameters in STM include the route of inoculation and the time-course of a particular infection. Also, certain gene products, important directly or indirectly for initiation or maintenance of the infection, may be niche-dependent or expressed specifically in certain animal or plant tissues only. If the duration of the infection in STM in vivo selection is short, genes important for establishment of the infection will be found, and, if the duration is long, genes important for maintenance of infection will be identified ***(8)***. Several routes of inoculation and different animal models can be used.
10. Keep homogenates on ice.
11. Each STM attenuated mutant has to be confirmed by a second round of STM screening ***(9)***, comparisons between in vivo bacterial growth rate of mutants vs

growth of the wild-type in single ***(10)*** or competitive ***(11)*** infections, or estimation of median lethal dose ***(2)***.

12. More than one endonuclease or partial digestion can be used to obtain more DNA fragments, ranging from 1 to 4 kb, which are easier to clone in pTZ18r.
13. Only clones that contain plasmid with chr fragments and the mini-Tn*5* marker are obtained.

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16

High-Throughput Scanning Mutagenesis by Recombination Polymerase Chain Reaction

Stefan Howorka and Hagan Bayley

1. Introduction

Site-directed in vitro mutagenesis is a powerful tool for investigating the structure–function relationships of proteins and the expression of genes. Often, it is desirable to subject the protein or gene of interest to scanning mutagenesis. The genetic analysis of scores of single or multiple point mutations contributes to a better understanding of *cis* elements in gene expression ***(1)***. In the case of proteins, cysteine (Cys) ***(2,3)*** and alanine scanning mutagenesis ***(4)*** are popular means for investigating the structure and function of proteins and the interactions between proteins. Cys-scanning mutagenesis is particularly useful, since the introduction of Cys allows targeted chemical modification with a wide variety of sulfhydryl-reactive reagents ***(5,6)***.

The generation of large numbers of mutants requires an efficient, fast, reliable, and simple high-throughput mutagenesis method. In the authors' studies, several site-directed mutagenesis (SDM) protocols did not meet these criteria ***(7,8)***. Often encountered problems included low mutational efficiency or low fidelity, resulting from polymerase chain reaction (PCR) errors. The following describes an improved version ***(9)*** of the recombination PCR-based mutagenesis method ***(10–12)***. The mutagenesis method is simple and fast, because it involves only two steps: two PCR reactions and one *Escherichia coli* transformation (**Fig. 1**). The method does not require specialized plasmids, convenient restriction sites, or time-consuming purification, modification, or subcloning steps. This simplicity makes the technique ideal for high-throughput mutagenesis studies.

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ

The improved protocol, which is based on the authors' findings during the Cys-scanning mutagenesis of a 100 kDa protein ***(13,14)***, features a high mutational efficiency of 92%, high fidelity (because of a low number of PCR cycles) and allows substitutions of up to 6 nucleotides (nts) ***(9)***. These improvements were brought about primarily by the use of mutagenic primers with an improved design. The improved primers had the mutagenic stretch positioned near the 5' end, as opposed to conventional primers with the mutagenic stretch in the middle of the primer. This improvement resulted in a drastic increase in the proportion of successful PCR reactions to 94%. In addition, high PCR yields allowed the number of PCR cycles to be decreased to 10, which in turn reduced undesired PCR errors.

2. Materials

1. PCR primers (100 pmol/µL) (*see* **Subheading 3.1.** and **Note 1**).
2. Plasmid template (*see* **Subheading 3.2.**).
3. 10 m*M* deoxyribonucleoside triphosphate mix (10 m*M* each of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate).
4. PCR reaction buffer (Expand Long Template PCR kit, Roche Molecular Biochemicals) (*see* **Note 2**).
5. *Taq*/*Pwo* DNA Polymerase Mix (Expand Long Template PCR Kit, Roche Molecular Biochemicals) (*see* **Note 2**).
6. Thin-walled PCR reaction tubes, 8-tube strips or 96-well tube plates (*see* **Note 3**).
7. PCR thermocycler.
8. Mineral oil (*see* **Subheading 3.2.**).
9. Agarose gel electrophoresis supplies and apparatus.
10. *E. coli* competent cells (*see* **Note 4**).

3. Methods

3.1. Primer Design

3.1.1. Mutagenic Primers

The gene-specific mutagenic primers (1 and 2 in **Fig. 1**) introduce the desired nt change(s) in the gene of interest. Design the primers, following these guidelines.

1. The desired nt change(s) should be located toward the 5' end of the primer.
2. Not more than six adjacent nts should be substituted.
3. Matching primers of one pair have complementary 5' ends and should form an overlap of 12 bp (**Fig. 2**). The nt changes should be placed at the center of the overlap. This means, e.g., that two adjacent single nt changes are best positioned 5 nt downstream from the 5' end of the primer (**Fig. 2A**). Similarly, a mutagenic stretch encompassing 6 nt exchanges is optimally positioned 3 nt downstream

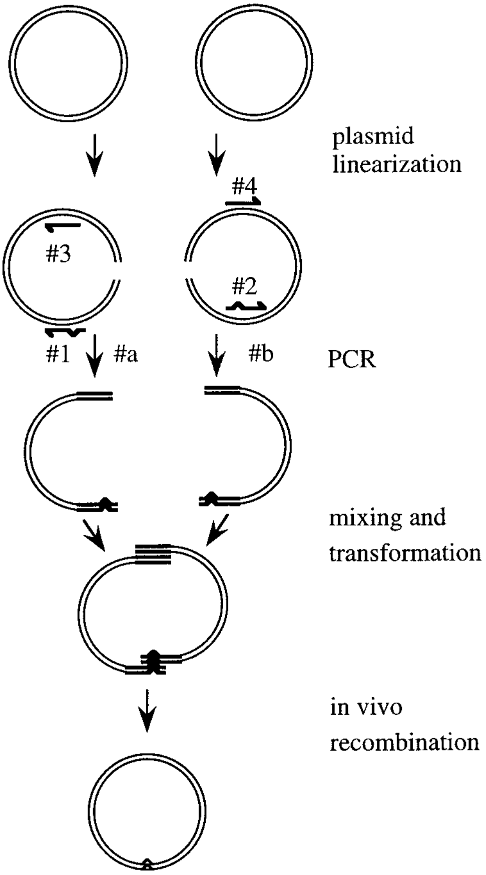

Fig. 1. Scanning mutagenesis by recombination PCR. Two plasmid halves are amplified by PCR, by using two pairs of primers: a mutagenic primer (arrows with peak) and a nonmutagenic primer (arrows without peak). The mutagenic primers 1 and 2, and the nonmutagenic primers 3 and 4 are, respectively, partly and completely complementary. Thus, the two PCR products have overlapping termini. The two PCR fragments recombine upon transformation into *E. coli* cells, giving rise to a plasmid with the desired mutation.

from the 5' end of the primer (**Fig. 2B**). Designing the mutagenic primers this way is important; It will result in a high PCR success rate, with high PCR yields at a low number of PCR cycles (*see* **Note 5**).

4. The overall length of the mutagenic primers should lie between 24 and 34 nt. Mutagenic primers with a mutagenic stretch of 4–6 nt should be longer than 30 nt.
5. The melting temperature should range between 65 and 69°C (calculated with an algorithm based on thermodynamic data, such as (www.idtdna.com/html/analysis/Calculator.html).

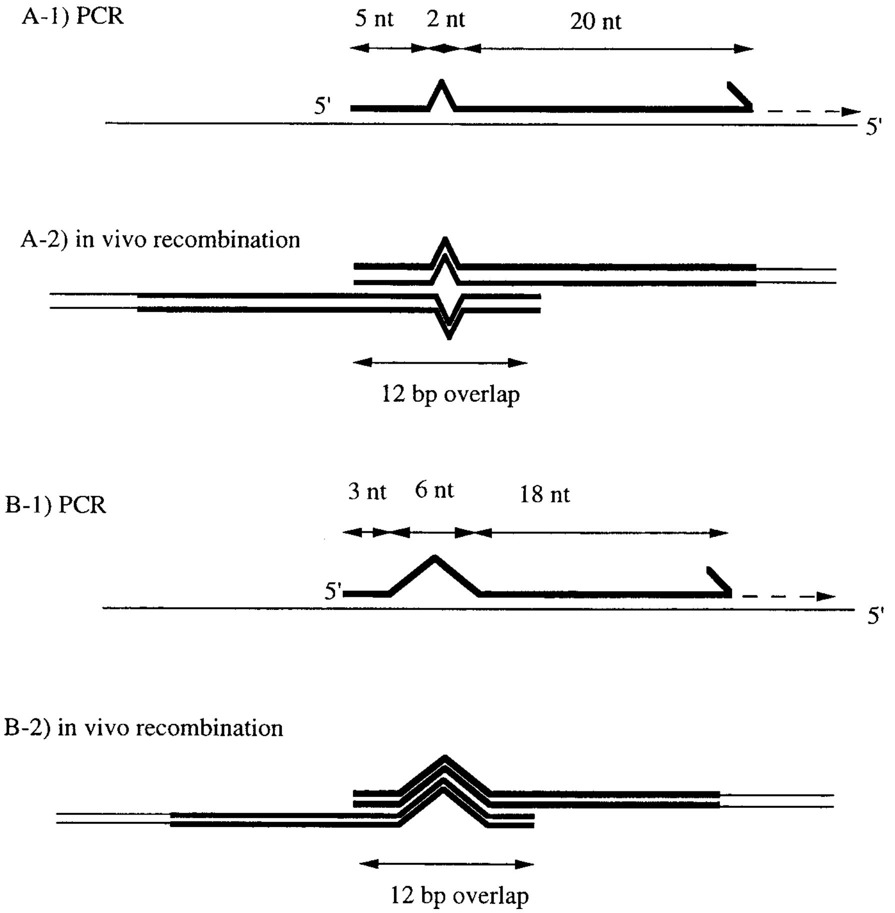

Fig. 2. Design of mutagenic primers for recombination PCR scanning mutagenesis for (A) 2-nt substitutions and (B) 6-nt substitutions. Depicted are priming events of mutagenic primers to the plasmid template during PCR (A-1 and B-1), and overlap formation by the partly complementary, mutated termini of the PCR products during in vivo recombination in E. coli cells (A-2 and B-2). Thick arrows with a peak represent mutagenic primers, and thick lines without an arrow depict primer-derived sequences. (A) To allow 2-nt substitutions, the mismatches should be positioned 5 nts downstream from the 5'-end of a pair of matching mutagenic primers. (B) For 6-nt substitutions, the mismatches should be located 3 nts downstream from the 5'-end of the mutagenic primers. The complementary termini of the primers result in overlaps of 12 bp during in vivo recombination.

6. If necessary, a unique restriction enzyme recognition site can be introduced with the mutagenic primer (*see* **Note 6**). This restriction site can facilitate the identification of clones containing the desired mutation, or can be used later to clone

larger inserts into the engineered site. Alternatively, for the identification of desired clones, the restriction site can be engineered into the nonmutagenic primers, which may be preferable in most cases (*see* **Subheading 3.1.2., step 7**).

3.1.2. Nonmutagenic Primers.

Design the nonmutagenic primers 3 and 4 (**Fig. 1**), according to these recommendations.

1. The nonmutagenic primers should anneal to a region of the plasmid lying outside the target gene. Depending on the total size of the plasmid, this precautionary step should help avoid the need to generate long PCR fragments (>4 kb), which might be difficult to amplify.
2. A preferred region for primer annealing is the sequence close to, or between, the origin of replication and the ampicillin (AMP)-resistance gene. The PCR products should not carry a functional origin of replication along with the resistance gene. A PCR fragment carrying both might yield a functional plasmid without recombination with the second fragment.
3. The melting temperature should be similar to that of the mutagenic primers.
4. The nonmutagenic primers can be completely complementary to the template DNA.
5. Designing a different set of nonmutagenic primers for each set of mutagenic primers is not necessary: One pair of nonmutagenic primers can be used for all pairs of mutagenic primers.
6. The pair of nonmutagenic primers used in the authors' studies annealed 0.2 kb upstream from the stop codon of the AMP-resistance gene in a pBR322-derived plasmid ***(15)***, and had the sequences: Primer 3: 5'-ATAAAGTTGCAG GACCACTTCTG-3'. Primer 4: 5'-CAGAAGTGGTCCTGCAACTTTAT-3'.
7. A restriction site can be introduced into the nonmutagenic primers, if it is not desirable to introduce the site into the target gene (*see* **Subheading 3.1.1., step 6**). The restriction site facilitates the isolation of clones carrying the desired mutation (*see* **Note 6)**. To avoid the possibility that the introduction of the restriction site affects plasmid replication or the expression of a functional AMP-resistance gene product, the site should be placed between the resistance gene and the origin of replication or incorporated into the resistance gene without altering its protein sequence.

3.2. Preparation of PCR Templates and PCR Reactions

1. To prepare the templates for PCR reactions #a and #b (**Fig. 1**), linearize the plasmid DNA in two separate digests (*see* **Note 7**) yielding templates a and b. The restriction enzymes must cut outside the amplified region (**Fig. 1**), and the digestions should be complete (*see* **Note 8**).
2. If possible, heat inactivate the restriction enzyme. Dilute the digest with sterile H_2O, to a final concentration of 10–20 ng/µL. No purification of the template is necessary.

Table 1
Preparation of Master Mixes A and B

Component	Master mix A, volumes for each PCR reaction #a	Master mix B, volumes for each PCR reaction #b
Nonmutagenic primer (100 pmol/µL) (*see* **Note 9**)	2 µL of #3	2 µL of #4
10 m*M* deoxyribonucleoside triphosphate	7 µL	7 µL
Template	2 µL of a	2 µL of b
Buffer 1	5 µL	5 µL
Sterile distilled H_2O	32 µL	32 µL
Taq/*Pwo* Enzyme mix (3.5 U/µL) (*see* **Note 3**)	0.4 µL	0.4 µL

3. Pipet 2 µL mutagenic primers (100 pmol/µL) #1 and #2 into separate PCR reaction tubes. For the generation of scores of mutants, it is advisable to use thin-walled, 8-tube strips or 96-well plates. Pipette the first 8 mutagenic primers 1 into the tubes of the first row, and the complementary primers 2 into the second row. Following the alternating sequence, complete pipeting the other primers.
4. Prepare master mix A and B for the PCR reactions #a and #b (**Fig. 1**). Depending on the number, *N*, of mutants to be made, make up (*N* + 1) × 48 µL master mix A and (*N* + 1) × 48 µL master mix B (**Table 1**). Keep the solutions on ice.
5. By using an 8-channel pipetor, add 48 µL master mix A to the rows of mutagenic primers 1, and 48 µL master mix B to the rows of mutagenic primers 2. Mix thoroughly.
6. Overlay the reactions with mineral oil, or, alternatively, use the hot top mode of the PCR thermocycler.
7. Perform the PCR amplification reactions, using the following cycling profile. One cycle of 2 min at 94°C, then 10 cycles of 1 min at 94°C, 1 min at 49°C, and 3–5 min at 72°C (1 min/kb) (*see* **Note 9**).
8. Confirm the formation of the PCR products by analyzing 5-µL samples of the PCR reaction mixtures on an agarose gel. Given the high efficiency of the procedure, this step can be done parallel with the transformations (*see* **Subheading 3.3.**).

3.3. E. coli *Transformation and In Vivo Recombination*

1. Thaw *E. coli* competent cells on ice (*see* **Notes 4**).
2. Mix portions of the crude, unpurified PCR mixtures of the two matching fragments (5 µL each, approx 40 ng DNA), and add to the cells (*see* **Note 10**).

3. Swirl gently, and incubate for 30 min on ice.
4. Heat shock the cells for 1 min at 42°C.
5. Streak out the cells on Luria-Bertani agar plates, supplemented with AMP (120 µg/mL), and incubate overnight at 37°C.
6. Pick two colonies of each mutant and grow in Luria-Bertani or TB medium ***(16)*** supplemented with AMP (120 µg/mL). Isolate plasmid DNA (*see* **Note 11**).

4. Notes

1. Primers can be purchased either in individual tubes or in the 96-well format. The latter format allows the use of a multichannel pipetor, decreasing the likelihood of pipeting mistakes.
2. The authors highly recommend the use of PCR kits designed for the amplification of long fragments, such as Long Template PCR Kit (Roche Molecular Biochemicals) or Taq Plus Long PCR System (Stratagene) (*see* **Note 9**).
3. 0.2-mL thin-walled PCR reaction tubes are recommended by the supplier of the Expand Long Template PCR kit, because they reduce the heat-transfer time during temperature cycling. A further advantage of the 0.2-mL tubes is that they are available in different formats, such as 8-tube strips and 96-well tube plates (available from Stratagene, La Jolla, CA, or Phenix Research, CA). These formats facilitate the parallel handling of dozens of PCR reactions, and reduce pipeting mistakes.
4. *E. coli* strains, such as DH5α, XL1B, JM109, HB101, and other *rec*A$^-$ strains, can be used. Competent *E. coli* cells can be either purchased or prepared by a protocol using polyethylene glycol and dimethylsulfoxide ***(17)***. When using XL1B cells made competent with polyethylene glycol and dimethylsulfoxide, an average yield of 200–400 colonies per in vivo recombination reaction can be expected.
5. The position of the mismatches in the mutagenic primer strongly influences the PCR success rate (the number of successful PCR pairs divided by the total number of PCR pairs). Primers with mismatches in the middle of the primer had, on average, a PCR success rate of 58%, compared to 94% of the primers with mismatches toward the 5' end ***(9)***.
6. In the authors' studies involving the Cys-scanning mutagenesis of a 3.0-kb gene ***(9)***, a *Nsi*I restriction recognition site (ATGCAT) was introduced with each cysteine codon (underlined). This restriction site was used to facilitate the identification of successfully mutated genes, and, later, to clone inserts into the modified gene. The introduction of the *Nsi*I site forces the amino acid, following Cys, to be either isoleucine or methionine, which may not be desirable in all situations.
7. The distance between the primer annealing site and the terminus of the linearized plasmid should be larger than 0.2 kb. A shorter distance gave low, or no, PCR yields.
8. The use of linear instead of circular plasmid DNA increases the PCR yield, because of relief of topological constraints. In any case, it is important to use completely linearized plasmid DNA. Contaminating undigested, supercoiled plasmid DNA results in a high background level of transformation during in vivo recombination. To ensure complete digestion, use high-grade purified plasmid DNA (purified over a $CsCl_2$ gradient, or with Qiagen Miniprep or Midiprep kits), rather than DNA purified by phenol/chloroform extraction.

9. The very low annealing temperature of 49°C, together with the high primer concentration and the use of a PCR kit designed for the amplification of long templates ensures a high PCR yield, even with 10 PCR cycles.
10. Heating the mixture of two PCR fragments, prior to addition to *E. coli* cells is not necessary.
11. To speed up the isolation of plasmid DNAs for scores of mutants, the authors recommend the use of kits designed for the parallel generation of large numbers of plasmids, such as Qiagen's QIAprep 8 or QIAprep 96 Turbo Miniprep kits.

Note added in press: The improved protocol described in this chapter has been used by the authors to perform a Cys-scanning mutagenesis of a 100-kD bacterial surface protein. The results of this study have been published recently ***(18)***.

Acknowledgments

This work was supported in part by the Army Research Office and the Robert A. Welch Foundation Grant A-1335. Stefan Howorka is currently the recipient of a fellowship from the Max-Kade Foundation and was supported by a postdoctoral scholarship from the Austrian Science Foundation (Fonds zur Förderung der wissenschaftlichen Fouschung) during the eariler stages of the work.

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In Vitro Scanning-Saturation Mutagenesis

**Jennifer A. Maynard, Gang Chen, George Georgiou,
and Brent L. Iverson**

1. Introduction

Scanning mutagenesis strategies have proven to be a useful approach to structure–function and protein evolution studies ***(1)***, yet great effort is required to generate comprehensive data for a given protein. In these experiments, one or several amino acid residues are changed (i.e., to alanine), and the resulting mutant proteins analyzed for changes in function. Successful applications include the mapping of functional binding epitopes ***(2,3)***, confirmation of low-resolution X-ray or computer-generated structural models ***(4)***, and investigation of the energetic contributions of contact residues ***(5,6)***. However, a stumbling block to the wider application of these techniques is that each desired mutation must be introduced into a gene, cloned into bacteria, and the protein produced then purified ***(7)***. This is a resource and time intensive process that precludes routine use.

Here is described a rapid variation on scanning mutagenesis strategies, which eliminates the need for cloning the mutant gene or purifying the resulting protein, and is amenable to automation. The process was developed for analysis of single-chain antibodies (scFv), and uses binding affinity as a measure of function. Minor variations should allow enzymes and receptor–ligand pairs to be analyzed, as well. Its utility has been demonstrated through the development of “scanning-saturation mutagenesis,” in which each specificity-determining residue of a scFv was individually mutated to all 19 other amino acid residues, as well as a stop codon control ***(8,9)***. When the protocols are optimized, the procedure is rapid. For example, in the scFv study, it only required 1 wk to analyze quantitatively, in duplicate, all 20 mutants per specificity-determining residue.

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ

The large pool of analyzed mutants provided a comprehensive set of structure-activity relationship (SAR) data, which was used to assess the general plasticity of the scFv's binding interface. In addition, by analyzing which mutants retained activity, and which ones did not, activity was correlated to the chemical attributes of the mutant side chains. What emerged was a proposed model of the roles played by each contact residue in the scFv binding site.

In the in vitro scanning mutagenesis procedure, mutations are created by a three-step overlap polymerase chain reaction (PCR) protocol. The crude PCR product is used as DNA template in a bacterial coupled in vitro transcription/translation reaction, and the reaction mixture containing mutant protein is analyzed for function by enzyme-linked immunosorbent assay (ELISA) and/or surface plasmon resonance (SPR), which measures off-rate kinetics. The advantages of this technique are as follows:

1. Coupled in vitro transcription/translation systems can produce protein from large numbers of mutants.
2. ELISA and SPR are convenient and accurate means of assaying the binding affinity resulting from a single mutation to within a factor of two.
3. ELISA-based quality-control measures can discriminate between differences in expression level, folding, and affinity of mutants.

Similar applications of in vitro transcription and translation have been demonstrated for structure–function studies ***(10–13)***, epitope mapping ***(14,15)***, screening open reading frames for function ***(16)***, biochemical characterization ***(17–20)***, and library screening ***(21–23)***.

2. Materials

2.1. Generate Mutants

2.1.1. Digest Plasmid

1. The gene of interest should be in a vector with a T7 promoter and terminator, a ribosome binding site, ATG start codon, a C-terminal epitope tag (e.g., HSV), and no periplasmic leader sequence (e.g., Novagen's pET 25b(+), with the gene of interest cloned into the *Nde*I and *Xho*I sites).
2. Three restriction enzymes. Two are required to digest the plasmid at regions flanking the gene, but internal to the vector primer locations. The third enzyme may cut anywhere external to the vector primer locations (*see* **Fig. 1**).
3. Agarose gel, 1.5% w/v.
4. Gel purification strategy (e.g., Qiagen gel purification columns).

2.1.2. Mutagenic PCR

1. Taq polymerase, 5 U/µL. Store at –20°C.
2. 10 m*M* total deoxyribonucleoside triphosphate. Store at –20°C.

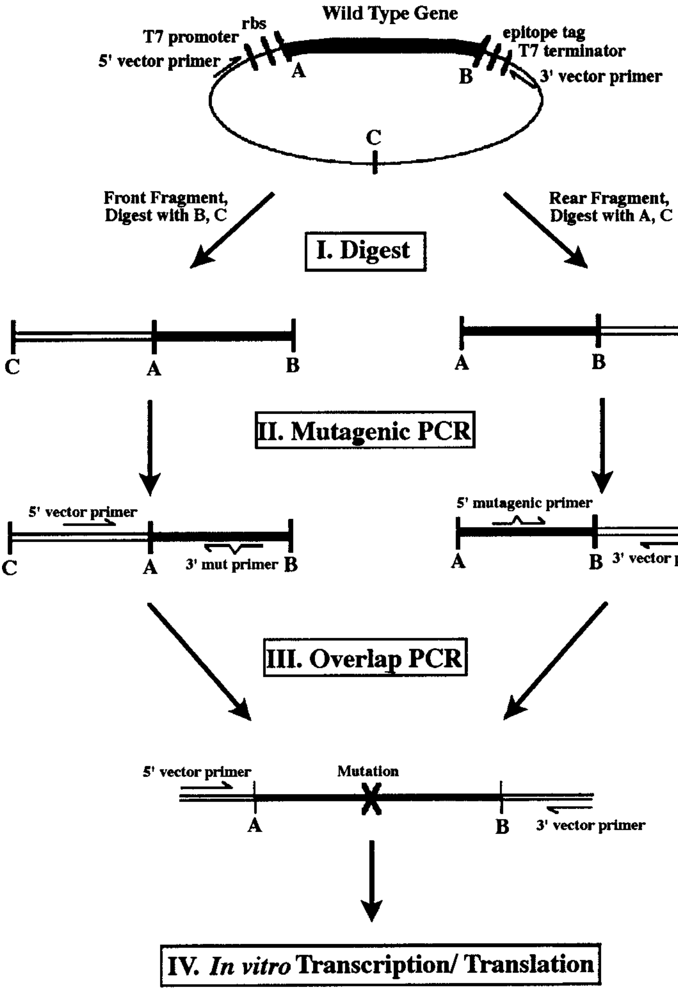
Wild Type Gene
rbs
T7 promoter
5' vector primer
A
epitope tag
T7 terminator
B
3' vector primer
C
Front Fragment, Digest with B, C
Rear Fragment, Digest with A, C
I. Digest
C
A
B
A
B
C
II. Mutagenic PCR
5' vector primer
C
A
3' mut primer
B
5' mutagenic primer
A
B
3' vector primer
C
III. Overlap PCR
5' vector primer
Mutation
A
B
3' vector primer
IV. In vitro Transcription/ Translation

Fig. 1. Diagram of the mutagenesis strategy. (I) The plasmid containing WT gene is digested to yield two fragments, each containing the full-length WT gene, and which cannot be amplified using the 5' and 3' vector primers. (II) Front and rear fragments encoding the mutation are produced by PCR with one vector primer and the corresponding mutagenic primer. (III) The full-length gene with a single mutation is generated by the overlap of the front and rear mutagenic fragments and the 5' and 3' vector primers. This mutant DNA can then be used directly in an in vitro transcription/translation reaction.

3. 10X PCR buffer containing 15 m*M* $MgCl_2$.
4. 100 µ*M* 5' vector primer: upstream of the T7 promoter; store at –20°C.
5. 100 µM 3' vector primer: downstream of T7 terminator (*see* **Note 1**).

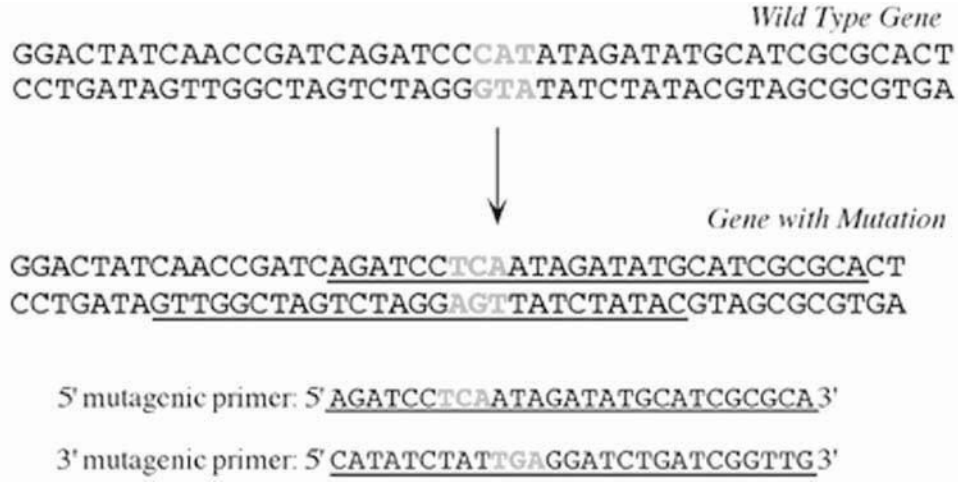
Wild Type Gene
GGACTATCAACCGATCAGATCCCATATAGATATGCATCGCGCACT
CCTGATAGTTGGCTAGTCTAGGGTATATCTATACGTAGCGCGTGA
Gene with Mutation
GGACTATCAACCGATCAGATCCTCAATAGATATGCATCGCGCACT
CCTGATAGTTGGCTAGTCTAGGAGTTATCTATACGTAGCGCGTGA
5' mutagenic primer: 5' AGATCCTCAATAGATATGCATCGCGCA 3'
3' mutagenic primer: 5' CATATCTATTGAGGATCTGATCGGTTG 3'

Fig. 2. Sample strategy for designing 5' and 3' mutagenic primers. Beginning with the WT gene sequence, the codon *(shown in gray)* is identified, which will be mutated. This codon is changed to one encoding the desired mutation *(also shown in gray)*, and primer sequences are chosen from the flanking DNA segments, so that 18 bp of each primer match the target sequence 3' of the mutation, and the two mutagenic primers have at least 18 bp of overlapping sequence.

6. 100 μ*M* 5' and 3' mutagenic primers, encoding desired mutation plus a stop codon control (one pair of primers for each mutation; for sample design) (*see* **Fig. 2**).
7. 2% w/v agarose gel.
8. Gel purification columns.

2.1.3. Overlap PCR

Same as above, plus materials to sequence the PCR product.

2.2. In Vitro Coupled Transcription/Translation

Escherichia coli S30 and rabbit reticulocytes are equally suited to the expression of scFvs ***(24)***, but S30 is simpler experimentally, because the transcription and translation steps are coupled. Kits are commercially available for bacterial coupled in vitro transcription/translation with linear templates (*see* Promega and Ambion for reliable reagents), but it is also possible and inexpensive to prepare these reagents in house. For S30 preparation, *see* **ref. *25***; for T7 RNA polymerase preparation, *see* **ref. *26***. Assay each batch for the optimal amount to use in an in vitro reaction (*see* **Notes 2–4**).

1. *E. coli* transfer RNA, 20 mg/mL in H_2O (Sigma). Store at –20°C (see **Note 5**).
2. S30 bacterial lysate, prepared without dithiothreitol (DTT) in the dialysis buffer. Aliquot in 0.5-mL volumes, and store at –80°C (*see* **Note 6**).

3. 10X salt preparation: 0.16 *M* magnesium acetate, 0.8 *M* ammonium acetate, 2.1 *M* potassium glutamate (glutamic acid plus one molar equivalent potassium hydroxide). Store at –20°C.
4. 5X Mix (for 1 mL): 286 µL 1 *M* HEPES-KOH, pH 7.5, 16 µL 0.55 *M* DTT, 63 µL 0.1 *M* adenosine triphosphate, 43 µL 0.1 *M* guanosine triphosphate, 43 µL 0.1 *M* cytidine triphosphate, 43 µL 0.1 *M* uridine triphosphate, 63 µL 2.7 mg/mL folinic acid, and 446 µL H_2O; store at –80°C.
5. Amino acids, 1 m*M* each (20 m*M* total) in water with 2 m*M* DTT, pH 7.0. Store at –20°C (*see* **Note 7**).
6. T7 RNA polymerase, 20 mg/mL in 50% glycerol; store at –20°C.
7. Cyclic adenosine monophosphate, 0.1 M in H_2O; store at –80°C.
8. Phosphenol pyruvate, potassium salt (PEP·K), 1 *M* in H_2O. Store at –80°C (*see* **Note 8**).
9. Reduced (10 m*M*) and oxidized glutathione (GSSG) (100 m*M*) in H_2O. Must be prepared fresh for each experiment, and concentrations optimized for each protein (only required for proteins containing disulfide bonds).
10. Mutant genes produced by PCR, 30–100 ng/µL. Store at 4°C or –20°C.
11. Autoclaved, double-distilled H_2O.
12. Autoclaved microcentrifuge tubes.

2.3. Functional Analysis of Mutations

2.3.1. ELISA

1. Chemiluminescent horseradish peroxidase (HRP) substrate (Pierce). Store at 4°C (*see* **Note 9**).
2. 50 m*M* sodium carbonate, pH 9.6. Autoclave, and store at 4°C indefinitely.
3. Polyclonal antisera to protein of interest (for scFvs, rabbit anti-$[\text{Fab}]_2$ from Pierce). Store at 4°C.
4. Goat antirabbit–HRP conjugate (Pierce). Store at 4°C.
5. Biotinylated antigen (Ag), at a concentration ~5× the *Ka*. Store as for Ag. (Convenient reagents for biotinylation of proteins, i.e., biotin-XX-succidimidyl ester, can be purchased from Molecular Probes.)
6. Avidin–HRP conjugate. Store at –20°C.
7. 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) as block and assay diluent. Prepare fresh.
8. PBS–0.05% Tween-20, wash buffer.
9. Chemiluminescent ELISA plate reader, i.e., Dynatech Laboratories' MLX Luminometer.
10. Microtiter plates. 96-well plates for chemiluminescent studies (opaque white, untreated) (Pierce).
11. 8-channel multipipetor.

2.3.2. Surface Plasmon Resonance

Instruments based on surface plasmon resonance, such as the BIAcore, chart the on- and off-rate kinetics of soluble antibody (Ab) with immobilized Ag-based

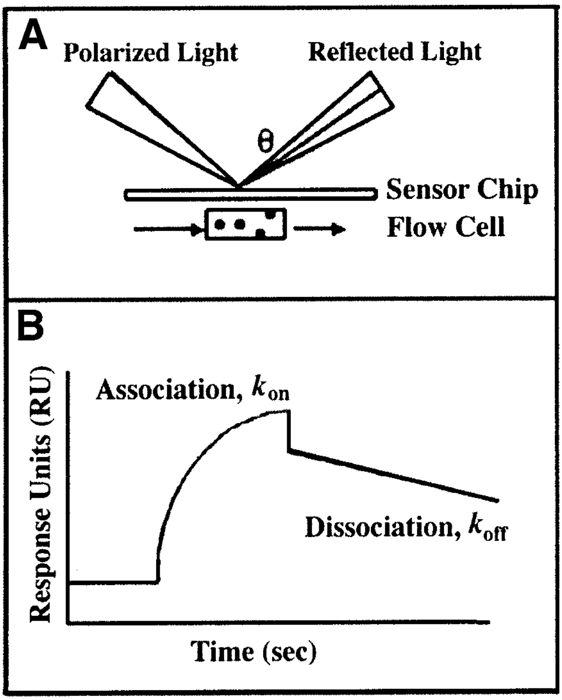

Fig. 3. Affinity measurement by surface plasmon resonance (BIAcore). **(A)** physical configuration: Ag is covalently bound to a sensor chip, the Ab flows over the surface. Binding is detected by a decrease in light intensity for a given incident angle. **(B)** A typical sensorgram: During the association phase, Ab flows under the chip and binds immobilized Ag, RUs increase. During dissociation, buffer with free Ag flows over the chip, and Ab dissociates from Ag, resulting in exponential decay of the RU signal with a characteristic rate constant k_{off}.

on changes in refractive index (*see* **Fig. 3**). This technique has been used extensively by the Ab engineering community to monitor the affinity of Abs in purified preparations, and also in crude periplasmic fractions ***(27)***. The technique is based on the change in mass bound to the surface, and it is possible to subtract nonspecific binding events because of transcription/translation reaction components with a no-DNA control reaction, and/or by running the reaction across a control surface without Ag. After blanking, the data can be fit to a 1:1 (or other appropriate) dissociation model to determine k_{off}. Instructions presented here are only guidelines: Please refer to the instrument manuals for more details ***(28)***.

1. Machine which measures changes in surface plasmon resonance, such as BIAcore.
2. CM5 chip for BIAcore.
3. NHS.
4. EDC.
5. Ethanolamine.
6. HBS-EP buffer: 0.15 *M* NaCl, 0.01 *M* HEPES, pH 7.4, 3 m*M* EDTA, 0.005% surfactant P-20 (Amersham Pharmacia). Filter and degas.
7. Regeneration buffer: Try several different regeneration buffers, as suggested in the instrument literature, to identify one that will remove bound scFv without compromising antigen integrity (e.g., 2 *M* filtered $MgCl_2$, polyethylene glycol, pH 10.0, HCl).
9. Ag to covalently couple to chip via primary amines on lysines (*see* **Note 10**). The Ag must be coupled at low density (≤1000 response units [RU]) to minimize avidity effects caused by rebinding.
10. BIAcore program to measure dissociation rates. To minimize the nonspecific binding of reaction components, and to prevent rebinding, run at 100 µL/min; monitor off-rates for 3 min.
10. In vitro transcription and translation reactions producing mutant proteins (*see* **Note 11**).

3. Method

3.1. Generate Mutants

Introducing the desired point mutations is a three-step process, which is very rapid when the mutagenic primers have been designed well (*see* **Fig. 2** for a sample design strategy). First, the plasmid containing the wild-type (WT) gene is digested to produce template for the mutagenesis, the resulting front and rear fragments are used as PCR template to produce mutated fragments, and finally, the full length gene containing the desired mutation is produced by overlap PCR (*see* **Fig. 1**) ***(29)***. It is essential that all full-length DNA is removed from the front and rear fragments, to prevent WT contamination of the mutant genes.

3.1.1. Digest Plasmid

1. Prep plasmid DNA from 5 mL overnight culture.
2. Choose restriction sites on the plasmid, so that the front fragment will not include the 3' vector primer’s target sequence. Similarly, choose sites for the rear fragment that will exclude the 5' vector primer recognition sequence (*see* **Fig. 2**).
3. Digest the plasmid, and gel-purify the desired bands.
4. Confirm that the fragments are not contaminated by any linear plasmid that could give rise to WT DNA at the overlap PCR step: Set up a PCR reaction for each fragment and plasmid with the 5' and 3' vector primers. The front and rear fragments should produce no product; the plasmid should produce product. If the fragments allow amplification of the full-length gene, repeat the purification until this negative control is successful.

3.1.2. Mutagenic PCR

1. To create the 5' front mutant fragments, set up a 50-µL PCR reaction with the 5' vector primer, 3' mutagenic primer, and the front fragment from the digest. Similarly, create the 3' rear mutant fragments in an amplification reaction containing the 5' mutagenic primer, 3' vector primer, and rear fragment (*see* **Note 12**).
2. Run the entire reaction on a 1.5% w/v agarose gel (confirm that bands run at expected sizes), and excise with a razor blade. Purify DNA either with a commercial kit or with homemade spin columns ***(30)***.

3.1.3. Overlap PCR

1. Set up a 50-µL overlap PCR reaction, with approximately equal concentrations of each pair of mutant front and rear fragments, and the 3' and 5' vector primers (*see* **Note 13**). For a positive control, generate a WT overlap product by combining WT digested fragments (from **Subheading 3.1.1., step 4**) in a PCR reaction.
2. Confirm generation of product of desired size by running 5 µL on a 1.5% agarose gel. If contaminating bands are not present, the DNA may be used directly in the transcription/translation assay (*see* **Note 14**). Concentration should be 30–100 ng/µL (*see* **Note 15**).
3. Sequence PCR product to confirm that only the desired mutation was introduced.
4. Fragments and overlap products can be stored at 4°C for several weeks, and at –20°C for longer periods.

3.2. Coupled Transcription/Translation Assay

In a 1.5-mL microcentrifuge or glass tube, combine X µL H_2O, X µL mutant DNA (~100 ng), 3 µL 10X salt solution, 6 µL 5X, 1.5 µL 10 m*M* amino acids, 0.2 µL 0.1 *M* cyclic adenosine monophosphate, 1 µL 1 *M* PEP, 1.5 µL 40% polyethylene glycol, 1 µL 20 mg/mL *E. coli* tRNA, 6 µL 5X, 0.1 µL 20 mg/mL T7 polymerase, 1 µL 30X GSSG, 1 µL 30X reduced glutathion (GSH), and 5 µL S-30 prep, for a total of 30 µL each reaction.

1. Put DNA and water in labeled 0.7- or 1.5-mL microcentrifuge tubes.
2. Thaw reagents on ice, mix before using.
3. Combine all reagents in the order listed. If preparing multiple samples, making a master mix allows for more accurate pipeting of small volumes.
4. Add S30 to master mix; immediately aliquot into tubes (*see* **Note 16**).
5. Vortex gently, and place in a 37°C water bath for 15–60 min.
6. Quench reaction by placing on ice.

3.3. Functional Analysis of Mutations

3.3.1. ELISA

Each sample will require four wells: duplicates of the specific coat, and duplicates of negative no coat wells (*see* **Fig. 4** for cartoon of ELISA strategy).

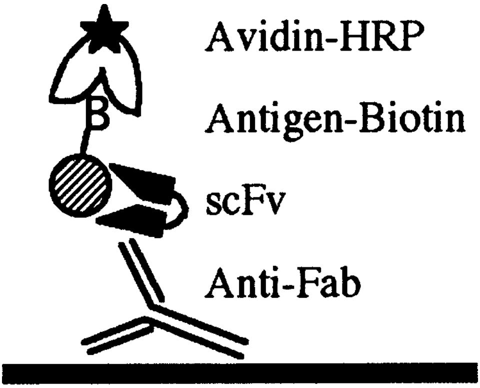

Fig. 4. Cartoon of ELISA scheme. Coating with a limiting amount of polyclonal anti-$(Fab)_2$ results in the capture of equal amounts of properly folded scFv from the in vitro transcription/translation reaction. Relative affinities of mutant scFv for Ag can then be measured by incubating with biotinylated Ag followed by avidin-HRP detection.

1. Coat ELISA plate with 50 µL of appropriate concentration of anti-$(Fab)_2$ or Ag (*see* below) in 50 m*M* sodium carbonate, pH 9.6. Store overnight at 4°C.
2. Remove coat and wash 3× with PBS-0.05% Tween.
3. For each 30-µL in vitro reaction, add 180 µL blocking buffer, mix by pipeting, and place 50 µL in each ELISA well.
4. Incubate overnight at 4°C (*see* **Note 17**).
5. Remove, and wash 3×.
6. Incubate with 50 µL biotinylated Ag, diluted in blocking buffer to a concentration of ~$5*K_{d,\text{ wild-type}}$; 1 h at room temperature.
7. Remove, and wash 3×.
8. Incubate with avidin-HRP, 1:1000 dilution in blocking buffer, 1 h at room temperature.
9. Remove, and wash 3×.
10. Add chemiluminescent substrate: 25 µL/well (12.5 µL each component). Allow ~5 min to develop.
11. Read plate in luminometer. Use a glow-endpoint (not kinetic) assay.

Controls that must be performed to establish optimal conditions:

1. Serially dilute WT overlap DNA from 10–200 ng/30 µL reaction, to determine the optimal amount to add in a reaction. (for the authors' Abs, 70–100 ng gives maximal signal, but as low as 10 ng is detectable) ***(7)***.
2. Serially dilute the polyclonal Ab as coat: twofold dilutions, starting at 1:100, with 50 µL/well. For each concentration, prepare one 30-µL reaction with WT overlap DNA. After quenching, add 180 µL 2% BSA in PBS; mix well. Aliquot 50 µL into each of four wells (two with anti-Fab dilutions, two BSA-only nega-

tive controls), and incubate overnight at 4°C. Develop as above. A saturation curve should be seen; the lowest concentration at which the signal is saturated should be used as coat in subsequent studies with mutants. This will provide a limiting amount of capture Ab, and will normalize the mutants with respect to concentrations of properly folded Ab, so that ELISA signals will directly correlate to affinity ***(31)***.

3.3.2. Surface Plasmon Resonance

Studies with WT are necessary to determine the size of reaction necessary (generally, 5X or 150 µL reactions), and to confirm that the off-rates measured with transcription/translation reaction are similar to those measured for purified WT protein. Controls for nonspecific binding include a reference surface (i.e., uncoupled chip), and transcription/translation reactions with no DNA added, or DNA encoding an irrelevant scFv. The benefits of SPR are that kinetic data can be determined without labeling either binding partner; ELISA provides (at best) information on relative equilibrium constants. For example data, please *see* **Fig. 5**.

1. Prepare 5X (150 µL), 4X (120 µL), and 3X (90 µL) reactions for each mutant in a 1.5-mL microcentrifuge tube. In addition, prepare positive (WT DNA) and negative (no DNA and/or irrelevant scFv DNA) reactions (for more robust data, perform replicate injections of each concentration).
2. Dilute each reaction to 330 µL with HBS-EP, transfer to a BIAcore tube, and cap.
3. Place tubes in machine, and initiate program.
4. To analyze data, open in BIAevaluation, zero the baseline and beginning of the injection.
5. Subtract the signal for a 5X negative control reaction from all 5X reactions. Do the same with 4X and 3X reactions.
6. Zoom in on the beginning of the dissociation phase, and choose a region ≥100 s, which is fairly linear. Model the dissociation using pseudo-first order kinetics (*see* **Fig. 5**):

$$\ln (R_t/R_n) = k_{\text{off}}(t_n - t)$$

   where R_t = response units at time, t, and R_n = response units at point t_n on curve.
7. Dissociation constants obtained for different concentrations and replicate injections should agree. Nonfunctional mutants will not bind to the surface. Mutants with off-rates faster than 0.02/s cannot be measured.
8. If assuming the change in on-rate will be minimal ***(32)***, an approximation can be calculated of the ΔΔG of binding for WT compared with mutant scFvs:

$$\Delta\Delta G = +RT \ln (K_{d,\text{ mutant}}/K_{d,\text{ wild-type}}) = +RT \ln(k_{\text{off, mutant}}/k_{\text{off, wild-type}})$$

3.4. Quality Control

An important issue in these studies is to normalize for expression and folding differences between mutants. This can be assayed with a variety of tech-

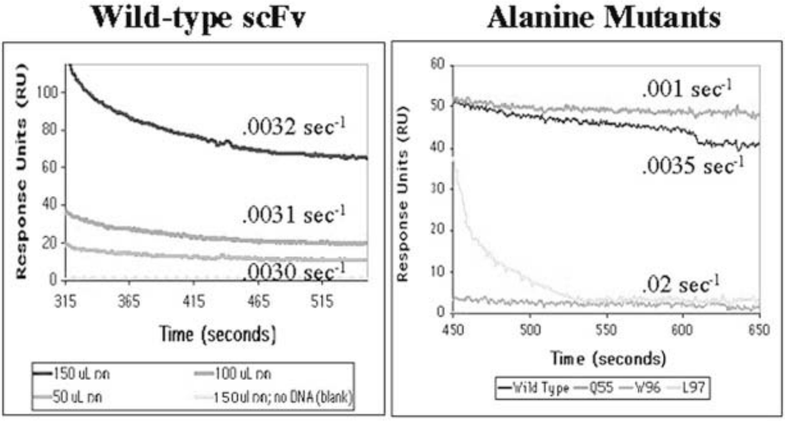

Fig. 5. Sensorgram illustrating BIAcore off-rates obtained for in vitro transcription/translation reaction products. Shown are WT (k_{off} = 0.003/s), HS30A (0.003/s), HL97A (0.002/s), and HW33A (undetectable binding).

niques, including autoradiography and Western analysis, to assess full-length protein yeilds by densitometry. For a strategy that is easily incorporated into the functional assay, the authors have employed a capture ELISA, using polyclonal anti-$(Fab)_2$ and biotinylated Ag. Polyclongal serum has been used frequently as a sensitive probe of changes in conformation brought about by protein engineering (more sensitive than low-resolution spectroscopic methods, such as circular dichroism) ***(33)***, and can detect changes invoked by nonconservative amino acid substitutions or substitutions of conserved residues. Similar information can be obtained by using a radioimmunoassay scheme (substituting ^{35}S Met or ^{14}C Leu for the non radioactive isotope in the transcription/translation reaction), with three different capture coats. Ab recognizing the epitope tag can measure total soluble protein yields; polyclonal Ab can measure the fraction of properly folded scFv; Ag coat can be used to assess affinity. The ratio of signals for Ag coat/polyclonal coat provides a measure of relative affinity for the mutants. Furthermore, to confirm the results of the in vitro transcription/translation study, it may be wise to subclone and express one or two mutants.

4. Notes

1. The design of the vector primers is important: to prevent downstream problems, ensure that a single, strong band of the expected size is produced during amplification of WT plasmid with the vector primers. Extra bands, especially ones smaller than the desired fragment, will be preferentially generated during the overlap, drastically reducing yields of the full-length mutant gene.

2. The S30 must be produced from an endonuclease and protease minus strain (e.g., BL21), to permit expression of PCR templates.
3. If the protein of interest contains disulfide bonds, DTT should be excluded from the reaction mixture and S30 dialysis steps; it may also be necessary to optimize the redox potential of the reaction by the addition of GSSG and GSH (try 10:1 and 3:1 ratios with a final concentration of 1–3 m*M* GSSG). Several groups ***(19,34)*** have also found that the addition of chaperones greatly increases the fraction of soluble, active material.
4. Many protocols for bacterial transcription and translation add rifampicin (1 µL 5 mg/mL in ethanol/30 µL reaction) to inhibit endogenous bacterial RNA polymerases. However, the authors have found that the elimination of rifampicin does not affect final yields.
5. S30 contains tRNA, but the authors find it necessary exogenous tRNA to both commercial and homemade S30 mixtures.
6. The S30 is the most sensitive reagent in this mix; avoid repeated freeze-thaw cycles, and characterize each new batch carefully. If reactions work poorly or not at all, suspect the S30 quality (all other reagents seem quite stable).
7. Cysteine, arginine, and tryptophan hydrolyze rapidly at 37°C; 1 m*M* of each may be added every 20 min during the reaction for improved yields.
8. Recent research suggests that PEP is poorly suited to be the energy source for these reactions, as hydrolysis produces a known inhibitor of the reaction, inorganic phosphate. A proposed substitute is pyruvate (1 µL 1 *M* stock/30 µL reaction), with pyruvate oxidase (1 µL 200 U/mL stock), thiamine pyrophosphate/co-carboxylase (1 µL of 0.1 *M* stock) and KH_2PO_4 (0.4 µL 1 *M* stock) added to regenerate the energy source. This reaction proceeds more slowly (2 h to completion), but may result in greater yields than the PEP-based reaction.
9. Chemiluminescent substrates are preferred, instead of chromogenic substrates, because they are more sensitive (to pmol, and even fmol levels), and have a much wider dynamic range (from 0 to 10,000, which assists in discrimination between mutants).
10. Alternatively, if the Ag is available with a His_6 tag, one can purchase a chip that will bind the tag and properly orient the Ag, so that the binding surface is not sterically inhibited. If a small molecule is being used, it may be necessary to couple it to BSA before immobilizing on a chip.
11. Because the active scFv concentration is low in the transcription/translation reaction, scale up to provide enough material to see a strong signal (90–150 µL is suggested to see ≥50 RU of binding for a BIAcore 1000, ≥15 RU for a BIAcore 3000).
12. The presence of a T7 terminator hairpin loop has been shown to stabilize the mRNA in an in vitro transcription/translation reaction, resulting in greater yield ***(35)***.
13. Low concentrations of each fragment generally results in better yield (<< 1 ng). If the reaction is poor, amplifying for seven cycles without primers, then adding primers for the remaining 23 cycles, may assist. Regarding mutagenic primer design: Complementarity of at least 15 bases between the front and rear mutagenic primers is necessary for good overlap yields.

14. Primer–dimer will not affect the transcription/translation reaction, because there will be no T7 promoter. If extraneous bands are present, gel-purify the desired band.
15. If yields from the overlap reaction are lower than desired, it may be helpful to purify the desired band, and perform PCR on the full-length template. This will usually result in homogeneous product of sufficient concentration.
16. Allowing the S30 to incubate with the reaction mixture, for as little as 10 min, even in the absence of DNA, has been shown to reduce protein yields significantly (J. Swartz, personal communication).
17. Because small amounts of protein are produced in the reaction, an overnight incubation allows free and Ag-bound scFv to reach equilibrium.

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18

Random Transposon Mutagenesis of Large DNA Molecules in *Escherichia coli*

Wolfram Brune

1. Introduction

Mutagenesis of large plasmids, such as bacterial artificial chromosomes (BACs) or P1-based artificial chromosomes (PACs), can be difficult for various reasons. Because of their large size (up to 300 kbp), unique restriction enzyme sites are usually not available. Even if they are, modifications based on restriction digest and ligation are mastered only by scientists with extensive experience in handling large DNA molecules. Moreover, sequence information is not available for many BACs and PACs derived from eukaryotic genome libraries, which further complicates mutagenesis.

Random transposon (Tn) mutagenesis can help to overcome some of these problems. It allows generation of a large number of mutant plasmids with minimal effort. This is particularly useful for sequencing large DNAs that require more than one sequencing reaction to determine the entire sequence, and provides a faster alternative to primer walking, random subcloning, and nested deletion methods. It can also be used for mapping projects.

Mutant BACs are also useful for studying the function of genes encoded on them. The entire genomes of large DNA viruses (e.g., herpes viruses or baculoviruses) have been cloned as single BACs in *Escherichia coli*, in which the individual viral genes can be modified by site-directed or random mutagenesis ***(1)***. In a similar way, mutant BACs have been used to generate transgenic animals ***(2)***.

A few different approaches for Tn mutagenesis of BACs and PACs have recently been published ***(3–5)***. The protocol described in detail in this chapter combines several advantages and makes the procedure fast, efficient, and easy to perform ***(3)***, even for the novice.

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ

1. The Tn used is a derivative of Tn*1721*, a member of the Tn*3* family ***(6,7)***. It has a strong preference for insertion into plasmids (or BACs or PACs), and inserts less often into the *E. coli* genome. Procedures to enrich for useful clones are not required.
2. The Tn donor plasmid has a temperature-sensitive mechanism of replication. It replicates at 30°C, but does not at temperatures above 42°C. Therefore, it is easily eliminated form the BAC host, simply by a temperature shift.
3. Because of Tn immunity, single Tn insertions are usually found in the target plasmid. Multiple insertions are rare.
4. The Tns contain binding sites for M13 forward and reverse primers, which facilitate sequencing under standard conditions.

2. Materials

1. The Tn donor plasmids, pTsTM2, pTsTM8, and pTsTM13 ***(3)***, encode a temperature-sensitive mechanism of replication and an ampicillin (AMP)-resistance gene ***(8)***. They further contain the transposable element with an antibiotic-resistance gene (erythromycin [EM], kanamycin [KM], or tetracycline [TET]), and the genes for transposase and resolvase, the enzymes necessary for transposition. These plasmids are available from the author.
2. The target BAC in its *E. coli* host strain (e.g., DH10B or DH5α).
3. LB agar plates with chloramphenicol (CHL) (13.6 μg/mL) and AMP (100 μg/mL).
4. LB agar plates with CHL (13.6 μg/mL) and KM (50 μg/mL), TET (5 μg/mL), or EM (250 μg/mL), depending on the Tn used.
5. Electrocompetent *E. coli* DH10B, e.g., GeneHogs (Research Genetics) or ElectroMAX DH10B (Clontech).
6. Columns and buffers for BAC DNA purification, e.g., NucleoBond AX100 columns (Macherey-Nagel or Clontech).

3. Methods

The following procedures describe mutagenesis of a BAC encoding a CHL-resistance gene, using the Tn donor plasmid, pTsTM8 (encoding a KM-resistance gene). When using a different target plasmid or a different Tn donor, the antibiotics need to be adjusted accordingly.

3.1. Introduction of the Tn Donor Plasmid

1. Grow *E. coli* bacteria containing the target BAC, and prepare competent cells (chemically competent or electrocompetent).
2. Transform with the Tn donor plasmid, and spread on Luria-Bertani (LB) agar plates containing CHL and AMP. Incubate at 30°C, the permissive temperature for replication of the Tn donor plasmid (*see* **Note 1**).
3. Pick a colony and grow a small (3 mL) overnight culture in LB medium with CHL and AMP at 30°C.

As an alternative to **steps 1** and **2**, electrocompetent DH10B cells can be co-transformed with the BAC and the Tn donor plasmid. Use undiluted DNA

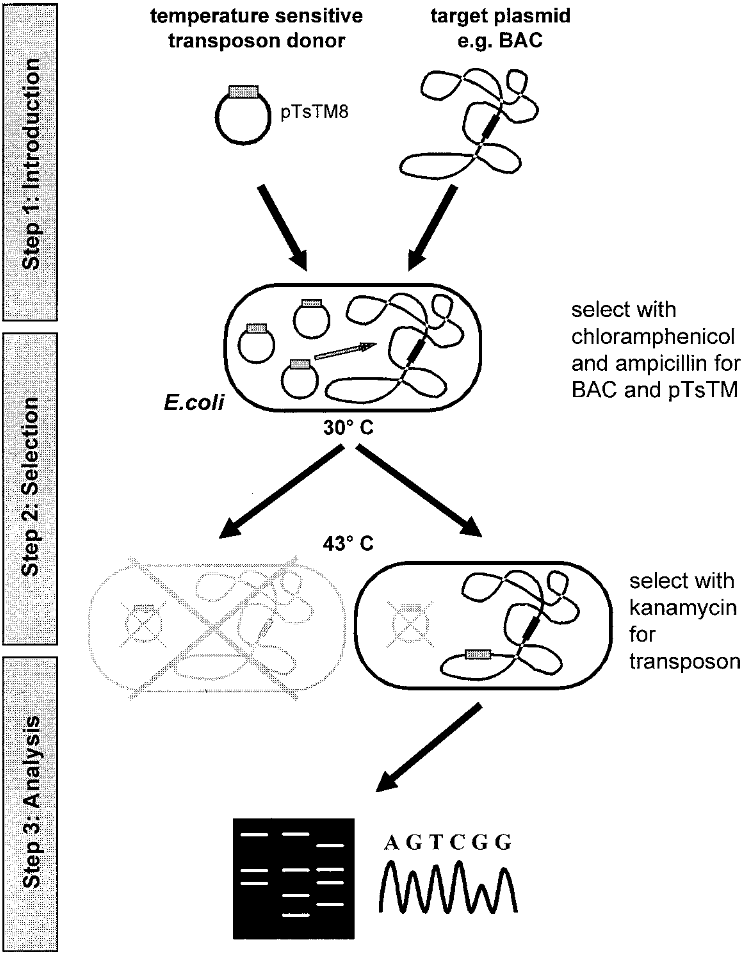
Step 1: Introduction
Step 2: Selection
Step 3: Analysis
temperature sensitive transposon donor
target plasmid e.g. BAC
pTsTM8
E.coli
select with chloramphenicol and ampicillin for BAC and pTsTM
30° C
43° C
select with kanamycin for transposon
A G T C G G

Fig. 1. Overview of the Tn mutagenesis protocol. The donor and the target plasmid are introduced into *E. coli*. A temperature shift from 30 to 43°C, in the presence of KM eliminates the donor plasmid and selects for transpositions. BAC DNA from resistant clones is analyzed by restriction digest or sequencing. (Reprinted with permission from **ref.** ***3***.)

samples for co-transformation. Make a miniprep to check for the integrity of the BAC.

3.2. Selecting Tn Insertion Mutants

Dilute a small aliquot (e.g., 2 µL) of the dense overnight culture in 100 µL LB, and spread on an LB agar plate containing CHL and KM (50 µg/mL). Incubate plate overnight at 43°C. At this temperature, the donor plasmid can-

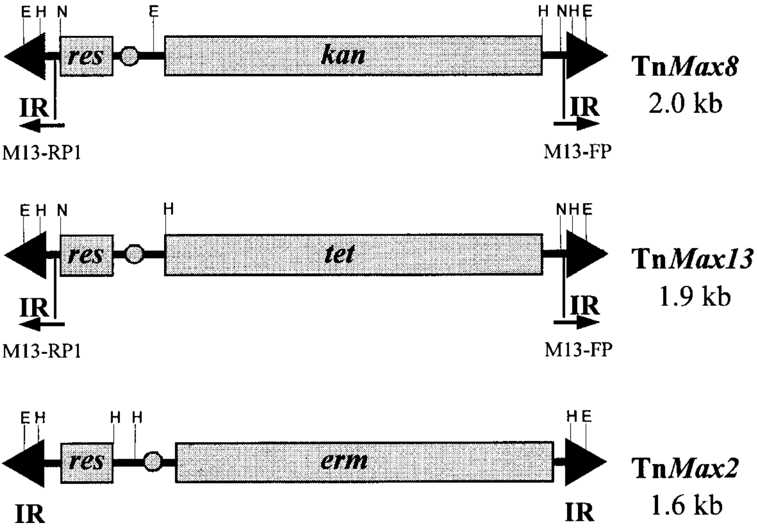

Fig. 2. Structure of the transposable elements. Three Tns, Tn*Max2*, Tn*Max8*, and Tn*Max13*, with different antibiotic-resistance genes, are encoded on the donor plasmids, pTsTM2, pTsTM8, and pTsTM13, respectively. Restriction sites for *Eco*RI (E), *Hin*dIII (H), and *Not*I (N), and M13 primer binding sites are located within or near the inverted repeats (IR). *kan*, *tet*, *erm*, resistance genes for KM, TET, and EM, respectively; *res*, resolution site necessary for transposition. (Reprinted with permission from **ref.** ***3***.)

not replicate, and will be lost. Only those bacteria in which a transposition has occurred will retain the antibiotic resistance gene encoded on the Tn and will thus be able to form colonies.

Try different dilutions of the overnight culture to determine the amount that gives a suitable number of colonies. When using the Tn donor plasmids, pTsTM13 or pTsTM2, use TET (5 µg/mL) or EM (250 µg/mL), instead of KM (*see* **Notes 2** and **3**).

3.3. Analysis of Tn Insertion Mutants

1. Pick individual colonies and grow them as liquid cultures overnight at 37°C. Select with CHL only, because selection with two antibiotics may result in slower growth.
2. Prepare BAC DNA from 100 mL culture, using NucleoBond AX100 columns (Clontech), according to the manufacturer's instructions for BACs, or using a preferred BAC purification protocol.
3. Digest BAC DNA (0.5–1 µg, depending on the size of the construct) with a suitable restriction enzyme, and separate on a large 0.6% agarose gel. The Tns contain *Eco*RI, *Hin*dIII, and *Not*I restriction sites within or close to the inverted

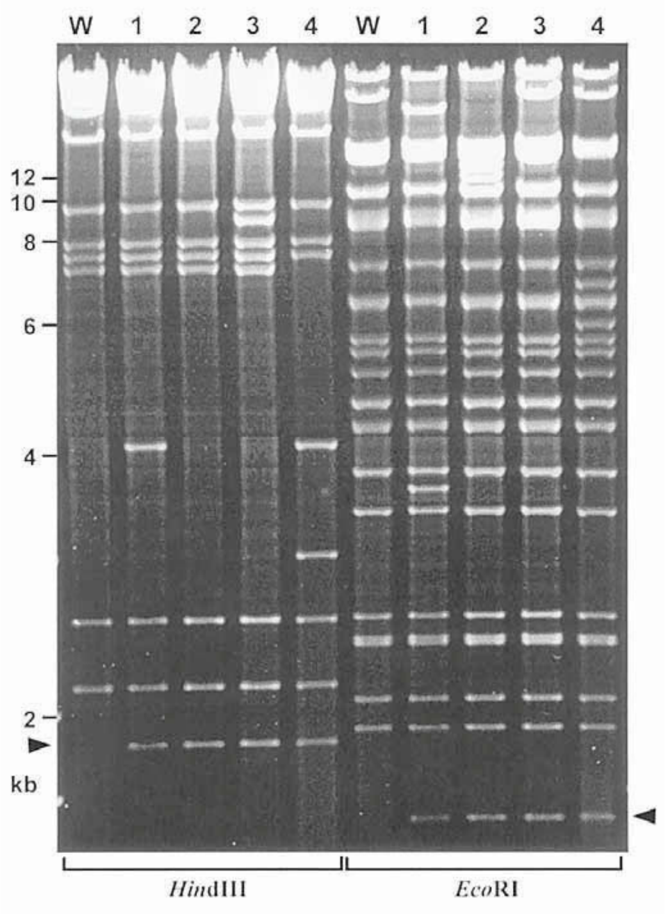

Fig. 3. Analysis of Tn insertion mutants by restriction digest. A 240-kb BAC was mutated by Tn*Max8* insertion. DNA of the wild-type BAC (W) and four mutants (1–4) was digested with *Hin*dIII and *Eco*RI. Tn insertion leads to a change of the restriction pattern and appearance of a Tn-specific band (▶) at 1.9 and 1.5 kb in the *Hin*dIII and *Eco*RI digest, respectively. (Reprinted with permission from **ref.** ***3***.)

repeats. Digestion with one of these enzymes results in excision of the Tn. The Tn will be visible as a distinct band at 1.5–2 kb, depending on the Tn and restriction endonuclease used. The vast majority of BAC clones should contain a Tn insertion. Using two different 240-kb BACs as targets, the author et al. found insertions in ~90% of clones analyzed ***(3,9,10)***, and using a 140-kb BAC, found insertions in ~80% of clones (W. Brune and H. Adler, unpublished results). Less than 5% of clones should have more than one insertion (*see* **Note 4**).

4. Determine the Tn insertion site and the adjacent BAC sequence, by sequencing, using M13 forward or reverse primers. High-quality BAC DNA is required to

obtain good and long sequence reads. Contact a sequencing facility for their recommended protocol. The sequence obtained should begin with the IR sequence of the Tn (AAG CTA GCG TCG ATT TTT CCA ATT CGC GGT CCC CC), and continue with the adjacent BAC sequence. If the sequence of a BAC is known, the precise insertion site can be determined by aligning the sequences (*see* **Notes 5** and **6**).

4. Notes

1. No CHL/AMP colonies obtained after transformation of the BAC host with the Tn donor plasmid. *Suggestions*: Be sure that plates were incubated at 30°C. It may take 18–24 h for colonies to grow at 30°C. Check competence of cells by transforming with a different plasmid (e.g., pBluescript). Check quality of the Tn donor plasmid by gel electrophoresis and transformation of a different batch of cells. Note that plasmids with a pSC101 origin of replication cannot be used as target plasmids, because the donor plasmids contain this origin.
2. Large and very small colonies are found on CHL/EM plates, using pTsTM2 as donor plasmid. *Suggestion*: *E. coli* is only moderately sensitive to EM. Thus, the tiny colonies are usually “false-positives”, and the large ones are the truly resistant ones. Increasing the EM concentration, or using a different Tn donor and antibiotic, may help, if this is a problem.
3. A considerable number of mutants obtained using pTsTM13 have more than one Tn insertion; some have deletions. *Suggestion*: Resistance to TET is dependent on the gene copy number. Therefore, use of only low TET concentrations (5 μg/mL), is critical, if the target plasmid is single-copy, such as a BAC. Selecting with higher TET concentrations may result in selective outgrowth of mutants with more than one Tn inserted. Do not serially passage the bacteria containing the donor and target plasmids at 30°C. If needed for later use, store at 4°C for a few days, or as glycerol stock at –70°C.
4. Upon gel electrophoresis of the digested BAC, individual bands are not clearly visible with ethidium bromide staining, because of high backround. *Suggestion*: Perform alkaline lysis exactly as recommended. It is critical to incubate the bacterial suspension no more than 5 min with the lysis buffer. Prolonged lysis and vigorous mixing results in release of bacterial genomic DNA, which accounts for the high background.
5. Poor sequencing results. *Suggestion*: High-quality BAC DNA (*see* **Note 4**) is needed to obtain good results. Because of the large size of BACs, more template is needed for the sequencing reaction than with small plasmids. Contact a sequencing facility for their recommendations. BACs containing more than one Tn cannot be sequenced, because the primer binding sites are no longer unique in the template. The Tn on pTsTM2 (containing an EM-resistance gene) does not contain M13 primer binding sites, and is therefore not recommended for sequencing applications.
6. Tn insertions are not homogeneously distributed over the entire target BAC plamid. *Suggestion*: Although Tn insertions are random, it is not uncommon that

insertions are more frequently found in one region of the target than elsewhere. The reason for this is unknown, and it varies from target to target, but it is usually not a problem. If Tn insertions in specific regions of a large target are needed, a PCR-based library screening approach is recommended ***(9)***.

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Random Chromosomal Gene Disruption Using Cassette Mutagenesis

French A. Lewis, III and Brian A. Dougherty

1. Introduction

Random mutagenesis of DNA has been an essential tool for investigation of bacteria, fungi, and other organisms. Given the recent explosion in genomic sequence information (e.g., over the past 5 yr there have been 30 published microbial genomes and over 100 microbial genome sequencing projects), there is a growing need for reliable methods of random DNA mutagenesis to allow for functional genomics work. The authors were studying *Haemophilus influenzae*, the first organism to have its genome completely sequenced ***(1)***. *H. influenzae* is a Gram-negative facultative anaerobe, which resides in the human upper respiratory tract and causes disease. *H. influenzae* is also capable of taking DNA from its environment and integrating it into the bacterial chromosome ***(2,3)***. A number of genes required for DNA transformation have been identified in *H. influenzae*. These studies have used a variety of molecular methods to identify transformation genes, including complementation of transformation-deficient mutants derived by chemical mutagenesis ***(4)***, a "poison-DNA" selection method ***(5)***, mini-transposon (Tn)*10* mutagenesis ***(6)***, Tn*916* mutagenesis ***(7)***, and other genetic selection techniques ***(8)***. In order to identify the remaining transformation genes by insertional mutagenesis, while overcoming potential biases inherent in Tn-based screens (e.g., hot-spotting), the authors decided to investigate the utility of the cassette mutagenesis protocol ***(9)***. In this procedure, an antibiotic-resistance cassette is ligated to restriction endonuclease-digested chromosome DNA, which has been treated so that the original gene order is maintained during the process. The resulting cassette-mutagenized DNA is then transformed into competent bacteria, resulting in the insertion of the cassette marker throughout the chromosome and the generation of mutants by gene disruption.

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ

Molecular analysis of *H. influenzae* has been greatly aided by the determination of the complete 1.83-Mb genome sequence. Although genome sequence facilitates directed gene knockouts of suspected transformation genes, the authors reasoned that phenotypic screening of mutagenized cells for transformation deficiencies would provide a more balanced, unbiased answer of what genes are involved in DNA transformation. The authors therefore used cassette mutagenesis in conjunction with a phenotypic screen for transformation-deficient (tfo$^-$) mutants and sequencing of PCR-generated genome sequence tags, to rapidly map mutations in transformation genes of *H. influenzae*.

This chapter describes the method developed to allow for phenotypic screening of random gene disruptions on the *H. influenzae*-chromosome. This technique requires an antibiotic-resistance cassette to be ligated to restriction endonuclease-digested chromosome DNA that has been treated so that the original gene order is maintained during the process. The resulting cassette-mutagenized DNA is then transformed into competent bacteria, resulting in the insertion of the cassette market by a double-recombination event throughout the chromosome and the generation of mutants by gene disruption. The cassette mutagenesis procedure is presented in the context of the authors' screen for transformation genes in *H. influenzae*.

2. Materials

1. Streptomycin (SM), erythromicin (EM), kanamycin (KM), hemin, α-nicotinamide adenine dinucleiotide (NAD), and cyclic adenosine monophosphate (cAMP) were obtained from Sigma (St. Louis, MO). Brain–heart infusion (BHI) media was obtained from Difco (Detroit, MI). TE buffer (10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.5), buffer-saturated phenol, 3 *M* sodium acetate (pH 5.2), and glycogen were obtained from Life Technologies (Gaithersburg, MD).
2. All strains are derived from the original Rd strain ***(10)***. The *H. influenzae* strains, KW20 ***(11)*** and MAP7, a SM-resistant, EM-sensitive derivative of MAP ***(12)***, were grown in BHI broth (Difco) supplemented with 10 µg/mL hemin and 2 µg/mL NAD (sBHI), as described elsewhere ***(13)***. Mutant strains of KW20 were constructed by transformation with cassette mutagenized DNA (*see* **Subheading 3.1.**). For antibiotic selection, SM was added to media at 250 µg/mL, and KM at 7 µg/mL. All chemicals were purchased from Sigma.
3. Plasmid pUC4K (Pharmacia, Piscataway, NJ) was the source of the KM-resistance (Km^r) cassette.
4. Qiagen miniprep columns (Qiagen, Chatsworth, CA) were used for all plasmid DNA preps.
5. *Sau3*AI, *Eco*RI, *Bam*HI, and *Ssp*I restriction endonucleases were obtained from New England Biolabs (NEB) (Beverly, MA).
6. DNA modification enzyme, T4 DNA ligase, was obtained from NEB.
7. All PCR primers were obtained from Life Technologies. The following PCR primers are necessary: primer 1, 5'-GAATTTAATCGCGGCCTCGAG-3'; primer

2, 5'-CCGAATTCAGGTGAAAATATT-3'; primer 3, 5'-AGGAATTCTGCCAGCGCATCAAC-3'; primer 4, 5'-GATTCAGATCTGGTATGAGTC-3'; BSPout primer (5'-CCCTGCAGGTCGACGGATC-3').

8. PCR reagents were obtained from Life Technologies.
9. DNA-sequencing reagents were obtained from Applied Biosystems (Foster City, CA).

3. Methods

3.1. Mutagenesis Technique

H. influenzae Rd strain KW20 was mutagenized by inserting the 1.2 kb Km^r DNA cassette into various *Sau*3AI restriction sites throughout the chr. The in vitro procedure for cassette-mutagenizing chromosome DNA ***(9)*** involves five steps: cleavage of the DNA with a suitable restriction enzyme (e.g., *Eco*RI); intramolecular ligation of the restriction fragments to form circles; partial digestion of the circles with a second enzyme (e.g., *Sau*3AI) that produces ends compatible with the Km^r cassette (e.g., *Bam*HI ends); ligation of cut circles and cassette DNA; and recutting the ligated DNA with the first enzyme (*Eco*RI), to linearize the circle. These five steps are outlined in **Fig. 1** and have been modified as described below.

1. *H. influenzae* Rd DNA of approx 50-kb size, was cleaved in a 1-mL reaction mixture containing 60 µg DNA and 50 U *Eco*RI for 2 h at 37°C. The mixture was phenol-extracted and the DNA recovered by ethanol precipitation, redissolved in 150 µL TE buffer, and adjusted to 500 µL in 1X ligation buffer.
2. Intramolecular ligation of DNA fragments is favored at low DNA concentrations. Therefore, the intramolecular ligation (to form circles of chr DNA) can be carried out by diluting the DNA to a final concentration of 50 ng/µL by adding 50 U T4 DNA ligase, and allowing the reaction to proceed at 25°C for a minimum of 3 h. The reaction is heat-inactivated at 65°C for 10 min, one-tenth vol 3 *M* sodium acetate is added, along with 1 µL 20 µg/µL glycogen (Life Technologies, cat. no. 10814-010) and 2.5 vol absolute ethanol. The reaction is incubated at –20°C for 30 min, and the DNA is recovered by centrifugation at 14,000*g* for 15 min, washed in 1 mL 70% ethanol, and redissolved in 50 µL TE buffer (*see* **Note 1**).
3. Circles were digested with *Sau*3AI in a reaction mixture (100 µL) containing approx 10 µg circles and 2 U *Sau*3AI. Incubation was at 23°C, and aliquots were removed every minute, from 2 to 8 min, into a tube containing 10 µL 0.5 *M* EDTA, pH 8.0. The pooled samples were extracted with phenol, precipitated, and redissolved in 50 µL TE buffer.
4. The 1.2-kb Km^r cassette was excised from 10 µg of pUC4K DNA, using *Bam*HI, gel-purified, and reconstituted in 50 µL TE buffer. *Sau*3AI-cut circles (5 µL) and cassette DNA (2 µL) were ligated together in a 20-µL reaction containing 2 U T4 DNA ligase at 16°C, for 75 min. The reaction was terminated by treatment at 70°C for 10 min.
5. 1 µL 2 *M* NaCl and 1 U *Eco*RI were added to the ligation mixture from **step 4**, and incubated at 37°C for 30 min, followed by 10 min at 70°C. The mutagenized

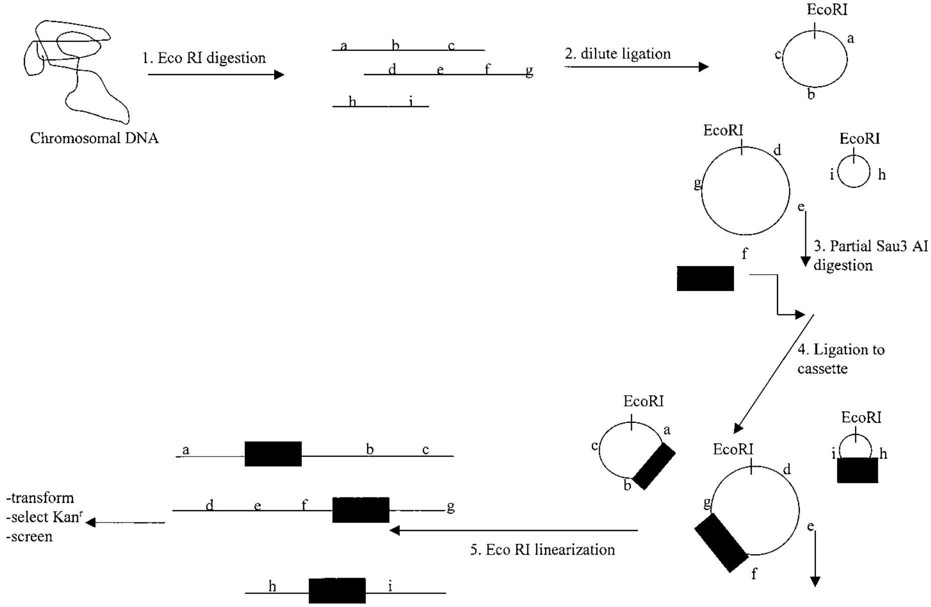
Chromosomal DNA
1. Eco RI digestion
a b c
d e f g
h i
2. dilute ligation
EcoRI
a
b
c
EcoRI
d
e
f
g
EcoRI
h
i
3. Partial Sau3 AI digestion
4. Ligation to cassette
EcoRI
a
b
c
EcoRI
d
e
f
g
EcoRI
h
i
5. Eco RI linearization
a b c
d e f g
h i
-transform
-select Kan^r
-screen

Fig. 1. Cartoon overview of the cassette mutagenesis procedure. The steps are described in greater detail in **Subheading 3.**

DNA was stored at –20°C. 1 µL yielded more than 40,000 Km^r clones using frozen competent KW20 cells.

3.2. cAMP-DNA Plate Screen for Transformation Mutants

(**Note:** This assay, along with the MIV and DNA-binding assays, are specific for determining the transformation phenotype in *H. influenzae*: they are not required for cassette mutagenesis.) *H. influenzae* strain KW20 was made competent by the MIV procedure ***(13,14)***. Frozen competent cells were transformed with cassette mutagenized DNA and transformants (approx 200 colonies/plate) were immediately plated on KM-supplemented sBHI, and incubated overnight at 37°C. The colonies were subsequently replica-plated, first onto a KM-supplemented sBHI plate, then onto sBHI plates supplemented with 1 m*M* cAMP, onto which 50 µg MAP7 chr DNA had been freshly spread and allowed to dry (modified from **ref. *15***). After overnight growth, colonies from the cAMP-DNA plates were replica-plated onto SM-supplemented sBHI plates. Colonies absent after overnight growth on the SM plates were noted, and the corresponding colony from the KM-supplemented replica plate was recovered and frozen for use in subsequent studies.

3.3. Inverse PCR and DNA Sequencing

Primers were designed to be complementary to the Km^r cassette (accession no. X06404), and included tailored restriction sites. The primer sequences were the following: primer 1, 5'-GAATTTAATCGCGGCCTCGAG-3'; primer 2, 5'-CCGAATTCAGGTGAAAATATT-3'; primer 3, 5'-AGGAATTCTGCCAGC GCATCAAC-3'; primer 4, 5'-GATTCAGATCTGGTATGAGTC-3'. A fifth primer, used only for sequencing, was the BSPout primer (5'-CCCTGC AGGTCGACGGATC-3'), designed to read out from the *Bam*HI-*Sal*I-*Pst*I polylinker flanking the Km^r cassette.

The inverse polymerase chain reaction (PCR) scheme is diagramed in **Fig. 2**. Template for the PCR reaction was prepared by *Ssp*I digestion of chr DNA, followed by dilute ligation (<1 μg/mL). Similarly treated KW20 chr DNA was used as a negative control. PCR reactions were performed in a reaction mixture (100 μL) containing 100 ng circularized chr DNA, 1 μ*M* primers, 80 μ*M* deoxyribonucleoside triphosphates, 2.5 m*M* $MgCl_2$, and 1X Gibco-BRL *Taq* DNA polymerase buffer. The PCR reactions were performed using a DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT). *Taq* DNA polymerase (Life Technologies) was added to the PCR reactions, upon reaching 93°C, and the reaction conditions consisted of 30 cycles of denaturing (93°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 3 min), followed by a 5-min final extension (72°C).

3.4. DNA Sequence Analysis

PCR templates were sequenced using the fluorescent dideoxy terminator method of cycle sequencing ***(16)*** on a Perkin-Elmer/Applied Biosystems 373A automated DNA sequencer ***(17)***, following the Applied Biosystems protocols. DNA sequence information from the inverse PCR templates was mapped to the *H. influenzae* genome sequence using search tools at The Institute for Genome Research (TIGR) World Wide Web site (http://www.tigr.org/tdb/mdb/hidb/hidb.html.). In addition to using the *H. influenzae* annotation ***(1)***, open reading frames surrounding the Km^r insertions were identified using DNA Strider, Basic Local Alignment Search Tool (BLAST) homology alignments to the National Center for Biotechnology Information nonredundant protein database, and visual inspection. In the interest of space, the DNA and predicted amino acid sequences are not presented; readers are referred to the L42023 accession number, the genome sequence publication, and TIGR World Wide Web site uniform resource locator cited above.

3.5. Results

3.5.1. Screen for Transformation-Deficient Mutants

In order to insert Km^r cassettes throughout the *H. influenzae* Rd genome, the cassette mutagenesis protocol ***(9)*** was used to mutagenize strain KW20 (**Fig. 1**;

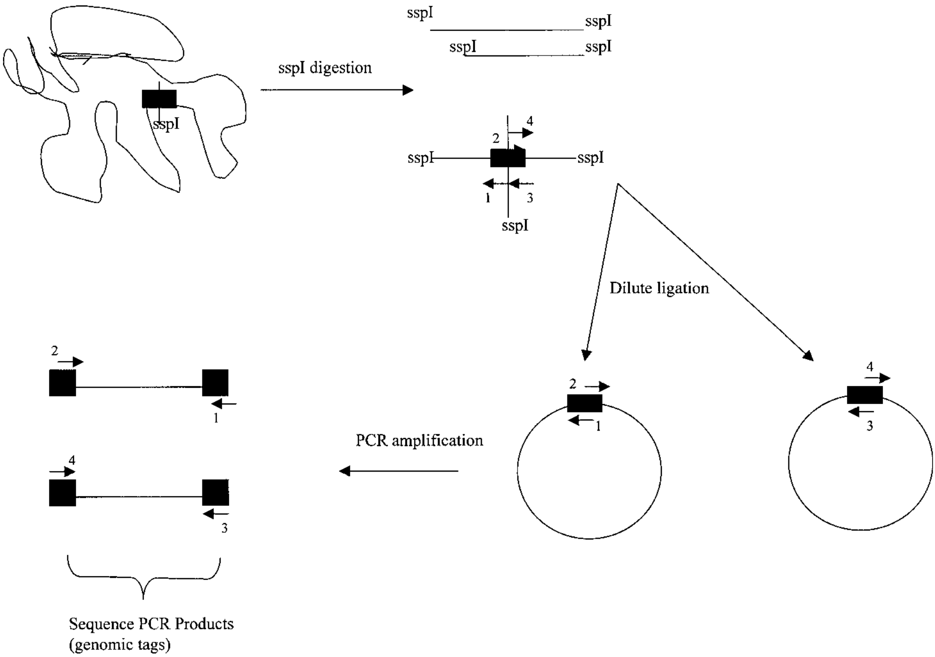
sspI
sspI digestion
sspI
sspI
sspI
sspI
4
2
sspI
sspI
1
3
sspI
Dilute ligation
2
1
4
3
PCR amplification
2
1
4
3
2
1
4
3
Sequence PCR Products
(genomic tags)

Fig. 2. Cartoon overview of inverse-PCR strategy, which amplifies DNA adjacent to the inserted Km^r cassette. The black box represents the cassette DNA. Primers 1–4 correspond to the PCR primers in **Subheading 3.3.**

see **Methods**). Approximately 8000 Km^r colonies were then screened for a tfo^- phenotype. This involved a replica-plate-based assay (the cAMP-DNA plate assay), to identify tfo^- mutants by exposing the colonies to chr DNA from a SM-resistant strain, then challenging the colonies on media containing SM. Mutants that failed to grow in the presence of the antibiotic were deemed candidate tfo^- mutants, and were recovered from a replica plate. Following a rescreening of candidate mutant colonies to reduce false-negatives, 26 tfo^- clones were identified. These strains were then further analyzed by the MIV and DNA binding assays.

3.5.2. Characterization of tfo^- Mutants by DNA Transformation and DNA-Binding Assays

To confirm and further characterize the tfo^- phenotypes of the mutant strains, each mutant was made competent by the MIV procedure ***(13,14)*** and assayed for transformation efficiency. 3/26 mutants failed to grow sufficiently to be assayed, and three other mutants displayed wild-type levels of transformation by the MIV procedure (data not presented). Of the remaining 20 mutants, the MIV transformation and DNA binding efficiencies of 15 were further examined, along with wild-type KW20 and one tfo$^+$ transformant (**Table 1**). The transformation efficiencies of the tfo$^-$ mutants ranged over six orders of magnitude, from slightly deficient to extremely deficient (i.e., background levels of transformation).

The MIV-competent cells were then assayed for DNA binding, using ^{32}P-labeled chr DNA. All mutants were at least one log down in DNA binding, with 11/16 mutants displaying background levels of DNA binding (data not shown). These experiments confirmed that the mutants identified in the cAMP-DNA plate assay screen, and subsequently by MIV-transformation assays, were deficient in transformation, because of a deficiency in the ability to bind or take up DNA.

3.5.3. DNA Sequence Analysis of the Transformation-Deficient Mutants

In order to identify the genes inactivated by the Km^r cassettes, chromosome DNA from tfo$^-$ mutants was amplified by an inverse PCR strategy (**Fig. 2**), then directly sequenced. The mutations were mapped by searching the DNA sequence of *H. influenzae* Rd (http://www.tigr.org/tdb/mdb/hidb/hidb.html), and the genetic organization surrounding insertions in five regions of the *H. influenzae* chr is presented below.

3.5.4. Penicillin-Binding Protein Region

One set of cassette insertions was found in the 29–42 kb region of the KW20 chromosome containing two clusters of genes apparently involved in cell wall biosynthesis. A previous screen for tfo$^-$ mutants resulted in a mutant (JG58) ***(18)***, with an insertion in the *pbp*2 gene found in this gene cluster. All three mutants identified here (BD314, BD345, and BD350), had similar DNA binding and uptake values (**Table 1**) (approx 4–8% DNA binding but background levels of DNA uptake), and had somewhat lowered transformation efficiencies, compared to wild-type (approx 50- to 400-fold reduced). Analysis of the DNA immediately adjacent to the *Sau*3AI site formed by the cassette-chr junction fragment in BD314, BD345, and BD350 indicated that, compared to the wild-ype KW20 sequence, there were deletions of 52, 2651, and 2174 bp, respectively. This apparently resulted from digestion at a second *Sau*3AI site in the *Eco*RI, circle and is addressed in more detail in **Subheading 3.6.** Strain BD345 also contained a 505-bp chimeric *Sau*3AI fragment between the left terminus of the cassette and the chromosome *pbp*2 gene. This chimera was from a region of the KW20 chromosome approx 530 kb away from the cell wall biosynthesis region, and was apparently ligated along with the Km^r cassette during the cassette-ligation reaction. The deletion of BD314 was within

Table 1
Comparison of MIV Transformation and Transformability Values (as Asessed by MIV- and DNA-Binding Assays) for Wild-Type (KW20) and Cassette Mutant Strains

Strain	Class of knockout	Transformable?	Point of chromosomal insertion (kb)[a]
KW20	Wild-type	+	
BD300	(Unknown)	+	
BD306	*com*	–	442
BD307	*MDR*	–	896
BD309	*pil*	–	334
BD310	*pil*	–	334
BD314	*pbp*	–	32
BD316	*com*	–	457
BD331	*com*	–	457
BD335	*pil*	–	334
BD339	*pil*	–	334
BD341	*pil*	–	334
BD342	*com*	–	457
BD343	*MDR*	–	951
BD344	*pgs*A	–	138
BD345	*pbp*	–	32
BD350	*pbp*	–	39

BD300 is a cassette mutant with normal levels of transformation. The region into which each Km^r cassette inserted is given for each transformation-deficient mutant.

[a] Location-based distance from the designated bp1 of the *H. influenzae* chromosome (the unique *Not*I site on KW20 chromosome).

the *rod*A homolog; the deletions associated with BD345 and BD350 resulted in the loss of several genes in these strains (**Fig. 2**).

3.5.5. com *Operon*

A second set of insertions were found in a genetic locus identified by a mini-Tn*10* screen for tfo$^-$ mutants ***(6)*** and a complementation study ***(19)***, and was further characterized as the competence-induced *com* operon ***(18)***. Three of the insertions (mutants BD316, BD331, and BD342) were in the same *Sau*3AI restriction site in the *com*E gene. The tfo$^-$ phenotypes, and lack of DNA binding, agree with previously published data ***(18)*** for *com*E mutants. BD316 and BD342 were the only other tfo$^-$ mutants listed in **Table 1** that harbored chimeric DNA adjacent to the cassette DNA.

The fourth cassette insertion mapping near the *com* operon was BD306, located approx 3 kb upstream of the *com*A–F operon. The Km^r cassette was associated with a 476-bp deletion of both *com*ORFJ and HI0442. Since insertions in *com*A–F are the only published mutations in the *com* operon associated with background levels of transformation, to date ***(18)***, it will be of interest to determine the gene(s) responsible for the loss of transformation in the BD306 mutant.

3.5.6. pil *Operon*

The mutant class with the most insertions was the type IV pilin-like protein class (strains BD309, BD310, BD335, BD339, and BD341). All had background levels of transformation and DNA binding/uptake (**Table 1**), and had insertions falling within a 4-kb region, which includes an operon of genes similar to other type IV pilin genes. Homology searches indicated that the operon consists of a type IV pilin subunit gene (*pil*A), two pilin secretion and/or assembly genes (*pil*B and *pil*C), and a prepilin peptidase (*pil*D). A dyad symmetry element found upstream of multiple *H. influenzae* competence-induced operons ***(20)*** is found upstream of the *pil* operon, and each of the five mutants found in the *pil* region disrupt this genetic element. The *H. influenzae pil* operon ORFs are similar to a set of genes required for natural transformation in other bacteria ***(21–24)***. Taken together, the most likely explanation for the tfo$^-$ phenotype of the five *pil* region mutants was lack of a functional pilin-like protein.

3.5.7. Drug Efflux Transporter Region

One class of tfo$^-$ mutants consisted of two insertions, BD307 and BD343, in a cluster of genes with convergently oriented directions of transcription. The *mtr*R, *acr*A, and *acr*B genes are homologous to components of multiple drug-resistance-efflux transporters ***(25,26)***, representing a transcriptional regulator, a membrane fusion protein, and a efflux subunit, respectively. The BD307 mutant, which was severely affected in MIV transformation, harbored a 5276-bp deletion that removes DNA from part or all of four open reading frames in this region (HI0894-0897). Consistent with *E. coli acr*A mutants ***(27)*** and *H. influenzae acr*A mutants ***(28)***, the BD307 mutant is hypersensitive to a number of hydrophobic compounds and antibiotics (**Table 2**); the *acr*A$^+$ BD343 mutant is similar to wild-type KW20 (data not shown). The BD343 insertion mutated only the *fts*N gene, and was 25-fold reduced for MIV transformation. BD307 and BD343, both *fts*N mutants, showed moderate filamentous growth (4–20 cells in length) at 37°C, compared to KW20, which is consistent with the growth characteristics of *fts*N mutants ***(29)***.

3.5.8. pgs*A Gene*

An insertion in the *pgs*A gene of KW20 was responsible for a 650-fold reduction in MIV transformation and background levels of DNA binding and uptake (**Table 1**). In *E. coli*, *pgs*A is an essential gene catalyzing the committed step to the synthesis of acidic phospholipids ***(30,31)***. The membrane of *H.*

Table 2
Comparison of MICs of Dyes, Detergents, and Antibiotics for KW20 (Wild-Type) and BD307 (Deleted for Several Genes Involved in Drug Efflux)

	MIC (µg/mL)	
Compound	KW20	BD307
Acridine Orange	50.00	1.56
Congo Red	100.00	1.56
Crystal Violet	3.13	<0.19
Ethidium bromide	1.56	0.78
Sodium dodecyl sulfate	50.00	6.25
Erythromycin	3.13	<0.19
Streptomycin	3.13	0.78
Tetracycline	0.39	<0.19
Vancomycin	>100.00	25.00

influenzae is composed of the phospholipids, phosphatidylglycerol (15%) and phosphatidylethanolamine (85%), and the development of competence did not affect these ***(32)***. A null mutation in *pgs*A would presumably decrease membrane levels of phosphatidylglycerol, but not phosphatidylethanolamine.

3.6. Discussion

The present study investigated the suitability of the cassette mutagenesis ***(9)***, using a model system of a transformable bacterium with a completely sequenced genome. The mutagenesis system was used to rapidly identify genes required for transformation in *H. influenzae* by random insertional mutagenesis, followed by phenotypic screening, inverse PCR, sequencing of mutants, and comparison to genomic sequence data. The authors decided to use the cassette mutagenesis technique, to take advantage of the high transformation efficiency of *H. influenzae*, and to investigate the usefulness of the technique to identify transformation genes not mutated in previous screens ***(4–6,8)***. Cassette mutagenesis techniques based on similar approaches have been described for other transformable organisms, such as *Bacillus subtilis* ***(33)*** or yeast ***(34)***. The current study represents a detailed analysis of the cassette mutagenesis procedure in *H. influenzae*.

Apparently the cassette mutagenesis method, combined with the phenotypic screen, was effective, because both previously identified (i.e., *com*E and *pbp*2) and newly identified transformation genes were found in this screen. The Km^r cassette inserted into distinct *Sau*3AI sites in all the mutants studied here, with the exception of the three *com*E mutants (BD316, BD331, and BD342). One complication of the procedure, for the authors, was that of deletions caused by digestion of the *Eco*RI circle at a second *Sau*3AI site. A byproduct of this reaction is the generation of small *Sau*3AI fragments, which occasionally ligate along with the cassette to create chimeras (e.g., BD316, BD342, and BD345). Apparently a more limited *Sau*3AI digestion would remedy the deletion/chimera problem. The publication of complete genome sequence data allows the cassette mutagenesis strategy to be refined to a high degree; however, the randomness of the insertions is still ultimately limited by restriction enzyme sites. In this study, although the screen was fairly extensive (~8000 colonies were screened), other known transformation genes were not isolated, possibly as the result of factors such as the position of genes relative to the nearest *Eco*RI sites, the presence of uptake signal sequences ***(35,36)*** on the *Eco*RI fragment, and the presence of *Sau*3AI sites in the central portion of the gene-coding region. It is unclear why known transformation genes such as the recombination genes *rec*-1 and *rec*-2 ***(5)***, have not been identified in subsequent screens, such as the mini-Tn*10* screen ***(6)*** or the present study. Also missing from the present study were mutations in regulatory transformation genes such as *sxy* ***(37)***, also known as *tfo*X ***(38)***, which resides on a 3146-bp *Eco*RI fragment, and *crp* ***(39)***, which was identified in the mini-Tn*10* screen ***(6)*** and resides on a 1820 bp *Eco*RI fragment. Possibly these small *Eco*RI fragments might have been less efficiently taken up by competent cells and integrated into the genome. In the latter case, an *Eco*RI fragment containing an insertion into the *sxy* or *crp* genes would have less flanking DNA outside the Km^r cassette for integration. Optimization of mutagenesis procedures for a higher order of randomness is underway by investigating alternate mutagenesis procedures, such as in vitro transposition ***(40)*** and integration of plasmids with randomly sheared DNA ***(43)***.

This study identified five loci involved in *H. influenzae* transformation (**Table 1**). Mutants in the *com* operon have been isolated in other screens for transformation genes ***(4,6)*** and, like the *com* mutants in this study (**Table 1**), have the background DNA binding/uptake ***(18)***. The *pbp* region may be involved in transformation, if the cell wall poses an impediment to DNA transformation, and alteration in expression of *pbp* proteins appears to correlate with the ability to transform DNA ***(41)***. The type IV pilin-like protein operon mutants have a loss of DNA-binding phenotype, which has been found in other bacteria in which type IV pilin and pilin-like proteins have a demonstrated role in natural transformation ***(21–24)***. This suggests that for all naturally transformable bacteria studied to date, type IV pilin-like proteins are a component of the DNA receptor/DNA channel that allows for transfer of DNA from the environment to the host cytoplasm. The effect of the phospholipid biosynthesis and multidrug-efflux transport mutations on *H. influenzae* transformation rates is less clear. It is conceivable that alterations in membrane phospholipid content or the cell membrane structure itself will result in less efficient transformation. The MDR efflux transporters are known to pump a broad range of toxic molecules out of the cell ***(25,26)***, and mutations of MDR genes result in increased sensitivity to these compounds in *H. influenzae*, as well (**Table 2**) ***(28)***. There are several possible mechanisms by which the BD307 deletion/mutation could result in the observed tfo$^-$ phenotype. Individual, nonpolar disruptions of the genes in each region will be necessary to provide the ultimate answer regarding the relative contribution of each gene to transformation.

Cassette mutagenesis is a technique that can be added to the list of random insertional disruption techniques, such as classic Tn mutagenesis, in vitro transposition, and insertion/deletion mutagenesis. The most critical factors for success of these techniques is the efficiency of transformation (determined by the host system and delivery technique) and the randomness of insertion (determined by the mutagenesis system). This chapter has demonstrated that cassette mutagenesis works as efficiently as previously employed methods for the naturally transformable *H. influenzae*. Although sequencing of hundreds of random, unselected Km^r mutants would have been preferred, to quantify randomness of the technique, cassette mutagenesis was sufficiently random to identify genes not tagged in previous mutagenesis screens, and only 2/15 insertions appeared to have a similar disruption.

4. Notes

1. One method used to avoid dilution of DNA in subsequent steps involves the use a “circle generator” (H. O. Smith, unpublished) to efficiently circularize the *Eco*RI fragments. (**Note:** the circle generator is not required if dilute DNA can be efficiently recovered, such as by ethanol precipitation in the presence of glycogen.) The mixing chamber of the generator was a 17 × 100-mm (Falcon 2059) plastic tube holding the ligation mixture (500 µL) containing 50 U T4 DNA ligase and a magnetic flea turning at about 60 rpm on a magnetic stirring plate. The *Eco*RI–cut *H. influenzae* Rd DNA (60 µg/500 µL) was introduced at ~100 µL/h, using a syringe pump and a 1-mL plastic tuberculin syringe. The reaction was carried out at 23°C for about 5 h. The circularized DNA was phenol-extracted, ethanol-precipitated, and redissolved in 50 µL TE buffer. Thus, intramolecular ligation was achieved using the circle generator, by slowly introducing substrate (unligated *Eco*RI-generated ends) to the ligation reaction chamber, rather than simply diluting the DNA to low concentrations and adding ligase.

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20

Transplacement Mutagenesis

A Recombination-Based In Situ *Mutagenesis Protocol*

Knut Woltjen, M. W. Todd Unger, and Derrick E. Rancourt

1. Introduction

The introduction of subtle mutations into genomic or cDNA regions of interest provides a powerful approach to the study of gene expression and function. Such manipulations of DNA permit numerous small physical changes, and their effects, to be examined. Unfortunately, most mutagenesis procedures require either tedious subcloning schemes to generate gene constructs, or the use of mutagenic polymerase chain reaction (PCR) methods that are not easily applicable to large DNA clones. Meanwhile, recombination-based procedures have readily become adapted for the introduction of mutations, ranging from single base-pair alterations to the introduction of large disruption cassettes or reporter genes. This chapter describes a recombination-based method using genetic positive-negative selection procedures for the deposition of subtle mutations from plasmids into phage clones.

Early applications of homology-directed phage–plasmid recombination events were designed for the isolation of phage clones from genomic libraries ***(1)***. Over the past two decades, phage–plasmid recombination methods have evolved to allow procedures such as gene modification, as well as incorporating a wide variety of phage vectors, probe plasmids, and selection mechanisms. The recombination screening procedure of Brian Seed ***(1)*** demonstrated that homologies as low as 50 bp were sufficient to promote specific phage–plasmid recombination events in *Escherichia coli*, even among a mixed population of phage clones. Phage–plasmid recombination events were later shown to proceed with as little as 20 bp homologous DNA ***(2,3)***. Improvements on Seed's method came with the generation of a more recombinogenic phage vector,

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ

Syrinx2A, which carried the recombination adept with plasmid (*rap*) gene, and a high-copy-number plasmid, πAN13 ***(4)***. Recombinant phages are selected on the basis of phenotypic shifts, driven by genetic markers in the incorporated probe plasmid. Traditional methods ***(1,4–6)*** utilize the suppressor tRNA gene, s*upF*, as a genetic marker in the probe plasmid. Acquisition of the plasmid, through single-crossover recombination between the phage clone and a plasmid-harbored region of homology, confers a SupF^+ phenotype on the appropriate phage clones, allowing them to grow in a SupF^0 host, despite the presence of amber mutations in essential phage genes. Selection procedures for phage–plasmid co-integrants typically utilize $supF^0$; $lacZ^{am}$ mutant *E. coli* strains as indicators, allowing recombinant phages to form blue plaques, following the addition of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and isopropyl-β-D-thio-galactopyranoside (IPTG) to the media.

Numerous modifications have been made to the components of the classic phage–plasmid recombination protocols, including adaptations to allow similar selection procedures for phage–phage ***(7)*** and plasmid–cosmid ***(8)*** recombination. Modifications to the phage vectors and probe plasmids have allowed the procedure to be applied to most phage cloning vectors ***(6,9)***. In phages that do not contain amber mutations, phage–plasmid recombinants are isolated in DK21 ($dnaB^{am}$; $lacZ^{am}$), via suppression of an amber mutation in the host *dnaB* gene, which is required for phage replication ***(6)***.

Perhaps the most valuable modifications to the recombination screening procedure involves methods of selection for reversion of the phage–plasmid co-integrant, to allow the phage clone to revert to its native configuration. This second recombination event, which occurs through the duplicated region of homology generated in the first integrative event, can be selected for based on either plasmid or host genetic markers. Perry and Moran ***(5)*** demonstrated that a mixed lawn of sup^0; $lacZ^{am}$ and *supF; lacZ E. coli* cells could be used to indicate condensatants-phages that had undergone a second single-crossover event to excise the plasmid. Phages retaining the plasmid (and therefore *supF*) were capable of growth on both hosts, forming blue plaques; those phages that had lost the plasmid sequences were only capable of growth in the s*upF* host, and therefore formed turbid white plaques. This approach is simplified in phage recombinants that do not bear amber mutations, because revertants can be selected indirectly on LG75, a sup^0; $lacZ^{am}$ mutant on which condensatants appear as white plaques.

In practice, the indirect identification of condensatants is problematic, because the frequency of reversion can be extremely low when small regions of homology are used for recombination. As a result, the authors developed a direct method for selecting revertants, using sensitivity to P2 interference (spi) selection ***(10,11)***. This approach arose from work in developing a method for

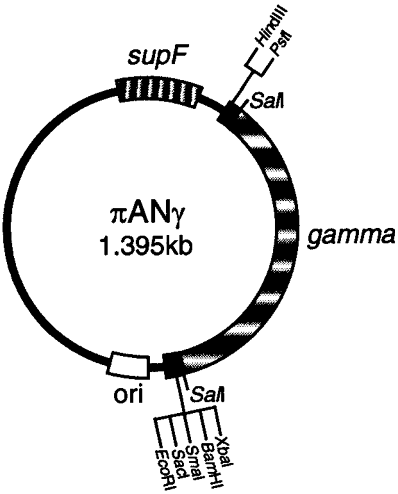

Fig. 1. Schematic map of the πANγ recombination plasmid. This πAN13 derivative plasmid has been modified by the introduction of the 500-bp λ *gam* gene into the *Sal*I site of the polylinker ***(10)***. Its small size allows it to accommodate large MOs, and still be integrated into a viable phage particle. The *supF* gene acts as a positive selectable marker for the presence of the plasmid, following integration; the *gam* gene may be selected against, following subsequent condensation of the plasmid. Note that the plasmid contains no antibiotic-resistance markers of its own, and therefore must be maintained in a host, such as MC1061[p3], which carries amber mutations in certain antibiotic resistance genes.

generating gene targeting vectors (TVs) in bacteriophage λ, using phage–plasmid recombination ***(12)***. This work first demonstrated that selectable marker cassettes could be delivered to an amber-bearing, *rap*$^+$ phage vector by double-crossover recombination, using a method of positive–negative selection. By positioning the λ *gamma (gam)* gene outside of the region of homology, double-crossover events that transferred the *supF-neo* cassette to the phage could be enriched by eliminating single-crossover recombinants in a *sup*^{0} P2 lysogen. This work has been extended by generating the recombination plasmid πANγ (**Fig. 1**), a derivative of πAN13 ***(4)***, which contains the negative selectable marker, *gam*. Based on λSyrinx2A, the phage cloning vector λTK (**Fig. 2**) was generated. The authors have also developed an isogenic λTK gene targeting library bearing 10–12 kb genomic inserts, which are ideal for generating TVs ***(11)***. Isolation of specific clones from the library is easily accomplished using retro-recombination screening (RRS), in which recombinant phages are first selected by *supF* plasmid integration, after which condensatants are selected by killing all of the *gam*$^+$ recombinants by spi selection on a P2 lysogenic host ***(11)***.

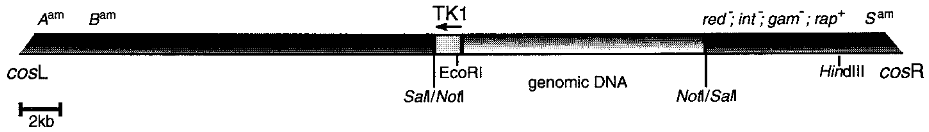

Fig. 2. Diagramatic representation of the λTK genomic cloning vector. All relevant genetic markers are indicated. The phage is derived from the Syrinx2A cloning vector. For a description of its construction, *see* Woltjen et al. ***(11)***. The λTK vector contains amber mutations in three essential genes (A^{am}; B^{am}; S^{am}), and thus requires the presence of *supF* activity in *cis* or *trans* for growth. The vector is deficient in all phage-encoded recombination pathways (red^-; int^-; gam^-), but, does contain the *rap* gene, which has been shown to increase the frequency of phage–plasmid recombination significantly. The herpes simplex virus thymidine kinase gene (TK) has been included, since these phage clones, once modified, are used directly as TVs in gene-targeting experiments in mouse.

The ability to select directly for condensatants, following phage–plasmid recombination, has led to development of a method for introducing small mutations and rare restriction sites in phage TVs using transplacement. First demonstrated in yeast ***(13)***, transplacement is a method by which exogenous genes may be placed into the yeast chromosomal DNA, through a cycle of integrative and excisive recombination, usually monitored by auxotrophy. This method has since been applied to the deposition of reporter genes, promoter regions, and even point mutations in bacterial cosmids ***(14,15)***. This recombination-based approach to DNA subcloning obviates the requirement for unique restriction sites in the clone, and simplifies the addition of gene sequences. Unfortunately, because of their large size and plasmid-like qualities, large genomic regions in cosmids are often unstable in $recA^+$ hosts. Also, because of the overly large size of these clones, aberrant recombination events are not an uncommon occurrence ***(15)***. This problem of rearrangements in plasmids and cosmids does not readily occur in bacteriophage λ, and is the principle reason why the authors developed a system of recombination-based gene TV construction in λ.

The authors' work with phage-derived TVs ***(12)*** and the generation of precise mouse models of human disease has led to exploration of means by which subtle mutations can be introduced into the mouse genome. Along these lines, the authors developed a phage–plasmid recombination system, similar to RRS and transplacement, referred to as "transplacement mutagenesis" (TM) ***(10)***. The selection procedure for incorporation and excision of πANγ into a λTK genomic clone is similar to RRS, but there are some key differences between these methods. First, a purified phage clone is used as the substrate for mutagenesis, rather than the entire genomic library. This ensures that only one clone for the genomic region of interest incorporates the plasmid, rather than promoting phage–plasmid recombination with a series of overlapping phage clones. Second, the probe region contained in πANγ harbors a mutation of interest, flanked by short regions of homology to the desired region. This mutation is usually generated by oligonucleotide synthesis, followed by subcloning into πANγ. In TM, the second excisive recombination event is critical, because the location of the crossover within the duplicated region of homology determines whether the mutation is retained in the plasmid, or deposited within the phage clone. The TM protocol provides the means by which, among other possibilities, unique restriction sites may be generated or destroyed, amino acid changes may be induced through codon shifts, and subtle changes to promoter regions may be explored.

2. Materials

2.1. Bacteriophages

1. λTK (**Fig. 2**) mouse genomic clone or other recombination proficient phage clone (*see* **Note 1**) (**Table 1**). The generation of the λTK library has been described elsewhere ***(11)***.
2. As an excellent resource for bacteriophage manipulation techniques, consult **ref. *16***.

2.2. Mutagenic Oligonucleotides

For considerations in the design of the mutagenic oligonucleotide (MO), *see* **Notes 4–6**.

2.3. Bacterial Strains and Plasmids

1. MC1061[p3]: *rec*$^+$; *supF*^{0}. The p3 episome in MC1061 carries genes for kanamycin (KM), ampicillin (AMP), and tetracycline (TET) resistance, however, both the *amp*^{r} and *tet*^{r} genes carry amber mutations. This bacterial strain is the host for the recombination plasmid πANγ. Kindly provided by Dr. D. M. Kurnit, University of Michigan, Ann Arbor ***(1)***.
2. LG75: *rec*$^+$; *supF*^{0}; *lacZ*^{am}. Requires the presence of exogenous *supF* activity for *lacZ* expression. Kindly provided by Dr. D. M. Kurnit, University of Michigan, Ann Arbor ***(3)***.
3. LE392: *rec*$^+$; *supE*; *supF*. Used for growing phages under relaxed conditions (Stratagene, La Jolla, CA) ***(16)***.
4. P2392: *rec*$^+$; *supE*; *supF*. Lysogenic for bacteriophage P2, and thus immune to subsequent infection by *gam*$^+$ (spi$^+$) bacteriophages (available from Stratagene) ***(16)***.
5. A large stock of plating cells may be harvested by centrifugation (2000*g* for 20 min) of a 10-mL culture (OD_{600} = 1.0), and resuspension in 4 mL 10 m*M* $MgSO_4$. Stored at 4°C, the cells will remain viable for an average of 2 wk (1 $OD_{600} = 8 \times 10^8$ cells/mL).

Table 1
Phage Vectors and Plasmid Constructs Amenable to TM

Phages	Relevant genotypes	Modifications to the TM protocol
Charon4A	A^{am}; B^{am}; S^{am}; gam^{-}; rap^{+}	N/A
Syrinx2A	A^{am}; B^{am}; S^{am}; gam^{-}; rap^{+}	N/A
λTK	A^{am}; B^{am}; S^{am}; gam^{-} recombinants; rap^{+}	N/A
λ2TK	A^{am}; B^{am}; S^{am}; gam^{-} recombinants; rap^{+}	N/A
Charon21A	W^{am}; E^{am}; gam^{-}	*rap* may be supplied in *trans*
EMBL3A	A^{am}; B^{am}; gam^{-}	*rap* may be supplied in *trans*
EMBL3, 4	No ^{am} mutations; gam^{-} recombinants	Co-integrate selection on DK21
λFIX	No ^{am} mutations; gam^{-} recombinants	Co-integrate selection on DK21
λDASH I, II	No ^{am} mutations; gam^{-} recombinants	Co-integrate selection on DK21
λGEM 11,12	No ^{am} mutations; gam^{-}	Co-integrate selection on DK21
λgt 10,11	No ^{am} mutations; gam^{+}; red^{+}; $rap^{+/-}$	Co-integrate selection on DK21; condensatants indirectly selected on LG75; *rap* may be supplied in *trans* (λgt 11 is rap^{-})

The essential characteristics of the phage vectors are as follows: rap^{+}; gam^{-}; $supF^{0}$; $lacZ^{-}$; inactivated phage recombination machinery (red^{-}; gam^{-}; int^{-}); phages may or may not have amber mutations; no homology between phage and plasmid, including: stuffer DNA, multiple cloning sites, selectable markers, phagemids; must be capable of packaging after ~2-kb insertion. The bacteriophage cloning vector, λ2TK, is similar to λTK, but two thymidine kinase genes flank the genomic insert ***(12)***.

6. πANγ. Recombination plasmid generated by Unger et al. ***(10)***. This 1.4-kb plasmid is a derivative of πAN13 ***(4)***, and is used as the vector for the MO (**Fig. 1**).

2.4. Antibiotics and Selection Materials

1. AMP: Inactivated by heat (>60°C). Dissolve in deionized H_2O, and filter-sterilize through a 20-µm filter (Millipore). Store at –20°C (25 mg/mL stock).
2. KM: Inactivated by heat (>60°C). Dissolve in deionized H_2O, and filter-sterilize through a 20-µm filter (Millipore). Store at –20°C (10mg/mL stock).
3. TET: Inactivated by heat (>60°C) and the presence on Mg^{2+} ions. Dissolve in 50% ethanol. Store at –20°C (12.5 mg/mL stock).
4. IPTG: Artificial inducer of the β-galactosidase operon. Store at –20°C (dissolve in deionized H_2O, 20 mg/mL stock).

5. X-gal: Artificial substrate for the β-galactosidase enzyme. Cleavage produces an indigo blue color. Store at –20°C in the dark, because X-Gal is light-sensitive (dissolve in *N*,*N*-dimethylformamide, 20 mg/mL stock).

Note: Antibiotics or other selection chemicals must be added to precooled (~50°C) media or top agar. Heating any of these compounds above 60°C will inactivate them.

2.5. Media, Buffers, and Solutions

1. Agar (VWR).
2. NZY media (Gibco-BRL) (per L): 21 g NZY contains 10 g NZ amine, 5 g NaCl, 5 g Bacto-yeast extract, 2 g $MgSO_4.7H_2O$. Used for the growth of phages on bacterial hosts, because of the Mg supplementation. For solid media, add agar at 15 g/L NZY media. For top agar, add 7.5 g/L NZY media.
3. Luria-Bertani (LB) media (per L): 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl. LB media is used if the media is to contain TET, because LB does not contain Mg. For solid media, add agar at 15 g/L NZY media. For top agar, add 7.5 g/L NZY media.
4. SM phage buffer (per L): 5.8 g NaCl, 2 g $MgSO_4.7H_2O$, 50 mL 1 *M* Tris-HCl, pH 7.5, 5 mL 2% (w/v) gelatin solution. Recommended practice is to periodically test working stocks of SM for phage contamination, by plating an aliquot of the stock buffer on a nonselective host.
5. Magnesium sulfate ($MgSO_4$): 10 m*M* Store at 4°C.
6. Dimethylsulfoxide (DMSO): To generate frozen permanent stocks of bacterial cells, add DMSO at a concentration of 7% (v/v) to 1 mL fresh bacterial culture. Freeze immediately, and store at –80°C.

2.6. Other Laboratory Materials

1. Petri dishes (VWR): For general phage and bacterial growth, 100-mm plastic Petri dishes are used. If larger numbers of phages need to be screened at any stage in the protocol, 150-mm dishes with 200 µL plating cells may be used.
2. Pasteur pipets: These pipets are excellent for phage collection from the surface of plates. The wide-bore variety may be used to core plaques to minimize cross-contamination.
3. Glass pipets: Manipulations of phage and bacterial stocks should be done with sterile glass pipets to maintain aseptic technique.
4. ART® tips (Molecular Bio-Products): If micropipets must be used for phage and bacterial manipulations, the use of aerosol-resistant tips greatly reduces the chance of contamination. However, if cautious pipeting techniques are used, ART tips are not essential.
5. Glass culture tubes: These are the preferred container for small-scale (<5 mL) bacterial growth.
6. 15/50 mL polypropylene tubes: These plastic tubes may be used for growing cultures over 5 mL. They are especially convenient, because they may be centri-

fuged directly, without requiring the transfer of the culture. Leave the caps loose on the tubes while growing (they may be secured with a piece of tape), to ensure sufficient aeration of the culture.

7. Parafilm™: An invaluable laboratory material, useful for reducing moisture loss in plates stored at 4°C.

3. Methods

The TM protocol alone may be completed in a mere 2 d, and is technically uncomplicated, pending experience working with bacteriophages and bacterial strains (**Table 2**). Familiarity with **ref. *16*** is strongly suggested. Although there are other resources available, this remains, to date, an invaluable comprehensive source of information on bacteriophage manipulation. Prior preparation for the TM protocol involves the isolation of a phage clone, and construction of the MO. Postprocedural work requires the preparation of DNA from potentially altered phages, and analysis of these clones. The TM procedure is typified by the successful transplacement of 2-bp mutation into a genomic clone of the mouse tissue inhibitor of metalloproteinase 3 (*Timp3*) gene ***(10)***. Although the protocol has been used to transplace a number of different mutation types, the *Timp3* mutation is used here to exemplify some of the finer points of the TM protocol.

3.1. Isolation of a Phage Clone

Although the TM protocol may potentially be applied to different bacteriophage vectors (*see* **Note 1**), clones derived from a Charon4A ***(17)***, Syrinx2A ***(4)***, or λTK library ***(11)*** are most amenable to this procedure. The DNA clone must be able to accommodate the transient integration of the 1.4-kb recombination plasmid, plus the length of the MO, without forcing the overall phage length above the maximum packaging length (~50.9 kb) (*see* **Note 3**).

Genomic regions of interest harbored in bacteriophages may be isolated by conventional Benton and Davis plaque-hybridization screening ***(18)***, or alternative means ***(11)***. Plaque–purifying the starting genomic clone is essential, so that only one species of phage is present for later manipulations. The DNA region to be altered by mutation may lie anywhere within the clone, and it is necessary to obtain sequence data for the area, if the MO is to be constructed synthetically. Restriction maps of the surrounding genomic environment are useful for later analysis, but are not absolutely necessary (*see* **Notes 10–12**).

3.2. Creation and Propagation of the Recombination Plasmid

3.2.1. Preparation of the MO

Critical design of the MO is of extreme importance. The mutation to be deposited in the target DNA is most often centered evenly within the region of

Table 2
Outline of TM Protocol

Isolation of a phage clone

Preparation of the MO

Generation of MC1061[p3; pANg] cells

- The MO is introduced into the πANγ recombination plasmid via conventional restriction/ligation techniques.
- The plasmid may then be introduced into competent MC1061[p3] cells, using electroporation, chemical transformation, or heat shock. Positive colonies are selected for by plating on LB-AKT.
- Positive colonies are identified by plasmid miniprep and restriction analysis.
- A single positive is used to innoculate a 10-mL LB-AKT liquid culture, which is grown overnight to an OD_{600} of 1.0.
- The cells are pelleted by centrifugation at 2000*g* for 20 min and resuspended in 4 mL 10 m*M* $MgSO_4$.

Plasmid integration

- ~1 × 10^4 pfu of the original phage clone and 100 µL of transformed MC1061[p3; πANγ] cells are incubated together at 37°C for 20 min.
- Following the addition of 4 mL NZY-AMP (50 µg/mL) top agar, the mixture is plated and grown on NZY-AMP (50 µg/mL) overday.
- Phages are collected in 4 mL SM buffer, followed by a 2-mL wash. A few drops of chloroform are added to ensure lysis of bacterial contaminants.
- Cellular debris is removed by centrifugation at 2000*g* for 20 min.

Selection for phage-plasmid co-integrates

- 100 µL (depends on probe length/mismatch length) lysate are mixed with 100 µL LG75 plating cells at 37°C for 20 min.
- 4 mL NZY top agar containing X-gal and IPTG (200 µg/mL) are added, and plated on NZY plates.
- The plates are incubated at 37°C overnight. Only blue plaques should be found on the plates following incubation.

Plasmid excision

- A blue plaque is cored into 1 mL SM (rocking, 1 h) buffer, plus chloroform.
- 50 µL of each plaque lysate is added to 100 µL LE392 plating cells, and incubated at 37°C for 20 min.
- 2 mL NZY media is added, and the culture grown at 37°C for 6–9 h.
- Following chloroform treatment, the cellular debris is removed by centrifugation at 2000*g* for 20 min.

Selection for condensation

- 100 µL lysate are mixed with 100 µL P2392 plating cells at 37°C for 20 min.
- 4 mL NZY top agar are added to the phages and bacteria, and the mix is plated on NZY at 37°C overnight.
- Plaques that grow on this lawn are each cored and placed in 1 mL SM buffer plus a few drops of chloroform.

Phage amplification and DNA preparation

Phage clone analysis

homology, but this is dependent on the base-pair composition of the surrounding DNA (*see* **Note 6**). As a general rule, the overall length of the MO should be no less that 50 bp, to allow for recombination to occur at reasonable frequencies ***(3)*** (*see* **Note 5**), and the mutation should be centered within this homology. Mutations to be engineered may range from single base-pair changes to the introduction of new restriction sites (*see* **Note 15**). Despite the origin of the DNA or the nature of the mutation, the MO must not be refractory to subcloning. The orientation of the MO in the πANγ recombination plasmid is unimportant, because it will not affect recombination or excision.

3.2.2. Generation of MC1061[p3; πANγ] Cells

The MO may be introduced into the πANγ recombination plasmid via conventional restriction/ligation reactions. Following transfection of MC1061[p3] by any number of procedures, MC1061[p3; πANγ] cells must be selected for in the presence of AMP, KM, and TET (all at 50 μg/mL working concentration). The p3 episome of MC1061[p3] harbors genes encoding resistance to each of these three antibiotics; however, the amp^r and tet^r genes also contain amber mutations, which are suppressed in a s*upF* host. Therefore, in MC1061[p3; πANγ] cells, Km^r selects for the presence of the p3 episome; the amp^r and tet^r genes serve the double role of maintaining both p3 and πANγ.

Multiple drug resistant MC1061[p3; πANγ] colonies are grown in liquid culture, to provide plating cells that will host the phage–plasmid recombination event. Typically, a 2.5-mL liquid culture grown by shaking (250 rpm) at 37°C to an OD_{600} of 0.6–1.0 (spectrophotometry using visible light at 600 nm), followed by resuspension in 1 mL 10 m*M* $MgSO_4$, will provide enough plating cells to passage the phages over the host cells on 10 individual plates.

3.3. Plasmid Integration

The initial single-crossover recombination event, which incorporates the recombination plasmid into the phage clone, is completed through the infection of MC1061[p3; πANγ] with the phage of interest, under minimal antibiotic selection. To achieve this, ~1×10^4 pfu of the phage clone are mixed with 100 μL MC1061[p3; πANγ] plating cells at 37°C for 20 min. The cell–phage mixture is then plated on NZY-AMP media in 3–4 mL NZY-AMP top agar. Foregoing the addition of TET and KM to the media in this step allows the bacterial cells to grow at a more competitive rate, compared to phage growth (*see* **Note 7**). After an overday incubation at 37°C (*see* **Note 14**), phages are collected from the surface of the confluent plate in SM buffer (rocking, 1 h). During lytic growth in MC1061[p3; πANγ], single-crossover recombination occurs between the matching homologies in the phage clone and plasmid, resulting in plasmid integration and the duplication of this homology (**Fig. 3**).

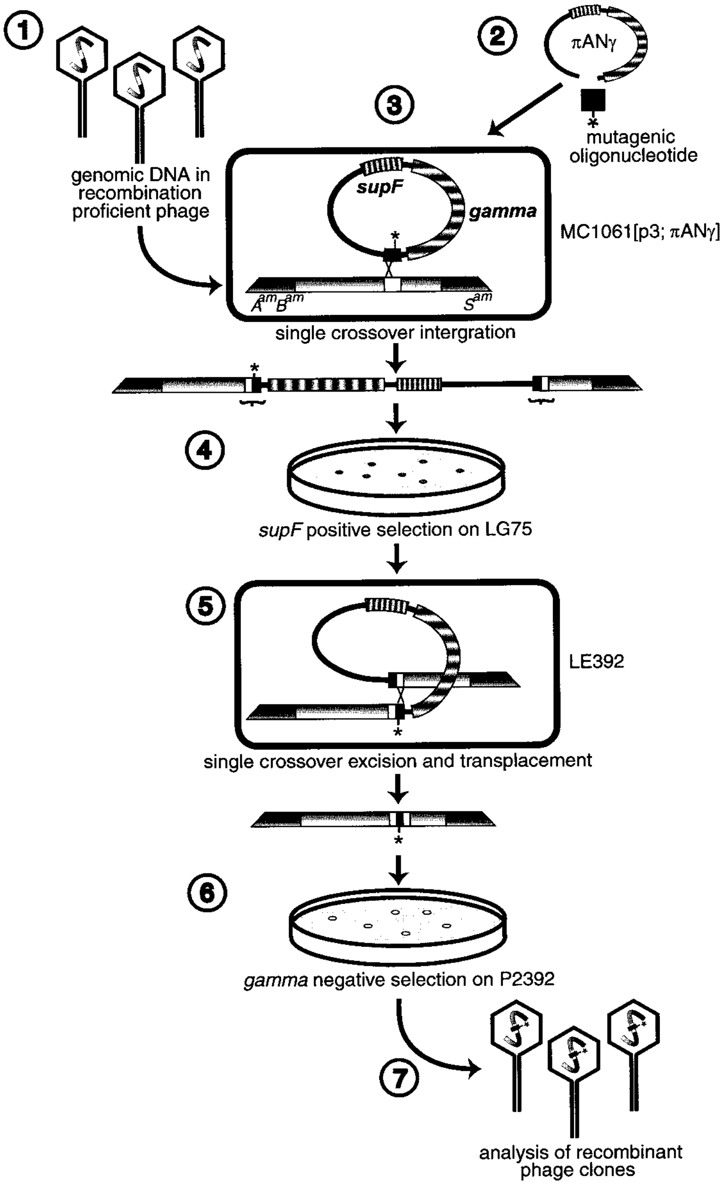
①
genomic DNA in recombination proficient phage
②
πANγ
mutagenic oligonucleotide
③
supF
gamma
MC1061[p3; πANγ]
A^am B^am
S^am
single crossover intergration
④
supF positive selection on LG75
⑤
LE392
single crossover excision and transplacement
⑥
gamma negative selection on P2392
⑦
analysis of recombinant phage clones

Fig. 3. Flow diagram of the TM protocol. **(1)** A phage clone is chosen on the basis of the genomic insert, and purified to homogeneity. This clone must be able to accommodate the incorporation of the probe plasmid. **(2)** The MO, bearing the desired mutation and flanked by regions of homology to the target genomic location, is introduced into the πANγ recombination plasmid. This plasmid construct is subsequently introduced into MC1061[p3], the host cell line in which the initial recombination event

Uptake of the two genetic selectable markers by the phage shifts its genotype to *supF* and *gam*$^+$. The plate lysate collected here should be titered, although, with sufficient confluency, a titer of approx 10^{10} pfu/mL can typically be expected.

3.4. Selection for Phage–Plasmid Co-Integrates

The phage clone adopts the SupF phenotype, and consequently the capability of suppressing its own amber mutations, growing independently on a s*upF*^{0} host, such as LG75. By virtue of this genetic marker, those phages can be selected for that are also capable of suppressing chromosomal amber mutations in these *supF*-deficient bacterial cells. LG75 is *lacZ*^{am}, allowing bacteriophage clones that have incorporated the plasmid to form blue plaques on this host in the presence of X-gal and IPTG. Here, ~10^4 pfu of phages (*see* **Note 8**) is mixed with 100 µL LG75 plating cells at 37°C for 20 min, to allow for phage adsorption to the cell surface. The mixture is plated on NZY plates in 4 mL NZY top agar containing X-gal and IPTG (200 µg/mL, i.e., 10 µL each of a 20 mg/mL stock/1 mL top agar) (*see* **Note 9**). Because the *supF* gene is absolutely required for phage growth, only blue plaques should be apparent on the bacterial lawn following ~16 h growth (**Fig. 3**). At this stage,

Fig. 3. *(continued)* will occur. **(3)** The MC1061[p3; πANγ] cells are infected with the phage clone. During lytic growth, phage–plasmid recombination is mediated by host recombination machinery. The entire plasmid is incorporated into the phage clone through single-crossover recombination, resulting in a duplication of homology, which will be important for later condensation and plasmid excision. **(4)** Phages that have incorporated the recombination plasmid are now s*upF*, and can therefore be selected on LG75 for growth and the formation of blue plaques. Since all phages growing on LG75 contain the plasmid, only one blue plaque need be chosen. **(5)** Under relaxed conditions (i.e., in a *supF* host), the plasmid-borne *supF* gene is no longer required for phage growth, since *supF* activity is available in *trans*. Therefore, the phage–plasmid co-integrant may undergo condensation, in which a second single-crossover recombination event effectively removes the plasmid. Depending on which side of the mutation the crossover occurs, either the mutation is retained in the plasmid, or deposited in the phage. For diagrammatic purposes, only the later event is shown. **(6)** Phages that have excised the plasmid, and therefore the *gam* gene, will not be growth-inhibited on the P2 lysogenic host. However, if the plasmid is not excised, the phages will be unable to form plaques. Here, it is important to isolate a number of plaques, since, even though the plasmid may be excised, the point mutation may not have been transplaced into the phages. **(7)** Analysis of phage clones obtained through the protocol may follow a number of different approaches. The number of transplaced phage clones obtained will be dependant on the MO utilized, and the properties of its homology. **Note:** Plasmid and phages are not drawn to scale.

there are only two species of plasmid-bearing phages, depending on which side of the MO underwent recombination. Both species bear the mutation in one of the two duplicated homologies. Realistically, it is only necessary to core one plaque into 1 mL SM buffer, and to use this individual clone for subsequent relaxation/plasmid excision steps. The bacterial plates can be sealed with Parafilm and stored at 4°C for later extraction of other positive phages, if required.

3.5. Plasmid Excision

In TM, the nature of the second recombination step is of utmost importance, because it determines whether the mutation is deposited within the phage or retained in the recombination plasmid. This recombination event is induced via passaging the phage through a nonrestrictive host, such as LE392. Because the host encodes s*upF*, the plasmid need no longer be retained within the phage clone, and excision is mediated by the duplicated homology generated by the initial recombination event (*see* **Note 13**) (**Fig. 3**). Here, 50 µL blue plaque lysate is used to infect 100 µL LE392 plating cells for 20 min at 37°C. The phages and cells are then propagated in 2 mL NZY, to allow condensation of the plasmid to occur. If the second single-crossover recombination event occurs on the same side of the homology as the integrative single-crossover recombination, the point mutation will be retained within the plasmid. However, if the second recombination event occurs on the opposite side of the mutation, the phage clone receives the engineered mutation in place of the wild-type sequence. Following clearing of the lysate with chloroform and centrifugation, an aliquot of the lysate is screened genetically for the absence of plasmid sequences. Again, measuring the titer of the relaxed lysate is important; a titer of ~10^9 pfu/mL is generally achieved.

3.6. Selection for Condensation

Removal of the plasmid from the phage clone by a second recombination event is monitored by the loss of the *gam* gene, noted genetically by the loss of the spi^+ phenotype. Thus, phages that have undergone condensation will be able to grow on the restrictive host, P2392. P2392 plating cells (100 µL) are mixed with 100 µL phages (*see* **Note 8**) from the prior liquid lysis step, at 37°C for 20 min, followed by plating in 4 mL NZY top agar on NZY plates. The only phages capable of forming plaques on a lawn of this bacterial host are those that have excised the recombination plasmid. Note, however, that these spi^- phages are a mixture of the native clone conformation and those clones that have acquired the mutation. Therefore, at this point, numerous phage plaques should be cored into 1 mL SM, to ensure that at least one chosen represents the desired mutant phage.

3.7. Preparation and Analysis of Recombinant Phage

3.7.1. Phage Amplification and DNA Preparation

Phages from the plaque lysates may be amplified by growth to confluency on a lawn of P2392 or LE392. This typically requires plating 100 µL phage plaque (~10^4–10^5 pfu) with 100 µL plating cells. If the phages from a confluent plate are collected in SM buffer, this provides a high titer stock that may be used to isolate phage DNA. Alternatively, phage may be collected from a liquid culture. It is recommended that putative positive phage clones are prepared on a small-scale ***(16)*** for initial analysis. Confirmed positives may then be prepared on a large-scale ***(16)*** for further manipulations.

3.7.2. Phage Clone Analysis

Analysis of potentially altered phage clones is dependent on the nature of the mutation introduced. The most useful mutations for clone analysis are those that generate or eliminate informative restriction sites (e.g., *see* **ref. *10***). In this case, simple restriction endonuclease digests of the transplaced clones, compared to the original clone, will distinguish between the native and mutant conformations (*see* **Note 10**). If the changes made to the clone do not result in the alteration of restriction sites, then competitive oligonucleotide priming (COP) ***(19)*** (*see* **Note 12**) may be used to monitor the presence or absence of a mutation as subtle as a single base-pair change. As a definite confirmation, directly sequencing the specific region of the clone ensures that the mutation is present. It is also possible to use radioactive hybridization to improve the chance of isolating a positive clone (*see* **Note 11**), taking advantage of the differential reassociation kinetics displayed by the normal and mutant oligonucleotides.

4. Notes

1. The important genotypic status of the phage vector to be applied to the TM protocol is summarized in **Table 1**. Primarily, the phages must have all phage-borne recombination machinery inactivated, and be unable to lysogenize the host. If λ *gam*-negative selection is to be used for the isolation of condensatants, the phage vector itself must be spi$^-$ (*gam*$^-$). The *rap* gene is not absolutely essential, but, in its absence, recombination efficiencies may suffer over 100-fold ***(4)***. This beneficial gene may, however, be supplied in *trans* on yet another plasmid, as demonstrated by Kurnit and Seed ***(6)***. The presence of amber mutations in some essential phage genes simplifies the TM protocol, because this directly mediates the selective growth of plasmid-harboring phages on the s*upF*^{0} host LG75. Essentially, this allows the utilization of phage of Charon4A derivation, as well as some members of the European Molecular Biology Laboratory (EMBL) class of phage vectors. Alternatively, the DK21 *dnaB*^{am} selection scheme circumvents the need for amber mutations within the phage itself ***(6)***, allowing a wider array of phage vectors to be utilized.

2. A lack of homology between the phage vector and the recombination plasmid is extremely important to prevent aberrant recombination events.
3. Bacteriophage λ displays a precise mechanism by which only phage vector DNA, ranging from 38 to 51 kb, is packaged within the phage capsid. Thus, it is important to keep in mind the size of the bacteriophage clone, in addition to its increase in size with the introduction of the πANγ plasmid and MO.
4. The design of the MO will affect all aspects of the protocol, including the nature of the mutation, and even the frequency and accuracy of recombination. The origin of the MO is also of important consideration. Isolation of the MO from a source of isogenic DNA (i.e., DNA from the same strain of organism, compared to the phage clone) is important, because even small divergences in homology can cause large alterations in the recombination frequencies. Recombination studies, using DNA approx 10% divergent over their homologies, were noted to recombine at a 40-fold lower level ***(3)***. The origin of the MO may be from a PCR preparation, expressed sequence tags, or a restriction digest if the mutation is naturally occurring, or from oligonucleotide synthesis or mutagenic PCR ***(20)*** if the mutation is to be engineered.
5. The overall homology length is of great importance. Although the minimum homology length possible has been reported to be 20 bp, longer homologies are more beneficial, with recombination frequencies increasing exponentially over the 20–74 bp range, and, above that, in a linear fashion ***(2)***. Mutations larger than single base-pair alterations will have a serious impact on the effective homology of the MO, splitting it into two flanking homologies. In this case, the MO is best designed with at least 20 bp homology on either side of the mutation.
6. In the authors' experiences with the protocol, the actual base-pair composition of the MO will have an effect on recombination preferences. Thus, if one side of the MO has a higher GC content, homologous annealing and recombination may preferentially occur on one side of the MO, reducing significantly the efficiency of transplacement. In this case, it is possible to remedy the situation by slightly shifting the homology around the mutation, reducing the length of homology on the otherwise preferred side.
7. Phage–plasmid recombination is affected mostly by the design and nature of the MO, but, there are some aspects of culture conditions that can also have an effect. The use of AMP alone, during passage of the phage clone over the MC1061[p3; πANγ] plating cells, gives the bacteria a growth advantage over the regular AMP/KM/TET selection, while still acting to maintain both the p3 episome and πANγ plasmid. Thus, the bacteria will not be annihilated by the phages upon infection, and amplification of the recombinant phage clones is possible. This is also related to the overall ratio of phages:plasmid. The πAN13 plasmid was modified to high copy number, to ensure that these ratios would be optimized. Low-copy-number plasmids result in few recombinant phages, because of the unavailability of plasmid DNA for incorporation. Therefore, it is important to monitor the multiplicity of infection at the initial recombination step. If the MO inherently demonstrates low recombination efficiencies, PCR- or hybridization-based approaches may be utilized to select for rare recombinant phages.

8. With longer regions of homology, both the integrative and excisive recombination events can be expected to occur at higher frequencies. Thus, the amount of phages from the plate (MC1061) and liquid (LE392) lysates that will need to be screened on LG75 and P2392, respectively, will be dependent on homology length. As an example, during transfer of the 2-bp mutation into the TIMP3 phage clone ***(10)***, an integrative recombination frequency of 5×10^{-3} was noted, while excision occurred at a frequency of 1.5×10^{-2}, presumably because the second recombination event was occurring intramolecularly, rather than intermolecularly. For newly designed MO, it may be required to titer the phage lysates on LE392, and either LG75 or P2392, prior to plating on the selective host. Note that these numbers will provide the recombination frequency, when the titer on the selective host is divided by the titer on LE392. Rational judgements may then be made on how much of the lysate should be screened, so that isolated plaques, especially in the case of the P2392 screen, are obtained.
9. Occasionally, during selection on LG75, the blue plaques obtained are not readily visible. Using the amounts of X-gal and IPTG indicated (*see* **Subheading 3.4.**) should give a strong blue signal, however, on occasion the staining is faint. Here, the signal may be enhanced by increasing the amount of X-gal included in the top agar (80 µL of a 20 mg/mL stock is more than sufficient), or even by refrigerating the plates for a few hours prior to analysis. It is important to observe only blue plaques on these plates, since white plaques suggest contamination of the phage stock with nonamber phages.
10. The direct analysis of phage clones obtained through this procedure is necessary, because the final spi$^-$ phages growing on P2392 may or may not carry the mutation. In the case of the introduction or elimination of restriction sites within the clone, the analysis simply involves an informative restriction endonuclease digestion reaction. Similarly, with the introduction of larger cassettes, restriction digestion, followed by Southern hybridization to the newly introduced DNA, should provide a reasonable confirmation. Small mutations that do not affect the restriction pattern of the phages are more difficult to detect. Here, it is more reasonable to simply sequence the relative region of the phage clone.
11. In the authors' experience, the chance of obtaining a mutant phage clone may be enhanced by applying a hybridization step prior to the coring of randomly chosen spi$^-$ phages ***(18)***. The isolated plaques on the P2392 lawn may be lifted onto a nylon or nitrocellulose membrane, and probed with the MO. Under stringent conditions, those phages containing the mutation will bind more strongly to the radiolabeled MO; the unaltered phages will hybridize to a lesser extent. Usually, strong signals will correctly indicate positive phage clones.
12. Alternatively, one may apply COP ***(19)*** to reveal which phage clones have obtained the mutation. This protocol makes use of the specificity of short oligonucleotide binding when two primers, one wild-type and one carrying the mutation, are in competition for the same binding site. Following labeling of one competitive primer, PCR reactions, containing both competitive primers and a universal downstream primer, are used to distinguish the nature of the phages.

13. Recently, the authors reported that the passage of the *supF; gam*$^+$ phages through relaxed conditions in LE392 is not a required step for condensation ***(11)***. Lysates from a blue LG75 plaque, when plated directly onto P2392, gave rise to a small number of spi$^-$ plaques. Presumably, condensation may be occurring among the linear concatamers of phage DNA generated during late replication. Growth of these phages is supported by the presence of the *supF* gene in *trans*, from the noncondensed phage. Although this step may be omitted, it is not recommended practice if the MO is short or disrupted by a large nonhomologous region. In these situations, it is more beneficial to facilitate condensation under relaxed selection conditions, rather than select for a few spontaneous condensatants.
14. It may also be possible to reduce the length of the initial recombination passage. Poustka et al. ***(8)*** reported that recombination times ranging from 1–3 h were sufficient. However, these experiments used large cosmids, which are extremely unstable in the *recA*$^+$ hosts used for the recombination step. Therefore, the shortened incubation times may have been an attempt to strike a balance between homologous recombination and cosmid rearrangements. In this protocol, the authors suggest the passage of the phages over MC1061[p3; πANγ] cells on plates. This step could, however, be completed in liquid media, a scenario that should provide more phages during a reduced incubation time, thereby making it feasible to obtain recombinant phages from a liquid culture in 1–3 h. This remains to be tested.
15. Mutations that may be introduced into phage clones using TM are nearly limitless. These range from amino acid changes through codon shifts, changes to splice donor and acceptor sites, alterations to promoter regions, the generation of fusion proteins, the attachment of reporter genes, the introduction of new promoters or enhancers, the deletion of small regions of DNA, changes to regions resulting in RNA secondary structure, and of course restriction endonuclease recognition site removal or introduction. TM may be used to insert large DNA fragments, similar to the double-crossover insertion method ***(12)***. The authors have applied the TM method to the development of TVs carrying subtle mutations. In many cases making minor changes to the genome would be more desirable, rather than gross alterations, so that they more accurately mimic the true in vivo scenario. Thus, the TM protocol is widely applicable to the introduction of diverse mutations into genomic and cDNA clones in bacteriophages. Recombination-based gene alteration protocols provide a simple, yet valuable strategy by which to construct custom alleles for diverse roles.

Acknowledgments

The authors thank Gerard Bain for the generation of the λTK murine genomic library, and his initial contributions to this work, and would also like to thank Shiying Liu for her technical assistance in the isolation of the *timp3* phage clone, and Frans van der Hoorn, Gerard Bain, and Torben Bech Hanson for their critical review of this manuscript. Knut Woltjen is supported by the National Sciences and Engineering Research Council and the Alberta Heritage

Foundation for Medical Research. D. R. is a scholar of the Alberta Heritage Foundation for Medical Research. This work was supported by the Canadian RP Research Foundation and the Medical Research Council of Canada.

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21

Preparation of Transposon Insertion Lines and Determination of Insertion Sites in *Arabidopsis* Genome

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1. Introduction

Arabidopsis thaliana is now recognized as a model organism for research in plant biology, especially genetics and molecular biology, and is used for the functional analyses of various genes. However, genomic DNA sequence data reveal that a large number of *Arabidopsis* genes remain functionally uncharacterized, that means that a reverse-genetic approach will become important for characterizing gene functions. One of the most promising strategies for reverse genetics is based on insertional mutagenesis.

For the purpose of insertional mutagenesis, the authors prepare transposon (Tn) insertion lines of the *Arabidzopsis* genome, using a maize nonautonomous *Dissociation* (*Ds*) Tn as an insertional mutagen ***(1,2)***. The Tn DNA not only introduces a mutation, but also tags the responsible gene. The strategy for *Arabidopsis* insertional mutagenesis is as follows:

1. Preparation of a large number of *Ds*-transposed lines. The authors use the *Activator* (*Ac*)/*Ds* local transposition system described in **ref. *3***, and have confirmed that most of the transposed lines contain only one *Ds* insertion/genome.
2. Amplification of genomic DNA flanked by *Ds*, using the thermal asymmetric interlaced (TAIL) polymerase chain reaction (PCR) technique ***(4)***, and reading the partial sequence of the amplified DNA.
3. Identification of the insertion sites by reference to published genomic DNA sequences.
4. Construction of an insertion site database to search for gene-knockout lines.

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ

This protocol describes preparation of *Ds*-transposed lines with modifications of the method described in **ref. *3***, and a TAIL–PCR technique, with modifications of the method described in **ref. *4***.

2. Materials

2.1. Tn Mutagenesis

2.1.1. Parental Lines

1. Nae*Ac*380-16: A transgenic line with a 35S promoter::*Ac* transposase chimeric gene.
2. *Ds*-GUS-T-DNA lines: *Ds* donor lines. Start loci of these lines are described in **ref. *3***.

All these transgenic lines contain only one T-DNA insert, and genotypes of these lines are homozygous for a T-DNA insert ***(3)***. These lines are available from the Arabidopsis Biological Resource Center (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/ABRCHOME.HTM).

2.1.2. Stock Solutions

1. Hygromycin B (Hyg) (Boehringer Mannheim, Mannheim, Germany, cat. no. 843.555): Stock has already been sterilized as 50 mg/mL–phosphate-buffered saline solution. Store at 4°C in the dark.
2. α-Naphthaleneacetamide (Nam) (Sigma, St. Louis, MO, cat. no. N-0250): Prepared as 10 m*M* stock in dimethylsulfoxide. Store at –20°C.
3. Chlorsulfuron (Chl) (Chem Service, West Chester, PA, cat. no. PS-1065): Freshly prepared as 200 μg/mL stock solution, by dissolving powder in 0.01 *M* NH_4OH solution, and sterilized by 0.22-μm filter.

2.1.3. Media

1. 5% 2-[N-morpholino] ethanesulfonic acid (MES)-KOH (pH 5.7 at 25°C) stock buffer.
2. Germination medium (GM): Murashige and Skoog basal salt mixture; 3% sucrose; MES-KOH stock buffer, to a final concentration of 0.05%; 0.8% agar. Sterilize at 120°C for 20 min, and pour into disposable Petri dish (ϕ 90 × 20 mm). Store the plates at 4°C.
3. GM (Hyg): Sterilize GM at 120°C for 20 min, and add the Hyg stock solution to a final concentration of 10 μg/mL, after cooling to approx 60°C. Pour into disposable Petri dish (ϕ 90 × 20 mm), and solidify agar. Store the plates at 4°C.
4. Top agar: Murashige and Skoog salts; 1% sucrose; MES-KOH stock buffer to a final concentration of 0.05%; 0.7% agar. Sterilize at 120°C for 20 min, and add the stock solutions to the medium to final concentrations of 20 μg/mL (Hyg), 6 μg/mL (Chl), and 5 μ*M* (Nam), after autoclaving and cooling to approx 60°C. Keep molten until use.

5. Bottom agar: Same as top agar, except for agar concentration of 2%. Pour into disposable Petri dish (ϕ 150 × 15 mm), and solidify agar. Store the plates at 4°C.

2.1.4. Other Equipment

1. 15-mL tubes.
2. Disposable Petri dish (ϕ 90 × 20 mm).
3. Disposable Petri dish (ϕ 150 × 15 mm).
4. Growth chambers for plate culture.
5. Soil (e.g.m Promix No. 2, Kakiuchi, Tokyo, Japan) and pots (e.g.m Arasystem, bvba Beta-Tech, Gent, Belgium) for plant growth.
6. Shelves for plant growth.
7. Parafilm M (American National Can, Chicago, IL).
8. Micropore surgical tapes (3M Health Care, St. Paul, MN, cat. no. 1530-0).
9. Envelopes for seed stock.
10. Sterilization solution: 1% antiformin; 0.02% Triton X-100. This solution must be made fresh every 2 wk. Store at 4°C.

2.2. TAIL–PCR

2.2.1. Kits

1. DNeasy Plant Mini Kit (Qiagen, Hilden, Germany).
2. QIAquick Gel Extraction Kits (Qiagen).
3. ABI PRISM BigDye Terminator Cycle Sequencing Kits, FS (Applied Biosystems, Foster City, CA).

2.2.2. Reaction

1. Ex *Taq* DNA polymerase (5000 U/mL, Takara, Shiga, Japan).
2. Supplied 10X Ex *Taq* DNA polymerase reaction buffer.
3. Supplied deoxyribonucleoside triphosphate (dNTP) mix: Mixture of 2.5 m*M* each deoxyadenosine, deoxythymidine, deoxyguanisine, and deoxycylidine triphosphates.
4. Degenerate primers (*see* **Note 1**):
   AD2: 5'-NGT/CGT/SWG/ANA/WGA/A-3', melting temperature (T_m) = 46.6°C;
   AD5: 5'-SST/GGS/TAN/ATW/ATW/CT-3', T_m = 46.7°C;
   W4: 5'-AGW/GNA/GWA/NCA/NAG/A-3', T_m = 45.3°C
   (N: mixture of G, A, T, and C; W: mixture of A and T; S: mixture of G and C).
5. Tn-specific primers (*see* **Fig. 3** and **Note 2**):
   Ds5-1: 5'-GGC/CAT/ATT/GCA/GTC/ATC/CCG/A-3', T_m = 62.1°C;
   Ds5-1a: 5'-ACG/GGA/TCC/CGG/TGA/AAC/GGT-3', T_m = 63.7°C;
   Ds5-2: 5'-TCC/GTT/CCG/TTT/TCG/TTT/TTT/AC-3', T_m = 57.1°C;
   Ds5-3: 5'-TAC/CTC/GGG/TTC/GAA/ATC/GAT-3', T_m = 57.9°C;
   Ds3-1: 5'-ACC/CGA/CCG/GAT/CGT/ATC/GGT-3', T_m = 63.7°C;
   Ds3-1a: 5'-GGT/TCC/CGT/CCG/ATT/TCG/ACT-3', T_m = 61.8°C;
   Ds3-2: 5'-CGA/TTT/CCG/TAT/TTA/TCC/CGT/TC-3', T_m = 58.9°C;
   Ds3-3: 5'-CCG/TCC/CGC/AAG/TTA/AAT/ATG-3', T_m = 57.9°C

2.2.3. Other Equipment

1. Microtubes (1.5 mL) (Treff, Schweiz, Switzerland, cat. no. 96.7246.9.01).
2. Pestles fitted with the microtubes (Treff, cat. no. 96.7339.9.01).
3. Mini-gel electrophoresis apparatus (submarine-type).
4. 1% Agarose gel.
5. Scalpels.
6. Liquid nitrogen.
7. Peltier Thermal Cycler PTC-200 (MJ Research, Watertown, MA).
8. Suprec-02 (Takara, cat. no. 9041): Filter cartridges designed for rapid concentration of DNA samples.
9. Model 3700 DNA Analyzer (Applied Biosystems).

3. Methods

3.1. Preparation of Ds-Transposed Lines

A scheme for *Ds* Tn mutagenesis is represented in **Fig. 1**. Parental Nae*Ac*380-16 and *Ds*-GUS-T-DNA are outcrossed and F_1 seeds are obtained. During F_1 generation, germinal transposition events occur, and transposed lines of F_2 generation are selected as both excision marker (Chl)- and Tn marker (Hyg)-resistant plants. Nam is used to select transposed lines without the *Ac* transposase gene. If not mentioned especially, seeds are sterilized by mixing in a 1.5-mL tube with 1 mL sterilization solution for 10 min, washed 3× with sterilized H_2O, and sowed onto GM plates. Seedlings are grown in a growth chamber at 23°C, under continuous illumination of 50 µmol photons/s/m^2 for 2–3 wk, and transferred to soil for further growth.

1. Outcross Nae*Ac*380-16 pistils with *Ds*-GUS-T-DNA pollens, and set F_1 seeds.
2. Grow F_1 plants (*see* **Note 3**), self-pollinate, and harvest F_2 seed sets from individual F_1 plants.
3. Add 8 mL molten top agar to a 15-mL tube with sterilized F_2 seeds (10 mg $\cong$ 500 seeds), suspend, and pour onto a bottom agar plate.
4. Seal the plates with Parafilm, and grow seedlings in a growth chamber at 23°C under continuous illumination of 50 µmol photons/s/m^2 for 1 wk.
5. Transfer 8–10 Chl-,, Hyg-, and Nam-resistant seedlings to GM (Hyg) plates, seal the plates with micropore surgical tape (3M Health Care), and grow for further 2 wk (*see* **Note 4**).
6. Transfer the Hyg-resistant seedlings to soil. The authors examined the number of independent germinal transposition events per single F_1 plant, by Southern analysis. **Figure 2** showed that 1–3 independent germinal transpositions have occurred per single F_1 plant. Therefore, the authors transferred more than 2 Hyg-resistant F_2 seedlings derived from a single F1 plant.
7. Store self-pollinated F_3 seed sets in envelopes under dry conditions.

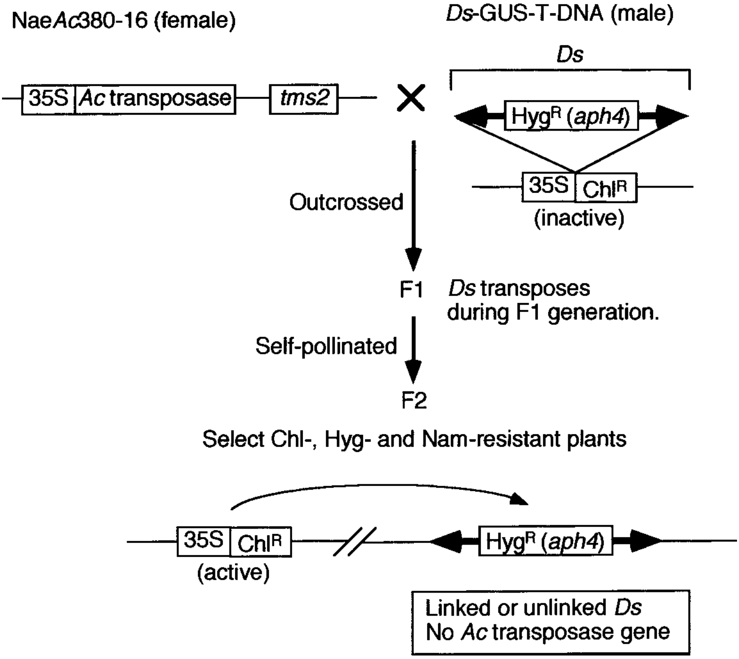
NaeAc380-16 (female)
Ds-GUS-T-DNA (male)
Ds
35S
Ac transposase
tms2
HygR (aph4)
35S
ChlR
(inactive)
Outcrossed
F1
Ds transposes during F1 generation.
Self-pollinated
F2
Select Chl-, Hyg- and Nam-resistant plants
35S
ChlR
(active)
HygR (aph4)
Linked or unlinked Ds
No Ac transposase gene

Fig. 1. Schematic representation of the *Ds* transposon mutagenesis. Because *Ds* is inserted between 35S promoter and a coding sequence for a Chl^R gene in the *Ds*-GUS-T-DNA, the excision marker is inactive, and the *Ds*-GUS-T-DNA lines are sensitive to Chl. When *Ds* is excised from the donor locus, the excision marker becomes active and confers resistance to Chl. In the Nae*Ac*380-16 line, the *Ac* transposase flanks to a marker gene, *tms2*, which confers sensitivity to Nam. Therefore, F_2 plants without the *Ac* transposase gene are obtained by the Nam selection. Chl^R, resistance to Chl; Hyg^R, resistance to Hyg.

3.2. Isolation of Genomic DNA from Arabidopsis Leaves

1. Cut 2–3 leaves from each F2 transposed lines (approx 1 mo old), and store in a 1.5-mL microtube at –80°C until genomic DNA isolation.
2. Pour liquid nitrogen into the microtube, and grind leaves to a fine powder, using a pestle.
3. Isolate genomic DNA, using DNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions (*see* **Note 5**).
4. Estimate approximate DNA concentration upon λ-DNA reference by electrophoresis.

3.3. TAIL–PCR

TAIL–PCR, inverse PCR, and plasmid rescue are techniques for isolating target DNA segments adjacent to known sequences. However, TAIL–PCR

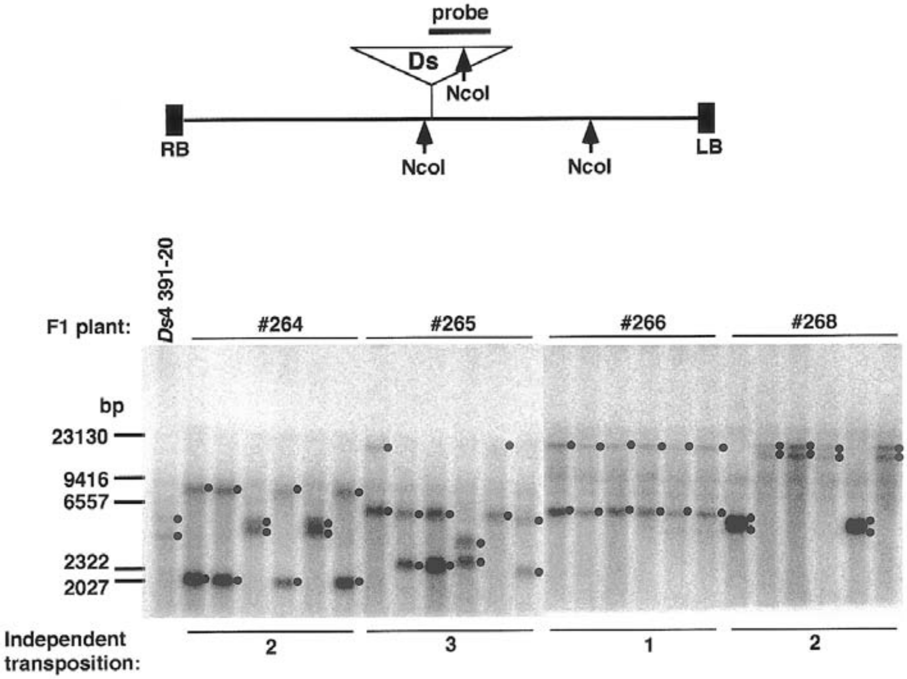

Fig. 2. Southern analysis for determination of the number of independent germinal transposition events per single F1 plant. Six Chl-, Hyg-, and Nam-resistant F_2 plants, derived from a single F_1 plant, were examined. Genomic DNA was digested with *Nco*I enzyme, and fractionated by 1% agarose gel. DNA was transferred to Hybond N+ filter (Amersham), and hybridized with a probe of *aph4* gene conferring Hyg resistance. (*Upper*) Schematic diagram of a *Ds*-GUS-T-DNA. The probe contains a *Nco*I site. (*Lower*) Results of Southern analysis. Dots show specific bands. *Ds*4 391-20 is one of a *Ds*-GUS-T-DNA parental line.

requires only the PCR procedure. Because of this simplicity, it has become an important method for genome analysis ***(5,6)***.

Ds5-1, Ds5-1a, Ds5-2, and Ds5-3 are used as a set of nested sequence-specific primers for amplification of *Ds* 5'-flanking genomic DNA, as shown in **Figure 3**. Ds3-1, Ds3-1a, Ds3-2, and Ds3-3 are used for amplification of 3'-flanking genomic DNA. These specific primer sets are used in combination with three degenerate primers.

3.3.1. Primary PCR

1. For a 20-µL reaction combine on ice:
    a. 15.1 µL genomic DNA (10–100 ng) and H2O.
    b. 2 µL 10X Ex *Taq* DNA polymerase reaction buffer.

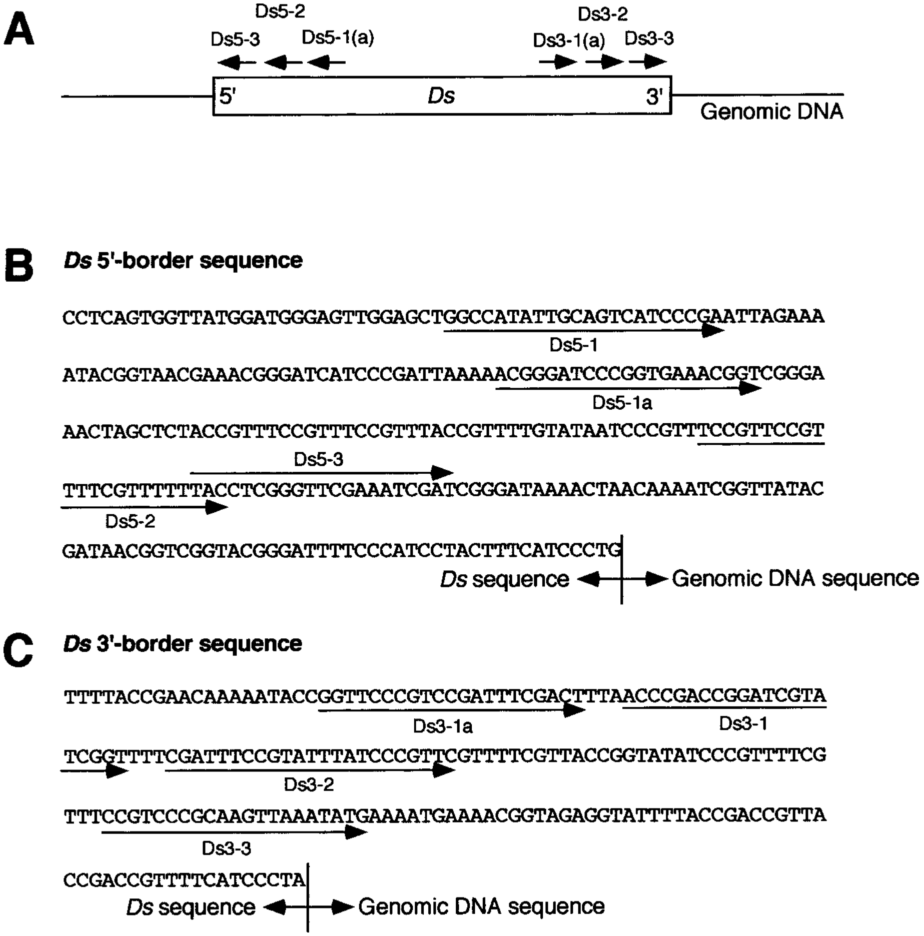
A
Ds5-2
Ds5-3
Ds5-1(a)
Ds3-2
Ds3-1(a)
Ds3-3
5'
Ds
3'
Genomic DNA
B
Ds 5'-border sequence
CCTCAGTGGTTATGGATGGGAGTTGGAGCTGGCCATATTGCAGTCATCCCGAATTAGAAA
Ds5-1
ATACGGTAACGAAACGGGATCATCCCGATTAAAAACGGGATCCCGGTGAAACGGTCGGGA
Ds5-1a
AACTAGCTCTACCGTTTCCGTTTCCGTTTACCGTTTTGTATAATCCCGTTTCCGTTCCGT
Ds5-3
TTTCGTTTTTTACCTCGGGTTCGAAATCGATCGGGATAAAACTAACAAAATCGGTTATAC
Ds5-2
GATAACGGTCGGTACGGGATTTTCCCATCCTACTTTCATCCCTG
Ds sequence
Genomic DNA sequence
C
Ds 3'-border sequence
TTTTACCGAACAAAAATACCGGTTCCCGTCCGATTTCGACTTTAACCCGACCGGATCGTA
Ds3-1a
Ds3-1
TCGGTTTTCGATTTCCGTATTTATCCCGTTCGTTTTCGTTACCGGTATATCCCGTTTTCG
Ds3-2
TTTCCGTCCCGCAAGTTAAATATGAAAATGAAAACGGTAGAGGTATTTTACCGACCGTTA
Ds3-3
CCGACCGTTTTCATCCCTA
Ds sequence
Genomic DNA sequence

Fig. 3. Specific primers used for TAIL-PCR. **(A)** Schematic representation of the *Ds* inserts, indicating the position and direction of the specific primers. **(B)** Nucleotide sequence of the *Ds* 5'-border sequence and positions of the specific primers. **(C)** Nucleotide sequence of the *Ds* 3'-border sequence and positions of the specific primers. *Ds* border sequences are described in **refs. *8*** and ***9***.

   c. 1.6 µL dNTP mix (2.5 m*M*).
   d. 1 µL of a given degenerate primer (100 pmol/µL).
   e. 0.2 µL Tn-specific primer Ds5-1 or Ds3-1 (20 pmol/µL) (*see* **Note 6**).
   f. 0.1 µL Ex *Taq* DNA polymerase (5000 U/mL).
2. The PCR program (*see* **Note 7**) is as follows:
   a. 94°C, 1 min; 95°C, 1 min (1×).
   b. 94°C, 1 min; 65°C, 1 min; 72°C, 3 min (5×).
   c. 94°C, 1 min; 25°C, 3 min; ramping to 72°C at 0.2°C/s; 72°C, 3 min (1×).

   d. 94°C, 30 s; 68°C, 1 min; 72°C, 3 min; 94°C, 30 s; 68°C, 1 min; 72°C, 3 min (2 high-stringency cycles); 94°C, 30 s; 44°C, 1 min; 72°C, 3 min (1 reduced-stringency cycle) (15×).
   e. 72°C, 5 min (1×).

3.3.2. Secondary PCR

1. For a 20-µL reaction, combine on ice:
   a. 1 µL 50-fold dilutions of the primary PCR products.
   b. 2 µL 10X Ex *Taq* DNA polymerase reaction buffer.
   c. 1.6 µL dNTP mix (2.5 m*M*).
   d. 0.8 µL of the same degenerate primer as in primary PCR (100 pmol/µL).
   e. 0.2 µL Tn-specific primer Ds5-2 or Ds3-2 (20 pmol/µL).
   f. 0.1 µL Ex *Taq* DNA polymerase (5000 U/mL).
   g. 14.3 µL water.
2. The PCR program (*see* **Note 7**) is as follows:
   a. 94°C, 30 s; 64°C, 1 min; 72°C, 3 min; 94°C, 30 s; 64°C, 1 min; 72°C, 3 min (2 high-stringency cycles); 94°C, 30 s; 44°C, 1 min; 72°C, 3 min (1 reduced-stringency cycle) (12×).
   b. 72°C, 5 min (1×).

3.3.3. Tertiary PCR

1. For a 100-µL reaction, combine on ice:
   a. 1 µL 10-fold dilutions of the secondary PCR products.
   b. 10 µL 10X Ex *Taq* DNA polymerase reaction buffer.
   c. 8 µL dNTP mix (2.5 m*M*).
   d. 4 µL of the same degenerate primer as in primary and secondary PCRs (100 pmol/µL).
   e. 1.5 µL Tn-specific primer Ds5-3 or Ds3-3 (20 pmol/µL).
   f. 0.5 µL Ex *Taq* DNA polymerase (5000 U/mL).
   g. 75 µL water.
2. The PCR program is as follows:
   a. 94°C, 1 min; 44°C, 1 min; 72°C, 3 min (30×).
   b. 72°C, 5 min (1×).

*3.3.4. Agarose Gel Analysis of TAIL–PCR Products (*see ***Fig. 4****)*

7 µL of secondary and tertiary PCR products are loaded on 1% agarose gel, and fractionated by electrophoresis. The specificity of the tertiary PCR products is verified by the size shift (20 bp in *Ds* 5'-flanking bands and 32 bp in *Ds* 3'-flanking bands) of the secondary and tertiary PCR products. The size shift is derived from the priming sites of Ds5-2 and -3 (*Ds* 5' border) and Ds3-2 and -3 (*Ds* 3' border), respectively. Visible bands of the primary PCR products in **Fig. 4** are not specific, and are not amplified during secondary PCR.

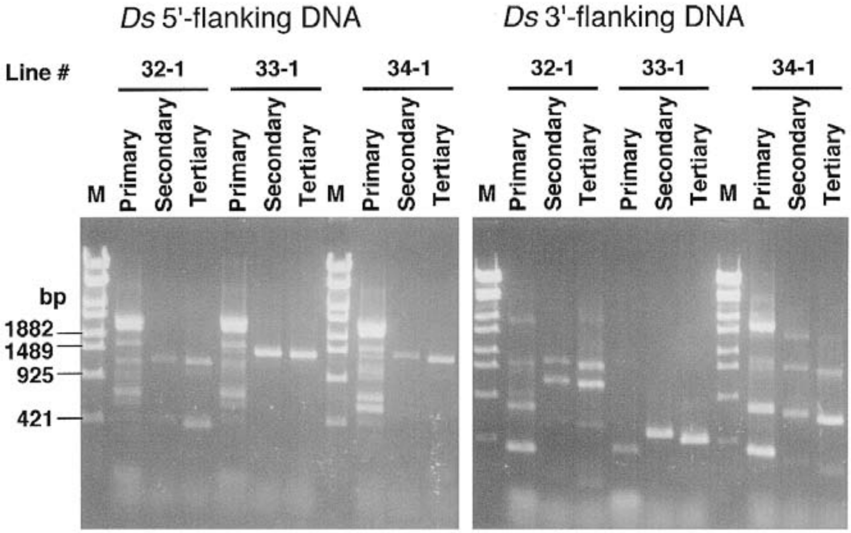

Fig. 4. Agarose gel analysis of TAIL-PCR products amplified from transposed lines. W4 was used as a degenerate primer in this experiment. Each 7 μL of the PCR product was electrophoresed. M indicates size marker.

3.4. Purification of the Specific TAIL–PCR DNA Fragments

1. Tertiary PCR products are concentrated to approx 10 μL, by using the Suprec-02 filter (*see* **Note 8**), and loaded on 1% agarose gel, then DNA fragments are fractionated by electrophoresis.
2. The gel is stained with ethidium bromide, then the specific band fraction is excised using a scalpel under UV illumination.
3. Extract the specific band from the gel, using QIAquick Gel Extraction Kits (Qiagen), according to the manufacturer's instructions.

3.5. Partial Sequencing of Specific Bands

The sequencing reaction is carried out with the ABI PRISM BigDye Terminator Cycle Sequencing Kit, FS (Applied Biosystems). The purified TAIL-PCR DNAs are used as templates, and Ds5-3 and Ds3-3 are used as sequencing primers for *Ds* 5'- and 3'-flanking DNA sequences, respectively. The DNA sequencing is carried out with the 3700 DNA Analyzer (Applied Biosystems), according to the manufacturer's instructions. After determining the partial sequence flanking Tn, examining homology with the Genbank/European Molecular Biology Laboratory/DDBJ database, using the BLASTN program, identifies the insertion sites (*see* **Note 9**). We have identified a knockout mutant of a gene encoding a ribosomal protein S13, using this strategy ***(7)***.

4. Notes

1. Degenerate primers can anneal only at reduced-stringency cycles in the primary and secondary PCRs. T_m for degenerate primers must be between 44 and 48°C. T_m is calculated with the formula $69.3 + 0.41(\%GC) - 650/L$, where L is primer length.
2. Tn-specific primers can anneal at both reduced- and high-stringency cycles in the primary and secondary PCRs. T_m for Tn-specific primers must be between 57 and 68°C.
3. By sowing F_1 seeds on GM (Hyg) plates, and selecting Hyg-resistant seedlings, self-pollinated seeds of the Nae*Ac*380-16 line can be eliminated, because only pollens from *Ds*-GUS-T-DNA lines contain a gene conferring resistance to Hyg.
4. Chl-sensitive seeds do not germinate; Nam-sensitive seedlings exhibit stunted roots and short hypocotyl, and do not fully develop cotyledons; Chl-resistant and Nam-resistant seedlings can be easily identified, because of these features. On the other hand, it is difficult to distinguish completely Hyg resistance from sensitive, after a week culture. For this reason, Hyg resistance of the candidates on GM (Hyg) plates is confirmed after further 2 wk culture.

   At least one triple marker (Chl, Hyg, and Nam)-resistant seedling was obtained from more than half of the F_1 plants.
5. The authors use the kit to isolate genomic DNAs from a large number of samples in a short time. Without many samples, one can use the cetyltrimethylammonium bromide (CTAB) method successfully for TAIL–PCR. The CTAB method is described as follows:

   a. Add 0.5 mL CTAB buffer (3% CTAB, 1.4 *M* NaCl, 20 m*M* EDTA, 0.2% 2-mercaptoethanol, 100 m*M* Tris-HCl [pH 8.0 at 25°C]; filter-sterilize, and store at room temperature) to ground leaves, and incubate at 60°C for 30 min, with occasional mixing.
   b. Add 0.5 mL chloroform/isoamyl alcohol (24:1), mix thoroughly, and centrifuge at 7000*g* (12,000 rpm) in a microcentrifuge for 5 min.
   c. Transfer the upper layer to a new 1.5-mL microtube.
   d. Add an equal volume of isopropanol, mix, and incubate at room temperature for 15 min.
   e. Recover the pellet by centrifugation at 7000*g* for 5 min.
   f. Dissolve the pellet with 100 µL TE buffer (10 m*M* Tris-HCl [pH 8.0 at 25°C], 1 m*M* EDTA) containing 20 µg/mL ribonuclease A, and incubate at 37°C for 60 min.
   g. Add 200 µL TE buffer, 100 µL 7.5 M ammonium acetate, 400 µL phenol/chloroform/isoamyl alcohol (25:24:1), and mix thoroughly.
   h. Centrifuge at 11,000*g* (15,000 rpm) in a microcentrifuge for 5 min, and transfer the upper layer to a new 1.5-mL microtube.
   i. Add 2 vol ethanol (room temperature), mix, and recover the pellet by centrifugation at 11,000*g* for 10 min at 4°C.
   j. Wash the pellet with 70% ethanol, and dissolve in 50 µL TE buffer.
6. Even if no specific PCR bands were detected by using Ds5-1 or Ds3-1 primers, use of the alternative Ds5-1a or Ds3-1a, respectively, often proved effective.

7. These programs are suited for the Peltier Thermal Cycler PTC-200. For other apparatus, it will be necessary to change parameters, especially denaturing conditions.
8. Without many samples, one can concentrate the products by the ethanol precipitation method.
9. One can search at the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/blast/).

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22

Evolutionary Molecular Engineering by Random Elongation Mutagenesis

Tomoaki Matsuura, Tetsuya Yomo, and Itaru Urabe

1. Introduction

Technological advancement in molecular biology set in motion the directed evolution of the properties of biomolecules, such as protein and nucleic acids. Toward the improvement of the properties in general, events in nature are imitated. The approach, commonly called "directed evolution," involves the alternating process of diversification, which is the generation of libraries, and subsequent selection for the fittest. The serial cycles of consecutive diversification and selection processes eventually lead to the accumulation of beneficial mutations, driving the improvement of targeted properties.

There are many selection or screening systems for attaining molecules with improved properties, such as affinity or activity, from different types of libraries. However, the mutant libraries generated should be effective, to warrant selection for better properties. If none of the molecules in the library exhibit a higher property, the chance to further improve the property under study will be slim. Because of its importance for directed evolution, several methods for generation of mutant libraries were developed, to the extent that structural information of the molecules no longer serve as a requirement for the process. Among the methods are random substitution mutagenesis ***(1)***, DNA shuffling ***(2)***, staggered extension process (StEP) ***(3)***, random priming in vitro recombination (RPR), incremental truncation for the creation of hybrid enzyme ***(4)***, and random elongation mutagenesis (REM) ***(5)***.

Here is described a new method for generating mutant libraries, in which the adapted concept is different from that of the conventional substitution mutagenesis. The method was named "random elongation mutagenesis" because the

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ

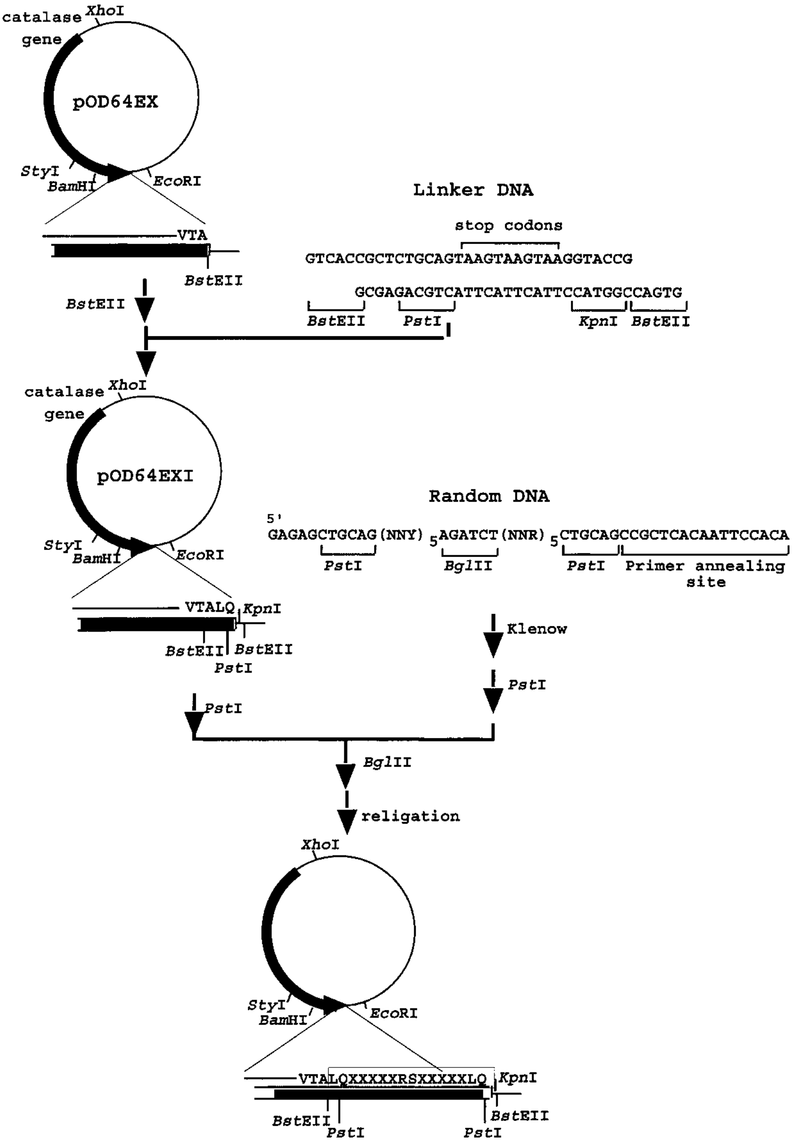
catalase gene
XhoI
pOD64EX
StyI
BamHI
EcoRI
VTA
BstEII
BstEII
Linker DNA
stop codons
GTCACCGCTCTGCAGTAAGTAAGTAAGGTACCG
GCGAGACGTCATTCATTCATTCCATGGCCAGTG
BstEII
PstI
KpnI
BstEII
catalase gene
XhoI
pOD64EXI
StyI
BamHI
EcoRI
VTALQ
KpnI
BstEII
BstEII
PstI
Random DNA
5'
GAGAGCTGCAG(NNY) 5AGATCT(NNR) 5CTGCAGCCGCTCACAATTCCACA
PstI
BglII
PstI
Primer annealing site
Klenow
PstI
PstI
BglII
religation
XhoI
StyI
BamHI
EcoRI
VTALQXXXXXXRSXXXXXXLQ
KpnI
BstEII
PstI
BstEII
PstI

Fig. 1. Construction of plasmids for random elongation mutagenesis. pOD64 contains the catalase gene in the *Pst*I-*Eco*RI fragment. The unique *Pst*I site was converted to *Xho*I site by site-directed mutagenesis, and the plasmid obtained was named pOD64EX.

process involved the attachment of a random peptide tail at the C-terminal of the target protein (**Fig. 1**). Common to all, this method also does not require structural information.

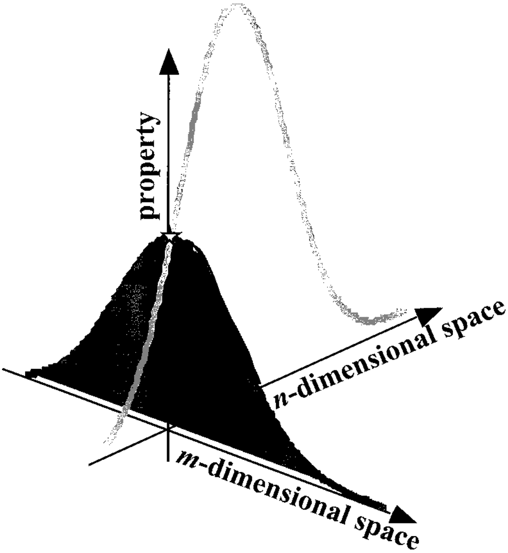

Fig. 2. Sketch of the landscape of a property of an enzyme in the *m* and *n*-dimensional sequence space. Landscape in the *m*-dimensional space is illustrated by plotting the values of the property of all mutants with *m* amino acid residues as obtained by substitution random mutagenesis on the axis for *m*-dimensional space. Landscape in the *n*-dimensional space illustrate all the elongation mutants prepared by adding a peptide tail with *n* amino acid residues to the C-terminal end of the enzyme with *m* amino acid residues, which was located at the highest peak of the *m*-dimensional landscape. The *m*- and *n*-dimensional sequence spaces are degenerated to the corresponding axes.

Basically, the concept of REM encompasses the theory of the landscape in a sequence space. For the protein with a length *m* amino-acid residues, all possible 20^m sequences are located in the *m*-dimensional sequence space. Population of the mutant libraries generated from a protein with *m* amino acid residues via substitution mutagenesis, including DNA shuffling, StEP, and RPR, then, will always be located in the same dimensional sequence space, because there will be no change in the length of the target protein. Consequently, serial cycles of consecutive substitution mutation and selection are restricted to pursue for the best sequence of a property in the *m*-dimensional sequence space. On the contrary, since elongation mutagenesis extends the peptide by the terminal attachment of certain length of a random peptide, e.g., *n*, the length of *m* is expanded to $m + n$ (**Fig. 2**). Therefore, acquiring a longer length of peptide from the original one would mean an expansion of dimension, and thus a search for better properties in higher dimensional sequence space.

The position of the target protein in both *m*- and (*m* + *n*)- dimensional sequence space is very unlikely to be similar, because the property of the proteins in the new (*m* + *n*)-dimensional space will have no means to be optimized, either naturally or artificially. If the property of a protein is nearly optimized, either naturally or artificially, by substitution mutagenesis, reaching a local or global optimum in the *m*-dimensional sequence space, REM then is useful for to further diversifying the property, because REM offers a higher dimensional sequence space, and relocates the protein from the optimum (**Fig. 2**). Once the protein is provided with a higher dimensional sequence space, substitution mutagenesis can be adapted to further optimize the property. In short, alternating the processes of substitution mutagenesis and REM would be one of the best approaches for diversification.

To demonstrate the efficiency of REM, the authors used the catalase I of *Bacillus stearothermophilus*, a member of the bacterial catalases with broad-spectrum peroxidatic activity, and as yet no defined structural information, as the model protein for the study. Previous studies ***(1)*** on the landscape of the enzyme revealed that both catalatic and peroxidatic activities can easily be improved by random substitution mutagenesis, but not thermostability. Analysis of the thermostability landscape of the enzyme showed that the probability of obtaining a mutant with a much higher stability is very low, because the thermostability is near the local optimum level. However, through the REM method, it was shown that a fraction of higher thermostable mutants occurring in the library was approx 10-fold higher than that of random substitution mutagenesis ***(5)***.

2. Materials

2.1. Escherichia coli *and Plasmid*

1. UM228 competent cell: UM228 is HB101 catalase hydrogen peroxidase I mutant ***(6)***. Prepare competent cell by calcium chloride method, as described in **ref.** ***7***.
2. Plasmid pOD64EXI [accession number AB020234]: pOD64 is a derivative of pUC19, containing the gene of catalase I from *B. stearothermophilus* ***(8)***. A synthetic linker DNA, containing the stop codons for all three frames between *Pst*I and *Kpn*I sites, was ligated to pOD64EX, which had been digested with *Bst*EII. The resulting plasmid was named pOD64EXI ***(5)*** (**Fig. 1**).

2.2. Media

1. Luria-Bertani (LB) medium: 10 g bacto-tryptone, 5 g bacto-yeast extract, and 10 g sodium chloride/L (pH 7.0). Sterilize by autoclave.
2. LB agar medium: LB medium with 15 g/L agar. Sterilize by autoclave.
3. 2X YT medium: 16 g bacto-tryptone, 10 g bacto-yeast extract, and 5 g sodium chloride/L (pH 7.0). Sterilize by autoclave.
4. 100 m*M* isopropyl thiogalactose in H_2O: Sterilize by filtering through a 0.22-µm filter.
5. 50 mg/mL Ampicillin in H_2O: Sterilize by filtering through a 0.22-µm filter.

2.3. Solutions for Electrophoresis

1. TAE buffer: 40 m*M* Tris-acetate and 2 m*M* EDTA. Adjust pH to 8.0.
2. TBE buffer: 50 m*M* Tris-borate and 10 m*M* EDTA. Adjust pH to 8.0.
3. 1% Agarose in TAE buffer.
4. 15 % polyacrylamide gel (PAG) in TBE buffer.
5. 12% Sodium dodecyl sulfate (SDS)-PAG.
6. SDS-PAGE running buffer: 25 m*M* Tris, 250 m*M* glycine, and 0.1 % SDS (pH should be 8.3 after mixing).
7. 2X SDS gel loading buffer: 100 m*M* Tris-HCl (pH 6.8), 100 m*M* dithiothreitol, 4% SDS, 0.1% bromophenol blue, and 10% glycerol.
8. 6X DNA gel loading buffer: 0.25% bromophenol blue and 30% glycerol.
9. Agarose gel electrophoresis apparatus (Mupid-2 electrophoresis apparatus [Cosmo Bio]).
10. Apparatus for SDS-PAGE and PAGE (Nihon Eido).
11. Power supply (Nihon Eido).

2.4. Solutions for DNA Extraction and Purification

1. TE buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA (pH 8.0) (sterilize by autoclave).
2. 3 *M* Sodium acetate (sterilize by autoclave).
3. 100% and 70% ethanol.
4. 1 mg/mL linear polyacrylamide.
5. Genclean gel extraction kit (Bio-Rad).
6. Glass bar: for crushing the gel in the tube.
7. Elution buffer (0.5 *M* ammonium acetate, 10 m*M* magnesium acetate, 1 m*M* EDTA, and 0.1% SDS [pH 8.0]).

2.5. DNA Modifying Enzymes and Buffers

1. 10X restriction endonuclease digestion buffer: 10X H-buffer and 10X M-buffer (Toyobo).
2. 10X Klenow buffer (Toyobo).
3. 10X Ligation buffer (Toyobo).
4. 1 m*M* deoxyribonucleoside triphosphates (dNTPs) (Toyobo).
5. Restriction endonucelase: *Pvu*II, *Kpn*I, *Pst*I, and *Bgl*II (Toyobo).
6. Klenow fragment (Toyobo).
7. Bacterial alkaline phosphatase (BAP) (Toyobo).
8. Ligase (Toyobo).
9. 0.2 m*M* TakoP5 primer (5'-TGTGGAATTGTGAGCGG-3').

2.6. Buffers for Activity Assay

1. 20 m*M* H_2O_2 in 0.1 *M* potassium phosphate (pH 7.0).
2. 20 m*M* H_2O_2, 4.1 m*M* 2,4-dichlorophenol, and 0.67 m*M* 4-aminoantipyrine in 0.1 *M* potassium phosphate (pH 7.0).

2.7. Other Equipment

1. DNA synthesizer (Applied Biosystems 381A DNA Synthesizer).
2. Spectrophotometer (Hitachi 220A).

3. Methods

This subheading is consists of three parts: First, the design and synthesis of random oligonucleotideis described; **Subheading 3.2.** describes on the cloning and generation of mutant libraries; the third subheading deals with characterization of the mutants, which alone depends on the property of interest of an enzyme or a protein, and the section is only briefly discussed. Refer to the original paper ***(5)*** for further information on the characterization of catalase I.

3.1. Design of Random DNA

The mixture of 70-mer single-stranded synthetic oligonucleotide (random DNA) is designed to contain two randomized portions, each of which is composed of 15-mer random oligonucleotides within *Pst*I fragment, and possesses a primer annealing region for synthesizing the antistrand (*see* **Fig. 1** and **Note 1**). Randomized portions comprised DNA sequence of (NNR) and (YNN), where N denotes any of A, T, G, and C nucleotide, R denotes G and C, and Y denotes A and T. All possible sequences of both NNR and YNN are free of stop codons, and NNR is a complementary sequence of YNN. Being complementary to each other allows the DNA fragments to have a reading frame in both orientations, and hence permits ease of cloning of the fragments in any orientation into the *Pst*I site at the C-terminus of calatase I gene (*see* **Notes 2** and **3**). *Bgl*II site was incorporated between two randomized portions, to avoid the problem of multimerization of oligonucleotides in the inserted random DNA (*see* **Note 4**). In cases when multimerization of the double-stranded random DNA occurred upon ligation to the C-terminus of catalase I, digestion with *Bgl*II and religation will eliminate the problem (**Fig. 1**). The oligonucleotides were synthesized conventionally by a DNA synthesizer.

3.2. Preparation of Random DNA

Preparation of double-stranded DNA from single-stranded random DNA can be done by Klenow fragment, PCR, or any other appropriate DNA polymerases. Here is described a method using the Klenow fragment (*see* **Note 5**).

1. Mix 1.45 nmol random DNA and 2.8 nmol TakoP5 primer, and add TE buffer to a final volume of 218 µL. Incubate the mixture at 70°C for 2 min and 37°C for 30 min, to allow the annealing of the TakoP5 primer to the random DNA.
2. Add 30 µL 10X Klenow buffer, 50 µL 1 m*M* dNTPs, and 2 µL Klenow fragment (16 U), and incubate at 37°C for 30 min.
3. To precipitate the DNA, add 2 µL linear polyacrylamide as a carrier for precipitation and 700 µL 100% ethanol, and centrifuge at 20,000*g* for 10 min at 4°C. To the pellet, add 500 µL 70% ethanol, and centrifuge at 20,000*g* for 10 min at 4°C. Decant the supernatant, and air-dry the pellet. Suspend the pellet in 355 µL TE buffer.

4. To digest the double-stranded DNA with *Pst*I, add 40 μL 10X H-buffer and 5 μL *Pst*I (50 U), and incubate at 37°C for 3 h.
5. For the analysis of restriction endonuclease digestion, add 70 μL DNA loading buffer to the digestion mixture, and load into 15% PAG. Run the electrophoresis for 5–6 h with constant voltage of 160 V, with TBE buffer. Visualize the digestion products by ethidium bromide staining.
6. Extract the band of interest by slicing out the portion of the gel containing the fragment of interest, and place into an Eppendorf tube. Crush the gel against the wall of the tube with a glass bar. Add 300 μL elution buffer (approx 1 vol gel volume), and incubate overnight at 30°C. Centrifuge at 20,000*g* for 10 min at 4°C. Transfer the supernatant to a fresh eppendorf tube. Be careful not to suck in any pieces of the gel. Add 1 μL linear polyacrylamide and 2 vol 100% ethanol, and proceed to ethanol precipitation as describe in **step 3**. Suspend the pellet in 30 μL TE buffer. This solution contains the random DNA, and can be readily used for ligation.

3.3. Preparation of Vector for Cloning

1. Mix 7.2 μg plasmid pOD64fl, 18 μL H-buffer, 30 U *Pst*I. Add TE buffer to a volume of 180 μL. Incubate for 3 h at 37°C.
2. Add 20 μL 1 *M* Tris-HCl (pH 8.0) and 2 μL BAP. Incubate for 1 h at 37°C.
3. Electrophorese on 1% agarose gel in TAE buffer for approx 40 min at 100 V.
4. Extract and recover the *Pst*I-digested vector DNA by the Geneclean gel extraction kit (Bio-Rad). Elute the DNA with 80 μL TE buffer. Eluted DNA is can be readily used for ligation.

3.4. Cloning and Transformation of E. coli

1. Mix the prepared 20 μL digested vector, 20 μL random DNA, 7.5 μL 2 μg/mL bovine serum albumin, 6 μL 10X ligation buffer, 2 μL ligase (2 U). Add TE buffer to a final volume of 60 μL. Incubate at 16°C overnight.
2. Purify DNA by ethanol precipitation, as described in **Subheading 3.2., step 3.** Suspend in 35 μL TE buffer.
3. Add 4 μL H-buffer and 1 μL *Bgl*II (10 U), and incubate at 37°C for 3 h. This step is required to ensure no multiple insertion of random DNA (**Fig. 1**).
4. Load into 1% agarose gel in TAE buffer and electrophorese for approx 40 min at 100 V.
5. Cut out the gel portion containing the band with the desired size of the plasmid and recover the DNA, using the Geneclean gel extraction kit (Bio-Rad). Elute the DNA in 30 μL TE buffer.
6. Religate the recovered digested plasmid by mixing 30 μL eluted DNA, 5 μL 2 mg/ml bovine serum albumin, 4 μL 10X ligation buffer, 1 μL ligase (1 U). Incubate at 16°C overnight.
7. Transform *E. coli* UM228 with ligation mixture, and plate out on an appropriate medium. Most of the clones should harbor the correct construct, and be readily used for subsequent selection or screening steps (*see* **Note 6**).

3.5. Characterization of Mutants

3.5.1. Thermostability

1. Grow *E. coli* UM228 cells harboring plasmid bearing a mutant gene of catalase I in 40 mL of 2X TY medium containing 50 µg/mL ampicillin at 37°C for 22 h.
2. Suspend the cells collected after centrifugation in 2 mL 0.1 *M* potassium phosphate (pH 7.0).
3. Disrupt the cells by sonication. The supernatant obtained after centrifugation is used as the cell lysate before heat treatment; the supernatant heated at 70°C for 10 min is used as the heat-treated lysate.
4. Analyze both lysates, before and after heat treatment, by SDS-PAGE.
5. Determine the catalase concentration from the intensity of the protein bands visualized by Coomassie brilliant blue ***(9)***. Thermostability (S) of catalase is calculated as the ratio of the concentration of residual protein after heat treatment (70°C, 10 min) to the protein concentration before heat treatment, because heat denaturation causes insolubilization and precipitation of catalase.

3.5.2. Catalatic and Peroxidatic Activity

1. For the catalatic activity (*see* **Note 7**), prepare substrate solution containing 20 m*M* H_2O_2 in 0.1 *M* potassium phosphate (pH 7.0). Add 10 µL cell lysate to 3 mL substrate solution, mix rapidly, and measure enzyme reactions at 30°C as the decrease in absorbance at 240 nm (H_2O_2 concentration). Reaction rate is calculated from the maximum slope, using the molar absorption coefficient of H_2O_2 of 43.6 *M*/cm ***(10)***. The value of the catalatic activity is expressed as half of the consumption rate of H_2O_2, because two molecules of H_2O_2 are consumed per catalytic cycle.
2. For the peroxidatic activity (*see* **Note 7**), prepare substrate solution containing 20 m*M* H_2O_2, 4.1 m*M* 2,4-dichlorophenol, and 0.67 m*M* 4-aminoantipyrine in 0.1 *M* potassium phosphate (pH 7.0). Add 10 µL cell lysate to 3 mL substrate solution, mix rapidly, and measure enzyme reactions at 30°C, as the increase in absorbance at 500 nm, because of the formation of a red dye. The reaction rate is calculated from the maximum slope, using the absorption coefficient of the red dye of 1.63×10^4 *M*/cm ***(11)***. Specific activity is calculated by dividing both activity values by the subunit protein concentration estimated from SDS-PAGE. Protein concentration was not estimated by measuring at OD_{280} (*see* **Subheading 3.5.1., step 5**).

4. Notes

1. Errors, such as deletions or insertions, which cause frame shift in the reading frame, may occur during the DNA synthesis. Therefore, it is important to include a stop codon with all three of its possible reading frames at the end of the inserted random DNA. Indeed, 20% of the resultant mutants contained a frame-shift.
2. There is a very weak, but statistically significant, correlation between thermostability and hydrophobicity (T. Matsuura, et al., unpublished result). Mutants with higher

hydrophobicity were less thermostable, perhaps because hydrophilic peptide tail increases the solubility of the overall protein, and therefore decreases the possibility to aggregate.

3. As mentioned in the Introduction, REM allows the search for better sequence in a higher dimensional sequence space than substitution mutagenesis can offer. In view of the concept of sequence space, attaching random amino acid sequence at the N-terminal of the protein is equivalent to that at the C-terminal. Although REM was not performed on the N-terminal of the protein, it is assumed that N-terminal elongation should have similar effect as in C-terminal elongation.
4. Length of the random peptides is a factor for the efficiency of REM. As reported ***(5)***, library generated with the attachment of approx 12 amino acid residues, of which 10 are randomized, to the C-terminal end, is efficient as desired. When the length was extended to contain 2 U random DNA (24 amino acid residues), the population of the mutant library contains more of the lower thermostability, compared to those with one unit random DNA (K. Miyai, et al., unpublished result). The results indicated that a random peptide that is too long has deleterious effect on thermostability of catalase I, compared to the shorter one. Nevertheless, characterization of mutant with longer peptides showed similar property distribution for catalatic activity and peroxidatic activity (K. Miyai, et al., unpublished result). Clearly, the length of the random DNA for REM depends on the protein and the property of interest. The authors therefore suggest a preliminary run with a shorter length, such as 10–15 amino acid residues, before trying the longer peptides.
5. Polymerase chain reaction can be used as an alternative method for the preparation of double stranded, random DNA sequence.
6. For screening, the authors characterized the single clones independently. However, a high-throughput screening or selection system, such as phage display or ribosome display, is recommended for big-batch screening, in consideration of the number of clones they handle per analysis.
7. The background catalatic activity detected from the host cell is assumed proportional to the protein concentration of the enzyme in the host cell, and was subtracted accordingly ***(9)***. There is no background peroxidatic activity detected from the host cell.

Acknowledgments

The authors thank Dr. Elizabeth P. Ko-Mitamura (Osaka University) for critical reading of the manuscript.

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Random Mutagenesis for Protein Breeding

Chris Fenton, Hao Xu, Evamaria I. Petersen, Steffen B. Petersen, and M. Raafat El-Gewely

1. Introduction

Mutagenesis is an essential process in evolution, generating an array of genetically unique organisms from which natural selection selects the best-suited to the immediate environment. Compatible and favorable mutations quickly converge; because offspring may inherit distinct advantageous mutations from both parents, and thus dramatically increase their survival rate. Parental crossing results in the accumulation of advantageous mutations in offspring. Evolutionary adaptation has allowed organisms to progressively specialize, and colonize, even in the most extreme of habitats.

The ability of any organism to survive is dependent on the evolutionary adaptation of its enzymes. Scientists, realizing the implications of harnessing unique and specialized enzymes to perform biological reactions, have searched from hot springs ***(1)*** to Antarctic ice sheets ***(2)***, for enzymes for use in industrial ***(3–5)***, pharmaceutical ***(6)***, environmental ***(7)***, or biotechnological ***(8,9)*** applications. Scientists have been forced to seek out, study, and clone enzymes derived from extremophiles ***(10)***. This is because sufficient knowledge is still lacking to rationally design proteins of similar function ***(11)***. Key qualities, such as stability, catalytic activity, substrate specificity, tertiary structure, or co-factor dependence or independence, cannot be predicted from the amino acid (AA) sequence ***(11)***; hence, scientists must search out and copy the evolutionary products of nature. This lack of knowledge becomes especially limiting when attempting to outdo nature and design enzymes for nonnatural environments ***(8)***. Enzymes taken from living organisms do not work well in nonnatural environments ***(12,13)***. Proteins taken from living organisms often

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ

perform highly regulated and specialized biological tasks within the context of a living cell, and therefore perform poorly outside the context of the cell ***(14)***.

Attempting to rationally design a protein, with a special catalytic activity, is a daunting and unpredictable process, at best. Even if a researcher is successful in enhancing a particular trait, it is most likely that another essential trait has been compromised or destroyed. Each AA substitution can alter, in a recursive fashion, the dynamic, structural, and mechanical properties of the protein, generating far too much data to be coherently analyzed using conventional technology. Even if a researcher managed to solve the computational nightmare of such a complex problem, the results would be, at best, an approximation ***(11)***, and certainly no guarantee that the protein would function as desired. Therefore, in order to successfully develop specialized proteins, scientists have had to mimic the nonrational process of evolution.

Directed evolution attempts to mimic the evolutionary process on a greatly accelerated time-scale ***(15)***. Evolutionary adaptation takes millions of years, but directed evolution may evolve molecules, usually proteins, with modified ***(16–18)*** or novel ***(19,20)*** functions, in weeks. In nature, the rate of natural mutations is extremely low. Directed evolution accelerates evolution by synthesizing huge numbers of heterogeneous mutant molecules, with the aid of laboratory random mutagenesis techniques. The mutated gene library is then cloned into an appropriate expression vector, using an appropriate host, e.g., bacteria or yeast.

Protein breeding is the optimization of enzymes through directed evolution. Mutagenesis is used to create genetic variation between enzymes. Selection of enzymes is based on the improvement of experimentally defined enzymatic activity. DNA is isolated and placed in a parental pool, then crossed or shuffled ***(21–24)***. The shuffling step allows the convergence of favorable diverse mutations into one molecule ***(12)***. Subsequent cloning and expression should produce recombinant molecules with even greater desired activity. Selected recombinant molecules then become the starting point for a new mutagenesis experiment, and the cycle can repeat itself until the desired level of activity is reached (in practice 3–4×). Thus, several generations of genetically unique enzymes can be produced, screened, recombined, and screened again in a short time ***(25)***. If a satisfactory level of modified activity is not reached, the random mutagenesis step may be replaced with other techniques, such as saturation mutagenesis ***(26)*** of suspected key catalytic sites. An overview of protein breeding (protein directed evolution/enzyme optimization) can be seen in **Fig. 1**. Note that each step shown in **Fig. 1** (error-prone polymerase chain reaction [PCR], cloning, functional expression and selection, and DNA shuffling) can and should be optimized for best results.

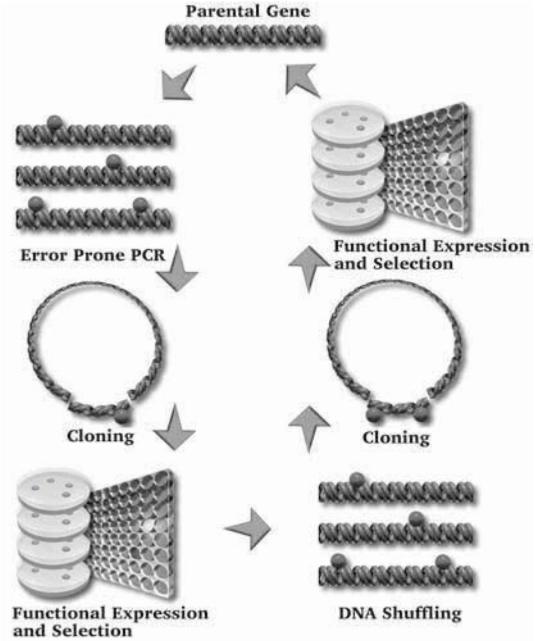
Parental Gene
Error Prone PCR
Cloning
Functional Expression and Selection
DNA Shuffling
Cloning
Functional Expression and Selection

Fig. 1. Protein breeding strategy (enzyme optimization).

Several techniques can be used to generate random mutagenesis libraries: chemical ***(27)***, staggered extension ***(28)***, random elongation ***(29)***, random priming ***(30)***, or error prone PCR ***(31–33,35,36)***. Regardless of the technique used, the measure of library quality is the number of nonidentical target gene sequences produced. Ideally, a library should contain all possible substitutions in the target gene. Unfortunately, the number of possible substitutions in a gene quickly scales beyond practical limitations, therefore, in practice, a library contains a small subset of all the possible substitutions, typically, only 1–4 substitutions/gene molecule.

The true power of directed evolution lies in the mutagenesis step, or the generation of diversity. Consider a single stretch of 100 nucleotides, substituting at each possible position (4^{100}), would produce ~1.6×10^{60} unique variants or solutions. Even 4 billion yr ***(34)*** of evolution is not enough time to generate every possible variant of every gene. Yet, in the laboratory, clever use of random mutagenesis and gene expression techniques allows the creation of billions of potential solutions in a simple experiment.

In fact, mutagenesis may discover adaptive enzymes not found in nature. Consider a solution involving multiple mutations, whose intermediary steps, 1–2 mutations, are detrimental to the organism. Because of the incremental nature of evolution, such solutions are unlikely to have survived the intermediary steps. Yet, by adjusting mutagenesis parameters to create and combine multiple mutations per molecule, unanticipated solutions can be derived that could not have evolved in nature.

Error-prone PCR is often used to generate random mutagenesis libraries. Unfortunately, most error-prone PCR protocols require the delicate balancing of triphosphate (deoxyadenosine, deoxycytidine, deoxyguanosine, and deoxythymidine) concentrations to be effective ***(35)***. Most error-prone PCR techniques also do not produce truly random mutations: They favor transitional point mutations over transversional ***(26)***. Transitional point mutations exchange one pyrimidine with another pyrimidine, or one purine with another purine; transversional point mutations exchange a purine with a pyrimidine, or a pyrimidine with a purine. Thus, exchanges of AT↔GC and TA↔CG are transitional point mutations, and AT↔CG, AT↔TA, GC↔CG, and GC↔TA are transversional point mutations. Only 5.7 AA substitutions have been documented ***(26)*** to be accessible through current error-prone PCR techniques from any given amino acid. Therefore, typical error-prone PCR is not a suitable method for the introduction of all 20 AA residues at each position, because of the transition:transversion ratio bias.

The authors have developed an error-prone random mutagenesis technique ***(36)*** that overlies the bias in transition:transversion ratio. Additionally the authors' PCR technique does not require the tedious optimization of each deoxyribonucleoside triphosphate (dNTP) concentration. In order to alleviate the transition:transversion bias inherent in error-prone PCR mutagenesis, this method is based on using two compensatory PCR steps (**Fig. 2**). During the first PCR, manganese is added to the PCR reaction buffer, causing the less-stringent *Taq* DNA polymerase to occasionally incorporate an incorrect base ***(37)***. In the second PCR reaction, the addition of deoxyinosine triphosphate (dITP), whose base-pairing preferences compensate for the transition:transversional bias of the first PCR reaction ***(36)***.

A comparison of results from manganese (Mn)–dITP error-prone mutagenesis to a typical Mn error-prone mutagenesis ***(32)*** can be seen in **Table 1**. The typical error-prone PCR procedure ***(32)*** produced 74.8% transitional mutants, and only 25.2% transversional mutants; Mn–dITP produced 58.3% transitional mutants and 41.7% transversional mutations, or an increase of 16.47%. Specifically, GC↔CG transversions increase from 1.5 to 8.3%. Such an increase in the number of transversions permits more than double the amount of AA substitutions at any given position, which dramatically increases the repertoire of AA substitutions available at each position. In particular this method should be ideal for the preparation of a random pool for a subsequent enzymatic optimization.

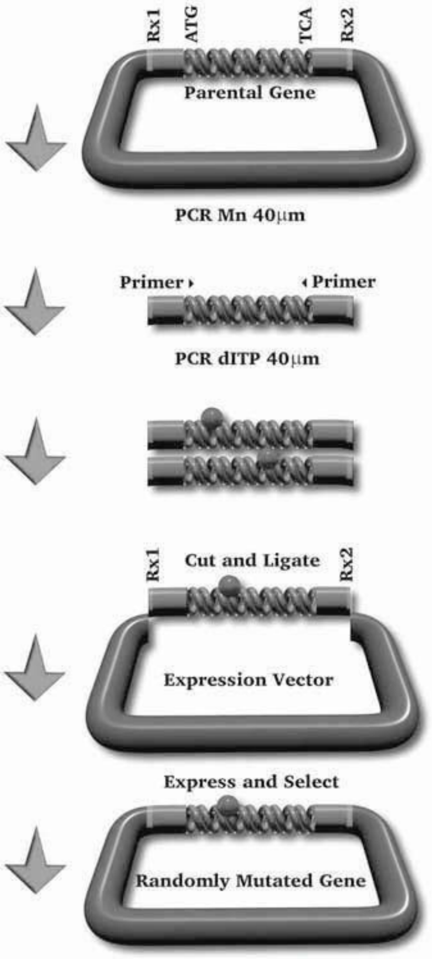
Rx1
ATG
TCA
Rx2
Parental Gene
PCR Mn 40μm
Primer ›
‹ Primer
PCR dITP 40μm
Rx1
Cut and Ligate
Rx2
Expression Vector
Express and Select
Randomly Mutated Gene

Fig. 2. Generation of a random mutagenesis library using two-step Mn–dITP error-prone mutagenesis. Rx1 and Rx2 indicate restriction enzyme recognition sites.

2. Materials

1. *Taq* polymerase (Promega, Madison, WI).
2. $MnCl_2 \cdot 4H_2O$ (Merck KggA, 64293 Darmstadt, Germany); 100 m*M* $MnCl_2$ stock solution.
3. PTC–200 Peltier Thermal Cycler (MJ Research, 02451 Waltham, MA).
4. 25m*M* $MgCl_2$ (comes with *Taq* polymerase kit).
5. *Taq* 10X reaction buffer without $MgCl_2$ (comes with *Taq* polymerase kit).
6. 5 m*M* dNTP (comes with *Taq* polymerase kit).

Table 1
Comparison of Mn–dNTP to Mn–dITP Error-Prone PCR Techniques

		Mn and reduced dNTP method[a]			Mn–dITP method[b]			
	Type	Cols	Exp	% Tot	Cols	Exp	% Tot	Changes
Transversions	AT↔TA	44	45	16.3	2	2	16.7	–0.4
	GC↔CG	4	45	1.5	1	2	8.3	–6.8
	AT↔CG	11	45	4.0	1	2	8.3	–4.3
	GC↔TA	9	45	3.3	1	2	8.3	–5
	Totals	68	180	25.2	5	8	41.7	16.4
Transitions	AT↔GC	176	45	65.2	3	2	25	31.8
	TA↔CG	26	45	9.6	4	2	33.3	–15.4
	Totals	202	90	74.8	7	4	58.3	–16.4

[a] Data from **ref. *32***.
[b] Data from **ref. *36***.
Exp, the number of expected colonies given a random distribution; Col, the number of actual colonies; % Tot, the number of actual colonies divided by the total number of colonies times 100; Changes, the % Tot of the Mn and reduced dNTP technique minus the % Tot of Mn–dITP technique.

3. Method

3.1. First PCR Reaction

1. Add 5 µL of 10X standard PCR buffer.
2. Add dNTP to final concentration of 200 µ*M*.
3. Add $MgCl_2$ to final concentration of 2.0 m*M*.
4. Add Mn $MgCl_2$ to final concentration of 40 µ*M*.
5. Add 5 p*M* of each primer.
6. Add 100 ng plasmid containing gene of interest.
7. Add 1 µL *Taq* (5 U).
8. *Add* H_2O, to total of 50 µL.
9. Program and run thermal cycler as shown in **Table 2**.

3.2. Second PCR Reaction

1. In a new PCR tube, add 5 µL 10X PCR standard buffer.
2. Add 2 µL of the product of the first PCR reaction.
3. Add 5 pm of each primer.
4. Add dNTP to a final concentration of 200 µ*M*.
5. Add $MgCl_2$ to final concentration of 2.0 m*M*.
6. Add dITP to a final concentration of 40 µ*M*.
7. Add 1 µL *Taq* polymerase (5 U).
8. Add H_2O, to total of 50 µL.
9. Program and run thermal cycler as shown in **Table 3**.

Table 2
First PCR Reaction

Hot start	94°C for 3 min	
Denaturation	94°C for 1 min	
Annealing	48°C for 1 min	20 cycles
Extension	72°C for 1 min	
Additional extension	70°C for 10 min	
Product storage	4°C	

Table 3
Second PCR Reaction

Hot start	94°C for 3 min	
Denaturation	94°C for 1 min	
Annealing	48°C for 1 min	30 cycles
Extension	72°C for 1 min	
Additional extension	70°C for 10 min	
Product storage	4°C	

4. Notes

1. Although *Taq* DNA polymerase was used in the establishment of this technique, any similar DNA polymerase that lacks proofreading ability should work (*Tfl*, *Tth*, *Klentaq*, and *Stoffel*). However, the authors have only tested *Taq* DNA polymerase.
2. Several experiments were done to figure out the most suitable concentrations of Mn and dITP. Before studying the combined effects of adding Mn and dITP to a PCR reaction, the authors had to ascertain the individual amounts of Mn or dITP that could be used in a PCR reaction mixture, without jeopardizing the PCR product. The results are summarized in **Table 4**. The addition 50 µ*M* Mn or 100 µ*M* dITP to the PCR reaction buffer resulted in a smeared PCR product. After establishing working ranges for Mn and dITP, experiments were attempted using both in the same PCR reaction mix: As summarized in **Table 5**, these attempts yielded no viable PCR product. Therefore, the Mn and dITP PCR reactions had to be run separately.
3. Both steps of Mn-dITP error-prone PCR reaction had to be optimized, in order to consistently introduce mutations into the PCR product. The PCR product of the first Mn PCR reaction had to be stable throughout the second dITP PCR reaction. **Table 6** shows that the addition of 40 µ*M* dITP to the second PCR reaction mix generates a viable PCR product, but the addition of 60 µ*M* dITP does not.

Table 4
Optimization of dITP and Mn for PCR

dITP (μ*M*)	100	20	10	0	0	0
Mn^{2+} (μ*M*)	0	0	0	50	10	5
PCR Product[a]	~200 ng	~1 μg	~1 μg	~1 μg	~1 μg	~1 μg
PCR Quality	Smeared	Good	Good	Smeared	Good	Good

[a] Product: The amount of DNA in 50 μL PCR product, estimated through gel electrophoresis, by comparing the intensity PCR aliquots to the intensity of a known amount of DNA ladder.

Table 5
Effects of Adding Both dITP and Mn into PCR Mix

dITP (μ*M*)	Mn^{2+} (μ*M*)	PCR Product[a]	PCR Quality
100	50	None	None
	30	None	None
	20	None	None
70	50	None	None
	30	<200 ng	Smeared
	20	<200 ng	Smeared
50	50	<200 ng	Smeared
	30	<200 ng	Smeared
	20	<200 ng	Smeared
20	50	<200 ng	Smeared
	30	~200 ng	Smeared
	20	~200 ng	Smeared

[a] Product: The amount of DNA in 50 μL PCR product, estimated through gel electrophoresis, by comparing the intensity PCR aliquots to the intensity of a known amount of DNA ladder.

Table 6
Optimization of dITP and Mn Concentrations for Two-Step Mn–dITP PCR Technique

Mn^{2+} (μ*M*)	Product[a]	Quality	dITP (μ*M*)	Product[a]	Quality
40	250 ng	Good	40	~1 μg	Good
			60	None	
50	250 ng	Good	40	~1 μg	Good
			60	None	

[a] Product: The amount of DNA in 50 μL PCR product, estimated through gel electrophoresis, by comparing the intensity PCR aliquots to the intensity of a known amount of DNA ladder.

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24

DNA Shuffling and Family Shuffling for In Vitro Gene Evolution

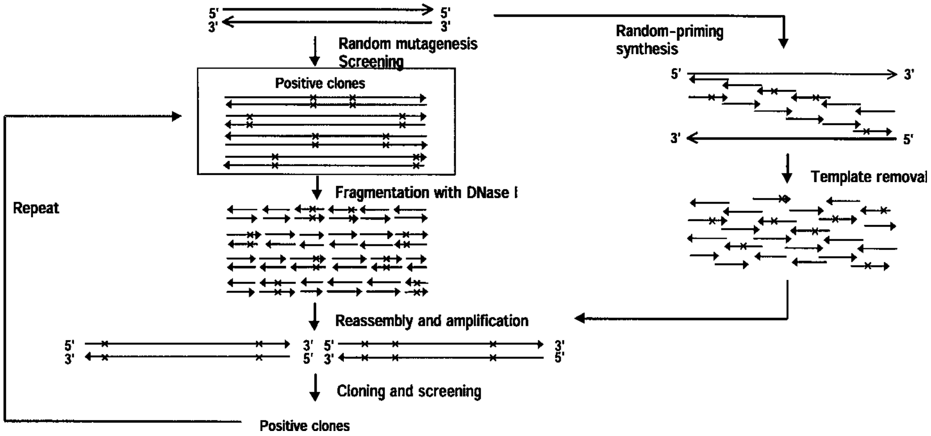
Miho Kikuchi and Shigeaki Harayama

1. Introduction

DNA shuffling is a developed technique that allows accelerated and directed protein evolution in vitro. In this method, the acquisition of genes encoding improved proteins is done in two steps: First, a single gene is mutagenized, and desired mutant genes are selected; second, the mutant genes are fragmented by DNase I, and subsequently recombined in vitro, by using polymerase chain reaction (PCR). Among the recombinants (i.e., the products of DNA shuffling), those producing most favored proteins are isolated ***(1**,**2)*** (**Fig. 1**). Modified versions of the DNA shuffling exist: Random priming was used to generate DNA fragment, instead of the DNase I digestion ***(3)*** (**Fig. 1**); PCR conditions, with very short annealing/extension steps, were employed to increase the frequency of recombination ***(4)***. Using these techniques, a number of improved enzymes have been obtained ***(1–9)***. When the DNA shuffling is done using a set of homologous genes, instead of a set of mutant genes derived from a single gene, this technique is called "family shuffling" (**Fig. 2**). Family shuffling utilizes naturally occurring nucleotide substitutions among family genes as the driving force for the in vitro evolution. The application of the family shuffling strategy has also provided many successful examples ***(10–15)***.

A potential problem of family shuffling is a low yield of recombinants. When two parental genes of 80% nucleotide sequence identity were shuffled, the frequency of hybrid formation was less than 1% ***(16**,**17)***. The low recombination yield may result from a lower frequency of the heteroduplex formation, compared to the frequency of the homoduplex formation ***(16)*** (**Fig. 3A**). Now, two improved techniques for the family shuffling have been devised. One is family shuffling using single-stranded (ss) DNA ***(17)***. In this method, ssDNAs of two

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ


5'
3'
5'
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Random mutagenesis
Screening
Positive clones
Random-priming
synthesis
5'
3'
3'
5'
Template removal
Repeat
Fragmentation with DNase I
Reassembly and amplification
5'
3'
3'
5'
5'
3'
3'
5'
Cloning and screening
Positive clones

Fig. 1. DNA shuffling. In vitro evolution of genes consists of repeated cycles of two steps: random mutagenesis and DNA shuffling of mutations. In the first step (random mutagenesis/screening), several mutants are isolated from a target gene. In the second step, the mutant genes are segmented by DNase I, then reassembled by PCR without primer, and amplified by PCR with primers. DNAs created by DNA shuffling are used to transform a recipient (usually *E. coli*), and transformed strains are selected for desired phenotypes. Instead of the DNase I segmentation, random-priming synthesis of short fragments can be used.

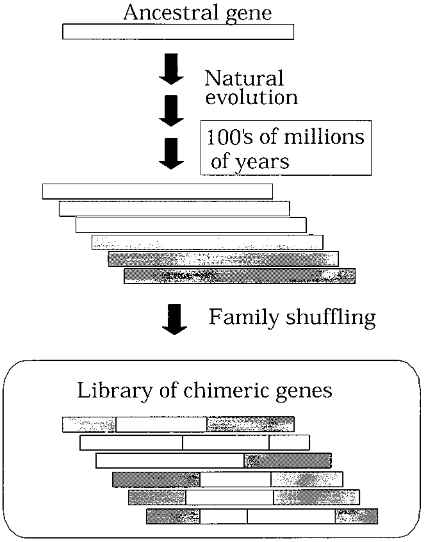
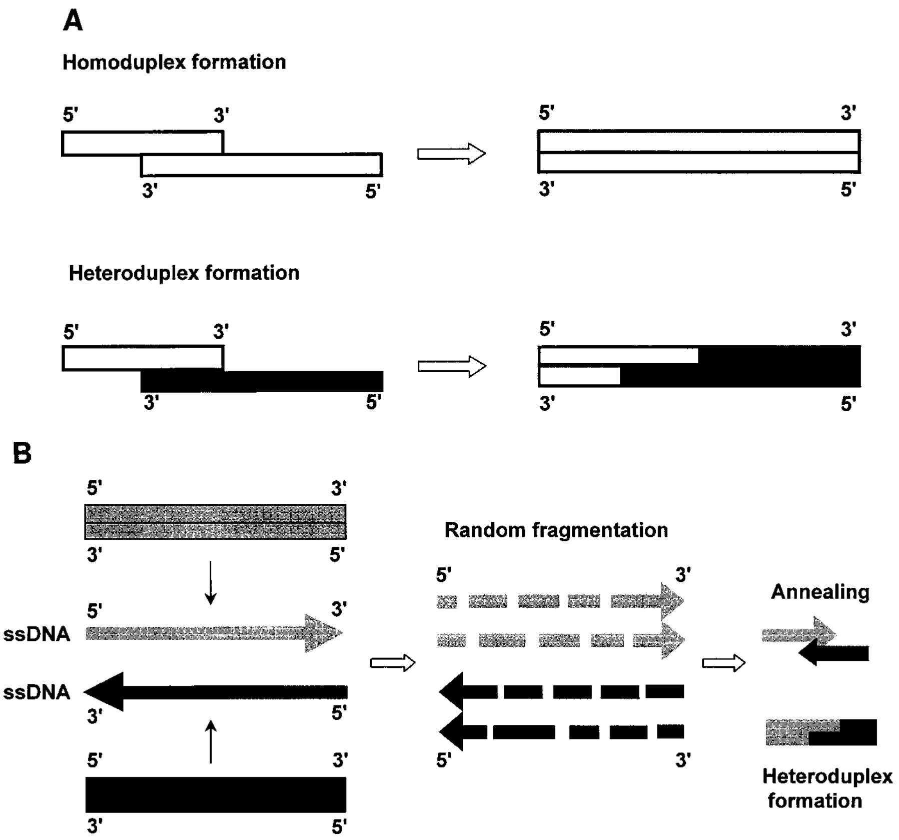

Ancestral gene
Natural
evolution
100's of millions
of years
Family shuffling
Library of chimeric genes

Fig. 2. Family shuffling. Contemporary genes belonging to the same gene family are derived from a single ancestral gene after repeated introduction of mutations through the natural divergent evolution processes. The shuffling of the family gene sequences creates a library of chimeric genes, from which desired chimera are selected.


A
Homoduplex formation
5'
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Heteroduplex formation
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3'
5'
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3'
ssDNA
ssDNA
3'
5'
5'
3'
3'
5'
Random fragmentation
5'
3'
5'
3'
Annealing
Heteroduplex formation

Fig. 3. DNA hybridization and extension in family shuffling. **(A)** DNA strands of two family genes are indicated by open and close boxes. In the family shuffling using two genes, two types of annealing occur: homoduplex (annealing of strands derived from the same gene) and heteroduplex (annealing of strands derived from two different genes). If the frequency of the homoduplex formation is higher than that of the heteroduplex formation, the frequency of the regeneration of the original gene sequences occurs preferentially, rather than the formation of chimeric genes. **(B)** To prevent the homoduplex formation in the first round of PCR, ssDNAs are prepared from two family genes. These ssDNAs are fragmented by DNase I, and only heteroduplex molecules are formed.

homologous genes are prepared. One ssDNA is the coding strand of one gene; another ssDNA is the noncoding strand of another gene. These two ssDNA molecules are digested by DNase I, and their fragments are used for family shuffling. In the first round of hybridization, the homoduplex formation is pre-

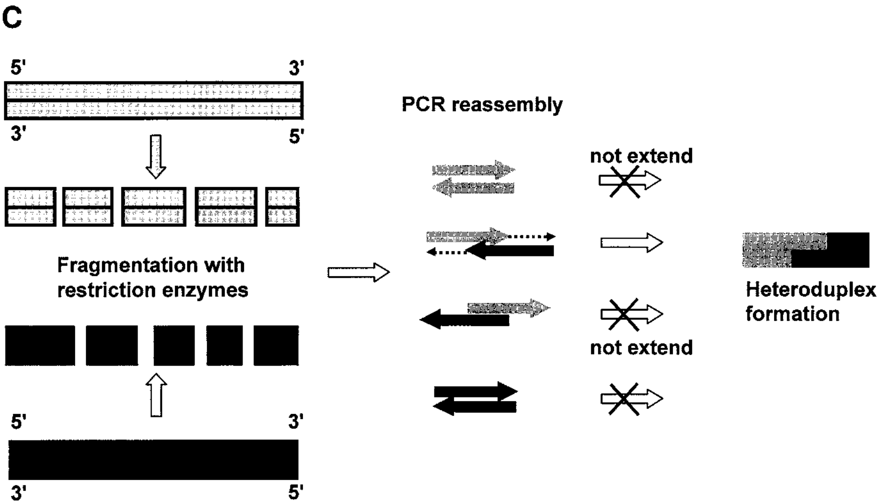


Fig. 3. *(continued)* DNA hybridization and extension in family shuffling. **(C)** In the family shuffling with restriction enzyme-cleaved DNA fragments, both homoduplex and heteroduplex are formed. On the homoduplex molecules, no DNA elongation occurs, but, on the heteroduplex molecules, the extension of DNA occurs, and chimeric DNA pieces are formed.

vented (**Fig. 3B**). The second technique is the shuffling of restriction endonuclease-digested DNA fragments, instead of the shuffling of randomly fragmented DNA ***(16)***. The annealing of endonuclease-digested DNA fragments would produce homoduplex at a high frequency, but significant DNA elongation only occurs on the heteroduplex molecules (**Fig. 3C**).

For the successful in vitro evolution of target proteins, the optimization of DNA shuffling processes is required. However, the design of screening methods for the selection of improved proteins is important. Furthermore, the probability of desired mutations should be estimated, to know the required size of mutant libraries.

2. Materials

2.1. DNA Shuffling

2.1.1. Mutant Isolation

1. TE buffer: 10 m*M* Tris-HCl (pH 7.4), 1 m*M* EDTA.
2. 10X *Taq* polymerase buffer: 100 m*M* Tris-HCl (pH 8.0), 500 m*M* KCl, 15 m*M* $MgCl_2$, 1% (w/v) Triton X-100; store at –20°C.
3. 1 *M* β-mercaptethanol; prepare freshly.
4. Dimethylsulfoxide (DMSO).

5. 5 m*M* $MnCl_2$; stable at room temperature for 2 wk.
6. Forward and reverse primers: 50 µ*M* stocks; store at –20°C (*see* **Note 1**).
7. 10 m*M* Deoxyguanosine triphosphate (dGTP); store at –20°C.
8. 2 m*M* Deoxyadenosine triphosphate (dATP); store at –20°C.
9. 2 m*M* Deoxythymidine triphosphate (dTTP); store at –20°C.
10. 10 m*M* Deoxycytidine triphosphate (dCTP); store at –20°C.
11. *Taq* DNA polymerase ; store at –20°C (*see* **Note 2**) .

2.1.2. Fragmentation of Genes by DNase I

1. DNase I: Store at –20°C; diluted enzyme solution must be prepared each day.
2. 10X DNase I digestion buffer: 500 m*M* Tris-HCl (pH 7.4) containing 100 m*M* $MnCl_2$.

2.1.3. Random Priming for the Synthesis Gene Fragments

1. Random hexamers can be purchased from many suppliers.
2. 10X random priming buffer: 900 m*M* HEPES (pH 6.6), 0.1 *M* $MgCl_2$, 20 m*M* dithiothreitol, and 5 m*M* each of dATP, dCTP, dGTP, and dTTP) (*see* **Note 3**).

2.1.4. PCR Reassembly and Amplification

1. Deoxyribonucleoside triphosphate (dNTP) mix: 2 m*M* each of dNTP; store at –20°C.
2. 10X *Pfu* DNA polymerase buffer: 200 m*M* Tris-HCl (pH 8.8), 20 m*M* $MgSO_4$, 100 m*M* KCl, 100 m*M* $(NH_4)_2SO_4$, 1% (w/v) Triton X-100, 1 mg/mL nuclease-free bovine serum albumin; store at –20°C (*see* **Note 1**).
3. Forward and reverse primers: 20 µ*M* stocks; store at –20°C (*see* **Note 1**).
4. *Taq*/*Pfu* DNA polymerase mixture: 1:1 (*see* **Note 2**).
5. *Pfu* Turbo DNA polymerase; store at –20°C.

2.1.5. Staggered Extension Process

1. dNTP mix: 2 m*M* each of dNTP; store at –20°C.
2. 10X *Taq* DNA polymerase buffer: 100 m*M* Tris-HCl (pH 8.0), 500 m*M* KCl, 15 m*M* $MgCl_2$, 1% (w/v) Triton X-100; store at –20°C.
3. Forward and reverse primers: 20 µ*M* stocks; store at –20°C.
4. *Taq* DNA polymerase; store at –20°C.

2.2. Family Shuffling Adopting the DNA Shuffling Method

Materials are indicated in **Subheadings 2.1.2.–2.1.4.**

2.3. Family Shuffling Using ssDNAs

2.3.1. Preparation of ssDNAs

The reagents for the ssDNA preparations are given in **ref. *18***.

1. 2X YT medium: 16 g Bacto tryptone, 10 g yeast extract, and 5 g NaCl/L.
2. Helper phage stocks can be purchased from several suppliers, and generally have titers higher than 10^{10} (*see* **Note 4**).

3. Phage precipitation solution: 20% (w/v) polyethylene glycol 8000 in 2.5 *M* NaCl; sterilize by filtration, stable at room temperature.
4. Phenol/chloroform/isoamyl alcohol (25:24:1).
5. Chloroform/isoamyl alcohol (24:1).
6. 7.5 *M* ammonium acetate (pH 5.2).
7. 100 and 70% (v/v) ethanol.

2.3.2. DNase I Treatment

Materials are indicated in **Subheading 2.1.2.**

2.3.3. PCR Reassembly and Amplification

Materials are indicated in **Subheading 2.1.4.**

2.4. Family Shuffling with Restriction Enzyme-Cleaved DNA Fragments

2.4.1. Fragmentation of Target Genes with Restriction Enzymes

1. Restriction enzymes; store at –20°C.
2. 10X Reaction buffer; store at –20°C.

2.4.2. PCR Reassembly and Amplification

Materials are indicated in **Subheading 2.1.4.**

3. Methods

3.1. DNA Shuffling

3.1.1. Mutant Isolation

A PCR-based method for the mutant isolation ***(19)*** is described in this section. Frequency of mutations by this method is expected to be ~1.4%. Factors affecting the fidelity of DNA synthesis are discussed in **ref. *19***. Other methods, including conventional chemical mutagenesis ***(20)*** could also be used.

1. Prepare DNA carrying the target gene.
2. Add in a 1.5-mL tube 10 μL 10X *Taq* DNA polymerase buffer, 10 μL each dNTP, 1 μL β-mercaptethanol, 10 μL DMSO, 10 μL $MnCl_2$, 1.5 μL forward and reverse primers, 2.5–4 U *Taq* DNA polymerase, 1 ng (a fmol range) DNA containing the target gene, and H_2O to the total volume of 100 μL (*see* **Note 2**).
3. Perform PCR under the following conditions: 25 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 4 min.
4. Purify PCR products by an appropriate method, e.g., agarose gel electrophoresis, to remove primers.
5. Subclone the PCR products in an appropriate vector.
6. Select desired mutants among transformants.

3.1.2. Fragmentation of Genes by DNase I

1. Prepare mutant genes to be shuffled.
2. Dilute 2 µg (or 3 pmol) of each DNA in 45 µL TE buffer, and add 5 µL 10X DNase I digestion buffer.
3. Incubate the solution at 15°C for 5 min, then add 0.3 U DNase I.
4. Incubate further for 2 min, then transfer to 90°C, and incubate for 10 min, to terminate the reaction (*see* **Note 5**).
5. Run a 2–3% low-melting agarose gel electrophoresis to purify fragments of 10–100 bp (longer fragments could also be used), using MERmaid SPIN Kit (Bio101).
6. Resuspend the DNA fragments in 50 µL TE buffer.

3.1.3. Random Priming for the Synthesis Gene Fragments

An alternative to the DNase I treatment for the segmentation of genes is random-priming using the Klenow fragment ***(3)*** (**Fig. 1**).

1. Prepare mutant genes to be shuffled.
2. Mix in a 1.5-mL tube, each 1 pmol DNA to be shuffled and 20 µg (10 nmol) dp(N)6 random primers.
3. After denaturation at 100°C for 5 min, add 10 µL 10X random priming buffer.
4. Adjust the total volume of the reaction mixture to 95 µL with H_2O.
5. Add 10 U (in 5 µL) of the Klenow fragment of *Escherichia coli* DNA polymerase I.
6. Incubate at 22°C for 3 h, then transfer on ice.
7. Add 100 µL ice-cold H_2O to the reaction mixture.
8. Purify the random primed products by filtering the whole reaction mixture through a Microcon-100 filter (Amicon), to remove the template, proteins, and large nascent DNA fragments. Filter the Microcon-100 filtrate through a Microcon-10 filter, to remove the primers and fragments smaller than 20 bases.
9. Dialyze the retained fraction (approx 65 µL) against PCR reaction buffer. Of this fraction, 10 µL was used for the whole gene reassembly, as described next.

3.1.4. PCR Reassembly and Amplification

The PCR reactions are done in two stages, the first PCR for reassembly of DNA fragments into longer pieces, and the second PCR for amplification.

1. Add to a 1.5-mL tube, 10 µL 10X *Pfu* DNA polymerase buffer, 10 µL dNTP mix, 10 µL of the purified fragments from **Subheading 3.4.2.** or **3.4.3.**, 2.5 U *Pfu* Turbo DNA polymerase, and H_2O to the total volume of 100 µL.
2. Without adding any primer, carry out PCR under the following conditions: 96°C for 2 min, then 25–30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The gene reassembly proceeds by the PCR.
3. Add to a new 1.5-mL tube, 1–5 µL reassembled PCR product (*see* **Note 6**), 10 µL 10X *Pfu* DNA polymerase buffer, 10 µL dNTP mix, 2 µL forward and reverse primers, 2.5 U *Taq*/*Pfu* DNA polymerase mixture, and H_2O to the total volume of 100 µL.

4. Perform PCR for 15–20 cycles, e.g., under the following conditions: 96°C for 2 min, followed by 20 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 60 s.
5. Purify a single band of the correct size from an agarose gel, using QIAEX II gel extraction kit (Qiagen), and subclone, after appropriate restriction endonuclease digestion (*see* **Note 1**), into an appropriate vector.
6. Select desired mutants among transformants.

3.1.5. Staggered Extension Process

The staggered extension process (StEP) is the repeated PCR cycles consisting of the denaturation step followed by extremely abbreviated annealing and elongation steps. In each cycle, growing fragments are denatured, then annealed and extended further. Due to the template switching, most of the PCR products contain sequence information from different parental sequences. This procedure can replace the above-mentioned procedures (**Subheadings 3.1.2.** and **3.1.4.** or **Subheadings 3.1.3.** and **3.1.4.**) ***(4)***.

1. Prepare DNAs to be shuffled.
2. Mix 0.15 pmol of each DNA, 10 μL 10X *Taq* DNA polymerase buffer, 10 μL dNTP mix, 1.5 μL forward and reverse primers, 2.5 U *Taq* DNA polymerase, and H_2O to the total volume of 100 μL.
3. Perform PCR under the following conditions: 95°C for 5 min, then 80 cycles of 94°C for 30 s and 55°C for 5 s.
4. Purify the PCR products, subclone them, and select desired mutants among transformants, as described in the previous subheading.

3.2. Family Shuffling Adopting the DNA Shuffling Method

1. Prepare DNAs of several homologous genes.
2. Using each 2 μg (or 3 pmol) of the DNAs, perform DNA shuffling under the same conditions as described in the **Subheading 3.1.2.** and **3.1.4.** or **Subheading 3.1.3.** and **3.1.4.**

3.3. Family Shuffling Using ssDNAs

3.3.1. Preparation of ssDNAs

1. Clone two homologous genes separately in phagemid vectors (e.g., pBluescript). The orientations of one gene, with respect to the origin of the ssDNA replication, should be the same; that of another gene should be opposite with respect to the origin of the ssDNA replication. The coding strand of one gene and the noncoding strand of another gene are then synthesized and packaged in phage particles. In other words, ssDNAs of the two homologous genes are complementary to each other.
2. Introduce the plasmids thus constructed into “male” *E. coli*, such as JM109 (because the infection by ssDNA phage requires F pili).
3. Inoculate a 1-mL culture of 2X YT medium supplemented with an appropriate antibiotic with a single JM109 transformant colony.

4. Grow the culture at 37°C with shaking (e.g., rotary shaking at 250 rpm) to an A_{600} of 2.
5. Inoculate 25 mL 2X YT containing an appropriate antibiotics in a 250-mL Erlenmeyer flask, with 0.5 mL of the above culture.
6. Incubate for 1 h, with shaking, at 37°C.
7. Infect with a helper phage (*see* **Note 4**) at an multiplicity of infection of 10–20.
8. Incubate overnight, with shaking, at 37°C.
9. Next day, harvest the cells by centrifuging at 12,000*g* at 4°C for 15 min.
10. Transfer the supernatant (containing phage particles) to a fresh tube. Do not introduce the pellet in the tube.
11. Spin the supernatant again, and transfer the supernatant to a fresh tube.
12 Add 0.25 vol phage precipitation solution to the supernatant. Leave on ice for at least 1 h, or overnight at 4°C.
13. Centrifuge at 12,000*g* for 20 min at 4°C. In the presence of polyethylene glycol, phage particles precipitate.
14. Remove the supernatant, and resuspend the pellet in 400 µL TE buffer, and transfer to a 1.5-mL tube.
15. Add 1 vol TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1) to the sample, vortex at least 1 min, and centrifuge at 12,000*g* for 5 min.
16. Transfer the upper phase (containing phagemid DNA) to a fresh tube, without disturbing the interface. Repeat the organic solvent extraction, until no visible material appears at the interface.
17. Add 0.5 vol (200 µL) 7.5 *M* ammonium acetate plus 2 vol (1.2 mL) 100% ethanol. Mix, and leave at –20°C for 30 min, to precipitate the phagemid DNA.
18. Centrifuge at 12,000*g* for 5 min, remove the supernatant, and carefully rinse the pellet with ice-cold 70% ethanol. If the pellet is disturbed, centrifuge again for 2 min. Drain the tube, and dry the pellet under vacuum.
19. By agarose gel electrophoresis, two major bands, corresponding to helper phage DNA and ssDNA from phagemid, are usually seen. A small amount of chromosomal DNA and RNA released by cell lysis may be present. Longer incubation during phagemid rescue increases the contamination of *E. coli* chromosomal DNA. ssDNA migrates faster than double-stranded (ds) DNA of the same length. The presence of the helper phage DNA does not interfere with the following reactions.

3.3.2. DNase I Treatment

1. Each 2 µg ssDNA is randomly fragmented with DNase I as described in **Subheading 3.1.2.** (*see* **Note 7**).
2. Fragments in the size range of 40 to 100 bases are isolated from 2–3% low-melting-point agarose gel, using QIAEX II gel extraction kit.
3. Resuspend the recovered DNA in 50 µL TE buffer.

3.3.3. PCR Reassembly and Amplification

1. Mix the ssDNA fragments in 100 µL reaction mixture as described in **Subheading 3.1.4., step 1**.

2. Perform PCR: 40 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and elongation at 72°C for 60 + 5 s/cycle (the duration of elongation is increased by 5 s after each cycle).
3. 1–5 μL reassembled PCR product is added in the PCR reaction mixture, as described in **Subheading 3.1.4., step 3** (*see* **Note 6**).
4. Perform PCR: 96°C for 2 min, followed by 25 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 45 + 20 s/cycle.
5. Separate the PCR products on an agarose gel, and recover a band corresponding to the size of the full-length gene.
6. Purify the PCR products, subclone them, and select desired mutants among transformants, as described previously.

3.4. Family Shuffling with Restriction Enzyme-Cleaved DNA Fragments

3.4.1. Fragmentation of Target Genes with Restriction Enzymes

1. Prepare DNAs of homologous genes.
2. Digest each DNA segment with one or more restriction enzymes.
3. Purify the digested fragment, to remove nondigested or partially digested DNA.

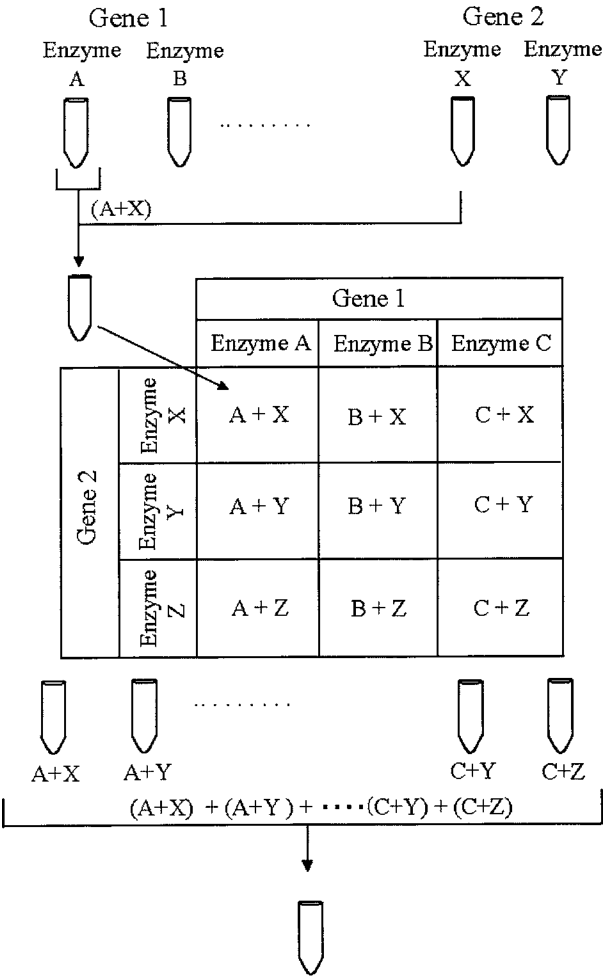
3.4.2. PCR Reassembly and Amplification

1. Mix the purified fragments, making all possible combinations of the mixtures (*see* **Note 8**) containing 100 ng of two different digested genes (*see* **Fig. 4**).
2. To each mixture from **step 1**, add 2 μL *Pfu* DNA polymerase buffer, 2 μL dNTP mix, 1.25 U *Pfu* Turbo polymerase, and H_2O to the total volume of 20 μL.
3. Perform PCR for each mixture: 96°C for 2 min, followed by 15 cycles (*see* **Note 9**) of 94°C for 1 min, 56°C for 1 min, and 72°C for 60 + 5 s/cycle.
4. Combine all reaction products into a single mixture up to 100 μL of the total volume.
5. Perform PCR: 25 cycles of PCR under the program indicated in **step 3**.
6. Add 1–5 μL reassemble PCR product into the PCR mixture, as outlined in **Subheading 3.1.4., step 3** (*see* **Note 6**).
7. Perform PCR as described in **Subheading 3.3.3., step 4.**
8. Purify the PCR products, subclone them, and select desired mutants among transformants, as described previously.

4. Notes

1. Primer design is crucial in DNA shuffling. Primer length and sequence should be carefully determined for a successful amplification, cloning and gene expression. Follow general guidelines on the primer design for PCR cloning, which are described in many textbooks. Sequences flanking the target gene can also be used for the primer design.

   Taq DNA polymerase adds a single 3'–dA overhang to a blunt dsDNA template. For the cloning of such PCR products, linearized plasmid vectors containing single dT overhangs (T-vectors) were developed. The cloning efficiencies of the



		Gene 1		
		Enzyme A	Enzyme B	Enzyme C
Gene 2	Enzyme X	A + X	B + X	C + X
	Enzyme Y	A + Y	B + Y	C + Y
	Enzyme Z	A + Z	B + Z	C + Z

Fig. 4. Family shuffling with restriction enzyme-cleaved DNA fragments. Two genes (gene 1 and gene 2) are cleaved by several restriction enzymes (A and B for gene 1; X and Y for gene 2). Two restricted DNA samples, one from gene 1 and the other from gene 2, are then mixed, and PCR reactions without primer are carried out. The combinations of the restricted DNA samples are indicated in the table in the middle of this figure. After this PCR step, all the PCR mixtures (A + X, A + Y, and so on) are mixed together, and the second PCR is carried out.

T-vectors, however, were variable. DNA polymerases, with 3'–5' exonuclease (proofreading) activity, remove mispaired nucleotides from 3' ends of dsDNA, and generate blunt-end PCR products. The PCR products generated by these proofreading polymerases thus can be cloned into vectors by blunt-end ligation.

However, blunt-end cloning of PCR products is less efficient than sticky-end cloning. Thus, it is advisable to introduce additional restriction sites at the 5' end of each of the primers. As amplification proceeds, these primers are incorporated into the PCR product. Thus, the PCR products can be digested by appropriate restriction enzymes, to clone in an appropriate vector.

2. *Taq* polymerase lacks a 3' 5' exonuclease activity. In other words, it lacks the proofreading function of DNA replication, and thus exhibits a high rate of replication errors. This enzyme showed higher replication errors when the concentration of $MnCl_2$ in the reaction mixture increased, or when the concentration of one nucleotide was lower than those of other three nucleotides in the reaction mixture. For this reason, PCR with *Taq* polymerase was often used for the mutagenesis of genes. *Pfu* and *Pfu* Turbo DNA polymerases, on the other hand, show high fidelity of DNA replication. Zhao et al. ***(21)*** have reported that using a proofreading DNA polymerase (*Pfu* or *Pwo*) in the reassembly step (*see* **Subheading 3.1.4.**) increased the frequency of active clones from the shuffling products. Therefore, PCR with the proofreading DNA polymerase (*Pfu* Turbo) is used here to reduce the negative mutation.

   The amplification by *Pfu* Turbo polymerase is not as powerful as that by *Taq* DNA polymerase. The replacement of *Pfu* Turbo polymerase by *Taq* DNA polymerase, or by the mixture of *Pfu* Turbo and *Taq* polymerases, often provides better results.
3. The high concentrations of random primers may allow the hybridization of many primer molecules to single DNA molecule inhibiting of the elongation of DNA replication and promoting the accumulation of short primed fragments. Under the reaction conditions described here, the length of random priming products was between 50 and 500 bp.
4. Helper phages, such as VCS-M13 (Stratagene) and M13KO7 (Pharmacia), are used for the rescue of ssDNA from phagemids. These helper phages can be prepared by propagating on a male *E. coli* strain.
5. The concentration of DNase I, the time, and temperature for the reaction should be optimized, because the digestion speed of DNase I is influenced by many factors, including the nature of DNA. After DNase I treatment and subsequent incubation at 90ºC, the brown precipitation may be produced. This is removed by centrifugation, then the supernatant is used for PCR.
6. The PCR amplification using primers (the second PCR step of DNA shuffling) seems to be inhibited when the concentrations of reassembled PCR products (i.e., the product of the first PCR without primers) are high. Check the concentrations of the first and second PCR products on an agarose gel. If the second PCR amplification is not efficient, reduce the concentration of substrates (i.e., the product of the first PCR) in the second PCR.
7. The authors used entire single-stranded plasmids for shuffling. As a consequence, the vector sequences are also shuffled. If necessary, ssDNA fragments carrying target gene sequences, but not vector sequences, could be amplified using asymmetric PCR technique.

8. The combination of any restriction enzymes can be used for the family shuffling with restriction enzyme-cleaved DNA. When no appropriate restriction enzyme is found to cleave a gene into appropriate sizes, use the combination of several enzymes. Preferably, prepare several sets of restriction fragments, and use them for the DNA shuffling.

   PCR amplification would fail under the following conditions. If a cleavage site (X) on one gene is close (e.g., <20 bp) to a cleavage site (Y) on another gene, the annealing of two gene fragments between site X and Y is not efficient, and the reassembly to full length is blocked at the X-Y site. If an end (X) of a restriction fragment of one gene is not strongly homologous to the corresponding region of another gene (Y), the annealing between the X and Y regions of two DNA fragments may not be effective, and the DNA elongation may not occur beyond this region. Similarly, if the 3' end nucleotide of one fragment does not form a base pairing with the corresponding nucleotide on another fragment, the DNA elongation does not occur at this site, because of this mismatch. The method using several sets of restriction enzyme digestions, however, can overcome such problems, because the problematic regions for DNA elongation are different in different sets of enzyme-cleaved fragments. The family shuffling using multiple sets of restriction enzyme-digested DNA is recommended, because this method also increases the variety of shuffling products.

   When more than two genes are used for shuffling, the number of mixtures (*see* **Fig. 4**) in **Subheading 3.4.1., step 1** increases exponentially. In that case, gene pairs showing lower homology would be mixed in this step. The recombination between genes of higher homology would occur after the mixing of all the gene fragments in **Subheading 3.4.2., step 1.**

   The mixing of two different restriction fragments derived from the same gene should not be mixed in **Subheading 3.4.1., step 1.** If they are mixed, the reassembly into the original sequence will occur preferentially.
9. PCR amplifications, especially in the first PCR, become difficult when the sizes of target genes are large. If the amounts of the first PCR products are small, the number of cycles in the first PCR should be increased. Especially in the restriction enzyme-based family shuffling, the 15 cycles in the first PCR may not be enough, when the size of the target DNA is larger than 1 kb.

Acknowledgments

The authors thank Kouhei Ohnishi for valuable discussion and Paul Baker for careful reading of the manuscript. This work was supported as a part of the Industrial Science and Technology Frontier Program supported by New Energy and Industrial Technology Development Organization.

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Mutagenic Polymerase Chain Reaction of Protein-Coding Genes for In Vitro Evolution

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1. Introduction

In vitro protein evolution is an efficient approach to study the structure and function of a protein, or to enhance its industrial utility ***(1)***. One round of evolution consists of random mutation of a protein-coding gene, expression the resulting library in a population of micro-organisms, and high-throughput screening or selection of clones that most strongly exhibit a desired phenotype ("winners"). After many rounds, mutations that confer the phenotype accumulate on a single allele, e.g., the authors have isolated an octuple mutant of the *Escherichia coli* β-glucuronidase with catalytic activity resistant to roughly 80-fold higher concentrations of glutaraldehyde than that of the wild-type enzyme ***(2)***. Here we describe a variation of the mutagenic polymerase chain reaction (PCR) ***(3,4)*** is described. The advantages of this method over other random mutagenesis techniques are explained.

The expression system and high-throughput assay should be developed prior to any random mutagenesis. The latter has utility only in the context of a screen that is precise, high-throughput, and sensitive enough to detect the desired activity in the ancestral protein, under stringent assay conditions. The following expression/assay system is used as an example: *E. coli* colonies, transformed with the wild-type β-glucuronidase expression vector, *gusA*-pBSΔ, turn green when induced on Luria-Bertani (LB) plates containing 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc). The stringency of this assay can increased by pretreating the colonies with glutaraldehyde before incubation with X-gluc ***(2)***.

From: *Methods in Molecular Biology, vol. 182: In Vitro Mutagenesis Protocols, 2nd ed.*
Edited by: J. Braman © Humana Press Inc., Totowa, NJ

In mutagenic PCR, manganese (Mn) ions, excess magnesium (Mg), and a nucleotide imbalance ***(4)***, or synthetic nucleotide analogs ***(5)***, are added to a PCR reaction to decrease the fidelity of Taq polymerase. Sequence diversity can also be generated by mutator strains ***(6)***, hypermutagenesis ***(7)***, cassette mutagenesis ***(8)***, DNA shuffling ***(9)***, or family shuffling ***(10)***. The virtues and disadvantages of each method is summarized in **Table 1**. The authors prefer mutagenic PCR ***(4)*** when mutating whole genes, because it enables the construction of a series of libraries that differ in mutation frequency simply by altering PCR conditions. This is important because libraries with too many wild-type or overmutated clones decrease the effective throughput of the screen. The appropriate mutation frequency is theoretically dependant on the length of the gene, the average number of random mutations that the protein can accept without unfolding, the mutation bias, and the throughput of the screen. This chapter shows that, in practice, the simplest way to evaluate a library of random mutants is to utilize the high-throughput assay under permissive conditions.

One drawback of mutagenic PCR is that not all types of nucleotide changes occur at equal frequency ***(11)***. The authors sometimes ***(2)***, although not always ***(12)***, observe a significant transition bias among winners. All PCR-generated libraries have similar transition biases, and selection will magnify or diminish this bias, depending on the nature of the beneficial mutations. Mutagenic PCR, using the commercially available nucleotide analogs 8-oxo-2'-deoxyguanosine triphosphate (8-oxo-dGTP) and 6-(2-deoxy-beta-ᴅ-ribofuranosyl)-3,4-dihydro-8H-pyrimido-[4,5-C][1,2]oxazin-7-one (dPTP), rather than Mn, can alter the bias and further increase sequence diversity ***(5)***. Cassette mutagenesis, based on oligonucleotides with random codons, does not exert a codon bias, but only permits randomization of a small number of nearby amino acid residues ***(8)***. Miyazaki and Arnold ***(13)*** have used cassette mutagenesis to randomize codons that were identified by sequencing winners generated by mutagenic PCR.

The mutation rates of the other methods are more difficult to control. In vitro random recombination by DNA shuffling is attractive, because it can theoretically unite all beneficial mutations from a screen in a single round. In practice, however, the 0.7% mutation rate associated with DNA shuffling ***(9)*** is too high for many applications, which necessitates multiple rounds of shuffling and screening ***(2,14–17)***, or labor-intensive procedures to reduce the mutation rate ***(18)***. The mutation rate of the family shuffling process has not been as well-characterized, but thus far seems even higher ***(10)***.

Propagation of the vector in a mutator strain, such as XL-1 Red ***(6)***, is also attractive, because this can obviate the labor of subcloning. The latter procedure induces a maximum mutation rate of 0.0005 mutations/bp, after propagation of a high-copy-number plasmid for 30 generations of bacterial growth,

Table 1
Comparison of Random Mutagenesis Methods

Method	Principle	Rate (%)	Bias	Application	Disadvantages	Ref.
Mutator strain XL-1 RED	*E. coli* lacking 3 DNA repair functions	0.05	None, according to manufacturer	Easy to obtain large libraries	Mutation rate low	***(6)***
Mutagenic PCR (Mn^{2+})	Low-fidelity PCR	0.02–0.7	Transition (*see* text)	Mutation frequency can be adjusted	*See* text	***(4)***
Mutagenic PCR (dPTP, 8-oxo-dGTP)	Low-fidelity PCR	0.02–0.7	Transition (*see* text)	Different mutation bias than Mn^{2+} PCR	Requires nucleotide analogs	***(5)***
Hypermutagenesis	Low-fidelity reverse transcription	0.5–10	Transition	High mutation rate for up to 200 bp	Mutation rate too high for whole-gene mutagenesis	***(7)***
DNA shuffling	Random fragmentation and PCR-like reassembly of winners	0.7	Transition	Brings beneficial mutations together	High mutation rate difficult to control	***(9)***
Family shuffling	DNA shuffling of natural homologs	>0.7?	Transition?	Randomly recombines divergent homologs	High mutation rate, technically difficult	***(10)***
Cassette mutagenesis	Extension of oligonucleotides with random nucleotides	0–75	None	Randomizes 1–8 nearby condons	Requires structural model	***(8)***

which is somewhat lower than the authors' target. Mutagenic PCR is therefore appropriate for most experiments in which the relationship between protein structure and function is unclear. The following protocol details the random mutation of a gene, the subcloning and transformation of the resulting library, and the evaluation of the library using a high-throughput assay.

2. Materials

2.1. Reagents and Buffers

1. DNA oligonucleotides can be purchased commercially from GenSet (La Jolla, CA) or Operon (Alameda, CA), and stored as a 100 µmol concentrated stock solution in 10 m*M* Tris-HCl, pH 9.5, at –20°C. These can be diluted in water to 1/10 µmol stocks, and stored at –20°C. The primers should complement the 5' and 3' ends of the gene, respectively, with a melting temperature (T_m = 4°C × [G + C] + 2°C × [A + T]) of 75–80°C. Primers should include restriction sites at the 5' ends so that the gene can be subcloned into the expression vector, in-frame, plus four or more additional nucleotides at the extreme 5' end.
2. Buffered deoxynucleotide triphosphates (dNTPs) (Amersham Pharmacia Biotech, Piscataway, NJ), should be mixed and diluted to 4 m*M* (each dNTP) in deionized water, and stored at –20°C.
3. 5X normal PCR buffer: 300 m*M* Tris-HCl, pH 8.5, 75 m*M* $(NH_4)SO_4$, 10 m*M* $MgCl_2$. If PCR yields are low, the DNA optimizer kit, containing a series of alternative buffers, can be purchased from Invitrogen (Carlsbad, CA).
4. Agarose (for 1–10-kb DNA fragments): 0.8% LE agarose (FMC, Rockland, ME) in 1X TAE (40 m*M* Tris-acetate, 1 m*M* EDTA), 0.5 µg/mL ethidium bromide.
5. 10X mutagenic addition buffer. The mutagenic PCR protocol is based on that of Cadwell and Joyce ***(3)***. Their buffer differs from normal (nonmutagenic) PCR buffer, in that it contains final (1X) concentrations an additional 0.8 m*M* deoxythymidine triphosphate (dTTP), 0.8 deoxycytidine triphosphate (dCTP), 4.8 m*M* $MgCl_2$, 0.5 m*M* $MnCl_2$. The authors therefore make a 10X cocktail of 8 m*M* dTTP, 8 dCTP, 48 m*M* $MgCl_2$, 5 m*M* $MnCl_2$ that can be added to otherwise normal *Taq* PCR reactions to make them mutagenic. Cadwell and Joyce ***(4)*** add the $MnCl_2$ last, to prevent precipitation, but the authors do not do this, and have not observed this problem. The cocktail is stored at –20°C.
6. Qiaquick PCR purification and gel extraction kits were purchased from Qiagen (Chatsworth, CA), and stored at room temperature.
7. 5X Gibco-BRL T4 DNA ligase buffer: 250 m*M* Tris-HCl, pH 7.6, 50 m*M* $MgCl_2$, 25% (w/v) polyethylene glycol 8000, 5 m*M* adenosine triphosphate (ATP), 5 m*M* dithiothreitol (DTT) ***(19)***. This buffer comes with the enzyme, if purchased from Gibco-BRL Life Technologies (Bethesda, MD). Store at –20°C.
8. SOC: Mix (per L) 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 2.5 m*M* KCl. Autoclave for 20 min (121°C, 14 atmospheres, slow cooling). Autoclave 1 *M* $MgCl_2$ and 20% (w/v) glucose, separately, and add 10 m*M* $MgCl_2$ and 20 m*M* glucose to the media, after it has cooled to room temperature.

9. TB buffer: Mix 10 m*M* PIPES, 15 m*M* $CaCl_2$, 250 m*M* KCl; pH to 6.7, using KOH, then add 55 m*M* $MnCl_2$. Filter sterilize and store at 4°C ***(20)***.
10. Chemically competent cells ***(20)***:
    a. Propagate *E. coli* cells in 50 mL liquid LB to midlog phase (OD_{600} = ~0.5) at 18°C (room temperature works almost as well). Incubate on ice for 10 min or more. Keep cells cold and sterile.
    b. Harvest cells by centrifuging at 2500*g* (3000 rpm in Beckman J-6B centrifuge) for 10 min at 4°C.
    c. Resuspend gently in a small volume of ice cold TB, bring up to 16 mL, and spin down again.
    d. Resuspend in 3.72 mL TB, add 0.28 mL dimethylsulfoxide (DMSO), aliquot 20 × 0.2 mL portions into microcentrifuge tubes in powdered dry ice.
    e. Store competent cells at –80°C.
11. Butterfly nitrocellulose membranes (Schleicher and Schuell, Keene, NH).
12. LB agar plates should be poured the evening before the transformation, and allowed to cool at room temperature overnight:
    a. Mix (per L) 10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar. Autoclave for 20 min (121°C, 14 atmospheres, slow cooling).
    b. Mix the following stocks in sterile water: 100 mg/mL ampicillin (AMP), 25 mg/mL kanamycin (KM), 100 m*M* isopropylthio-β-D-galactoside (IPTG). These can be stored at –20°C. Mix 50 mg/mL X-gluc in DMSO; this cannot be stored long; do not mix more than need.
    c. Let the LB agar media cool to 50°C (when flask can be held in one's bare hands). Add 1 mL AMP and 1 mL KM stocks/L LB agar plates. For some plates, also add 0.5 m*M* IPTG and 0.08% X-gluc. Unused plates can be stored at 4°C, but the X-gluc in plates is unstable, so they should be used within 1 wk.

2.2. Enzymes

All enzymes can be obtained from the indicated manufacturer, and stored at –20°C.

1. *Taq* polymerase: Perkin-Elmer (Foster City, CA) or Promega (Madison, WI).
2. Proteinase K: Boehringer-Mannheim (Indianapolis, IN). Mix small volumes (<100 µL) of 10 mg/mL stock solution in H_2O, and store at –20°C. Mix new stock, if precipitation is visible after thawing.
3. Restriction enzymes (New England Biolabs [NEB], Beverly, MA).
4. Shrimp alkaline phosphatase (Boehringer-Mannheim, Indianapolis, IN).
5. T4 DNA ligase (Gibco-BRL Life Technologies, Bethesda, MD). Just prior to use, add 1 µL of T4 DNA ligase (1 U) into 9 µL of 1X ligase buffer (1/10 dilution). Note that NEB uses a different unit definition of ligase activity, and does not provide a buffer containing polyethylene glycol.

2.3. Expression System

1. *gusA*-pBSΔ is an expression vector that uses the *lac* promoter to drive the expression of β-glucuronidase. It is based on pBluescript (Stratagene, La Jolla, CA), which confers AMP resistance ***(2)***.

2. The InvαF'/pREP4 strain *(2)* expresses little endogenous β-glucuronidase activity. The pPREP4 plasmid (Qiagen, Chatsworth, CA) is a constitutive *lac* repressor expression vector that stabilizes *gusA*-pBSΔ. It is based on pACYC184, which confers resistance to 25 µg/mL KM.

3. Method

3.1. Mutagenic PCR

1. Set up 50 µL PCR reactions containing 1X normal PCR buffer, 200 µmol dNTPs, 100–500 n*M* primers, 20 fmol template, and 0, 0.312, 0.625, 1.25, 2.5, or 5 µL 10X mutagenic addition buffer (*see* **Note 1**). Add water to 49 µL, then 1 µL (2.5 U) *Taq* polymerase.
2. Overlay a drop of light mineral oil, and amplify for 25 cycles of PCR. For primers with 75–80°C T_m, 25× (94°C for 30 s, 72°C for 1 min/kb of desired product) should be appropriate.
3. Check yield by running a microliter of each reaction in an agarose gel, with a known quantity of a mol wt standard. The total yield should be 1–10 µg (*see* **Note 2**).
4. Eliminate *Taq* polymerase. Add EDTA to 5 m*M*, sodium dodecyl sulfate to 0.5%, and proteinase K to 50 µg/mL, and incubate at 65°C for 15 min (*see* **Note 3**).
5. Purify PCR product, using Qiagen Qiaquick PCR purification kit, as directed by the manufacturer (*see* **Note 4**).

3.2. Subcloning

1. Set up 50-µL restriction digests: 1-10 µg of each purified PCR product, or 2 µg of the expression vector, separately, with the appropriate restriction buffer and an excess of restriction enzyme overnight (*see* **Note 5**).
2. Confirm digestion by running 1 µL of each reaction in an agarose gel, with a known quantity of a mol wt standard.
3. Dephosphorylate the vector by adding 1 µL of shrimp alkaline phosphatase to the restriction digest, and incubate at 37°C for another hour (*see* **Note 5**).
4. Gel purify the insert and vector:
    a. Pour a 0.8% agarose gel with one large well.
    b. Add loading dye to each digest, and run in separate gels.
    c. Visualize bands on UV light box, and excise desired bands, using a clean razor blade.
    d. Purify agarose-encased DNA, using the Qiagen Qiaquick gel extraction kit, as directed by the manufacturer (*see* **Note 4**).
5. Check the purification yield by running 1 µL of each reaction in an agarose gel, with a known quantity of a mol wt standard.
6. Ligate 20 fmol purified vector with 60 fmolf insert (*see* **Note 6**) in 20-µL reactions with 1X BRL T4 DNA ligase buffer + 0.1 U T4 DNA ligase *(19)*. Set up one ligation for each library, plus control reactions without insert, and without insert or ligase. Incubate at 16°C overnight.

3.3. Determination of Inactivation Rate (Using β-Glucuronidase as Example)

1. Transform library into chemically competent *E. coli* ***(20)*** (*see* **Note 7**):
    a. Heat kill ligase by incubating each ligation at 65°C for 10 min.
    b. Thaw frozen competent cells at room temperature, and place on ice after thawing has started.
    c. Add equal volumes of each ligation to competent cells, so that no 200-µL aliquot of cells receives more than 10 ng total DNA. Incubate on ice for 30 min.
    d. Heat shock cells by incubating tubes at 42°C for 40 s. Place tubes back on ice briefly, and add 800 µL SOC. Incubate at 37°C for 1 h.
    e. Plate serial dilutions of each transformation onto noninducing LB agar AMP/KM plates, in order to estimate the size of each library, and to propagate each library at an appropriate colony density for the high-throughput screen.
2. Assess the enzyme activity of each clone in the high-throughput screen, under permissive conditions. Adsorb the transformed colonies to a nitrocellulose filter, and transfer them (colony side up) to a second LB agar AMP/KM/IPTG/X-gluc plate, and incubate for 2 h at 37°C. The authors generally look for libraries that contain 70–99% clones exhibiting less than wild-type activity (*see* **Note 8**).

4. Notes

1. Each of these should yield a library mutated at a different rate, although the authors do not expect a linear relationship between Mn concentration and mutation rate ***(21)***.
2. If PCR in **step 2** fails, try the different PCR buffers in the PCR Optimizer kit in conjunction with 0 and 0.625 µL 10X mutagenic addition buffer. PCRs of longer templates (> 2 kb) generally do not amplify at higher concentrations of Mn, but these should not be mutated at such high rates.
3. *Taq* polymerase interferes with cloning by filling in restriction digested DNA. It binds tightly to the ends of PCR products, and cannot be eliminated through silica chromatography alone ***(22)***.
4. The Wizard PCR prep kit (Promega) and the Geneclean DNA purification kit (Bio101, Vista, CA) should also work.
5. In order to reduce the background of colonies transformed with uncut vector, the authors prefer long double digestions of small amounts of vector, followed by dephosphorylation using the thermolabile shrimp alkaline phosphatase.
6. 20 fmol of a 1-kb fragment of DNA is 13 ng ***(19)***.
7. If the library size is smaller than the throughput of the screen, try electroporation: Denature T4 DNA ligase by heating reaction for 65°C for 10 min. Remove salt, as follows. Add 30 µL H_2O and 500 µL butanol to each ligation. Vortex, and precipitate in microcentrifuge (13,000*g*) for 10 min. Carefully remove supernatant, air-dry pellet, and resuspend in 5 µL H_2O ***(23)***. Electroporate each ligation into *E. coli* ***(24)***. Plate small portions of each transformation (100 µL, diluted 1/10, 1/100, 1/1000) onto LB agar AMP/KM plates.

8. The authors conservatively consider any colony less green than the wild-type control to be inactivated. Most random mutations, particularly transitions, will be silent or selectively neutral ***(25)***. The actual numbers of mutations/allele will follow a Poisson distribution ***(4)***. If a library were made so that each clone contained an average of one deleterious mutation, 36.8% of the clones would be phenotypically wild-type. The authors' experience so far is that libraries containing 70–99% inactivated clones will yield winners for screens of 1000–10,000 clones. Higher mutation frequencies may be advantageous for higher-throughput screens and selections (>10^6) ***(26)***.

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SECOND EDITION

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ISBN 0-89603-910-2
90000
9 780896 039100

Methods in Molecular Biology™ • 182
IN VITRO MUTAGENESIS PROTOCOLS
SECOND EDITION
ISBN: 0-89603-910-2

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