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Haitao Guo
Andrea Cuconati
Editor

Hepatitis B Virus

Methods and Protocols

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Hepatitis B Virus

Methods and Protocols

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Cover Image: HBV infection in HepG2 cells reconstituted with the viral receptor NTCP (HepG2-NTCP). HepG2-NTCP cells were infected with HBV, on day 7 post infection, the core antigen of HBV (HBc) was stained with an anti-HBc monoclonal antibody (1C10) in green, HBc antigen is distributed both in cell nuclei and cytoplasm. Cell nuclei were stained with DAPI in blue. (modified from Figure 1 in Chapter 1)

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Preface

By any measure, hepatitis B is one of the world's most important infectious diseases, by which up to one third of the world's population may have been initially infected, with up to 400 million still suffering a chronic infection. The causative agent is hepatitis B virus (HBV), a virus that straddles the line between DNA and RNA viruses, with a DNA genome that replicates by reverse transcription. HBV and its relations in the family *hepadnaviridae* are solely liver tropic viruses and infect and replicate only in hepatocytes. The infectious virion particles contain a partially double-stranded, polymerase-linked circular DNA (termed relaxed circular, or rcDNA) molecule that is converted to an episomal covalently closed circular (ccc) DNA in the nucleus of the infected cell. This cccDNA genome is the "real," persistent virus genetic material, existing in multiple copies as extrachromosomal DNA that is continually transcribed during active infection into five mRNA species for the viral gene products; the longest form, namely pregenomic RNA (pgRNA), is a greater-than-genome length transcript that is encapsidated in the cytoplasm and then reverse transcribed into rcDNA by a complex process that includes the viral polymerase acting as a primer, the core protein, and host heat shock proteins. This "nucleocapsid" can then be enveloped by the three viral glycoproteins and secreted from the cell, or can be recycled to the nucleus to amplify the pool of cccDNA. This greatly simplified description of the intracellular life cycle does not capture many interesting aspects of HBV biology that appear to be important for the maintenance and propagation of the infection in a host, including mechanisms for modulating the host immune response. For example, the three envelope proteins, collectively known as hepatitis B surface antigen (HBsAg), are present in the serum to very high levels, a state that is thought to induce immunotolerance by HBsAg's possible effect in T-cell exhaustion, the titering out of antibodies, and so on. A secreted variant of the core protein, e antigen (HBeAg), which is detectable in many patients and correlates with a poorer prognosis, is also implicated in immune modulation. Even the core protein, X protein, and the polymerase have been reported to have activity in regulating innate immune signaling pathways and antigen. On the treatment front, the currently approved options for patients are limited to reverse transcription inhibitors (specifically, nucleoside/nucleotide analogues) and two forms of alpha interferon. There is much room for novel drug development and improvement of treatments.

Technically, the study of HBV has presented challenges that endure since its discovery in the 1960s. Even as the biology of many other viral species has systemically been unraveled, in some cases leading to effective therapies and even cures, the hepadnaviridae have stubbornly hung on to many of their secrets. Interspersed with many breakthroughs that have given us a good understanding of a complex life cycle, the details on many aspects of its life cycle and the disease it causes await elucidation. We still have an incomplete understanding of how the immune system of the host is affected to permit a chronic infection; the specifics of how the virus enters cells even after the discovery of the viral receptor; and most intriguingly, how the partially double-stranded relaxed circular genome is converted to cccDNA. The efforts to answer these questions have been hampered by the technical difficulties of studying this virus and the lack of truly robust, tractable systems that reproduce the full infection cycle in vitro and the most important immunological features of the disease process in vivo. Not surprisingly, the pace of discovery of new drugs and therapies has also suffered.

Nevertheless, recent technical progress in the field has been considerable, and this volume will hopefully serve as a reference for the dissemination of these advances. The authors' contributions span the gamut of the field, detailing protocols and techniques ranging from cell culture studies to in vivo and clinical immunology. Laboratory techniques for classical virology and genetic studies include thorough treatments of in vitro infection systems from the Li, Glebe, and Urban groups; analysis and quantification of cccDNA and its mutations from the Arbuthnot, Protzer, and Zhang groups; in vitro polymerase activity assays from the Hu and Tavis groups; the study of cellular trafficking of core protein from the Kann and Shih groups; effects on intracellular calcium metabolism by the Bouchard lab; detection, cloning, and sequencing of HBV markers in laboratory-generated and clinical samples by Dandri, Huang, Jilbert, Weiland, and Tong groups; new strategies aimed at exploiting novel mechanisms for drug discovery by Tavis and Arbuthnot groups; novel and already established animal and in vivo-derived models detailed by the groups of Chen, Lu, Menne, Ou, and Su; and methods contributed by the Robek lab for the study of T-cells in HBV mouse models. Finally the editors have also submitted chapters on the classic method for resolution of extracellular viral particles by native gel electrophoresis (Guo) and on the microtiter assay methods for detection of HBV antigens in drug discovery and other applications (Cuconati).

This project was made possible primarily by the very kind and patient cooperation of the chapter authors, and we thank them in earnest. We want to especially thank the senior series editor Dr. John Walker for the invitation to assemble this volume and his constructive guidance and support. A special thanks also goes out to Mr. David Casey for his excellent technical support. We believe the effort was very worthwhile and important to the advancement of this field, and we hope the readers will agree.

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Haitao Guo
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Chapter 1

NTCP-Reconstituted In Vitro HBV Infection System

Yinyan Sun, Yonghe Qi, Bo Peng, and Wenhui Li

Abstract

Sodium taurocholate cotransporting polypeptide (NTCP) has been identified as a functional receptor for hepatitis B virus (HBV). Expressing human NTCP in human hepatoma HepG2 cells (HepG2-NTCP) renders these cells susceptible for HBV infection. The HepG2-NTCP stably transfected cell line provides a much-needed and easily accessible platform for studying the virus. HepG2-NTCP cells could also be used to identify chemicals targeting key steps of the virus life cycle including HBV covalent closed circular (ccc) DNA, and enable the development of novel antivirals against the infection.

Many factors may contribute to the efficiency of HBV infection on HepG2-NTCP cells, with clonal differences among cell line isolates, the source of viral inoculum, and infection medium among the most critical ones. Here, we provide detailed protocols for efficient HBV infection of HepG2-NTCP cells in culture; generation and selection of single cell clones of HepG2-NTCP; production of infectious HBV virion stock through DNA transfection of recombinant plasmid that enables studying primary clinical HBV isolates; and assessing the infection with immunostaining of HBV antigens and Southern blot analysis of HBV cccDNA.

Key words HBV, NTCP, Cell culture, Viral infection, Receptor, cccDNA, Antivirals

1 Introduction

Human hepatitis B virus can infect primary human hepatocytes (PHHs) [1], primary Tupaia hepatocytes (PTHs) [2], and differentiated HepaRG cells [3]. Studies of HBV infection on these cell culture systems have greatly contributed to our understanding of the virus; however, the use of these culture systems has several limitations. PHH and PTH are difficult to obtain, they retain susceptibility to HBV infection only for a few days in culture, and exhibit high variability from different sources. HepaRG cells are a mixed population from liver progenitor cells, and they require 2 weeks of differentiation to become susceptible to HBV infection. Human hepatoma HepG2 derived cell lines such as HepAD38 [4], HepG2.2.15 [5], HepDE19 [6] are useful in studying HBV replication, but they are not a genuine infection system. The identification of NTCP as a bona fide receptor for HBV and its satellite

hepatitis D virus (HDV) significantly advanced our understanding of the viral infections [7, 8]. Importantly, HepG2 cells expressing NTCP (HepG2-NTCP) are susceptible to HBV and HDV infection, thus opening a new avenue for various studies from basic virology to drug development against HBV/HDV using the de novo HBV infection system. Here, we describe the methods for conducting HBV infection experiments with HepG2-NTCP cells. We provide detailed protocols for generating HepG2-NTCP single cell clone; producing HBV virion from HepDE19 cell line or through DNA transfection of recombinant plasmid harboring $1.05 \times$ viral DNA genome that enables studying primary clinical HBV isolates; assessing the infection with immunostaining of HBV antigens; and quantification of HBV-specific RNAs and analysis of HBV cccDNA using quantitative PCR (qPCR) and Southern blot. Some of the procedures may be adapted to or further developed for high-throughput screening purposes.

2 Materials

2.1 Cell Lines

Human hepatocellular carcinoma cell line HepG2 (ATCC HB-8065) [9]; Human hepatocellular carcinoma cell lines Huh-7 (JCRB0403) [10]; HepG2-NTCP stable cells (*see* below).

2.2 Medium for Regular Cell Cultures

1. Dulbecco's Modified Eagle Medium (DMEM).
2. Williams E medium.
3. DMEM/F-12.
4. DMEM/10% FBS/PS.
Add 50 mL fetal bovine serum (FBS) to 450 mL DMEM. Add 5 ml penicillin streptomycin (PS, 100 \times). Store at 4 °C.
5. DMEM/10% FBS/PS/G418.
Add 250 μ L 100 g/mL G418 to 50 mL DMEM/10% FBS/PS to a final concentration of 500 μ g/mL. Store at 4 °C.
6. Freezing buffer.
DMEM, 20% FBS, 10% dimethyl sulfoxide (DMSO).
Add 1 mL FBS and 1 mL DMSO to 8 mL DMEM/10% FBS.
7. DMEM/F-12, HEPES, 10% FBS/PS/G418/Dox.
Add 50 mL FBS, 5 mL PS stock solution (100 \times) and 2.5 mL 100 g/mL G418, 500 μ L 1 g/mL Dox to 450 mL DMEM/F-12.
8. DMEM/F-12, HEPES, 10% tet-free FBS, PS, G418.
Add 50 mL tet-free FBS, 5 mL PS stock solution (100 \times), and 2.5 mL 100 g/mL G418, to 450 mL DMEM/F-12.

2.3 Medium for HBV Infection

Culture medium composition has major impact on HBV infection efficiency in HepG2-NTCP cells. The medium we use for HBV infection is based on hepatocytes maintenance medium (PMM), containing 2% DMSO (*see Note 1*), EGF, and other components [7].

Stock Solutions

1. Dimethyl sulfoxide (DMSO), cell culture grade.
2. Transferrin: 5 mg/mL transferrin in Williams E medium. Store at -80°C aliquots.
3. Hydrocortisone: 18 mg/mL hydrocortisone in DMSO, 1000 \times . Store at -80°C aliquots.
4. Dexamethasone: 40 $\mu\text{g}/\text{mL}$ dexamethasone in DMSO, 1000 \times . Store at -80°C aliquots.
5. Sodium selenite: 5 $\mu\text{g}/\text{mL}$ sodium selenite in sterile ultrapure water. Store at -40°C aliquots (*see Note 2*).
6. Epidermal growth factor (EGF): 10 $\mu\text{g}/\text{mL}$ EGF in Williams E medium. Store at -80°C aliquots.
7. Insulin-Transferrin-Selenium (ITS-G) (100 \times): Formulation: 0.67 $\mu\text{g}/\text{mL}$ Sodium selenite, 1 mg/mL Insulin, and 0.55 mg/mL Transferrin (Life Technology).
8. Penicillin streptomycin (PS), 100 \times .
9. GlutaMAXTM Supplement, 100 \times .

Composition of PMM:

Williams E medium with: 5 $\mu\text{g}/\text{mL}$ transferrin, 10 ng/mL EGF, 3 $\mu\text{g}/\text{mL}$ insulin, 2 mM L-glutamine, 18 $\mu\text{g}/\text{mL}$ hydrocortisone, 40 ng/mL dexamethasone, 5 ng/mL sodium selenite, 2% DMSO, 100 U/mL penicillin and 2 mM L-alanyle-L-glutamine. Add 335 μL transferrin stock solution (5 mg/mL), 500 μL hydrocortisone stock solution (18 mg/mL), 500 μL dexamethasone stock solution (40 $\mu\text{g}/\text{mL}$), 299 μL sodium selenite stock solution (5 $\mu\text{g}/\text{mL}$), 500 μL EGF stock solution (10 $\mu\text{g}/\text{mL}$), 1.5 mL ITS-G, 5 mL PS stock solution (100 \times), 5 mL GlutaMAX stock solution (100 \times), 9 mL DMSO to 477 mL Williams E medium (*see Note 3*).

2.4 HBV Inoculum for In Vitro Infection

1. Recombinant HBV obtained from transfection of Huh-7 cells with 1.05 viral genome DNA (*see below*).
2. HepDE19 produced virus (*see below*).
3. HBV patients' sera (*see Note 4*).

2.5 Immunoassay Kits for Assessing HBsAg and HBeAg

ELISA or other immunoassay kits for HBsAg and HBeAg (from various vendors).

2.6 Immuno fluorescence Staining of Cells Infected by HBV

1. 10× PBS (pH 7.4): 80 g NaCl, 2.5 g KCl, 14.3 g Na₂HPO₄, 2.5 g KH₂PO₄. Working concentration 1× PBS.
2. 3.7% Formaldehyde: add 3.7 g formaldehyde in PBS to final 100 mL, store at -20 °C aliquots in 10 mL.
3. 0.5% Triton X100 in PBS, store at 4 °C.
4. 3.0% BSA in PBS, sterile by filtration.
5. Mounting medium, 1:1 glycerol: PBS, 0.1–0.5% N-propyl gallate, 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI), store at -20 °C.
6. Primary antibodies against HBV antigens (HHAJ) [7, 11]: mouse monoclonal antibody (mAb) against HBV core antigen (HBcAg), 1C10, 1 mg/mL; mouse mAb specific to HBV preS1 protein 2D3, 1 mg/mL; mouse mAb specific to HBV preS2 protein, 1F4, 1 mg/mL; mouse mAb specific to HBV S protein (HBsAg), 17B9, 1 mg/mL. Other specific antibodies can also be used. Secondary Antibody, Alexa Fluor 488 conjugated Goat anti-mice IgG.

2.7 Quantification of Viral RNA by qRT-PCR

1. TRIzol® reagent (Life Technology).
2. Reverse Transcriptase M-MLV (RNase H-).
3. SYBR Premix ExTaq™ Perfect Real Time (TaKaRa).
4. Primers: for HBV 3.5 kb transcripts, HBV2268F: 5'-GAGTGTGGATTTCGCACTCC-3', HBV2372R:5'-GAGGCGAGGGAGTTCTTCT-3'; For total HBV transcripts: HBV1803F: 5'-TCACCAGCACCATGCAAC-3', HBV1872R: 5'-AAGCCACCCAAGGCACAG-3'.

2.8 Analysis of HBV cccDNA by qPCR or Southern Blot

1. Cell lysis buffer: 20 mM Tris-HCl, 0.4 M NaCl, 5 mM EDTA, 1% SDS, pH 8.0, store at room temperature.
2. Proteinase K.
3. Phenol/chloroform/isoamylalcohol (25:24:1), pH 8.0.
4. Phenol/chloroform (25:24).
5. 70% ethanol.
6. Isopropanol.
7. TE buffer: 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA (0.5 M stock, pH 8.0).
8. **Plasmid-Safe™ ATP-Dependent DNase** (Epicentre Technologies) or T5 exonuclease (NEB).
9. SYBR Premix Ex Taq (Ti RNase H Plus) (TaKaRa).

10. HBV cccDNA specific primers [7, 12] forward: 5'-TGC ACTTCGCTTCACCT-3' and reverse: 5'-AGGGGCA TTTGGTGGTC-3'.
11. Hirt lysis buffer: 10 mM Tris-HCl, 10 mM EDTA, 0.6% SDS, pH 8.0.
12. Phenol, saturated with 10 mM Tris-HCl (pH 8.0).
13. 5 M NaCl.
14. *Hind*III and *Eco*RI enzyme.
15. 1×TAE buffer: 0.04 M Tris-HCl base, 0.04 M glacial acetic acid, 1 mM EDTA, pH 8.2–8.4. Prepare 50×stock solution, store at room temperature.
16. Depurinating solution: 0.2 N HCl.
17. Denaturing buffer: 0.5 N NaOH, 1.5 M NaCl.
18. Neutralization buffer: 1.5 M NaCl, 1 M Tris-HCl, pH 7.4.
19. 20×SSC: 3 M NaCl, 0.3 M sodium citrate.
20. Wash buffer: 0.1% SDS, 0.1×SSC.
21. Amersham Hybond™ -N+ membrane (GE Healthcare).
22. Whatman 3 MM chromatography paper.
23. EZ-DNA Extract kit.
24. pGEMT-HBV-D plasmid: one copy of full-length HBV-D type genome.
25. [α -32P]dCTP (250 μ Ci, NEG513H, Perkin Elmer).
26. PerfectHyb™ plus hybridization buffer (Sigma).
27. Random primer DNA labeling kit.
28. Carestream X-OMAT BT Film.

3 Methods

All procedures involving infectious HBV should be carried out in a BSL-2 facility and follow the national and institutional guidelines of handling infectious materials. Collagen I coated plates/dishes should be used for culturing HepG2 and its derivative clones.

3.1 Establish HepG2-NTCP Cell Line

1. Clone human NTCP coding sequence into an expressing vector (e.g., pcDNA3.1) to generate an NTCP expression plasmid.
2. Grow HepG2 cells in DMEM/10% FBS at 37 °C CO₂ incubator. Split the cells to a 10-cm plate 16 h before transfection, the cell density should reach 50% at the time of DNA transfection.
Transfect HepG2 cells with 15 μ g NTCP expression plasmid using Lipofectamine®2000, change the medium to DMEM/10% FBS after 6 h.

3. Split the cells (1:5) to five 10-cm plates 48 h after transfection, and grow the cells in DMEM/10% FBS/PS/G418, and change the cell medium every 2 days until single clones are easily visible (*see Note 5*).
4. Pick at least 50 single clones and transfer them individually to a 48-well plate by digestion with trypsin in cloning cylinders. Alternatively, stain the cell clones with an NTCP antibody and sort single cells with high NTCP expression level into the 48-well plate using a FACS sorter. Change the culture medium every 2 days till cells reach 100% confluence, grow the single clones in six-well plates, and freeze one aliquot in nitrogen liquid with freezing buffer.

3.2 Produce Recombinant HBV by DNA Transfection in Huh-7 Cells [7]

1. Clone 1.05× of HBV genome to a cloning plasmid, for HBV genotype D type virus, include nt1809 to 3182 and 1 to 1977 fragment. For HBV genotype B/C type virus, clone nt1809 to 3215 and 1 to 1990 fragment to a plasmid to generate HBV 1.05× viral DNA genome.
2. Grow Huh-7 cells with DMEM/10% FBS in an incubator with 5% CO₂ at 37 °C, and split cells to 25-cm plates 24 h before transfection, the cells should reach 90% confluence before DNA transfection.
3. Transfect 30 µg HBV plasmid with 60 µL Lipofectamine®2000 to a 25-cm plate, incubate at RT for 15 min, then add to Huh-7 cells drop by drop, gently shake the plate, add total 15 mL cell medium.
4. Change the medium to 25 mL PMM 5 h after transfection, and continue incubating the cells in 5% CO₂ incubator at 37 °C. Collect culture medium in 50 mL conical centrifuge tubes, and spin the supernatant at 2000×g for 15 min, transfer upper supernatant to new tubes and aliquot in 2 mL, store at -80 °C.

3.3 Produce HBV Using HepDE19 Cell Line [6]

1. Grow HepDE19 cells in a 10-cm plate with DMEM/F-12/PS/G418/Dox, and allow the cells to propagate for three to four generations.
2. Change the medium to DMEM/F-12 containing HEPES, 10% tet-free FBS, PS, and G418.
3. Collect cell supernatant every 2 days, replenish with fresh medium.
4. Measure HBV DNA copies in supernatant with primer HBV2268F/HBV2372R by qPCR (*see below*).
5. Pool the supernatant with HBV DNA copies over 1 × 10⁵/mL, add 25% sterile PEG8000 to a final concentration of 10%, keep the mixture on ice for 1 h.

6. Centrifuge the tubes at $2000 \times g$ at $4\text{ }^{\circ}\text{C}$ for 30 min, resuspend the pellet in 1/2 volume of PMM, and store at $-80\text{ }^{\circ}\text{C}$ aliquots.

3.4 Select HepG2-NTCP Cell Line with High Susceptibility to HBV Infection

1. Seed HepG2-NTCP single clones on to wells of 48-well plate (60,000 cells/well) using 200 μL DMEM/10% FBS/PS/G418.
2. Change the culture medium with 200 μL PMM 3 h after cell seeding.
3. Prepare virus for infection. Mix 2 mL Huh-7 produced virus with 500 μL 25% PEG8000. Add 200 μL virus mixture to each well, shake the plate at 350 rpm in 10 s intervals for 4 h at RT. Then move the plate to a 5% CO_2 incubator and incubate at $37\text{ }^{\circ}\text{C}$ for 16–24 h.
4. Discard the virus mixture and wash the cells twice with 200 μL DMEM and then change the medium to PMM, and put the plate back into the incubator (*see Note 6*).
5. Collect supernatant at 3, 5, 7 days postinfection.

Assess HBV infection using ELISA (or other immunoassay kits) for HBsAg and HBeAg and immunofluorescence staining (*see below*), and select cell clones with high HBV infection efficiency for future experiments. We selected a clone named HepG2-NTCP (AC12) for infection studies.

3.5 Testing of Secreted Viral Antigens of HBV

Collect supernatant samples in 1.5 mL tubes from HepG2-NTCP cultures at 3, 5, 7 days postinfection, spin at 3000 rpm for 5 min at a bench-top centrifuge, and transfer appropriate volume of the supernatant to a testing plate. Commercial kits for determining the level of HBsAg and HBeAg are readily available. Depending on the purpose of the experiment and budget, ELISA or other assays can be used. Testing HBsAg and HBeAg levels in supernatant offers a convenient way to assess HBV infection and is recommended as the first line assay.

1. Test HBeAg, following the manual of commercial kit.
2. Test HBsAg, following the manual of commercial kit.

3.6 Examination of HBV Infection by Immunofluorescence Staining

1. Wash cells with 200 μL $1 \times$ PBS twice on day 7 postinfection, add 200 μL 3.7% formaldehyde, and incubate at RT for 10 min.
2. Discard formaldehyde and wash the cells with 200 μL $1 \times$ PBS once, permeabilize cells with 150 μL 0.5% Triton X100 for 10 min.
3. Wash cells with 200 μL $1 \times$ PBS once and incubated with 3% BSA for 1 h.

4. Dilute anti-HBc, anti-preS1, anti-preS2, or anti-HBsAg monoclonal antibody (1C10, 2D3, 1F4 or 17B9, or other specific mAbs) with 1% BSA to 5 $\mu\text{g}/\text{mL}$, and add 150 μL to the cells, incubate at 37 $^{\circ}\text{C}$ for 1 h.
5. Wash the wells with 1 \times PBS for three times and add secondary antibody (2 $\mu\text{g}/\text{mL}$ of Alexa Fluor conjugated goat anti-mouse IgG, or other secondary antibody). Capture the images with fluorescence microscope or confocal (Fig. 1).

Immunofluorescence staining helps to estimate HBV infection rate on HepG2-NTCP cells, and can be used in high-content imaging analysis. Typical images of HBV core, preS1, preS2, and S staining of HBV infected HepG2-NTCP (AC12) are illustrated in Fig. 1.

3.7 Quantification of HBV-Specific RNAs

1. Wash the cells with 200 μL 1 \times PBS once on day 5 postinfection, add 200 μL Trizol reagent, and extract total RNA following the manual (RNA can also be extracted with a column based assay).
2. Digest 500 ng total RNA with 0.5 U DNAase I (Amp Grad) in 10 μL reaction, incubate at RT for 15 min, then add 0.8 μL 2.5 mM EDTA to the reaction, and heat for 10 min at 65 $^{\circ}\text{C}$ to stop the reaction.
3. Add 3 μL 5 \times Premix script buffer, 0.75 μL primer script RT mix, 0.75 μL 60 μM random primer, 0.5 μL reverse transcriptase to the above reaction, and incubate the mixture at 37 $^{\circ}\text{C}$ for 15 min, and heat at 85 $^{\circ}\text{C}$ 5 s to inactivate the reverse transcriptase.
4. Use cDNA derived from 20 ng total RNA as template for real-time qPCR amplification.
5. In a separate real-time qPCR reaction, add 20 ng of total RNA without reverse transcription as template to assess possible HBV viral DNA contamination in the RNA preparation. Real-time qPCR for HBV 3.5 kb and total HBV-specific transcripts are both conducted by denaturation at 95 $^{\circ}\text{C}$ for 30 s, followed by 40 cycles of 95 $^{\circ}\text{C}$ denaturation for 3 s, and 60 $^{\circ}\text{C}$ annealing/elongation for 30 s. Real-time qPCR is performed using SYBR Premix Ex Taq kit on an ABI Fast 7500 real-time system instrument. HBV RNA copy numbers can be estimated from a standard curve generated from diluted plasmid including HBV DNA sequence.

3.8 Examining HBV cccDNA

3.8.1 Quantification of HBV cccDNA by qRT-PCR

1. Wash HepG2-NTCP cells in a 48-well plate with 200 μL PBS once on day 7 postinfection.
2. Add 200 μL cell lysis buffer into the cells, gently mix several times, transfer the lysate into 0.5 mL Eppendorf tube and supply proteinase K (200 $\mu\text{g}/\text{mL}$), and then incubate for 4 h at 56 $^{\circ}\text{C}$.

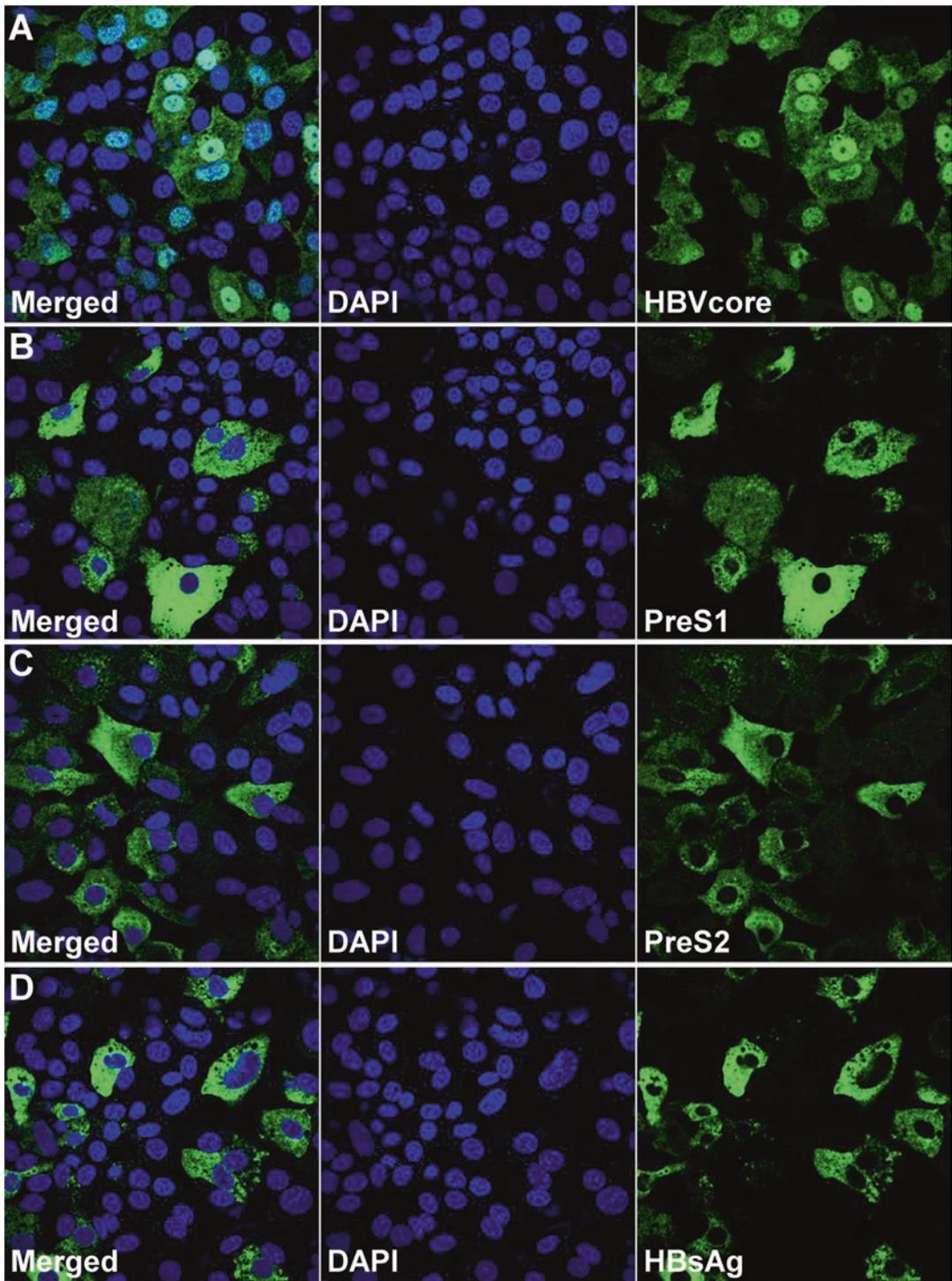


Fig. 1 HBV infection of HepG2-NTCP cells. HepG2-NTCP (AC12) cells were infected with HBV (genotype D) at an mge (multiplicities of genome equivalents) of 100 in the presence of 5% PEG8000, cells were fixed with 3.7% paraformaldehyde for 10 min on day 7 postinfection and permeabilized with 0.5% Triton X100 for 10 min, and then were stained with 1C10 (a: HBV core), 2D3 (b: PreS1), 1F4 (c: PreS2), or 17B9 (d: HBsAg) followed by Alex 488 conjugated rabbit anti-mice IgG

3. Isolate total DNA according to a standard genomic DNA isolation procedure. Briefly, add 200 μL Phenol/chloroform/isoamylalcohol, mix thoroughly by hand shaking for 10 s. Centrifuge at $8000\times g$ for 10 min at 4 $^{\circ}\text{C}$, and transfer the aqueous phase to a fresh 0.5 mL Eppendorf tube.
4. Add 200 μL Phenol/chloroform, mix thoroughly by hand shaking for 10 s. Centrifuge at $8000\times g$ for 10 min at 4 $^{\circ}\text{C}$, and transfer the aqueous phase to a fresh 0.5 mL Eppendorf tube.
5. Add an equal volume of isopropanol (approx. 200 μL) and mix thoroughly by inverting the tube several times. Incubate at -80°C for 2 h to precipitate DNA.
6. Centrifuge the tube at 10,000 rpm for 20 min at 4 $^{\circ}\text{C}$ and discard the supernatant. Add 400 μL 70% ethanol to wash the DNA pellet. Centrifuge at $6000\times g$ for 5 min at 4 $^{\circ}\text{C}$.
7. Discard the supernatant. Allow the pellet to air dry for about 10 min at room temperature. Dissolve the DNA pellet in 20 μL TE buffer.
8. Digest 500 ng of the extracted DNA with 0.5 μL plasmid-safeTM ATP-dependent DNase (PSAD) in 25 μL reaction for 8 h at 37 $^{\circ}\text{C}$ to allow removal of linear genomic DNA and HBV replication intermediates (rcDNAs, single-stand DNAs, linear double-strand DNAs). Inactivate DNase by incubating the reactions for 30 min at 70 $^{\circ}\text{C}$ (*see Note 7*).
9. Take 2 μL of the digested DNA to quantify HBV cccDNA. Perform the real-time qPCR using the SYBR Premix Ex Taq on Applied Biosystems 7500 Fast Real-Time qPCR System as the following reaction procedure: 95 $^{\circ}\text{C}$ for 5 min then 45 cycles of 95 $^{\circ}\text{C}$ for 30 s, 62 $^{\circ}\text{C}$ for 25 s, and 72 $^{\circ}\text{C}$ for 30 s. HBV cccDNA copy numbers can be calculated with a standard curve from pGEMT-HBV-D plasmid with known nucleic acid quantities.

3.8.2 Detection of HBV cccDNA from HBV Infected HepG2-NTCP Cells Using Southern Blot

1. Seed HepG2-NTCP cells in a collagen-coated six-well plate at approximately 90% confluence with DMEM complete medium supplemented with 500 $\mu\text{g}/\text{mL}$ G418 (*see Note 8*). Perform HBV infection assay as described above.
2. On day 7 postinfection, wash the cells with 2 mL PBS once and selectively extract HBV cccDNA by a modified Hirt method as previously described [13, 14]. Dissolve the extracted DNA pellet in TE buffer, and then digest the DNA sample with *Hind*III or *Eco*RI restriction enzyme for 2 h before analysis.
3. Prepare 3.2, 2.1, and 1.7 kb HBV DNA markers by PCR amplification of pGEMT-HBV-D plasmid containing 1.0 \times

HBV-D genome with appropriate primers, purify the PCR product using a DNA gel extraction kit.

4. Separate the DNA samples and 100 pg of each HBV DNA marker by electrophoresis through 1.2% agarose gel at 80 V for 3 h in 1× TAE buffer (*see Note 9*).
5. After electrophoresis is completed, add freshly prepared 0.2 N HCl and gently shake for 10 min at room temperature to depurinate the DNA samples. Rinse the gel with deionized water and add denaturing buffer. Gently shake for 1 h at room temperature. Rinse the gel with deionized water and then neutralize it in neutralization buffer by gently shaking for at least 1 h at RT.
6. Transfer DNA from the gel to Hybond™-N+ membrane with 20× SSC for 24 h by the capillary transfer method [15].
7. After gel transfer, fix the DNA to the Hybond™-N+ membrane by UV crosslinking at 1200 mJ for 1 min. Wash the membrane briefly in deionized water and allow to air-dry. Use the membrane immediately for hybridization, or store at 4 °C.
8. Prepare HBV DNA probes using random primed labeling method to incorporate [α -³²P] dCTP into 3.2 kb HBV DNA fragment by Klenow enzyme following manufacturer's instructions. Denature probes at 95 °C for 3 min and then cool on ice. Directly subject the labeled probes to hybridization, or store at -20 °C (*see Note 10*).
9. Place the crosslinked membrane in a hybridization tube to perform prehybridization in 5 mL PerfectHyb™ plus hybridization buffer for 1 h at 65 °C and then overnight hybridization in 5 mL fresh hybridization buffer containing 25 μ L HBV DNA probes at 65 °C. After washing twice in wash buffer at 65 °C, place the membrane with the DNA-binding side facing up on a cassette. Expose it to films for 24 h in the dark (*see Note 11*). A typical image of HBV cccDNA southern blot is illustrated in Fig. 2.

3.9 Infection of HepG2-NTCP Cell with Patient-Derived Viruses

1. Seed HepG2-NTCP cells on a 48-well plate at a density of ~60,000 cells/well, and change the medium to PMM after cells adhere to the plate (usually in 2–3 h).
2. Add 5 μ L patient serum to 200 μ L 5% PEG/PMM, mix well, and add to the cells on the plates.
3. Examine HBV infection at day 5–7 after the inoculation.

4 Notes

1. Cell culture grade DMSO should be used in PMM buffer. Including 2% DMSO in PMM is important for HBV cell infection and virus production in Huh-7 cells.

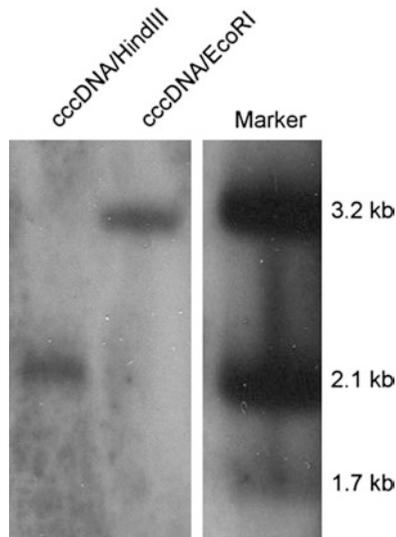


Fig. 2 Southern blot analysis of cccDNA from HBV infected HepG2-NTCP cells. HBV cccDNA is extracted from infected HepG2-NTCP (AC12) cells by Hirt method and analyzed by southern blot. The 3.2 kb HBV cccDNA migrates as 2.1 kb linear DNA, *HindIII* digestion did not alter its migration as there is no *HindIII* cutting site in the viral genome; the viral genome has a unique *EcoRI* cutting site and digestion with the enzyme linearizes cccDNA, it then runs as 3.2 kb genome-length double stranded DNA. Marker: HBV DNA marker, the size of each HBV DNA species is labeled on the right

2. Prepare sodium selenite stock solution at 5–10 g/mL, diluted with PBS or H₂O to 5 µg/mL, and store aliquots in –40 °C.
3. Prepare 500 mL PMM, store at 4 °C, the medium is stable for 2 months.
4. Measure HBV DNA copies in the samples by qPCR. Sera with HBV DNA copies less than 10⁷/mL may not infect cells efficiently without ultra-centrifugation. Not all HBV sera can yield appreciable infection on HepG2-NTCP cells by direct inoculation.
5. This is a key step to set up the stable cell lines. Test the transfection efficiency, if over 50% cells were successfully transfected, then split cells to no less than ten 10-cm plates, and change the cell culture every 2 days; otherwise, it will be difficult to select single clones at the end. This step will take about 2–3 weeks.
6. To remove residual HBsAg/HBeAg, infected cells must be washed thoroughly.
7. Alternatively, the extracted DNA can also be digested using T5 exonuclease.
8. Do not seed more or less cells than the recommended density, as doing so may reduce infection efficiency.

9. Be sure that a positive control (DNA markers containing HBV genome) is included on the gel.
10. Steps involving radioactive isotopes must be conducted in a designated place or room and follow institutional guidelines for handling radioactive materials.
11. Multiple exposures should be taken to achieve the desired signal strength.

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Hepatitis B Virus Infection of HepaRG Cells, HepaRG-hNTCP Cells, and Primary Human Hepatocytes

Yi Ni and Stephan Urban

Abstract

Investigations of virus-host interactions rely on suitable in vitro cell culture systems that efficiently support virus infection. Such systems should ideally provide conditions that resemble those of natural host cells, e.g., the cell-type specific signaling and metabolic pathways. For HBV infection, primary human hepatocytes (PHHs) are the most faithful system fulfilling these requirements but access to these cells is limited. Moreover, the reproducibility of experimental results depends on many factors including the preparation method or variability of the donors. The human liver cell line HepaRG, after differentiation, resembles PHHs with respect to many hepatocyte-specific markers including the expression of cytochrome P450 enzymes, liver-specific transcription factors, and transporter proteins like the HBV-specific receptor, sodium taurocholate co-transporting polypeptide (NTCP). HepaRG cells have also been shown to express key molecules of the innate immune system. So far, the HepaRG cell line is the only one allowing both studies on HBV/HDV infection and liver-specific drug toxicity and metabolism. The relative low susceptibility of HepaRG cells when compared with PHHs depends on various factors and can partially be overcome by constitutive expression of the receptor NTCP, allowing infection without full differentiation. Ectopic NTCP expression does not interfere with the ability of cell differentiation induced by DMSO. Here, we describe in detail how to technically perform HBV infection in vitro with these cells. The methods can be used to explore the mechanism of HBV infection and to build an antiviral screening platform suitable for evaluation of drug efficacy in cells that are metabolically close to primary human hepatocytes.

Key words HepaRG, NTCP, PHH, HBV infection, Authentic infection

1 Introduction

Human Hepatitis B virus (HBV) belongs to the family *Hepadnaviridae*, which comprises two genera: the genus *Orthohepadnavirus* infecting mammals and the *Avihepadnaviruses* infecting birds. Members of the *Orthohepadnaviruses* including WHV (Woodchuck Hepatitis virus) [1], GSHV (Ground squirrel hepatitis virus) [2], WMHBV (woolly monkey hepatitis B virus) [3], and HBV can efficiently replicate in the liver of their respective hosts showing a pronounced species specificity. This restriction is mostly determined by the differences in their envelope proteins

and recognition of receptors. Although in vitro cultured primary human hepatocytes (PHHs) had been successfully infected with HBV already in 1986 [4], the use of these cells for systematic studies became practical only after optimization of the cell culture conditions, namely the implementation of DMSO to the culture medium and the addition of polyethylene glycol (PEG) to increase the efficacy of infection [5, 6]. PHHs are accepted as the gold-standard in vitro model for HBV infection, in which nearly 100% of cells are reproducibly infectable under certain conditions (e.g., increasing multiplicity of genome equivalents) [7]. Since none of the commonly used immortalized hepatic cell lines (e.g., HepG2, HuH7) supported HBV infection, PHH for a long time was the only system to study the complete HBV replication cycle. This limitation was overcome after the discovery of a human hepatoma cell line called HepaRG. This cell line in culture behaves like liver progenitor cells bearing the potential to differentiate into hepatocyte-like granular cells and biliary cells following DMSO treatment [8]. In addition to their susceptibility to HBV and Hepatitis D Virus (HDV), HepaRG cells have been intensively investigated with respect to hepatocyte-specific functions, such as albumin secretion, formation of bile canaliculi, drug transporter activities, expression of cytochrome P450 and glutathione S-transferase, and other enzymes involved in drug metabolism [9, 10]. Compared to PHHs, the unlimited availability of HepaRG cells makes it a very important tool for pharmacological and toxicological studies besides in vitro HBV and HDV infection/coinfection. Interestingly, it turns out that sodium taurocholate cotransporting polypeptide (NTCP), one of the transporters expressed on HepaRG cells solely after differentiation, is the specific receptor for HBV [11, 12].

The identification of human NTCP (hNTCP) as the *bona fide* HBV receptor profoundly changed the field of HBV infection models. The permissive but non-susceptible HepG2 cell line can be infected with HBV upon expressing hNTCP. It is now widely used for infection studies including high-throughput drug screening approaches. The endogenous hNTCP level of HepaRG cells that can be achieved through differentiation is only ca. 10% of that in PHHs [13]. This may partially explain the observation that HepaRG cells cannot be infected to a similar percentage compared to PHHs. Accordingly, when hNTCP is stably expressed in HepaRG cells [12], the HBV infection efficacy is improved. Moreover, since the differentiation process that is required for hNTCP expression in naive HepaRG cells is no longer required, HepaRG-hNTCP cells can already be infected shortly after seeding although at less efficacy than those differentiated. In comparison to HepG2-hNTCP, fully differentiated HepaRG-hNTCP secretes higher levels of HBsAg upon infection, resembling the levels obtained in HBV-infected PHHs.

The addition of DMSO to the culture medium upregulates the expression of hNTCP during differentiation of HepaRG cells and enhances HBV replication in both infected cells [6, 12] and cells that express HBV transcripts from a chromosomal integrate [14]. Thus, DMSO apparently has multiple effects on HBV infection including receptor induction in HepaRG cells but also accelerating replication at post-entry steps. Although the underlying mechanisms are not well understood, the presence of DMSO in the medium is necessary for an efficient infection.

Although inoculation with less than ten virions established chronic HBV infection in chimpanzee [15], all the cell culture-based infection models, including the most susceptible PHHs, are not able to support unlimited virus spread (under certain conditions limited spread within the culture is observed). This is probably due to the lack of microarchitecture of hepatocytes in flat monolayer culture, where progeny viruses diffuse into large volume of culture medium instead of accumulating locally in the space of Dissé. In order to get higher infection rates it is necessary to enrich virus on the two-dimensional cell monolayer surface. The addition of 4% polyethylene glycol (PEG) during the inoculation with virus is one of these measures. PEG boosts the interaction of the virus with heparansulfate proteoglycans, which is a prerequisite for subsequent engagement of the NTCP receptor [16]. However, as a consequence, the viral inoculum is firmly associated with the cells within the first days after infection, leading to a strong background signal in many assays detecting viral nucleic acid or protein. This fact should be kept in mind for data interpretation, especially when aiming at quantification of early infection markers including cccDNA.

In this chapter, we describe the methods covering preparation of virus, infection, and immunofluorescence readout to judge the infection efficacy. The principle of this method can be used for study of infection or adapted to practical screening for antiviral drugs.

2 Materials

2.1 Virus Production

2.1.1 Preparation of HBV from HepAD38 Cells

1. HepAD38 cells [17].
2. Tet-off medium: DMEM/F-12 (1:1) medium supplemented with 10% heat-inactivated and selected fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin.
3. Tet-on medium: Tet-off medium supplemented with 0.3 µg/ml tetracycline.
4. 40% PEG: dissolve 400 g PEG (MW 8000) in water to a final volume of 900 ml and autoclave it. Add 100 ml sterile 10× PBS.

5. T-175 flask with a growth area of 175 cm².
6. Falcon® cell culture multi-flasks (5-layer with a growth area of 875 cm²) or CellSTACK® cell culture chambers (5-stack with a growth area of 3180 cm²).

2.1.2 Preparation of HBV from Transiently Transfected Cells

1. HuH7 cells.
2. Plasmids harboring over-length HBV genome, such as pCHT-9/3091 [18] or P26 [19].
3. Culture medium: DMEM medium supplemented with 10% heat-inactivated selected fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin.
4. 40% PEG: as mentioned above.

2.1.3 Preparation of HBV from Patient Serum

1. Patient serum with high viral load.
2. 20% sucrose/PBS (w/v): dissolve 20 g sucrose in PBS to a final volume of 100 ml, filtrate through 0.22 µm pore size filter.
3. Ultracentrifuge with SW28 rotor (or equivalents) and suitable centrifugation tubes.

2.2 Prepare Cells for Infection Assay

1. HepaRG [8], HepaRG-hNTCP cells [12] or primary human hepatocytes (PHHs).
2. Growth medium: William E medium supplemented with 10% heat-inactivated fetal bovine serum (*see Note 1*), 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin, 50 µM hydrocortisone.
3. Differentiation medium: Growth medium supplemented with 1.5–2% DMSO (*see Note 2*).
4. Collagen solution, type I from rat tail.

2.3 HBV Infection of HepaRG, HepaRG-hNTCP, or PHH Cells

1. Prepared HBV virus.
2. Differentiation medium: as mentioned above.
3. 40% PEG: as mentioned above.

2.4 Immunofluorescence Assay (IF) to Quantify Intracellular Viral Core/L-Protein

1. 4% PFA solution: Add 4 g of paraformaldehyde to 100 ml PBS, heat it up while stirring to approximately 60 °C. Adjust the pH to 7 after it is completely dissolved.
2. Rabbit anti-core polyclonal antibody (DAKO).
3. Purified MA18/7 antibody (1 mg/ml): mouse monoclonal antibody against preS1 [20].
4. Blocking buffer: dissolve BSA in PBS to a final concentration of 2%.
5. Permeabilization buffer: freshly prepare 0.5% Triton-100 in PBS (v/v).

6. Fluorescent-labeled secondary antibodies, such as Alexa Fluor 488 goat anti-rabbit or Alexa Fluor 546 goat anti-mouse antibody (ThermoFisher).
7. Hoechst stock solution (5 mg/ml): dissolve 100 mg Hoechst in 20 ml of water, store at -20°C .
8. Fluoromount-G, if the cells are seeded in wells with cover slips.

3 Methods

3.1 Preparation of HBV Stock for Infection

3.1.1 Preparation of HBV from HepAD38 Cells

For virus preparation, two factors have to be taken into consideration: scale and convenience. The protocol below using Falcon® multi-flasks with the culture medium of 150 ml produces 2–3 ml concentrated virus stock every 3 days. It can be simply scaled up to bigger sizes such as CellSTACK® cell culture chambers in proportion to their growth area.

1. Expand HepAD38 cells in Tet-on medium by splitting them every 2–3 days to get 2 T-175 flasks with 70% confluence ($\sim 1 \times 10^8$ cell in total) (*see Note 3*).
2. Trypsinize and seed all cells into one Falcon® multi-flasks with five layers, grow for 3–5 days until reaching 80% confluence (*see Note 4*).
3. Change to Tet-off medium (150 ml) and refer as day 0 post induction (*see Note 5*).
4. Change medium every 3 days (*see Note 6*).
5. Collect the supernatant starting from day 15 until day 60 (*see Note 7*).
6. Every time (or every second time) of collecting supernatant, start to concentrate the virus immediately since the infectivity might get gradually reduced overtime.
7. Clarify the supernatant by sterile filtering through a $0.45\ \mu\text{m}$ pore size filter (*see Note 8*).
8. Add 27 ml 40% PEG into 150 ml supernatant (final 6% PEG), mix by inverting 30 times, and store at 4°C overnight.
9. Centrifuge at $10,000 \times g$ for 1 h with fixed angle rotor at 4°C (*see Note 9*).
10. Remove the supernatant and use 1/50 to 1/100 of the original volume of PBS/10% FCS to suspend the pellet (*see Note 10*) and collect all suspension.
11. Shake or rotate the virus suspension at 4°C overnight.
12. Centrifuge at $3000 \times g$ for 10 min at 4°C , transfer the supernatant to a new tube, and centrifuge again at $3000 \times g$ for 10 min at 4°C to remove insoluble precipitate.
13. Aliquot and freeze the supernatant at -80°C after quantification.

3.1.2 Preparation of HBV from Transiently Transfected Cells

Both pCHT-9/3091 and P26 constructs contain 1.1 mer over-length HBV genomes driven by the CMV promoter. The protocol described here might need to be modified when using different constructs according to the kinetics of virus production.

1. Seed 3×10^6 HuH7 cells (*see Note 11*) in 10-cm dish with Culture medium.
2. On the next day, transfect proximally 70% confluent HuH7 cells (*see Note 12*) with transfection reagents such as lipofectamine 2000 or JetPrime according to the manufacturer's protocol and refer as day 0.
3. Refresh the medium at days 3 and 8.
4. Collect culture supernatant between day 3–8 and day 8–12. Centrifuge the collected supernatants at $3000 \times g$ for 15 min at 4°C to remove cell debris.
5. Add 40% PEG to a final concentration of 6% (w/v) and prepared virus as mentioned above for HepAD38 cells.

3.1.3 Preparation of HBV from Patient Serum

Some patient sera with high titers of HBV ($>10^9$ IU/ml) might be used directly for infection. However, sera could contain unknown inhibitors interfering with infection. Therefore, pelleting of virus through a sucrose cushion is strongly recommended, which not only concentrates virus but helps to remove inhibitory substances from human serum.

1. Place 10 ml 20% sucrose/PBS at the bottom of an ultracentrifugation tube for an SW28 rotor.
2. Carefully add serum on the top of the sucrose layer without disturbing the sucrose cushion (*see Note 13*), fill the tube with PBS if the serum volume is less than 23 ml.
3. Centrifuge at 28,000 rpm ($140,000 \times g$) for 4 h at 4°C .
4. Carefully remove the supernatant and add 1 ml PBS with 10% FCS to suspend the pellet. Cover the centrifugation tube with parafilm.
5. Shake the tube at 4°C overnight to allow resuspension of the virus.
6. Pipette up and down for >30 times, transfer virus to an Eppendorf tube, and centrifuge at $3000 \times g$ for 10 min at 4°C to remove insoluble precipitate.
7. Aliquot and freeze the supernatant at -80°C after quantification.

3.2 Prepare Cells for Infection Assay

3.2.1 Differentiate HepaRG or HepaRG-hNTCP Cells for Infection

The following protocol describes differentiation of HepaRG cells in a 24-well plate. It is also applicable for HepaRG-hNTCP cells and can be proportionally adjusted to different plate formats.

1. HepaRG cells are maintained in Growth medium with weekly splitting at a ratio of $\sim 1:8$.

2. Trypsinize and seed HepaRG cells at a density of 10^5 cells/well in Growth medium and refer as day 0.
3. Replace the Growth medium with 500 μ l Differentiation medium at day 14 (*see Note 14*).
4. Refresh the Differentiation medium every 2–3 days (*see Note 15*).
5. The HepaRG cells are ready for infection at day 28 (*see Note 16*).

3.2.2 Plating of PHHs for HBV Infection

PHHs can be prepared from the liver tissue if the respective infrastructure and technique are available on site. It might also be available from commercial vendors (e.g., Biopredic) providing plated or cryopreserved platable human hepatocytes. The cell viability should be above 75% before seeding. The following protocol describes the seeding of PHHs in a 24-well plate.

1. Dilute collagen in PBS to a final concentration of 100 μ g/ml.
2. Add 200 μ l diluted collagen solution to each well. Allow the collagen to bind for 1 h at room temperature.
3. Remove collagen solution. Leave the plate open in the culture hood for 10–30 min until it gets dry (i.e., there is no liquid visible on the plate).
4. If needed, wash PHH with Growth medium by centrifugation at $50\times g$ for 5 min at 4 °C (*see Note 17*).
5. Dilute PHH to 3×10^5 living cells/ml in Growth medium, and add 500 μ l to each well.
6. Four hours later, remove unattached cells and add 500 μ l Differentiation medium (*see Note 18*).
7. The next day the PHHs are ready for infection or they can be kept in serum-free Differentiation medium for up to 3 days with daily medium change (*see Note 19*).

3.3 HBV Infection of HepaRG, HepaRG-hNTCP, or PHH Cells

The genome equivalence of virus stock should be quantified by qPCR or DNA dot-blot [19] (*see Note 20*), which is not described in this chapter. The DNA quantification result should be carefully interpreted, since transfected cells might contain input DNA and cell culture might secrete large amount of naked nucleocapsid. A sucrose or CsCl gradient of prepared virus can be useful to precisely quantify the enveloped virus fractions. Typically, at least 100 MGE (multiplicity of genome equivalents) is required for an efficient HBV infection. Here, we describe the infection of HepaRG, HepaRG-hNTCP, or PHH cells in a 24-well plate.

1. For each well, mix 50 μ l 40% PEG with 450 μ l Differentiation medium. The final PEG concentration is 4% (*see Note 20*). Vortex for 5 s.
2. Add desired amount of HBV, and vortex again for 5 s. Usually, 20 μ l concentrated virus from HepAD38 can result in a well-detectable infection.

3. Aspirate cell culture medium, then add inoculum, and refer as day 0.
4. Four- to twenty-four-hours after infection (*see Note 21*), wash the cell with PBS for two times and then add 500 μ l Differentiation medium.
5. Refresh and/or collect medium every 2–3 days.
6. Monitor the viral markers and choose the end point of infection as needed (*see Note 22*).

3.4 IF Against Viral Core or L Proteins to Analyze Infected Cells (See Note 23)

The following protocol describes the IF staining of infected cells in a 24-well plate.

1. At 6–12 days after infection, wash cells once with PBS.
2. Add 250 μ l 4% PFA for 10 min at room temperature.
3. Aspirate the fixation buffer and wash once with PBS, then the cells in PBS can be stored at 4 °C (*see Note 24*) or stained as the following.
4. Add 250 μ l permeabilization buffer for 10 min at room temperature.
5. Remove permeabilization buffer and wash once with PBS.
6. Add 250 μ l first antibody (anti-core or Ma18/7 antibody, 1:1000 diluted in blocking buffer) for 1 h at room temperature or overnight at 4 °C.
7. Remove antibody and wash cells three times with PBS.
8. Add 250 μ l fluorescent labeled secondary antibody (diluted as recommended by manufacturer in blocking buffer) and Hoechst (1:1000 diluted) for 1 h at room temperature.
9. Wash cells three times with PBS, then the cells are ready for examination under a fluorescent microscope.
10. If cells are seeded in wells with cover slips, carefully take it out and mount it to a slide with Fluoromount-G. Leave it dry for 10 min before microscope analysis.

4 Notes

1. The differentiation process of HepaRG cells strongly depends on the serum used. Serum from different batches or manufacturers should be tested for differentiation with 2% DMSO.
2. 2% DMSO is preferred if it is tolerated. The sensitivity of cells to DMSO relies on the quality of serum more than that of DMSO. If a severe toxic effect is observed, the concentration of DMSO can be reduced to 1.5%.

3. HepG2-derived HepAD38 cells are prone to clump together during culturing and might be difficult to be trypsinized into single cells. Too many cell clusters after splitting impair the long-run viability of cell culture. Shortening the splitting period such as every 2 days helps to prevent this problem. Cell strainer with 40 μm pore size can be used to remove big clusters after trypsinization. If it is still unsatisfactory, add 5 $\mu\text{g}/\text{ml}$ insulin and 50 μM hydrocortison to the Tet-on medium during cell propagation.
4. Cells growing in multilayer can hardly be examined under microscope. Homogenous distribution is usually a good sign. When using a CellSTACK chamber, it is important to leave the chamber in a horizontal position so that all cells are completely covered by culture medium.
5. HepAD38 cells grow slowly in Tet-off medium since the transcription of viral pregenomic RNA is strongly induced.
6. Occasionally changing the medium after 4 days of culturing is tolerable. In this case, the volume of culture medium should be increased.
7. The secreted HBeAg should be monitored over the whole culturing, which is a good indicator for the virus production. HepAD38 cells produce high amount of naked nucleocapsids that may interfere with quantification of virions.
8. This step aims at clarifying the virus-containing medium from cell debris. Vacuum-driven filtration system facilitates this step. Centrifugation can be used as well, such as 5000 $\times g$ for 15 min at 4 $^{\circ}\text{C}$.
9. Centrifugation in a fixed rotor results in a thin layer of precipitate at the distal side of the centrifugation tube. This pellet can be then suspended more easily compared to a thick pellet formed at the bottom of the tube.
10. After pipetting up and down, the suspension is quite turbid.
11. HepG2.2.15 cells containing stably integrated HBV genomes can be seeded and cultivated in Culture medium as well. HepG2.2.15 cells constitutively produce virus after reaching confluence. The supernatant of HepG2.2.15 cells can be collected and viral particles can be concentrated by PEG precipitation as well. However, ~ 50 -fold lower virus concentration in comparison to HepAD38 cells should be expected.
12. HuH7 cells can be transfected with higher efficiency than HepG2 cells and produce higher levels of HBV.
13. Cut the end of 1 ml pipette tips and use them to slowly add the first 10 ml serum to avoid disrupting the sucrose solution.

14. It is not necessary to change the medium during the first 2 weeks. If there is a significant evaporation of medium, add 0.5 ml of Growth medium at day 7 post seeding.
15. During the DMSO-induced differentiation process, some cells inevitably undergo apoptosis; the majority differentiates into hepatic or biliary epithelial cells. The formed hepatic cell region, characterized by the formation of canaliculi between cells, should be resistant to DMSO. If the hepatic islands are not well formed or quickly disrupted, different sources of HepaRG cells or serum should be considered.
16. Over-expression of NTCP has no apparent impact on the differentiation of HepaRG cells. Bile canaliculi should be easily recognized when HepaRG-hNTCP cells get differentiated, which can also be stained with anti-MRP2 antibody [7].
17. PHHs are fragile and should be centrifuged at low force.
18. The unattached PHHs 4 h post seeding will not be able to attach firmly even after overnight incubation.
19. PHHs will be dedifferentiated in serum-containing medium and therefore down-regulate the NTCP expression and reduce their susceptibility to HBV infection. However, serum-free condition usually reduces the lifetime of PHH in culture and is not necessary as long as the cells are infected.
20. PEG enhances the infection efficacy by ~10-fold via promoting virus attachment to heparan sulfate proteoglycans [16].
21. HBV infection is a “slow” process. Overnight inoculation results in ~5-fold higher infection rate than 2–4 h inoculation.
22. cccDNA reaches plateau at day 4; core protein can be detected at day 5 p.i.; Envelope protein can be detected at day 7 p.i.; HBsAg can be measured between day 4–10; HBcAg can be measured between day 7–13.
23. The HBV X protein and polymerase are difficult to be detected by IF postinfection, which might be due to their very low expression level in the context of authentic infection.
24. The PFA-fixed cells are stable in PBS at 4 °C for at least 2 weeks.

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Live Cell Imaging Confocal Microscopy Analysis of HBV Myr-PreS1 Peptide Binding and Uptake in NTCP-GFP Expressing HepG2 Cells

Alexander König and Dieter Glebe

Abstract

To obtain basic knowledge about specific molecular mechanisms involved in the entry of pathogens into cells is the basis for establishing pharmacologic substances blocking initial viral binding, infection, and subsequent viral spread. Lack of information about key cellular factors involved in the initial steps of HBV infection has hampered the characterization of HBV binding and entry for decades. However, recently, the liver-specific sodium-dependent taurocholate cotransporting polypeptide (NTCP) has been discovered as a functional receptor for HBV and HDV, thus opening the field for new concepts of basic binding and entry of HBV and HDV. Here, we describe practical issues of a basic in vitro assay system to examine kinetics and mechanisms of receptor-dependent HBV binding, uptake, and intracellular trafficking by live-cell imaging confocal microscopy. The assay system is comprised of HepG2 cells expressing a NTCP-GFP fusion-protein and chemically synthesized, fluorophore-labeled part of HBV surface protein, spanning the first N-terminal 48 amino acids of preS1 of the large hepatitis B virus surface protein.

Key words HBV entry, NTCP, HBV preS1-domain, Endocytosis, Live-cell imaging confocal microscopy

1 Introduction

Infection of human hepatocytes with HBV is believed to be mediated by low-specific binding of viral surface proteins to heparansulfate proteoglycans (HSPG), followed by specific targeting of the NTCP. While interaction with HSPG has been shown to depend mainly on amino acids (aa) residues within the S-domain of HBV surface proteins [1], the N-terminal 48 aa of the preS1-domain of large hepatitis B virus surface protein (LHBs) show direct interaction with the NTCP and are critical for HBV and HDV infection [2]. Determining site-specific virus-receptor interactions is necessary to search for specific inhibitors targeting this process. The NTCP is a sodium-dependent bile acid (BA) transporter, predominantly expressed on the basolateral membrane of hepatocytes. The NTCP

recycles more than 90% of BAs from the portal blood due to symporting BAs (e.g., taurocholate) and sodium ions into hepatocytes. Thus, this transporter is a critical factor for the maintenance of the enterohepatic circulation of BAs [3]. The NTCP transport activity is regulated at both the transcriptional [4] and the post-translational level. Posttranslational regulation of NTCP transport activity includes translocation of NTCP from an intracellular pool to the basolateral membrane, leading to short-term increased BA-transport capacity [5]. Endocytotic internalization of NTCP from the plasma membrane (PM) is believed as a mechanism for rapid cellular downregulation of BA uptake. Endocytosis of the transporter from the basolateral membrane followed by lysosomal degradation is shown by expression of rat NTCP (rNTCP) in the human hepatoma cell line HepG2 [6, 7]. Furthermore, Sarkar et al. demonstrated recycling of endocytosed rNTCP from early endosomes back to the PM using primary rat hepatocytes [8]. Fusion of a fluorescent tag (e.g., GFP) to the cytosolic located C-terminus of the NTCP enabled visualization of NTCP at the PM and subsequent endocytosis and trafficking in living cells by fluorescence microscopy. To demonstrate direct interaction of the HBV infection-relevant N-terminal myristoylated (Myr) preS1-domain with the NTCP, a C-terminal fluorophore (Alexa594, AX594) labeled HBV Myr-preS1 peptide comprising AA 2–48 (Myr-preS1-AX594) can be used to visualize ligand-receptor interaction with NTCP-GFP. We demonstrated a membrane association of HBV Myr-preS1-AX594 to NTCP-GFP transfected HepG2 cells, followed by NTCP-directed uptake and trafficking of both markers into same vesicular compartments [9]. The mentioned live cell assay can be used as a tool to investigate the regulatory mechanisms associated with the NTCP/HBV internalization and trafficking.

2 Materials

2.1 Designing of HBV Myr-preS1 (2–48) Peptide, C-Terminally Conjugated with Alexa594

The HBV Myr-preS1-peptide was obtained from Bio Synthesis (Lewisville, TX, USA) comprising the sequence of N-terminal preS1-domain aa 2–48 of the LHBs from HBV genotype (gt) D. Similar to the composition of the LHBs in vivo, the first N-terminal Glycin (G) of the peptide (corresponding to aa position 2 of LHBs ORF) was modified with an myristic acid (Myr). For visualization in fluorescent microscopy, the C-terminus of the peptide was covalently conjugated with the fluorophore Alexa 594 (AX594; Life Technologies, Carlsbad, USA) via an artificially introduced Cystein (C).

Sequence and modifications of HBV gt D Myr-preS1-AX594 peptide (2–48):

myr-GQNLSTSNPLGFFPDHQLDPAFRANTANPDWDFN
PNKDTWPDANKVG C-Alexa594.

The peptide Myr-preS1-AX594 was obtained in a lyophilized form and can be dissolved in DMSO at a concentration of at least 1 mM and is stable over at least 6 months when stored at -80°C (*see Note 1*).

2.2 NTCP-GFP Fusion Protein Expression Plasmid

Expression plasmid coding for C-terminal GFP-tagged full open reading frames of human NTCP in a pcDNA5/FRT/TO-TOPO vector system (Invitrogen, Hamburg, Germany) was kindly provided by Barbara Döring and Joachim Geyer (Institute of Pharmacology and Toxicology, Justus Liebig University Giessen, Germany). Detailed information about this plasmid can be found elsewhere [9].

2.3 Preparation of Media to Culture Human Hepatoma Cell Line HepG2

Mix 430 mL HEPES buffered, high glucose, phenol-red free DMEM with 50 mL fetal calf serum (FCS), 5 mL penicillin/streptomycin (100 Units/mL Penicillin, 100 $\mu\text{g}/\text{mL}$ Streptomycin), and 5 mL Sodium Pyruvate (1 mM).

2.4 Cell Culture Vessels

For continuous subculturing of HepG2, 10 cm plates were used. To perform live cell confocal imaging, uncoated μ -Slide 8 Well were used.

2.5 Preparation of 1000 mL PBS

1. Weigh 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 , and 0.24 g KH_2PO_4 and transfer it to a 2 L flask. Add 800 mL deionized Milli-Q water and mix it with a magnetic stirrer to dissolve it.
2. Adjust the pH with HCl (37%) to 7.4.
3. Fill volume to 1000 mL with deionized Milli-Q water and transfer PBS to autoclavable bottles for sterilization by autoclaving.

2.6 Preparation of 1000 mL PBS++

Mix 495 mL sterile PBS with 5 mL 100 \times sterile stock solution of MgCl_2 , CaCl_2 .

For preparation of 100 \times sterile stock solution of MgCl_2 , CaCl_2 dissolve 1.32 g CaCl_2 and 2.133 g MgCl_2 in 100 mL PBS. Sterilize the stock solution by using 0.2 μm syringe filter.

2.7 Preparation of Wash Buffer

Supplement 50 mL phenol-red free DMEM with 0.1 g bovine serum albumin (0.2% BSA, Albumin Fraction V).

2.8 Preparation of Acidic Wash Buffer

Supplement 50 mL phenol-red free DMEM with 0.1 g bovine serum albumin (0.2% BSA, Albumin Fraction V) and adjust pH 3.5 using HCl (37%).

2.9 Preparation of 0.4 mg/mL Collagen Solution

Dilute Collagen Type I Rat Tail to 0.4 mg/mL in deionized Milli-Q water.

2.10 Preparation of 0.1 % Trypsin-EDTA/PBS Solution

Dilute 0.5 % Trypsin-EDTA 1:5 in sterilized PBS.

2.11 Confocal Microscope

Necessary technical requirements to track the membrane-bound transport of two markers are a laser scanning confocal microscope (CLSM) that allows optical sectioning and time-lapse imaging of multiple fluorescent markers and are able to acquire high resolution. For an efficient collection of light from a thin confocal slice of cell monolayers, inverted oil or glycerin immersion Plan-Neo 40× NA (numerical aperture) oil 1.3 or Plan-Neo 63× NA oil 1.4 should be used. While the cells are on the microscope stage it is important to maintain normal cellular functions over time. Therefore and to stabilize microscope focus, controlling the ambient temperature to 37 °C by, i.e., an air stream microscope stage incubator is necessary. For represented data in this article, an inverted Leica TCS SP5 VIS clsm was used, equipped with a galvo-z drive, x,y,z,t-scanner (1–2.800 Hz; zoom 0.7–40×), an AOBs system and with the following lasers: Diode 405 nm; Argon 458, 476, 488, 496, 514 nm; Diode 561 nm, HeNe 594 nm; HeNe 633 nm. Fluorescence signals were detected with Leica HCX PL APO objectives (20×/0.7multi-imm; 63×/1.3glyc and 63×/1.4oil) and by conventional PMTs set to the range appropriate for the respective fluorophores. Images were recorded and preprocessed using the LAS AF software (Leica, version 2.7.3). Experiments were conducted in an air stream microscope stage incubator (The Cube, The Box; Live Imaging Services, Switzerland) with the temperature set to RT for analysis of Myr-preS1-AX594 binding to NTCP and heated to 37 °C for the detection of endocytotic uptake of Myr-preS1-AX594 and NTCP.

3 Methods

3.1 Cell Culture

For optimized growth conditions during cultivating HepG2 cells, culture plates have to be freshly coated with collagen before seeding HepG2. Therefore, incubate 10 cm plates with 10 mL and Ibidi-slide with 200 µL of 0.4 mg/mL collagen solution for 5 min, remove collagen and dry the plates over 15 min in a cell-culture hood. For cultivation, use incubator settings of 95 % humidity and 5 % CO₂. To maintain actively and healthy growing of cells over time, passage the cells when reaching 70–90 % confluency.

3.1.1 Subculturing of HepG2 Cell Line

1. Discard culture medium from cells.
2. Use 10 mL glass or plastic pipette and a pipette boy to add 10 mL PBS on HepG2 monolayer in 10 cm plate.
3. Wash the cells by gently shaking PBS for 10 s.

4. Discard PBS.
5. Disperse 2 mL of 0.1 % Trypsin-EDTA/PBS solution over the cells and incubate cells at 37 °C and 95 % humidity.
6. When cells start to detach, add 8 mL of DMEM (10% FCS) to dilute cell suspension to 10 mL in total.
7. Rinse all cells from plate bottom and separate single cells from larger cell groups by carefully squeezing cell suspension through the pipette and plate bottom three times.
8. Subdivide cells by transferring 2–6 mL of cell suspension to a new freshly collagen coated 10 cm plate. To maintain HepG2 growth in a monolayer, distribute cells evenly by gentle shaking. Cells should be reattached after several hours incubation period.

*3.1.2 Transient
Transfection of NTCP-GFP
Expression Plasmids
into HepG2 Cells*

1. Plate HepG2 cells on a freshly collagen coated 10 cm plate. Culture cells in incubator for 24 h until 70–90 % density is reached.
2. For one 10 cm cell culture plate, prepare a transfection mastermix by adding 8 µg of purified plasmid-DNA to 300 µL DMEM in a 1.5 mL Eppendorf tube. For other cell culture vessels, the DNA quantity has to be adapted accordingly.
3. Mix by vortexing and short centrifugation.
4. Incubate for 5 min at room temperature (RT).
5. Add 32 µL of FuGene HD (Promega) (ratio of 4:1 FuGene HD (µL) versus plasmid-DNA (µg)) and repeat **steps 3** and **4**.
6. During incubation, discard the cell supernatant and wash the cells with 10 mL PBS++ by gently shaking the plate for 10 s.
7. Pipette 10 mL of 37 °C pre-warmed DMEM supplemented with 2 % FBS onto cells.
8. Evenly distribute the complete transfection mastermix over 10 cm plate and shake the plate gently for 10 s.
9. After overnight incubation at 37 °C and 95 % humidity in an incubator, exchange supernatant with fresh DMEM supplemented with 10 % FCS and Pen/Strep.

*3.1.3 Preparation
of GFP-NTCP Expressing
HepG2 for Live Cell
Imaging Microscopy*

1. Pipette 150 µL of DMEM (10% FBS, Pen/Strep) into each well of a freshly collagen coated Ibidi slide (*see* Subheading **3.1**).
2. Take a freshly trypsinized cell suspension of NTCP-GFP transfected HepG2 (**step 9**, Subheading **3.1.2**) and untransfected HepG2 (as control), add 150 µL of cell suspension to each Ibidi-well, and distribute the cells by gentle shaking. After overnight culturing at 37 °C and 95 % humidity 70–80 % final density should be reached (*see* **Note 2**).

3.2 Myr- preS1-AX594 Binding Assay

3.2.1 Preliminaries

1. Prepare wash buffers and PBS++ like described in Subheadings 2.6 and 2.7, respectively.
2. Precool wash buffers and DMEM in 50 mL Falcon tubes on ice.
3. Prewarm DMEM to 37 °C in 50 mL tube.
4. Prewarm microscope stage to 37 °C by switching on the air stream heater several hours before imaging to avoid focus shift.
5. Set up CLSM and its associated hardware, run CLSM software and activate lasers for warm-up at least for 30 min to avoid power fluctuation over imaging period (UV for nucleus staining, Argon laser line 488 nm for NTCP-GFP, He-Ne laser line 594 nm for Myr-preS1-AX594 peptide), adjust settings for specific light excitation, and emission of used fluorophores. Set pinhole to obtain optical slices of at least 1 µm thickness enabling detection and tracking of dynamic vesicles.

3.2.2 Nucleus Staining

1. Wash the cells twice with 400 µL wash buffer.
2. Dilute Hoechst 33342 in DMEM (10 µg/mL) and add 200 µL to each well.
3. Incubate cells for 15 min at 37 °C and 95 % humidity.
4. Wash cells three times with ice cold PBS++.

3.2.3 Peptide Binding

1. During incubation period of Subheading 3.2.2, prepare ice cold DMEM supplemented with 10–200 nM Myr-preS1-AX594.
2. Precool transfected and non-transfected cells for 15 min by incubating with ice cold DMEM in a fridge at 4 °C.
3. Discard supernatant and incubate transfected and non-transfected cells with 100 µL ice cold peptide solution for 30 min in a fridge at 4 °C (*see Note 1*).
4. Wash cells gently three times with 400 µL ice cold wash buffer.

3.3 Live Cell Confocal Microscopy

3.3.1 Live Cell Confocal Imaging of Myr- preS1-AX594 Binding to NTCP-GFP Expressing HepG2 Cells

1. Add 300 µL of ice cold imaging medium (bicarbonate and HEPES buffered phenol-red free DMEM or FluoroBrite DMEM Media) to the cells (*see Note 3*).
2. Seal the Ibidi slide and immediately transfer it into an air stream microscope stage incubator (The Box; Live Imaging Services, Switzerland) set to RT (*see Note 4*).
3. To obtain an overview of Myr-preS1-AX594 binding to NTCP-GFP expressing HepG2 cells like represented in Fig. 1a, choose a 20× magnification objective.
4. To identify cells or region of interest (ROI), choose the desired focus and use zoom operations (Fig. 1b).

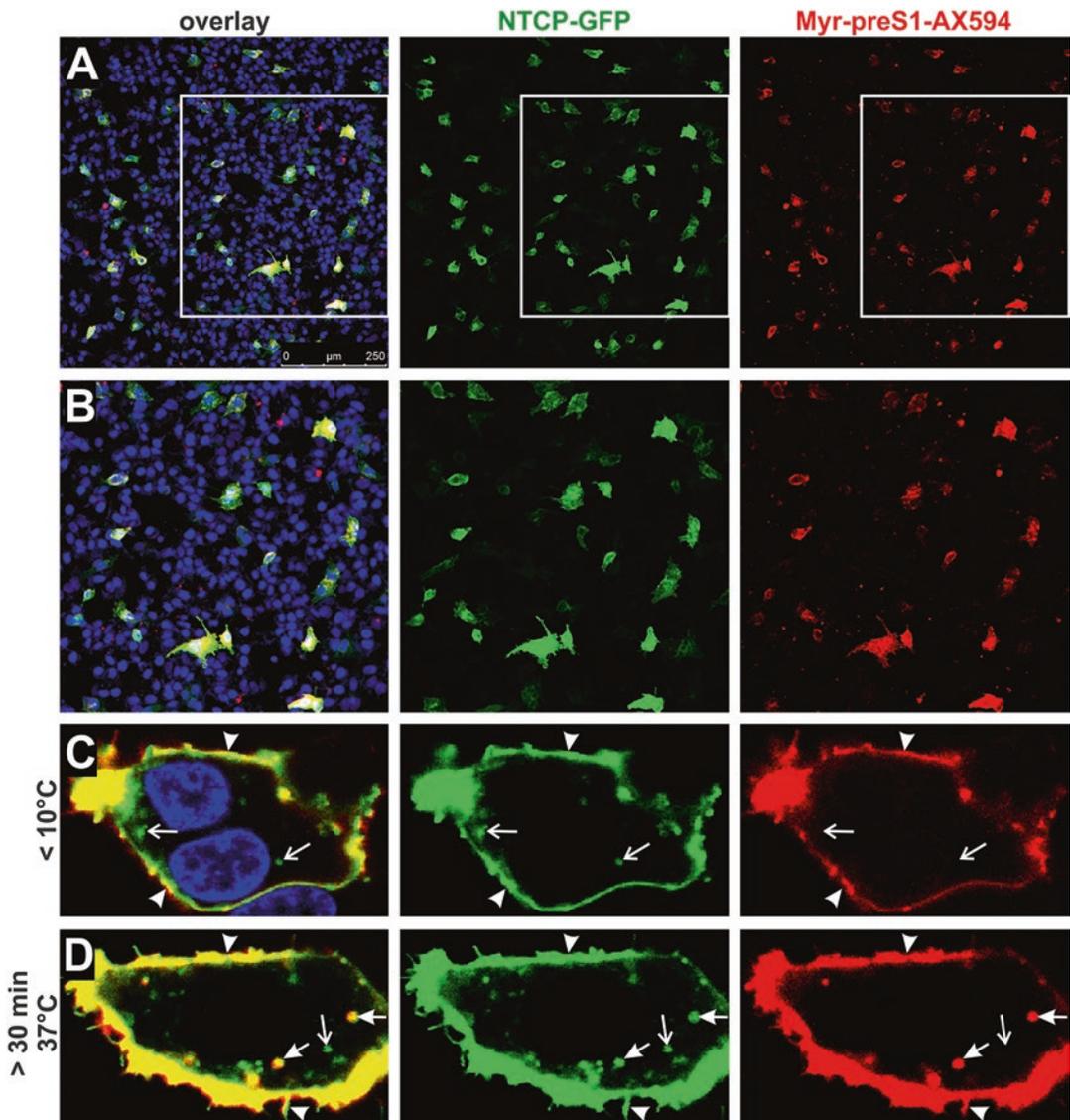


Fig. 1 Live-cell-imaging microscopy analysis of HBV Myr-preS1-AX594 binding and endocytosis in NTCP-GFP expressing HepG2-cells. HepG2-cells were transiently transfected with an expression plasmid encoding for hNTCP-GFP fusion-protein (*green*). After seeding and 2 days of culturing in Ibidi-chamberslides, cells were precooled below 10 °C and incubated with ice-cold Myr-preS1-AX594 (*red*) at 0.2 μ M for 30 min. After repeated washing, Myr-preS1-AX594 binding to NTCP-enriched plasma membrane (*arrow head*) and NTCP-enriched intracellular vesicle-like compartments (*opened arrow*) were detected immediately. Uptake of Myr-preS1-AX594 into intracellular vesicle-like NTCP-enriched compartments (*closed arrow*) was analyzed after 30–60 min at 37 °C by time-lapse live cell-confocal microscopy imaging

5. Scan several images with different settings of laser power, detector gain, and offset to obtain maximal fluorescence signal and minimal background noise (*see Note 5*).
6. To exclude detection of NTCP unspecific binding of Myr-preS1-AX594, use the chosen settings and observe a sample of

non-transfected HepG2 cells. Use laser power, smart gain, and offset to adjust settings of Myr-preS1-AX594 excitation and emission.

7. After exclusion of background noise and NTCP unspecific binding of Myr-preS1-AX594 to HepG2 cells, like described in **steps 5** and **6**, collect data sets of multiple spots in at least a duplicate setup of NTCP-GFP expressing HepG2 cells and repeat the experiment several times to confirm reproducibility.
8. For single cell analysis of peptide binding to NTCP-GFP, represented in Fig. 1c, use a 63× magnification objective and zoom operations and repeat **steps 4–7**.

3.3.2 Live Cell Confocal Imaging of Alexa594 Conjugated Myr-preS1-AX594 Uptake into NTCP-GFP Expressing Cells

1. To maintain normal cellular kinetics of endocytosis, heat ambient temperature of the cells to 37 °C by exchanging supernatant from Ibidi slide with fresh 37 °C prewarmed imaging medium and by heating the microscope stage incubator to 37 °C by switching on the air stream heater (The Cube; Live Imaging Services, Switzerland).
2. Approx. 30 min after the temperature shift from 4 to 37 °C, cellular uptake of NTCP-GFP and Myr-preS1-AX594 into vesicle like organelles is detectable like shown in Fig. 1d. Time-lapse imaging can be used to track vesicle dynamics (*see Note 6*).
3. To track the dynamics of NTCP-GFP and Myr-preS1-Alexa594 trafficking, imaging each 5 s over a time course of 3 min is sufficient (data not shown, movie resulting from time-lapse imaging experiment using this protocol is published in König et al. [9], <http://www.sciencedirect.com/science/article/pii/S0168827814003596>) (*see Notes 7–10*).

To subsequently analyze images for colocalization, marker quantification or to analyze dynamic tracking and the size of organelles, i.e., Leica microscope LAS AF, ImageJ or Imaris software can be used.

4 Notes

1. For handling pure peptides in solution (e.g., DMSO; DMEM), low bind Eppendorf tubes and low bind pipette tips should always be used.
2. Prior to imaging of fluorescent markers in living cells with confocal microscopy, cells must be attached to glass coverslips or chambers with coverslip-like bottom. To avoid image artifacts due to spherical aberration, thickness and quality of the cell

culture vessel bottom material should be considered and adapted to the assay and objectives used [10]. Appropriate sealable chambers for inverted microscopes are commercially provided by various companies (i.e., ibidi, Munich, Germany or Nunc, Thermo Scientific, USA).

3. To minimize background fluorescence, i.e., phenol-red free Dulbecco's modified Eagle medium (DMEM) and FluoroBrite DMEM Media (Gibco, Life Technologies, USA) should be used to image cells.
4. Using sealable cell culture vessels and a bicarbonate, HEPES buffered culture media is sufficient to stabilize pH over short time experiments. For longer experimental approaches, pH must be regulated by an increased atmospheric CO₂ concentration of 5–10% using an automated gas mixer system (i.e., The Brick; Live Imaging Services, Switzerland).
5. To perform living cell imaging microscopy, it is assumed that the investigator is familiar with basic handling of a confocal microscope, esp. concerning settings like laser power, detector gain or offset, pinhole, scan speed, image size, and collecting time series images.
6. Settings of time-lapse imaging depend on rapidity of vesicular trafficking process, concentration of the fluorophores within the organelles, and the size of fluorescently labeled organelles.
7. To avoid signal bleed through, imaging of markers can be separated in time by sequential scanning. For optimal detection of a possible colocalization of the two dynamic markers (GFP and Alexa594), they should be imaged simultaneously.
8. During the time course of repeated imaging, photobleaching of fluorescent labeled organelles might happen, and is dependent on fluorophore concentration, laser power, scan speed, and zoom. Use improved settings to determine imaging conditions with minimized bleaching.
9. To reduce photodamage of the cells by short wavelength light minimize UV-laser power. If a representation of nuclei is not required, UV-excitation should be stopped or usage of nucleic staining should be avoided.
10. For optimal discrimination between PM located and cytoplasmic organelle located markers, a so called acid stripping can be performed to decrease the amount of noninternalized, but membrane-bound Myr-preS1-AX594. Therefore, incubate cells three times with ice cold acidic wash buffer (Subheading 2.8) on a shaker (AMERSHAM ZLE.164 shaker) for 5 min before cells are neutralized immediately by washing with normal wash buffer three times for 5 min on a shaker.

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Intracytoplasmic Transport of Hepatitis B Virus Capsids

Quentin Osseman and Michael Kann

Abstract

The early steps of HBV entry remain largely unknown despite the recent discovery of an HBV-specific entry receptor. Following entry HBV capsids have to be transported through the cytoplasm to the nuclear periphery, followed by nuclear entry. These steps have to take place in a coordinated manner to allow delivery of the genome into the nucleus. Due to the viscosity of the cytoplasm, the intracytoplasmic translocation has to be active and directed.

Here, we describe protocols that can be applied to investigations of the HBV capsid with the cytoplasmic transport systems. We have chosen to present two independent experimental approaches, which allow avoiding artifacts. Aside of the specific capsid detection system, the protocols can be applied to any other viral structure.

Key words Hepatitis B virus, Capsids, Microtubules, Dynein, Retrograde transport

1 Introduction

HBV nucleocapsids contain mainly a relaxed circular genomic DNA (rcDNA) that had to be converted to a chromatinized covalently closed circular DNA (cccDNA) for allowing transcription. As the hepadnaviral polymerase is unable to mediate this conversion [1], HBV depends on the enzymatic activities provided by the nucleus. This requirement presupposes transport of the rcDNA genome from the cell periphery into the nucleoplasm. In analogy to other viruses, this translocation can be separated into three distinct steps: (1) passing the plasma membrane in vesicles (or by fusion), (2) consecutive escape from the vesicle into the cytoplasm, (3) intracytosolic transport to the nuclear periphery, and (4) transport of the genome. As DNA is not karyophilic per se viral proteins are needed to allow the attachment of cellular soluble nuclear transport receptors, which mediate translocation through the nuclear pore complex (NPC) into the nucleus. At one step the genome has to be released so that replication can occur. The site of genome-release depends upon the size to the genome surrounding

structure: large capsids as, e.g., that of adenoviruses [2] cannot pass the nuclear pore complex and thus disintegrate on the cytosolic side of the NPC. Alternatively, a direct, injection-like release of the genome through the NPC was exemplified for herpes simplex virus 1 [3]. A sort of “premature” genome release has been described for HIV, leading to liberation of the dsDNA from the capsid into the cytoplasm. However, there is growing evidence that HIV capsids also disassemble on the cytoplasmic side of the nuclear pore [4]. In fact, such transport inside a capsid could be important for escaping the cellular innate immune response initiated by pattern recognition receptors (PRRs).

HBV enters the cell using the Na⁺ taurocholate cotransporting polypeptide (NTCP; SLC10A1) as the specific HBV entry receptor [5]. Recent publications show that endocytotic uptake occurs through an endosomal pathway leading to capsid release into the cytoplasm [6, 7], which is subsequently followed by cytoplasmic transport towards the nuclear periphery. HBV capsids have a diameter of 36 nm, which is below the transport limit of the nuclear pore (39 nm, [8]) and thus pass the NPC intact. The passage is mediated by an interaction between the cellular transport receptors of the importin family [9] and the core protein from which 180 or 240 copies form the capsid. The capsids become arrested on the nuclear side of the NPC by strong interaction with a protein of the NPC called nucleoporin 153 (Nup153; [10]). Disassembly is restricted to capsids with an rc form of the genome and it is thought that genome release occurs at this place [10].

For arrival at the NPC the capsids have to pass the cytoplasm, which has a high viscosity some 100 times higher than water [11–13]. Although below the experimental diffusion limit of 50 nm, as it was shown by Luby-Phelps using latex beads [14], diffusion is ineffective for this size. Thus active translocation is required, using cellular transport principles.

There are two cellular systems for translocation of macromolecular structures: the movement caused by actin polymerization and depolymerization as it is used by intracellular bacteria as, e.g., *listeria* or baculoviruses [15–18], and the transport via microtubules. In fact, observations by Rabe et al. showed that depolymerization of microtubules by nocodazole prevents HBV capsid arrival at the nucleus but also release of hepadnaviral genomes [19].

Microtubules (MT) are composed of two tubulin isoforms that have similar structure. Both tubulin have a GTP binding domain in N terminus, one central domain and the C terminus part, which is implicated in interaction with Microtubules Associated Proteins (MAPs) [20]. Tubulin alpha and beta heterodimerize and form protofilaments and association of 13 protofilaments leads to the formation of cylinder of 16 nm in diameter. Microtubules are polarized with a highly dynamic plus end at the cell periphery. This extremity exhibits a high dynamic instability with polymerization and depolymerization phases, which are influenced by directly

MT-linked MAPs [21]. The minus end in the nuclear periphery is stable and fixed at the Microtubules Organizing Center (MTOC).

MT-mediated transport can occur via three mechanisms. Two of them are independent of motor complexes and they are based on direct interaction of the cargo with tubulin. Movement is relatively slow and occurs by tubulin polymerization or depolymerization. This is exemplified by the chromosome separation upon cell division during which the chromosomes attach to the extremity of the microtubules [22]. Another direct transport uses the so-called treadmill mechanisms, described for translocation of p53 to the nuclear periphery [23]. For this transport, the cargo attaches to tubulin and the dynamics of microtubules moves the tubulin molecules toward the MTOC.

The majority of cellular cargos (vesicles, proteins, and protein complex) and viral structures use molecular motors. The direction of the transport is determined by the sort of motor complex to which the capsids attach. The anterograde transport is mediated by the kinesin complex. Kinesins belong to KIFs (Kinesin Family members) comprising 45 members [24]. They are classified into three subtypes, having different functions: M KIFs are not mobile on microtubules but they act on MT depolymerization. C KIFs and N KIFs mediate transport of cargos along microtubules. They are also important for cytokinesis, mitotic spindle formation, microtubules dynamics, etc. [25, 26]. Of note, the velocity of C and N KIF transport is also affected by MAPs [27].

Retrograde transport needs cytosolic dynein, which differs from axonemal dynein despite their similar structures. Cytoplasmic dynein is ubiquitously distributed existing in two isoforms [28]. Both are complexes of 1.5 MDa and they are composed of 14 chains: two heavy chains (DYNHC) that contain motor domain, which “walk” on the microtubule, driven by ATP hydrolysis. Four Light Intermediate Chains (DYNLIC) modulate activity of the heavy chains and two Intermediate Chains (DYNIC) are essential for dynein assembly (scaffolding). The DYNIC dimer acts as a linker or platform between the DYNHC and three dimers of Dynein Light Chains (DLC). The latter are subdivided into three families: Tctex, Lc8, and roadblock. DLC are implicated in several interactions including cellular and viral cargos. It has been shown, for instance, that Tctex interacts with DocA/B allowing neurotransmitter release. It also interacts with RAD21, which is essential for DNA repair, or with CD155 needed for membrane constitution. A recent review of Merino-Gracia et al. listed several viral cargos including HSV UL34, UL9 and UL35 and adenoviral protein E3, the matrix protein from Mason Pfizer virus and with L2 and E4 from papillomavirus interacting with DLCs [29]. The cargo–Tctex interaction is mediated by a common motif (R/K) (R/K)XX(R/K) on the cargo.

For the members of the Lc8 family, 66 cellular and viral partners have been experimentally shown to interact [30, 31].

These proteins comprise either a KXTQTX or a XG(I/V)QVD consensus motive. The cellular interaction partners serve in various mechanisms like mitosis (NEK9, EML3), transcription regulation (BS69, NRS1), nuclear transport (Nup159, Pak1) but also cytoplasmic transport of dynein and other cytoplasmic transport components (DYNIC, Swallow, Myosin 5a). Aside its role in the dynein complex Lc8 seems to have also chaperoning activities supporting protein dimerization [31, 32]. Among the viral Lc8 interaction partners are the African swine fever virus (ASFV) structural protein P54, HSV UL9, and Ebola virus VP35 [30].

Roadblock is involved in the transport of three cellular partners, which are Rab 6-positive transport vesicles, and TGF β which is important in cell proliferation [33, 34].

However, several cargos do not directly interact with proteins of dynein but via an adapter complex termed dynactin [35]. Dynactin is composed of 11 different subunits subdivided into two substructures: a filamentous structure of 40 nm composed of Arp1 polymer with cap of capZ proteins at one side and Arp11 and P62 cap at the other side, which is scaffolded by dynamitin. The second structure is called the flexible arm and is composed of P150glued. Due to its binding capacity, this protein is particularly important in dynactin complexation as P150glued binds to dynein (DYNIC) and directly to microtubules. Aside of being an adaptor complex, dynactin is important for dynein processivity, microtubules stabilization, and organization [36].

HBV capsids can be expressed in various systems. Transfected cell lines produce HBV from which the viral surface proteins can be dissociated using nonionic detergence. Alternatively, capsids can be obtained by stripping off the surface proteins from plasma-derived virions. However, in our hands, the removal of surface proteins from these HBV is incomplete. Both sorts of capsids contain the mature rcDNA, while capsids derived from lysed cells mostly contain replication intermediates [9].

Alternatively, HBV capsids can be produced in heterologous system like *E. coli*. These capsids represent the immature RNA-containing capsids, having the C terminus of the core proteins inside capsids' cavity by interaction with the RNA [37]. The C termini become accessible upon genome maturation, which is important as they comprise the nuclear localization signal needed for importin binding [9, 38]. In fact, such (or a similar) structural change of the capsids due to genome maturation is also evident as only capsids with rcDNA but not with an immature genome become encapsidated for progeny virus synthesis [39]. An alternative to the use of mature capsids is the use of empty *E. coli*-expressed capsids as they also expose the core C terminus on the surface [40]. In fact, the use of empty capsids is not entirely artificial as empty virions were also found in patient sera [41, 42], supporting the assumption that "emptiness" mimics the structure of capsids with mature rcDNA genomes.

2 Material

2.1 *Co-immune Precipitation/Co-sedimentation*

1. Transport Buffer (TB), mimicking intracellular conditions: 20 mM HEPES, 2 mM magnesium acetate, 110 mM potassium acetate, 1 mM EGTA.
2. Phosphate Buffered Saline (10× PBS): 1.36 M NaCl, 26.8 mM KCl, 101 mM Na₂HPO₄, 17.6 mM KH₃PO₄ at pH 7.4.
3. Dynabeads M280 sheep anti Rabbit IgG.
4. 60% Sucrose/TB.
5. Cell lysate or purified proteins.
6. Polycarbonate tubes (230 μL for TLA100 rotor).
7. Dako (anti HBc antibody).

2.2 *In Vitro Polymerization of Microtubules*

1. Phosphate Buffered Saline (10× PBS): 1.36 M NaCl, 26.8 mM KCl, 101 mM Na₂HPO₄, 17.6 mM KH₃PO₄ at pH 7.4.
2. General tubulin buffer (GTB: 80 mM PIPES, 2 mM MgCl₂, 0.5 mM EGTA pH 7.0), Tubulin Glycerol Buffer: cushion buffer (TGB : 80 mM PIPES, 2 mM MgCl₂, 0.5 mM EGTA, 60% glycerol pH 6.9).
3. Purified tubulin and purified tubulin labeled with Rhodamine.
4. GTP.
5. Teflon glass slides.
6. Poly L-Lysine.
7. 0.2% BSA/1× PBS.
8. Rabbit Reticulocyte Lysate (RRL).
9. Anti-capsid antibodies (DAKO).
10. Epifluorescence microscope or confocal microscope at large pinhole.

2.3 *Microinjection*

Somatic cells:

1. Microdish grid 500 (Ibidi).
2. Eppendorf FemtoJet microinjection or similar device allowing microinjection under microscopical control.
3. Unrelated fluorophore labeled antibodies.
4. 1× PBS.
5. 4% PFA.
6. 1× PBS, 0.2% Triton X 100.
7. Blocking solution: 1× PBS, 10% FCS, 0.1% saponine.
8. Epifluorescence microscope or confocal microscope at large pinhole.

Xenopus laevis oocytes:

1. Microinjection device for *Xenopus laevis* oocytes.
2. Forceps and biocular for manually dissecting the oocytes.
3. 1× TE: 50 mM Tris–HCl, 0.5 mM EDTA, pH 8.7.
4. MBS (modified Barth’s saline buffer): 88 mM NaCl, 1 mM KCl, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 10 mM HEPES, pH 7.5.
5. 2 % glutaraldehyde in MBS.
6. 2 % glutaraldehyde in low-salt buffer (LSB): 1 mM KCl, 0.5 mM MgCl₂, 10 mM HEPES, pH 7.5.
7. 2 % low melting agarose.
8. 1 % OsO₄.
9. Ethanol in different concentrations.
10. Epon 812 (Fluka).
11. Thin section device of 50-nm sections.
12. EM grids.
13. 2 % uranyl acetate.
14. 2 % Pb citrate.
15. Electron microscope.

3 Methods

3.1 Preparation of Empty Capsids

1. Use purified HBV capsids expressed in *E. coli* in a concentration of 10 mg/mL in 1× PBS, stored at 4 °C.
2. For 50 µg of capsids add 1 µL of RNase A at 20 mg/mL.
3. Add nine volumes of H₂O.
4. Incubate at 42 °C for 10 min (*see Note 1*).
5. Proceed to second incubation for 30 min at 37 °C.
6. Adjust salt concentration to 1× PBS.
7. Leave capsids at 4 °C for at least 24 h (*see Note 2*).
8. Verify RNA degradation by ethidium bromide stain after NAGE [38] (*see Fig. 1a*).
9. Verify capsid re-formation by Western blot after NAGE [43] (*see Fig. 1b*).

3.2 Co-immune Precipitation (*see Note 3*)

1. Use 3.5 × 10⁶ magnetic beads per assay (*see Note 4*).
2. Wash three times in 1 mL 1× PBS at RT (*see Note 5*).
3. Add 25 µg of anti HBc and incubate overnight at 4 °C (*see Note 6*).

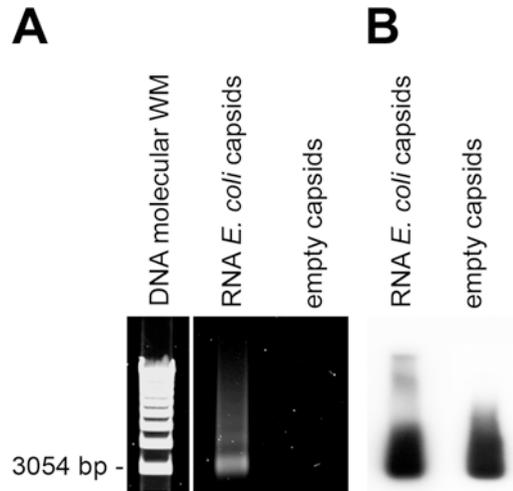


Fig. 1 (a) Ethidium bromide stains of *E. coli* capsids after native agarose gel electrophoresis. *Left*: DNA molecular weight marker, *middle*: purified capsids, *right*: capsids after permeabilization, RNaseA treatment and subsequent capsid reformation. (b) Western blot of *E. coli* capsids after native agarose gel electrophoresis. *Left*: purified capsids, *right*: capsids after permeabilization, RNaseA treatment and subsequent capsid reformation. Electrophoresis was done on 1% agarose/TAE gel

4. Wash three times in 1 mL 1× PBS.
5. Saturate beads with 1× PBS/0.25% BSA for 1 h at RT.
6. Wash beads three times with 1 mL 1× TB for 10 min at RT.
7. Add 100 ng of HBV capsids in 500 μL 1× PBS and incubate for 90 min at RT.
8. Wash three times with 1 mL TB.
9. Depending on the experiment, add purified proteins, cellular lysates (or both) (*see Note 7*).
10. Wash beads with 1 mL 1× PBS, 500 μL 1× PBS, and 250 μL 1× PBS. Change cups after each washing step.
11. After the last washing step suspend beads in 20 μL 1× PBS 0.1% NP40 prior to denaturation and loading on SDS PAGE.

3.3 Co-sedimentation (See Note 8)

This protocol allows verification of the co-precipitations avoiding unspecific binding to the matrix or antibodies used in co-precipitation (*see Note 9*). The described protocol has been established empirically (*see Note 10*) (for overview *see Fig. 2*).

1. Use 0.5–2 μg of HBV capsids with or without purified proteins or lysate (*see Note 11*).
2. Mark the polycarbonate centrifugation tubes by a pen allowing to have the orientation of tube inside the rotor.
3. Add 100 μL of 60% sucrose, 1× TB per tube.

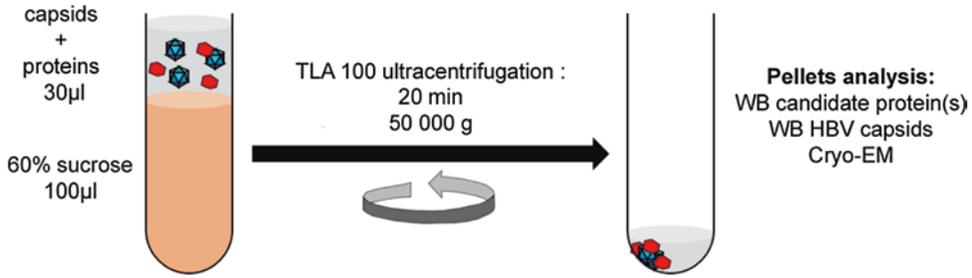


Fig. 2 Schematic presentation of co-sedimentation assays

4. Mix 0.5 µg capsids and cell lysate or purified proteins and add the sample in a final volume of 30 µL on the top of the cushion.
5. Spin at 50,000×*g* for 20 min at 4 °C.
6. Remove the supernatant carefully including the sucrose cushion using long tips.
7. Wash the pellet (very!) carefully by pipetting 20 µL of 1× TB around the pellet.
8. Suspend the pellet in 20 µL 1× PBS, 0.1 % NP40 and transfer the solution into Eppendorff LoBind cups.
9. Analyze sedimented proteins by SDS PAGE and subsequent Western blotting and/or by NAGE and Western blot for sensitive capsid detection [43].
- 9a. After NAGE, transfer capsids to a PVDF membrane using vacuum blot and 10× SSC. Immune detection is described elsewhere [38], allowing sensitivities in the sub-nanogram range (*see* Fig. 2).
- 9b. Immune detection of the capsid attach proteins depends upon the protein and the antibody and should be titrated before. Use a titration series and conditions allowing detection of one copy per capsid.

3.4 Binding to In Vitro Polymerized MT

We investigated capsids binding to in vitro polymerized microtubules by quantitative colocalization after microscopy. The tubulin used in our assays had a purity of at least 99% and was rhodamine labeled. The capsids were immune labeled (*see* below). For HBV capsids-MT interaction cell lysate had to be added to provide the dynein complex. Colocalization was quantified using ImageJ and in house plug-in. The plug-in quantifies automatically counted capsids by masks of microtubules and capsids. Importantly, proper quantification needs microscopy fields showing a similar percentage covered with MT.

3.4.1 MT In Vitro Polymerization

This protocol is based on cell nucleation that leads to cell's microtubules assembly. In vitro this process forces tubulin concentration.

1. Thaw a non-labeled tubulin aliquot of 10 μL (10 mg/mL) on ice and mix with rhodamine-labeled tubulin, freshly resuspended in 5 μL GTB was added (*see Note 12*).
2. Add GTP to a final concentration of 1 mM in a total reaction volume of 16 μL (*see Note 13*).
3. Add 4 μL of TGB (cushion tubulin buffer, *see Subheading 2*), which adjusts the glycerol concentration to 5% allowing tubulin polymerization.
- 4a. Incubate for 45 min at 37 °C.
- 4b. During this incubation: coat Teflon glasses slides with poly L-Lysine by adding it a concentration of 0.01% for 10–30 min at RT. Remove excessive poly L-Lysine afterward.
5. After MT polymerization, increase the glycerol concentration to 27.5% by adding 180 μL TGB.
6. Distribute 20 μL of this solution per well on the glass slide having a surface of 50 mm² (8 mm diameter).
7. Remove excessive fluid after 15 min incubation at RT.
8. Dry cover slips and wash the MT-covered wells three times with 1 \times PBS.
9. Block unspecific binding sites on the wells by 0.2% BSA/1 \times PBS for 30 min at RT.
10. Wash the wells three times with 1 \times PBS.
11. Verify MT binding and polymerization by fluorescence microscopy (*see Note 14* and Fig. 3).

3.4.2 Capsid Binding to In Vitro Polymerized Microtubules

This protocol can be modified by adding different inhibitors or competitors. It further allows confirmation of indirect binding of cargos to MT.

1. Mix capsids with cell lysate (100 ng of capsids and 10 μL of RRL in 100 μL final volume of transport buffer).
2. Add capsid solution for 90 min at RT.
3. Wash wells 3 \times using 1 \times PBS.
4. Samples may be fixed using 4% paraformaldehyde EM grade. However, this treatment had no effect in our hands.
5. Add anti HBc antibodies in a dilution of 1:1000 in 1 \times PBS to the wells for 45–60 min at RT.
6. Wash three times in 1 \times PBS.
7. Add secondary antibody (Alexa 488 labeled anti rabbit antibody, 1:1000 in 1 \times PBS) for 45 min at RT.
8. Wash three times in 1 \times in PBS.
9. Mount the slide and analyze the wells by fluorescence microscopy (e.g., using a Leica DMI 600 or Axioplan Zeiss). The instability of rhodamine favors the use of anti-bleaching solutions for mounting.

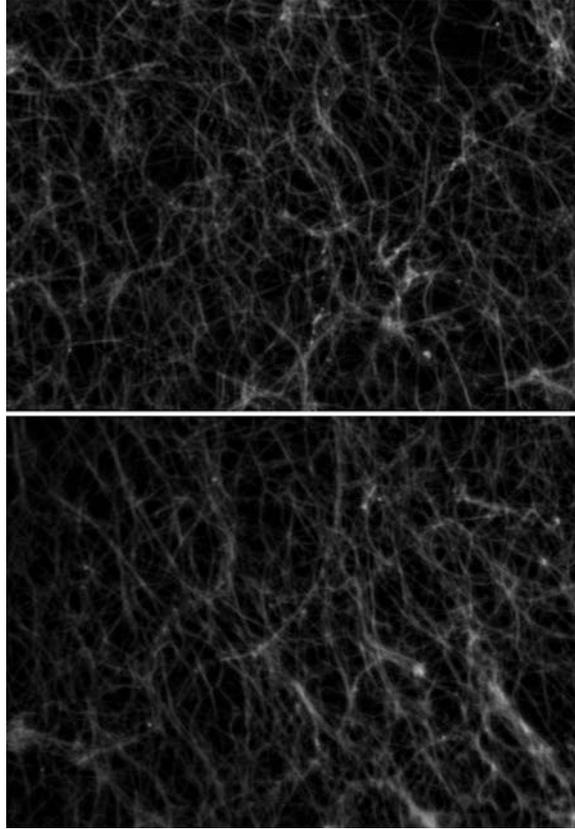


Fig. 3 Fluorescence microscopy of in vitro polymerized microtubules on a poly lysine-coated glass cover slip using a mixture of unlabeled and rhodamine-labeled tubulin

3.5 Microinjection: The Possibility of Competition

The protocols described before show the need and analysis of binding partners but no functional investigation on active transport. Microinjection, in contrast, allows such analyses. Lipofection was shown to be an alternative for HBV capsids [19] but is limited for detailed investigations.

For microinjection all sorts of cells can be used as cytoplasmic dynein is well conserved between species [44]. This allows the use of cells with a large cytoplasm, which are easier to manipulate (e.g. U2OS cells). Alternatively, *Xenopus laevis* oocytes can be used. The oocytes cells have the advantage that higher volumes can be injected (50 nL instead of 100 fL) allowing to work with lower capsid concentration and of less purity as aggregation is less frequent. Furthermore, the much larger size of *Xenopus laevis* oocytes makes it unlikely that the capsids arrive at the nucleus just by the turbulences due to microinjection [45].

The disadvantage of *Xenopus laevis* oocytes is that localizing the capsids by indirect immune fluorescence is difficult asking for

electron microscopy as readout. Electron microscopy however has the disadvantage that quantification is laborious as it has to be done on several sections.

Irrespectively, microinjections allow the use of competitors or inhibitors identified by in vitro assays. This can comprise the pre-injection of anti-tubulin antibodies interfering with transport, which block arrival of HBV capsids at the nuclear envelope efficiently. When using identified interaction partners of HBV capsids as competitors, attention has to be paid in interpretation of the results as they can interfere with capsid binding to dynein but also affect the general transport capacity. This asks for using other cargos using the same transport mechanism as controls.

For somatic microinjection, we use U2OS cell line as these cells exhibit a large cytoplasm facilitating microinjection.

1. Seed cells in microdish grid 500 (Ibidi) one day before experiment.
2. Perform microinjection using an Eppendorf FemtoJet microinjection or a similar device under microscopical control.
3. Inject capsid preparations (*E. coli*-derived full or empty, or from HepG2.2.15 supernatant after surface protein stripping) mixed with unrelated labeled antibodies (*see Note 15*).
4. After the required incubation time at 37 °C wash slides with 1× PBS and fix with 4% PFA for 30 min at RT.
5. Wash three times with 1× PBS.
6. Permeabilize fixed cells using 1× PBS, 0.2% Triton X 100 for 10 min at RT.
7. Wash three times with 1× PBS.
8. Block unspecific binding sites by incubating for 10 min at RT with blocking solution.
9. Immunostaining of capsids and cellular structures depends upon the individual antibodies and should be preevaluated. Pay attention that the primary antibodies do not react with the injected antibodies and that there is no bleed through of the fluorophores (depended upon the microscope).

Microinjection of Xenopus laevis oocytes. Detailed description on the microinjection and thin sectioning for EM is described in [9].

1. Inject oocytes with c. 50 nL of capsid preparation containing c. 5×10^8 capsids. Inject into the cytoplasm at the transitional zone between the animal and vegetal poles. For mock injections use 1× TE.
2. Incubate oocytes at RT in MBS for the required time (*see Note 16*).
3. Fix oocytes are fixed o.n. at 4 °C with 2% glutaraldehyde in MBS.

4. Wash oocytes with MBS.
5. Disect the oocytes at the animal poles and refix in 2% glutaraldehyde in low-salt buffer (LSB: 1 mM KCl, 0.5 mM MgCl₂, 10 mM Hepes, pH 7.5) for 1 h at RT.
6. Dissect oocytes and wash with LSB.
7. Embed the samples in 2% low melting agarose.
8. Post-fix the samples using 1% OsO₄.
9. Dehydrate samples sequentially by incubation in increasing ethanol concentrations and embed them in Epon 812 (Fluka) [45].
10. Cut the blocks in 50 nm sections and place the thin sections on EM copper grids.
11. Stain with 2% uranyl acetate for 30 min and 2% Pb citrate for 5 min.
12. Go for EM and image acquisition.
13. Quantify capsids at the NPCs by counting the number of capsids at the NPCs and the percentage of NPCs covered with capsids. Use different sections and images as the numbers vary.

4 Notes

1. 42 °C allows partial dissociation of purified *E. coli* capsids.
2. 4 °C favors re-association of disassembly intermediates to capsids.
3. All steps: incubations and washing steps were done using a rotating wheel for incubation to ensure good mixture of the component.
4. We used biomagnetic beads (dynabeads M280 sheep anti Rabbit IgG) as the reduced surface caused less unspecific binding of capsids than for instance sepharose. However, these beads stick to the surface of siliconized tube walls so we recommend the LoBind tubes after BSA saturation.
5. The beads were coated with anti-capsid antibodies (DAKO), which interact with core proteins assembled to at least hexameric form [46]. The disadvantage of this technique is that once bound of the antibodies no further detection of the capsids via immune blot following native agarose gel electrophoresis (NAGE) is possible for verifying the same capsid amount bound to the beads. NAGE using DAKO however is in our hands much more sensitive than the use of antibodies against denatured core proteins following PAGE and classical Western blot. Alternatively, radioactively labeled core/capsids can be used.
6. An essential negative control thus comprises samples without capsids but the other components.

7. Of note, the interpretation of the results in the presence of lysate can cause problems when a complete dissociation of MT to tubulin dimers for instance by incubation at 4 °C is not ensured. Avoiding a control experiment showing that has the risk of indirect precipitation.
8. The main problem is the low volume and requires more sensitive mechanical manipulation. However, aside of the advantages described before co-sedimentation is faster than co-immune precipitation. Further advantages are the parallel detections of intact capsids after NAGE and further input proteins but also the detection of capsids by electron microscopy excluding aggregates and allowing further structural analysis.
9. The sedimentation shows that the binding occurred to capsids and not to disassembly intermediates, which can be derived from capsid breathing [46, 47] during the incubation periods or washing steps.
10. Considering the sedimentation coefficient of HBV capsids assays by centrifugation using a Beckman Tabletop and a TLA100.
11. The quantity of proteins or lysate was tested before to avoid unspecific sedimentation due to aggregate formation.
12. The rhodamine labeled tubulin had a concentration of 20 µg and represent 6% of the total amount of tubulin. With a final volume of 20 µL we obtain a final glycerol concentration of 5%.
13. The high final glycerol concentration allows the maintenance of microtubules without stabilizing agents as paclitaxel®.
14. Cave: rhodamine bleaches rapidly.
15. We use Alexa594-labeled secondary antibodies in a final concentration of 0.4 mg/mL for labeling of the injected cells and for visualizing the proper injection site. Due to their MW of 150 kDa they diffuse in the injected compartment without passing the nuclear envelope. When the intensity is compared with a standard dilution series quantification of the injected capsids is possible. If required dilute your sample with transport buffer.
16. First capsids can be observed at the nucleus 15–30 min after microinjection. Accumulation occurs during the first 4 h.

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A Homokaryon Assay for Nucleocytoplasmic Shuttling Activity of HBV Core Protein

Ching-Chun Yang, Hung-Cheng Li, and Chiaho Shih

Abstract

Hepatitis B virus (HBV) core protein (HBc) can be present in both nucleus and cytoplasm. The arginine-rich domain (ARD) at the cytoplasmic tail of HBc contains both a nuclear localization signal (NLS) and nuclear export signal (NES). We established a homokaryon assay to detect the dynamic trafficking of HBc between nucleus and cytoplasm in hepatocytes. Using immunofluorescence assay (IFA) and PEG-induced cell-cell fusion, we demonstrated that a chimeric reporter protein of SV40 large T antigen, when fused in-frame with HBc ARD, can shuttle from a donor nuclei (*green*) to the recipient nuclei (*red*) in the context of binucleated or polynucleated hybrid cells. The shuttling activity driven by HBc ARD can be measured quantitatively by this IFA method.

Key words HBV core, Arginine-rich domain (ARD), Nuclear import, Nuclear export, Nucleocytoplasmic shuttling

1 Introduction

There are approximately 350 million carriers chronically infected with HBV. Chronic hepatitis B leads to the development of cirrhosis and highly malignant hepatocellular carcinoma (HCC). While chronic hepatitis B is treatable these days, it is most often not curable [1]. It is known that HBV core protein (HBc) can be found in both nucleus and cytoplasm. Based on immunofluorescence microscopic analysis (IFA), the pattern of cytoplasm-predominant HBc in the liver biopsy samples is often associated with more severe liver inflammation [2–8]. In contrast, the nucleus-predominant HBc pattern tends to be associated with less aggressive liver disease. To better understand HBV pathogenesis and the subcellular trafficking of HBc in chronic hepatitis, we developed a homokaryon assay to investigate the mechanism of nuclear import and export of HBc in hepatocytes.

HBc contains a capsid assembly domain at the amino terminus, and an arginine-rich domain (ARD) at the carboxyl terminus. This

ARD domain contains clustering arginine residues reminiscent of the clustering basic amino acids of the conventional nuclear localization signals (NLS) [9]. Different HBV research groups mapped the NLS-like signals of HBc to different positions of the ARD domain [10–12]. This long-standing discrepancy in the positions of the NLS-like signals in HBc was revisited by us in cell culture in the context of active HBV replication [13]. Surprisingly, we identified two nuclear export signals (NES) in addition to a bipartite NLS signal. Further investigation demonstrated that HBc can be exported as an RNP cargo via an NXF1-p15 complex mediated mRNA export pathway [14]. Being equipped with both NLS and NES, HBc appeared to be capable of shuttling between nucleus and cytoplasm as revealed by both heterokaryon and homokaryon assays [13].

The principle of heterokaryon and homokaryon assays is to produce somatic cell hybrids containing two nuclei from the same species (homokaryon) or two different species (heterokaryon). A protein containing both NLS and NES can in theory shuttle from the donor to the recipient nuclei. In the case of the heterokaryon assay, the protein shuttling activity from the donor to the recipient nuclei of a different species can be visualized through the species-specific DAPI staining patterns of the nuclei under confocal microscopy [15]. However, it often requires some experience and confidence in differentiating the nuclear staining patterns of one species (human) from the other species (mouse).

To circumvent this technical issue, we developed the “homokaryon assay” (Fig. 1). As for the heterokaryon assay, we generated the somatic cell hybrids between donor and recipient cells by PEG treatment. Unlike the heterokaryon assay, we did not differentiate donor from recipient nuclei by their species-specific DAPI staining patterns. Instead, we marked the recipient nuclei with one color (red) by transfection with a protein containing only NLS but

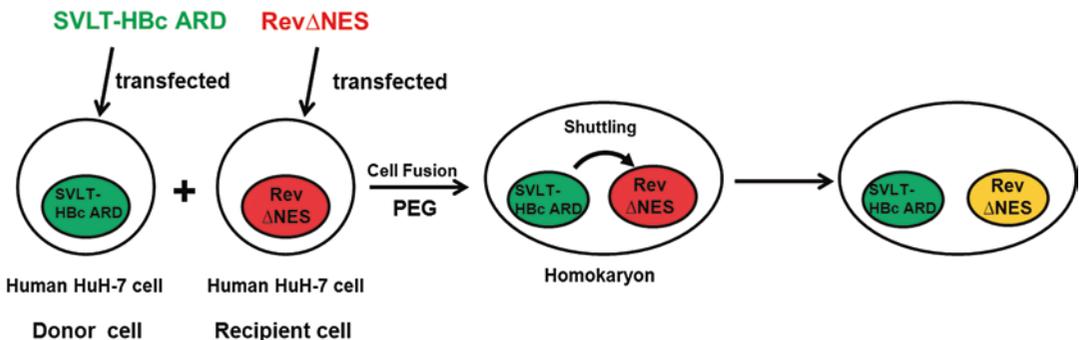


Fig. 1 Experimental design of the homokaryon assay to detect the shuttling activity of HBc. HuH-7 donor cells transfected with SV40LT-HBc ARD chimera (SVLT-HBc ARD) (*green*) were fused with HuH-7 recipient cells transfected with NES-deficient HIV-1 Rev (Rev Δ NES) (*red*). SVLT and Rev Δ NES are supposed to be localized exclusively to the nucleus. However, if the chimeric protein of SVLT-HBc ARD can be colocalized with Rev Δ NES protein (*yellow*), it indicates that the SVLT-HBc ARD protein can shuttle from donor to recipient nucleus

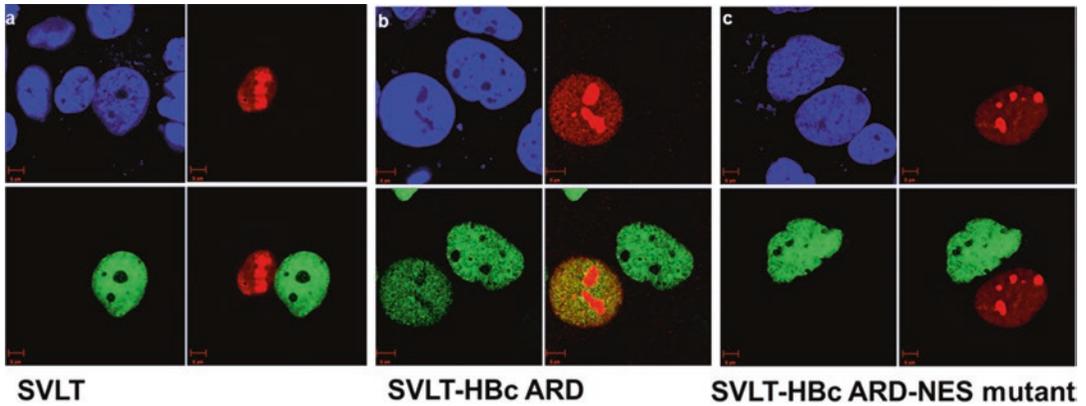


Fig. 2 Homokaryon analysis revealed the existence of two independent NES-like signals in HBc ARD domain. HuH-7 donor cells were transfected with wild type SVLT (*left panel a*), SVLT-HBc ARD chimera (*middle panel b*), or SVLT-HBc ARD-NES defective mutant (*right panel c*), respectively. These donor cells (*green*) were fused with HuH-7 recipient cells transfected with NES-deficient HIV-1 Rev (Rev Δ NES) (*red*). (*a*) Wild type SVLT contains only NLS and no NES, and is localized exclusively to the nucleus. There was no transport of SVLT from donor to recipient nucleus. (*b*) Chimeric protein of SVLT-HBc ARD appeared to shuttle from donor to recipient nuclei. (*c*) No apparent shuttling was observed for HBc ARD-NES mutant. This figure is adapted from Li et al. (2010) [13], with the permission from *PLoS Pathogens*

without any NES. In addition, we marked the donor nuclei with a different color (green), by transfection of cells of the same species with a putative shuttling protein, which contains both NLS and NES. In the context of a somatic cell hybrid, a green colored shuttling protein could exit the donor nucleus via its NES, and get imported into the red recipient nuclei via its NLS, which would then result in a yellow color in the recipient nuclei. Lack of the yellow color would indicate the lack of shuttling activity of the putative shuttling protein under test.

An example of the homokaryon assay and results are shown in Fig. 2. Specifically, we tested whether the putative NES of HBc ARD, when fused in-frame with an NLS-containing SV40 large T antigen (SVLT), could render such a chimeric protein SVLT-HBc ARD shuttling activity in the homokaryon assay (middle panel b, Fig. 2). As a control, when the NES of HBc ARD was ablated, the shuttling activity of the chimera protein was lost, and no yellow color was detected by confocal microscopy (right panel c, Fig. 2). The wild type SVLT contains only NLS and no NES. Therefore, SVLT is localized exclusively in the nucleus (left panel a, Fig. 2).

2 Materials

2.1 Cell Culture and Transient Transfection

1. Human hepatoma HuH-7 cells.
2. Dulbecco's modified Eagle medium (DMEM).
3. Fetal bovine serum (FBS).

4. Phosphate buffered saline (PBS).
5. A 37 °C, 5 % CO₂ incubator.
6. 6 and 3 cm dishes.
7. Transfection reagents: Lipofectamine 2000 (*Invitrogen, USA*) or PolyJet DNA transfection reagent (*SignaGen, USA*).

2.2 Homokaryon Assay

1. 0.5 % trypsin–EDTA (10×).
2. Glass coverslips: Round (18 mm in diameter) or square (18×18 mm).
3. Protein synthesis inhibitors: cycloheximide.
4. Polyethylene glycol (PEG) 6000.

2.3 Immunofluorescence Analysis (IFA)

1. 4 % paraformaldehyde.
2. 0.5 % Triton X-100 in PBS.
3. 3 % bovine serum albumin (BSA) in PBS.
4. Primary antibody specific for chicken polyclonal anti-Rev (HIV-1) antibody (*ICON-GeneTex, Taiwan*), mouse monoclonal anti-SV40 LT (P Ab416) antibody (*ICON-GeneTex, Taiwan*).
5. Secondary antibodies: Rhodamine-conjugated donkey anti-mouse polyclonal antibody (*Santa Cruz, USA*); and FITC-conjugated goat anti-chicken-IgY antibody (*ICON-GeneTex, Taiwan*).
6. Mounting reagent: ProLong Gold antifade reagent with 49-6-diamidino-2-phenylindole (DAPI) (*Invitrogen, USA*).
7. Confocal microscope.

3 Methods

3.1 Cell Culture and Transient Transfection

1. Seed human hepatoma HuH-7 cells to 6 cm dishes containing 10 % fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM) at 37 °C in the presence of 5 % CO₂.
2. Transiently transfect HuH-7 donor cells with SVLT, SVLT-HBc ARD or SVLT-HBc ARD-NES mutant chimera by using transfection reagents such as Lipofectamine 2000 or PolyJet DNA transfection reagent according to the vendor's protocols.
3. Transiently transfect HuH-7 recipient cells with NES-deficient HIV-1 Rev (RevΔNES).

3.2 Homokaryon Assay

1. Twenty-four hours post-transfection, trypsinize transfected donor and recipient HuH-7 cells.
2. Premix well HuH-7 donor and recipient cells at a 1:1 ratio in 2 mL DMEM containing 10 % FBS, before seeding onto glass coverslips (18×18 mm) in the 3 cm dishes (*see Note 1*).

3. Maintain the coculture at 37 °C for at least 18 h.
4. Treat the cocultured cells with cycloheximide at 100 µg/mL in 2 mL DMEM containing 10% FBS for 30 min prior to fusion.
5. To induce fusion, cover the cells with 50% (wt/vol) polyethylene glycol (PEG) 6000 in 2–3 drops of phosphate-buffered saline (PBS) for 2 min at 37 °C (*see Note 2*).
6. Rinse the PEG-treated cells for at least five times with 3 mL PBS (*see Note 3*).
7. Incubate these PEG-treated cells for 2 h in DMEM containing 10% FBS and cycloheximide at 100 µg/mL at 37 °C in the presence of 5% CO₂ prior to immunofluorescence analysis.

3.3 Immunofluorescence Analysis (IFA)

1. Wash the harvested cells with 3 mL PBS three times before fixation for 30 min at room temperature by using freshly prepared 4% paraformaldehyde in 2 mL PBS.
2. Wash the fixed cells again with 3 mL PBS three times.
3. To permeabilize the cells, incubate in 0.5% Triton X-100 in 2 mL PBS for 15 min at room temperature.
4. Wash the permeabilized cells with 3 mL PBS three times.
5. To block the washed cells, incubate in 2 mL PBS containing 3% BSA for at least 30 min at room temperature.
6. Remove blocking buffer and incubate with the primary antibody diluted in 1:500 for 1 h at room temperature.
7. Wash the cells with 3 mL PBS three times.
8. Remove PBS and incubate with the secondary antibody diluted in 1:200 for 1 h at room temperature.
9. Wash the cells with 3 mL PBS three times.
10. After immunostaining, mount the coverslips on slides in ProLong Gold antifade reagent with 49-6-diamidino-2-phenylindole (DAPI).
11. Use confocal microscope (*Zeiss-LSM 510*) to collect images. Images are averaged four times and processed with Zeiss software.

4 Notes

1. The transient transfection efficiency is the key to the homokaryon assay. The higher the transient transfection efficiency is, the more likely is the homokaryon assay to succeed.
2. 50% (wt/vol) polyethylene glycol (PEG) 6000 is cytotoxic. Do not incubate with cells for longer than necessary (2 min).
3. PEG is sticky, and difficult to wash away cleanly. Repeated washings may be necessary for best results.

Acknowledgments

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Analyses of HBV cccDNA Quantification and Modification

Yuchen Xia, Daniela Stadler, Chunkyu Ko, and Ulrike Protzer

Abstract

Covalently closed circular DNA (cccDNA) serves as the transcriptional template of hepatitis B virus (HBV) replication in the nucleus of infected cells. It ensures the persistence of HBV even if replication is blocked. Immune-mediated killing of infected hepatocytes, cell division, or cytokine induced non-cytolytic degradation of cccDNA can induce the loss of cccDNA. For studies on HBV control, the analysis of cccDNA integrity and its exact quantification is very important. Here, we describe different methods for HBV cccDNA quantification and modification.

Key words Covalently closed circular DNA, Quantitative PCR, Differential DNA denaturation PCR, Quantitative differential DNA denaturation PCR, Deamination

1 Introduction

After infection, hepatitis B virus releases its partially double strand DNA genome into the host nucleus to form covalently closed circular DNA (cccDNA). HBV cccDNA serves as a template for viral transcription and replication, and thus plays a central role in HBV infection [1]. Persistence of cccDNA is a crucial issue in hepatitis B treatment since a cure for HBV infection would require the elimination or large diminishment of the cccDNA pool. Thus, novel antiviral agents that eliminate cccDNA-containing hepatocytes or affect cccDNA integrity, stability, or transcriptional activity are required to promote a cure to chronic HBV infection. To this end, the analysis of cccDNA in a sensitive and specific way is very important for the study of virus biology and development of potential antiviral therapies.

We recently demonstrated an antiviral mechanism that interferes with cccDNA integrity and stability in infected hepatocytes by introducing base exchanges in cccDNA through cytosine deamination by APOBEC3 family deaminases after either interferon (IFN)- α , IFN- γ or tumor necrosis factor- α treatment, or lymphotoxin β receptor-activation [2–4]. These induced base excision

repair (BER) proteins that repair damaged DNA are responsible primarily for removing small, non-helix-distorting base lesions from the genome [5]. This mechanism is also involved in the host response to foreign DNA [6]. APOBEC3A or 3B expression resulted in the deamination of multiple cytidines in HBV cccDNA [2–4]. The resulting uracils are then excised by uracil-DNA glycosylase, forming nuclease-sensitive apurinic/apyrimidinic (AP) sites. Finally, cellular nucleases like APE1 cleave the damaged foreign DNA [2, 6].

Here, we describe protocols for (1) a quantitative PCR method that allows rapid and sensitive detection and quantitation of HBV cccDNA with high specificity and efficacy, (2) differential DNA denaturation and PCR detection of cccDNA deamination and modification, (3) quantitative differential DNA denaturation PCR quantifying deaminated cccDNA, and (4) other cccDNA modification or repair methods.

2 Materials

1. NucleoSpin® Tissue kit (Macherey-Nagel, Germany).
2. T5 exonuclease (New England Biolabs, USA).
3. PCR grade water (Roche, Germany).
4. NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, USA).
5. LightCycler 480 SYBR Green I master mix (Roche, Germany).
6. LightCycler® 480 Real-time PCR system (Roche, Germany).
7. PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Germany).
8. LightCycler™ 96 system (Roche, Germany).
9. GeneJET Gel Extraction Kit (Fermentas, Germany).
10. TA Cloning® Kit (Invitrogen, Germany).
11. GeneJET Plasmid Miniprep Kit (Thermo Scientific, Germany).
12. One Shot® Stbl3™ Chemically Competent E. coli (Invitrogen, USA).
13. TAE buffer: 40 mM Tris acetate, 1 mM EDTA, pH 8.2–8.4 (at 25 °C).
14. APE1 (New England Biolabs, USA).
15. PreCR Repair Mix (New England Biolabs, USA).
16. Interferon α (Referon A, Roche, Germany).
17. Entecavir (Sigma, USA).
18. HepaRG cells.

19. HepaRG cell culture medium: Williams E medium (Gibco) supplemented with 10% fetal calf serum Fetalclone II (Hyclone), 20 mM L-glutamin (Gibco), 50 U/ml penicillin/streptomycin (Gibco), 80 µg/ml gentamicin (Ratiopharm), 0.023 IE/ml human insulin (Sanofi-Aventis), and 4.7 µg/ml hydrocortisone (Pfizer).
20. HepaRG cell differentiation medium: HepaRG cell culture medium supplemented with 1.8% DMSO (Sigma).
21. Primary human hepatocytes (PHH), *see Note 1*.
22. Collagen type IV.
23. HepAD38 cells.
24. HepG2.2.15 cells.
25. HBV inocula were prepared as described before [4]; *see Note 2*.
26. PCR primers (*see Note 3*).
27. HBV cccDNA sample prepared from differentiated HepaRG cells (*see Note 4*).
28. HBV rcDNA sample prepared from HepG2.2.15 cells (*see Note 5*).

3 Methods

3.1 Quantitative PCR

Since HBV cccDNA is maintained at low copy numbers in the infected hepatocytes, traditional Southern blot detection relies on large amount of initial material and requires Hirt lysis to extract protein-free DNA [7]. Quantitative PCR (qPCR) with cccDNA selective primers provides a rapid, reliable, and quantitative method for HBV cccDNA detection with high sensitivity and good specificity. However, care needs to be taken because high amounts of HBV rcDNA will give a false-positive result.

1. Extract intracellular total DNA with “NucleoSpin® Tissue” kit according to manufacturer’s instructions (*see Note 6*).
2. Isolated intracellular total DNA is subjected to T5 exonuclease treatment (NEB, M0363). Mix 8.5 µl of total cellular DNA with 1 µl NEB buffer 4 (10×) and 0.5 µl T5 exonuclease (10 U/ul) to a final volume of 10 µl. Incubate the mixture at 37 °C for 30 min with subsequent heat inactivation at 99 °C for 5 min (*see Note 7*). After T5 exonuclease treatment, add 30 µl of distilled water to each sample for fourfold dilution (*see Note 8*). If rc and cccDNA amounts are low, this step can be omitted.
3. Prepare 1:2 serial diluted DNA as standard (*see Note 9*).
4. Mix 0.5 µl of cccDNA92fw (20 µM), 0.5 µl of cccDNA2251rev (20 µM), 4 µl T5 exonuclease treated DNA, and 5 µl LightCycler™ 480 SYBR Green I master (*see Note 10*).

Table 1
PCR program for HBV cccDNA quantification

Program name		Cycles	Analysis mode				
Denaturation		1	None				
Amplification		50	Quantification				
Melting		1	Melting curves				
Cooling		1	None				
Denaturation							
Target (°C)	Acquisition modes	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisitions (per °C)	Sec target (°C)	Step size (°C)	Step delay (cycles)
95	None	00:10:00	4.4		0	0	0
Amplification							
Target (°C)	Acquisition modes	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisitions (per °C)	Sec target (°C)	Step size (°C)	Step delay (cycles)
95	None	00:00:15	4.4		0	0	0
60	None	00:00:05	2.2		0	0	0
72	None	00:00:45	4.4		0	0	0
88	single	00:00:02	4.4		0	0	0
Melting							
Target (°C)	Acquisition modes	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisitions (per °C)	Sec target (°C)	Step size (°C)	Step delay (cycles)
95	None	00:00:01	4.4				
65	None	00:00:15	2.2				
95	continuous		0.11	5			

Three-step PCR reaction was programmed with LightCycler™ 480 software

5. Perform PCR on LightCycler™ 480 Real-time PCR system with the program shown in Table 1 (*see Note 11*).
6. Analyze melting curves of cccDNA PCR products to check specificity and standard curve to determine PCR efficiency (Fig. 1) (*see Note 12*).
7. To obtain a relative quantification of cccDNA, host genome Prnp gene can be used as reference and total HBV DNA can be determined. Mix 0.5 µl of Prnp fw and 0.5 µl of Prnp rev,

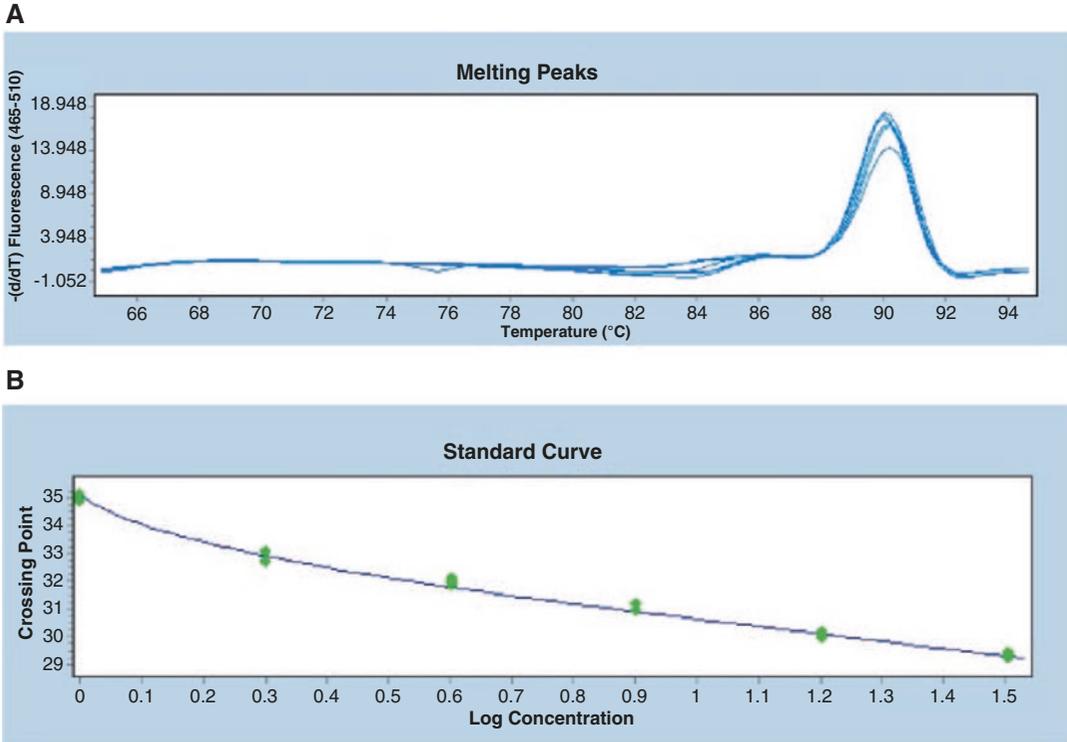


Fig. 1 Melting curve and standard curve. HBV cccDNA PCR was performed on Light Cycler™ 480 machine, melting curve (a) and standard curve (b) were analyzed

or 0.5 μ l of primer HBV1745 fw and 0.5 μ l of primer HBV1844rev (20 μ M each), 4 μ l template DNA and 5 μ l LightCycler 480 SYBR Green I master mix. The following cycling profile is recommended: 95 °C 5 min; (95 °C 25 s, 60 °C 10 s, 72 °C 30 s) \times 40; melting curve; with single acquisition at 72 °C in the amplification process and continuous acquisition (5/°C) during melting.

8. Validation of cccDNA qPCR:

Perform HBV cccDNA and rcDNA qPCR with serial diluted HBV rcDNA samples. Determine amplification curve, melting curve and analyze Cp value (Table 2) (*see Note 13*).

3.2 Differential DNA Denaturation PCR

Differential DNA denaturation PCR (3D-PCR) is a method that can be used to discover mutants with GC \rightarrow AT transitions [8, 9]. AT-rich DNA melts at lower denaturation temperatures than GC-rich DNA due to the two hydrogen bonds between A and T versus the three between G and C. Therefore, doing PCR with a lower denaturing temperature allows differential amplification of AT-rich sequences.

1. Dilute amplicons from cccDNA qPCR 1:50 with PCR grade water as 3D-PCR templates.

Table 2
Validation of cccDNA qPCR

rcDNA samples	Mean Cp value (HBV-DNA primer)	Mean Cp value (cccDNA primer)
10 ⁷ /reaction	14.57 ± 0.25	37.80 ± 0.44
10 ⁶ /reaction	16.76 ± 0.36	Non-detectable
10 ⁵ /reaction	20.07 ± 0.33	Non-detectable
10 ⁴ /reaction	23.68 ± 0.47	Non-detectable
10 ³ /reaction	27.65 ± 0.43	Non-detectable

Serial diluted HBV rcDNA samples were determined by both cccDNA and rcDNA qPCR. Mean Cp value and the variations are presented

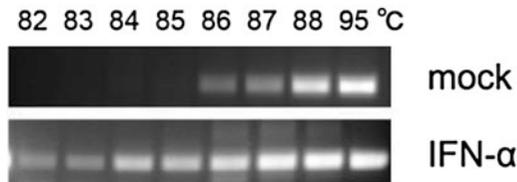


Fig. 2 3D-PCR of HBV cccDNA. HBV infected primary human hepatocyte cells were treated with 1000 IU/ml of IFN- α . 3D-PCR was performed to detect HBV cccDNA deamination 4 days after treatment

- Mix 0.5 μ l of 5'HBxin (20 μ M), 0.5 μ l of 3'HBxin primer (20 μ M), 1 μ l template DNA, and 23 μ l PCR grade water.
- Add the mixture into "PuReTaq Ready-To-Go PCR Beads" (GE Healthcare, Munich, Germany). Briefly centrifuge the tubes to bring down the liquid (*see Note 14*).
- Perform PCR amplifications in LightCycler™ 96 system (Roche Diagnostics, Mannheim, Germany) by using a gradient in denaturing temperature: (92–82 °C) for 5 min; then (92–82 °C for 1 min; 60 °C for 30 s; 72 °C for 30 s) \times 35, 72 °C for 10 min. Amplification at denaturing temperature of 95 °C was used as positive control (*see Note 15*).
- Amplicons were detected in a 2%-agarose gel electrophoresis (Fig. 2).
- Purify DNA from gel by using "GeneJET Gel Extraction Kit" (Fermentas, St. Leon-Rot, Germany).
- Ligate purified DNA into pCR®2.1 vector from "TA Cloning® Kit" (Invitrogen, Karlsruhe, Germany) and transform ligation product into competent *E. coli* Stbl3 cells according to manufacturer's instructions.
- Extract plasmid using "GeneJET Plasmid Miniprep Kit" (Thermo Scientific, Schwerte, Germany). Dilute DNA samples

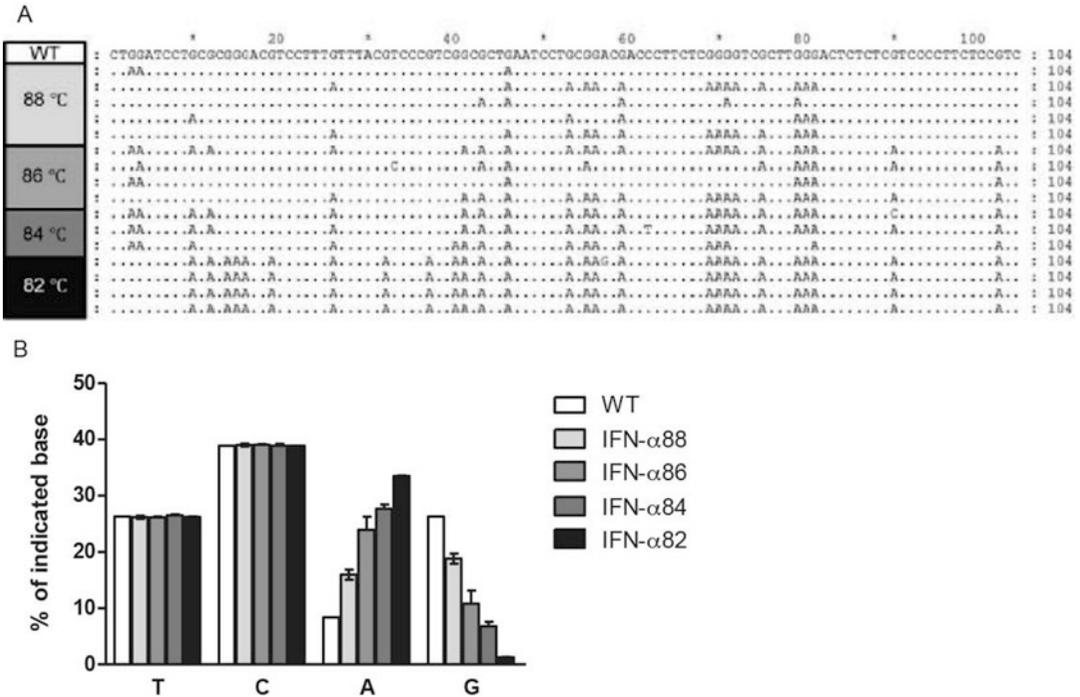


Fig. 3 Sequence analysis of 3D-PCR products. (a) PCR products with different denaturation temperatures were cloned and sequenced. (b) Different contents of nucleotides between IFN- α treated and untreated sample were summed up

to a concentration between 30 and 100 ng/ μ l and send for sequencing.

- Analyze DNA sequences by multiple sequence alignment (<http://www.ebi.ac.uk/Tools/msa/muscle>), GeneDoc and MEGA 5 (Fig. 3) (*see Note 16*).

3.3 Quantitative 3D-PCR

Quantitative 3D-PCR combines 3D-PCR with SYBR green dye-based quantitative PCR, provides the proportion of GC \rightarrow AT transitions in total DNA populations.

- Dilute cccDNA qPCR products 1:50 with PCR grade water as 3D-PCR templates.
- Mix 0.5 μ l of 5'HBxin (20 μ M), 0.5 μ l of 3'HBxin primer (20 μ M), 4 μ l template DNA and 5 μ l SYBR[®] Green Mix.
- Carry out PCR in LightCycler[™] 96 system by using a gradient in denaturing temperature: (95–84 $^{\circ}$ C) for 5 min; then (95–84 $^{\circ}$ C for 1 min; 60 $^{\circ}$ C for 30 s; 72 $^{\circ}$ C for 30 s) \times 35, 72 $^{\circ}$ C for 10 min. Amplification at denaturing temperature of 95 $^{\circ}$ C was used as 100% for the evaluation (*see Note 17*).
- Analyze the result with the software (Fig. 4) (*see Note 18*).

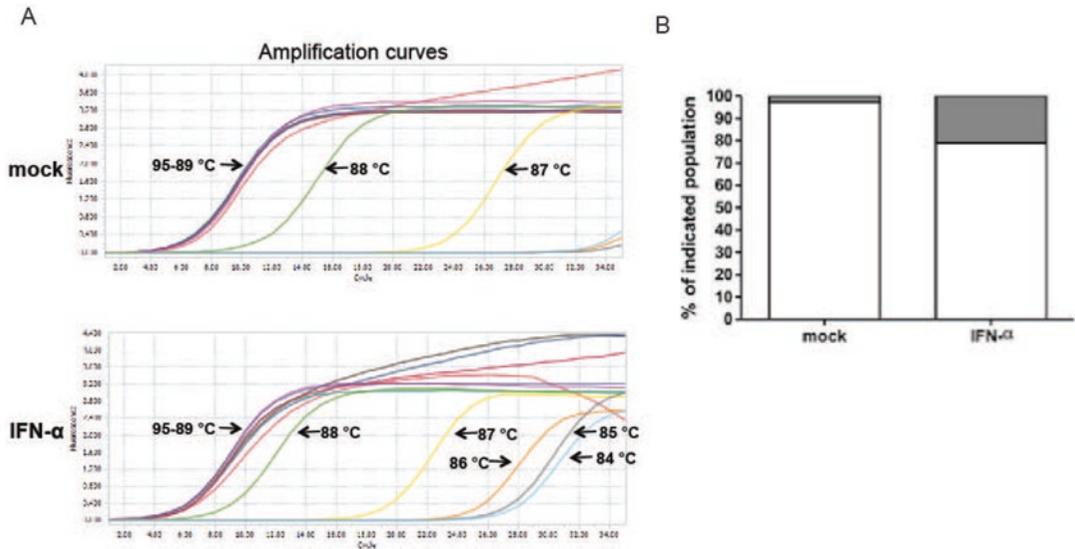


Fig. 4 Quantification of cccDNA deamination. HBV cccDNA qPCR products from mock or IFN- α treated HBV infected HepaRG were further analyzed by quantitative 3D-PCR. (a) Amplification curves were presented. (b) Proportion of deaminated cccDNA (gray) was calculated according to Cp values of different denatured PCR products

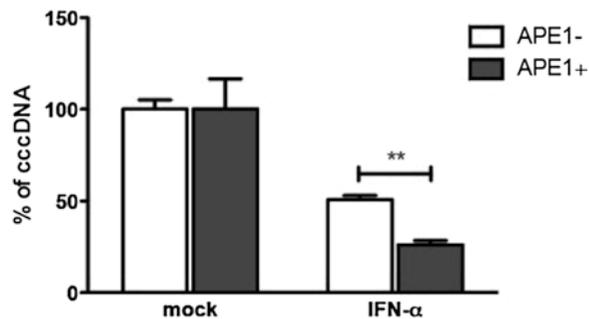


Fig. 5 Quantification of APE1 treated cccDNA. DNA extracts from HBV infected PHH cells were incubated with APE1. HBV cccDNA was quantified by qPCR

3.4 cccDNA Modification

3.4.1 APE1 Digestion

1. Mix 1 μ g of total cellular DNA with 2 μ l NEBuffer 4, 10 unit APE1 (M0282L, New England Biolabs) and H₂O up to 20 μ l.
2. Incubate the reaction at 37 $^{\circ}$ C for 2 h.
3. Inactivate the reaction at 70 $^{\circ}$ C for 5 min.
4. Analyze treated cccDNA by qPCR as in Fig. 5 (see Note 19).

3.4.2 PreCR Mix Treatment

1. Mix 1 μ g of total cellular DNA with 2 μ l ThermoPol Buffer, 100 μ M dNTPs, NAD⁺, 1 μ l of PreCR Repair Mix (M0309L, New England Biolabs) and H₂O up to 20 μ l.
2. Incubate the reaction at 37 $^{\circ}$ C for 20 min.
3. Analyze treated cccDNA by 3D-PCR as in Fig. 6 (see Note 20).

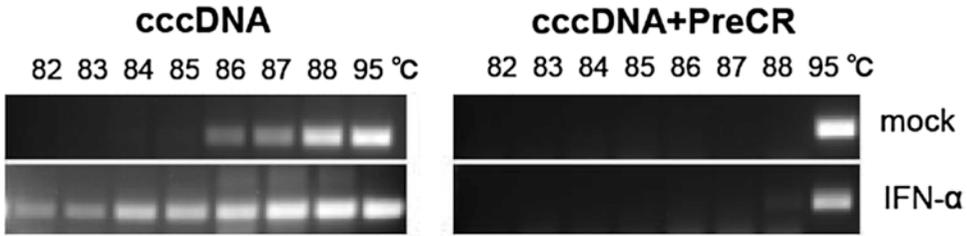


Fig. 6 3D-PCR of PreCR Repair Mix treated cccDNA. DNA extracts from HBV infected PHH cells were incubated with PreCR Repair Mix. 3D-PCR was used to detect HBV cccDNA deamination

4 Notes

1. Primary human hepatocytes were isolated from surgical liver specimens obtained during metastasis resection upon informed consent of the patient and seeded onto plastic dishes coated with collagen type IV in supplemented Williams E medium.
2. HBV infection was carried out with concentrated supernatant from HepAD38 cells by heparin affinity chromatography and subsequent concentration via sucrose gradient ultracentrifugation. Infection was performed (at a MOI of 100 vp/cell) with 5% PEG8000 and William's E medium contain 10% FBS, Penicillin/streptomycin, Human insulin (350 μ l, sigma I9278), Hydrocortison (5 μ g/ml, sigma H2270), and 1.8% DMSO (sigma, 2650).
3. HBV cccDNA selective primers:
cccDNA 92 fw: GCCTATTGATTGGAAAGTATGT
cccDNA 2251 rev: AGCTGAGGCGGTATCTA
Primers detecting all HBV DNA species:
HBV1745 fw: GGAGGGATACATAGAGGTTTCCTTGA
HBV1844 rev: GTTGCCCGTTTGTCTCTAATTC
Primers detecting the cellular prion coding DNA:
Prnp fw: TGCTGGGAAGTGCCATGAG
Prnp rev: CGGTGCATGTTTTACGATAGTA
Primers used to detect cccDNA modifications:
HBxin fw: ATGGCTGCTARGCTGTGCTGCCAA
HBxin rev: AAGTGCACACGGTYYGCCAGAT
4. HepaRG cccDNA sample was prepared as follows. HepaRG cells were maintained in HepaRG cell culture medium for 2 weeks and then for 2 more weeks in HepaRG cell differentiation medium with medium exchange two times per week.
Differentiated HepaRG cells were infected with HBV (MOI=200 vp/cell) for 8 days, and treated with 1 μ M Entecavir (ETV) for additional 7 days. Nuclei of the cells were isolated with NE-PER Nuclear and Cytoplasmic Extraction Reagents. DNA from cell nuclei was extracted with NucleoSpin®

Tissue kit as described above. DNA needs to be digested with T5 nuclease as indicated below for samples with total HBV-DNA > 10⁵/μl.

5. HepG2.2.15 cells were cultured in HepaRG cell differentiation medium to allow virus production. rcDNA prepared from the concentrated HepG2.2.15 cell culture supernatant was extracted with NucleoSpin[®] Tissue kit as described.
6. Tested samples: HBV infected PHH, HepaRG, HepG2-NTCP, Huh7-NTCP, and HepG2-H1.3. Please note that high infection efficiency obtained in some NTCP overexpressing cells results in false positive detection of cccDNA by PCR due to high numbers of rcDNA molecules. These samples need to be digested with T5 nuclease before further analysis.
7. Aforementioned reaction condition was based on Southern blot analysis as shown below. Intracellular DNA was isolated from HepAD38 cells either from HBV capsid or after Hirt lysis to deplete protein-bound DNA [10], and subjected to digestion using different concentrations of T5 exonuclease for 10, 30, or 60 min. Southern blot analysis shows that cccDNA remained intact when isolated DNAs were digested at 37 °C for 30 min at a concentration of 500 U/ml (lane 7), whereas all other forms of HBV DNA (i.e., rcDNA, double stranded linear (dl) DNA, and protein free (PF)-rcDNA [11, 12]) were completely digested (Fig. 7). Dose and incubation time, however, need to be adapted to the experimental conditions, because higher doses or longer incubation times will result in a loss of cccDNA (Fig. 7a). It is of importance to note that “plasmid safe DNase” is not suited to digest all rcDNA [13] (Fig. 7b) and thus cannot be used to diminish rcDNA in cccDNA PCR assays.
8. Presence of the T5 exonuclease buffer inhibits PCR reactions making it necessary to dilute treated samples with PCR grade water at least 1:4 (e.g., for HepaRG cells). Some cell lines (e.g., HepG2-NTCP cells) might require higher dilution due to total DNA amounts, for example 1:8 with PCR grade water.
9. To determine the amplification efficiency, use a 1:2 serial dilution of the sample in which you expect the highest cccDNA amount (for example, untreated HBV infected cells). Alternatively, use cccDNA sample as described in **step 4**.
10. For cccDNA PCR we used primers that selectively detected HBV cccDNA by spanning the nick and gap in the viral genomic DNA.
11. To specifically amplify cccDNA from other viral DNA forms, a protocol with fast ramp rate (4.4 °C/s) annealing conditions to avoid unspecific amplification from incompletely double-

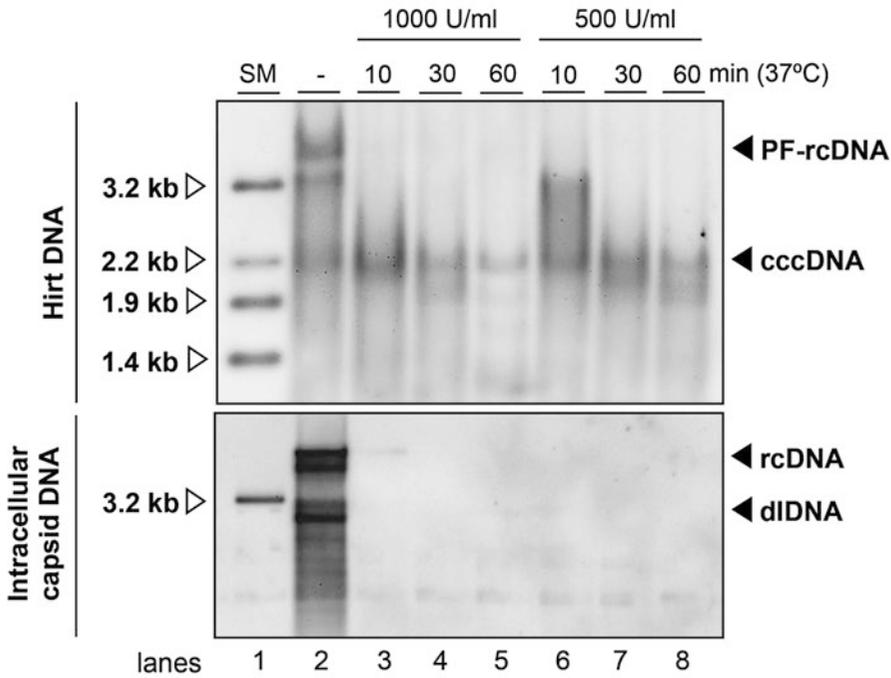
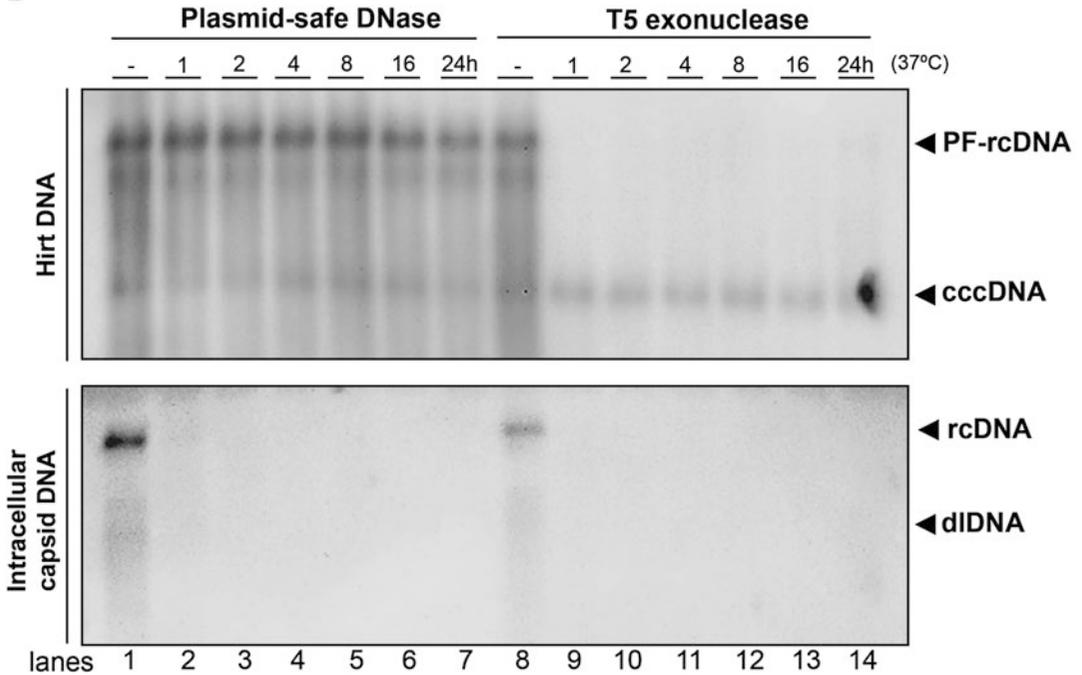
A**B**

Fig. 7 Southern blot analysis of viral DNA treated with T5 exonuclease or plasmid-safe DNase. HepAD38 cells were cultured in the absence of tetracycline for 11 days to induce HBV gene expression. Protein-free DNA after Hirt lysis and DNA from intracellular capsids were extracted. (a) Samples were treated with T5 exonuclease with indicated dose and time or (b) treated with 1000 IU/ml either plasmid-safe DNase or T5 exonuclease for indicated time, and subjected to gel electrophoresis. Southern blot analysis was performed to detect viral DNA using a digoxigenin-labeled HBV-specific probe

stranded forms of HBV DNA (e.g., using a Light Cycler™) is essential.

12. Specific PCR products show a melting peak at 90 °C while unspecific PCR product, if there is any, show a melting peak at 86 °C. Therefore, we set our acquisition temperature to 88 °C.
13. Although specific qPCR primers and an optimized PCR program are used for cccDNA quantification, there is still a chance to get unspecific amplification when HBV rcDNA levels are high. As shown in Table 2, 10^7 copies rcDNA per PCR reaction will give a false positive qPCR result. In this case, any PCR amplification with $C_p > 35$ should be considered as unspecific amplification. It is very important to determine the specificity of your own assay since this will vary largely with infection efficiency and primers as well as amplification protocols used.
14. Any other Taq DNA polymerase-based PCR reaction should also work. Do not include dUTP and uracil-DNA N-glycosylase in the PCR reaction [14].
15. In case there is no gradient thermal cycler, you can also perform independent PCR with different denaturing temperatures one by one, and run all PCR products on the same DNA gel. When deamination level is low, we only expect very small differences between samples, for example 0.5 °C, that makes precise temperature control very important.
16. Always include the wild-type sequence as control, which means the original virus strain you used. Deamination is the biological process that removes an amine group from a molecule. For DNA, the consequence is that a cytidine loses its amine group and converts to uridine. If deamination occurs in both DNA strands, C to U mutation will be observed in both strands. Since these two mutated strands are not paring to each other, during PCR amplification, there will be two different PCR products. When performing alignments, we compared the plus strand sequence of our PCR products with wild type. If the original deamination happens in plus strand, a C to T transition, otherwise, a G to A transition is observed. Since we only observed G to A transition, deamination only occurred in cccDNA minus strand as shown in the following picture (Fig. 8).

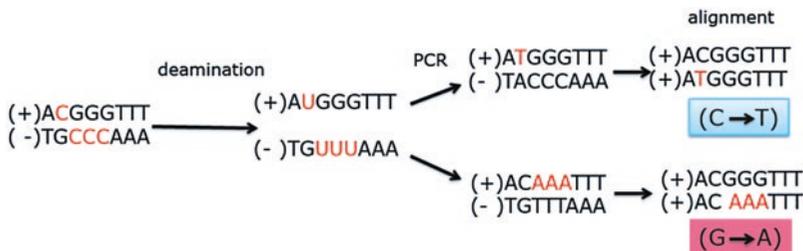


Fig. 8 Strand specific deamination. Plus strand deamination leads to C to T transition while minus strand deamination results in G to A transition

17. In case there is no gradient quantitative thermal cycler, you can also perform independent qPCR with different denaturing temperature one by one.
18. Amplification curves of 95–89 °C denaturation PCR products are clustered together and proved to be wild type (by sequencing); PCR products melting below 88 °C most likely are deaminated samples with the lower denaturing temperature indicating more G to A transitions (Fig. 3a). The proportion of deaminated cccDNA was calculated by: $2^{\Delta(Cp95\text{ }^{\circ}\text{C}-Cp88\text{ }^{\circ}\text{C})}$.
19. Presence of APE1 and buffer inhibits PCR reactions, so it is necessary to dilute APE1 treated samples with PCR grade water. Degradation of cccDNA by APE1 treatment indicates that cytokine treatment induces endonuclease-sensitive AP sites.
20. Presence of the PreCR Repair Mix inhibits PCR reactions, so it is necessary to dilute treated samples with PCR grade water. PreCR Repair Mix repairs deaminated DNA by cleaving the uracils and filling the AP sites as shown in Fig. 6.

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Detection of HBV cccDNA Methylation from Clinical Samples by Bisulfite Sequencing and Methylation-Specific PCR

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Abstract

Mapping of DNA methylation is essential in understanding the process of HBV covalently closed circular DNA (cccDNA) transcription. Here, bisulfite sequencing PCR and methylation-specific PCR, two PCR-based approaches used in determining and quantifying the DNA methylation pattern, are described.

Key words HBV cccDNA, DNA methylation, Bisulfite sequencing PCR, Methylation-specific PCR

1 Introduction

DNA methylation is one of the epigenetic modification which correlates with alteration in gene expression [1, 2]. The target sequence of methylation in mammalian genome is 5' cytosine of CpG dinucleotides, and clusters of CpGs are mostly found associated with gene promoters [3]. The 5-methyl-cytosine was recognized by CpG-binding proteins, including the methyl-CpG-binding domain (MBD), UHRF, and Kaiso protein families, which interact with histone-modifying and chromatin-remodeling enzymes, mediating stable repression of genes [4].

The first detection of virus DNA methylation was been reported in adenovirus in 1980 [5]. The inverse correlation between methylation level of virus DNA segments and their expression provides clues to the involvement of DNA methylation in inactivation of DNA virus genomes. Evidence demonstrates that several DNA virus infections will trigger an epigenetic response, including HIV, BLV, EBV and HBV [6–8].

Hepatitis B virus (HBV) is an enveloped hepatotropic DNA virus, containing a 3.2 kb partially double stranded circular DNA genome [9, 10]. Without interacting with DNA methyltransferase, the newly synthesized HBV DNA within the viral capsid and

Dane particle remain unmethylated. However, once in the nucleus of the infected hepatocyte, the relaxed circular DNA genome converts into covalently closed circular DNA (cccDNA), which is further organized into a viral minichromosome by histone and nonhistone proteins. CccDNA has been shown to be susceptible to DNA methylation mediated by DNMTs in the infected hepatocyte nuclei [11]. As the transcription template for the production of all viral mRNA, methylation of cccDNA results in the repressed transcription and consequently low level of viral replication. The HBV genome contains three major CpG islands. Among which, island I overlaps the start site of the small surface (S) gene, island II spans a region that overlaps enhancer I/II and is proximal to the core promoter, and island III covers the start codon of the polymerase (P) gene and upstream region of SP1 promoter. Among which, the CpG island II methylation has been shown to be associated with decreased pgRNA transcription and consequently viral replication [12, 13].

To understand further the biology that HBV cccDNA methylation state changes in HBV infection, here we report two methods used in determining the distribution of 5-methyl-cytosine, the bisulfite sequencing PCR (BSP) and methylation-specific PCR (MSP). Chemical conversion using sodium bisulfite is performed prior to both two approaches. Cytosine undergoes sulfonation, hydrolytic deamination, and alkali desulfonation and converts to uracil (the uracil amplified as thymine in PCR), while 5-methyl-cytosine remains as cytosine. For BSP, the primers are designed to amplify the bisulfite-treated DNA in the target region. The PCR product is cloned and sequenced; the selective reaction helps to discriminate between unmethylated cytosine (T) and methylated cytosine (C) (Fig. 1). For MSP, separate pairs of primers are designed to specifically amplify the methylated DNA or the unmethylated DNA, respectively. Due to the low copy number of cccDNA within infected liver cells, we applied nested PCR to amplify the CpG islands of HBV cccDNA.

2 Materials

2.1 DNA Isolation

1. Cell lysis buffer: 50 mM Tris-HCl (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.2 % NP-40, 0.15 M NaCl.
2. Nuclear lysis buffer: 6 % SDS, 0.1 N NaOH.
3. Neutralization buffer: 3 M KAc (pH 4.8).
4. Ethanol (100 %, 70 %).
5. 3 M NaAc (pH 5.5).
6. 10 mg/ml yeast RNA (Ambion).

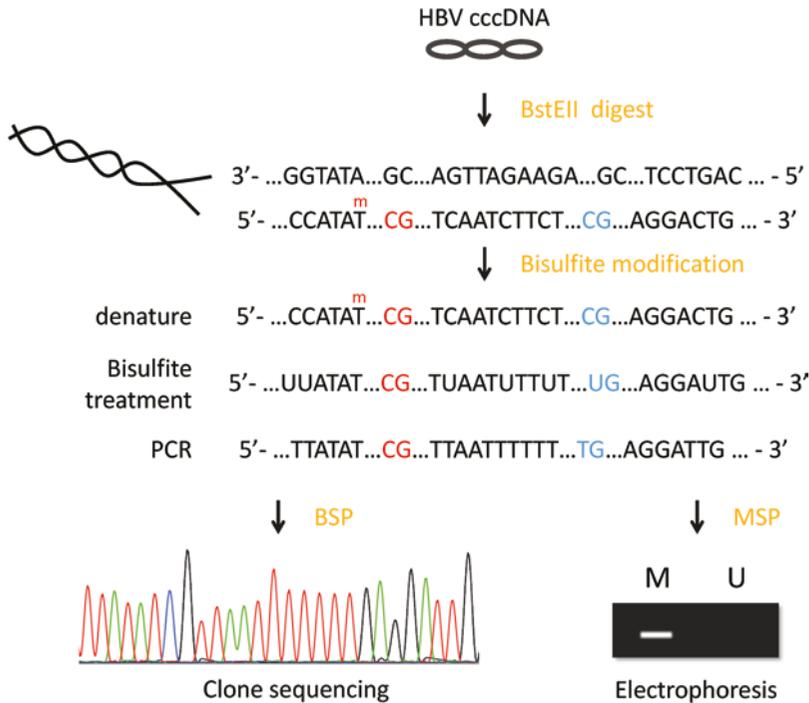


Fig. 1 Bisulfite sequencing of hepatitis B virus covalently closed circular DNA. HBV cccDNA are linearized with BstEII digestion prior to the bisulfite conversion, which converts the unmethylated cytosine (*blue*) to uracil (the uracil amplified as thymine in PCR), while 5-methyl-cytosine remains as cytosine (*red*). The converted DNA are PCR amplified and cloned into T-vectors. Methylated cytosine is discriminated from the unmethylated cytosine through the selective reaction

7. Phenol/chloroform: phenol/chloroform/isoamyl alcohol (25:24:1), saturated with 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA.
8. TE buffer: 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA.

2.2 Pretreatment of DNA

1. Plasmid-Safe DNase with appropriate buffer.
2. Restriction enzyme with appropriate buffer (Bst EII).
3. PCR Clean-Up Kit (Axygen).

2.3 Bisulfite Conversion

1. EpiTect Bisulfite Kit (Qiagen).
2. Dissolve the required number of aliquots of bisulfite mix with 800 ul RNase-free water.
3. Prepare buffer BL containing 10 µg/ml carrier RNA.
4. Ethanol (100%).

2.4 Primer Design

The primer can be designed manually or using the online software: MethPrimer (<http://www.uro-gene.org/methprimer/>) and MSP primer (<http://www.mspprimer.org/cgi-bin/design.cgi>).

**2.5 PCR
Amplification
of Bisulfite-Treated
DNA and DNA
Sequencing**

1. MightyAmp DNA Polymerase (TAKARA), PCR buffer, MgCl₂ solution, and dNTP (10 μM).
2. Primers (10 μM).
3. 1× TAE buffer: 0.04 M Tris base, 0.04 M glacial acetic acid, 1 mM EDTA, pH 8.2–8.4. Prepare 30× stock solution, and store at room temperature.
4. Agarose (molecular biology grade).
5. 10× DNA gel loading buffer: 10 mM EDTA (pH 8.0), 50 % (V/V) glycerol, 0.25 % (W/V) bromophenol blue.
6. Gel Extraction Kit.
7. T-vector (Takara).
8. T4 DNA ligase with appropriate buffer.
9. DH5α Chemically Competent Cell.
10. Ampicillin (100 mg/ml).

3 Methods

3.1 DNA Isolation

1. Lyse liver biopsy tissues (2–10 mg) by adding 400 μl pre-chilly cell lysis buffer [50 mM Tris–HCl (pH 8.0), 1 mM EDTA, 0.2% NP-40, 0.15 M NaCl]. Homogenize the tissue with Qiagen Tissue Ruptor on ice until the tissue disruption was efficient (*see Note 1*).
2. Centrifuge at 16,000×g for 10 min at 4 °C. Remove the supernatant. The pellet was treated with 400 μl nuclear lysis buffer (6% SDS, 0.1 N NaOH), followed by incubation for 30 min at 37 °C, vortex intermittently.
3. The lysate was then neutralized by 100 ul of neutralization buffer [3 M KAc (pH 4.8)], and centrifuged at 16,000×g for 15 min at 4 °C.
4. Transfer the supernatant to a fresh 2 ml EP tube, add equal volume of phenol to the supernatant, and mix thoroughly by hand shaking for 15 s. Centrifuge at 16,000×g for 15 min at 4 °C, and transfer the aqueous phase to a fresh 2 ml tube. Add equal volume of phenol–chloroform to the supernatant and mix thoroughly by hand shaking for 15 s. Centrifuge at 16,000×g for 15 min at 4 °C, and transfer the aqueous phase to a fresh 2 ml tube.
5. Add 1/10 volumes of 3 M NaAc, 2 volumes of 100% ethanol, and 2 μl tRNA, and mix thoroughly by pipette. Precipitate DNA at –20 °C overnight.
6. On the second day, centrifuge the tube at 16,000×g for 30 min at 4 °C, and discard the supernatant. Add 1 ml 70% ethanol and gently rotate the tube to wash the DNA pellet. Centrifuge at 16,000×g for 15 min at 4 °C. Discard the supernatant.

- Allow the pellet to air-dry for about 5 min at room temperature. Dissolve the DNA pellet in 50 μ l TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

3.2 Pretreatment of DNA

- Add DNA solution (<3.33 μ g, V_{max} = 50 μ l) to the following reaction to remove the potential contaminating host genomic DNA by the Plasmid-Safe DNase treatment.

Plasmid-Safe DNase preparation:

Sterile water	42 μ l
25 mM ATP	2 μ l
10 \times reaction buffer	5 μ l
Plasmid-Safe DNase (10 U)	1 μ l
Total reaction volume	50 μ l
Incubate at 37 $^{\circ}$ C for 1 h	

- Purify the cccDNA using a PCR Clean-Up Kit. Elute the DNA with 30 μ l of TE buffer.
- PCR amplification of GAPDH gene.

To exclude the possible contamination of the integrated HBV DNA, GAPDH (or other control gene) PCR amplification should be carried out before the CpG island amplification. Primer sequences are listed as follows:

Forward primer: 5'- ATTCCACCCATGGCAAATTC-3'.

Reverse primer: 5'-GGATCTCGCTCCTGGAAGATG-3'.

Amplification were performed with initial denaturation at 94 $^{\circ}$ C for 5 min, followed by 35 cycles of 94 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 60 s, with a final extension of 5 min at 72 $^{\circ}$ C.

- The conversion efficiency of plasmid DNA was poor due to the quick reannealing of the single-stranded DNA after the denaturation during the bisulfite conversion. Therefore, HBV cccDNA was linearized with BstEII (or other restriction endonuclease which would not affect the target region) before the bisulfite treatment to maximize the conversion efficiency (*see Note 2*). Digestion reaction components: Add 2 μ l BstEII, 5 μ l NEBuffer 3.1, 0.5 μ l 10 \times BSA, and 12.5 μ l sterile water into the cccDNA solution (30 μ l), and mix thoroughly. Incubate at 37 $^{\circ}$ C for 3 h.
- Purify the cccDNA using a PCR Clean-Up Kit. Elute the DNA with 40 μ l of TE buffer.

3.3 Bisulfite Conversion of HBV cccDNA

Bisulfite treatment of the HBV cccDNA from liver biopsy tissue samples was performed by using Qiagen EpiTect Bisulfite Kit (Qiagen) or EZ DNA Methylation Kit (Zymo Research) or [EpiJET Bisulfite Conversion Kit](#) (Invitrogen).

1. Bisulfite reaction components: Add 85 μl DNA bisulfite mix and 15 μl DNA Protect Buffer into the cccDNA solution (<2 μg , $V_{\text{max}}=40 \mu\text{l}$), and mix thoroughly (*see Note 3*).
2. Incubate in a thermocycler with the following conditions: Denaturation at 95 °C for 5 min → Incubation at 60 °C for 25 min → Denaturation at 95 °C for 5 min → Incubation at 60 °C for 85 min → Denaturation at 95 °C for 5 min → Incubation at 60 °C for 175 min → Hold at 20 °C.
3. Transfer the completed bisulfite reactions to clean 1.5 ml microcentrifuge tubes. Add 560 μl buffer BL (containing 10 $\mu\text{g}/\text{ml}$ carrier RNA). Vortex and centrifuge the tube briefly. Transfer the mixture into the spin column. Centrifuge at $16,000 \times g$ for 1 min.
4. Discard the filtrate, add 500 μl buffer BW, and centrifuge at $16,000 \times g$ for 1 min. Discard the filtrate, add 500 μl buffer BD, and incubate at room temperature for 15 min.
5. Wash the spin column with 500 μl buffer BW twice. Centrifuge at $16,000 \times g$ for 1 min and discard the filtrate.
6. Place the spin columns into a clean 1.5 ml EP tube, and elute the DNA with 30–40 μl of TE buffer. The bisulfite-converted DNA was stored at $-80 \text{ }^\circ\text{C}$, which should be used for PCR as soon as possible.

3.4 Design of BSP and MSP Primers

MethPrimer can be used to predict CpG islands within the HBV whole genome. The CpG islands were defined based on the following criteria: (1) a GC content of $\geq 50\%$, (2) an observed-to-expected CpG dinucleotide ratio ≥ 0.60 , and (3) a sequence window longer than 100 bp. As mentioned above, bisulfite modification converts the unmethylated “C” to “T,” while mC remains “C.” The modified DNA turns into two DNA strands which are not complementary. The primers can be designed according to either strand. We designed the primers according to the negative strand (Table 1).

BSP: (1) PCR products should be less than 500 bp; (2) primers should not contain CpG dinucleotides; (3) and the non-CpG “C” can be included at 3′ of the primers ensure that complete converted DNA being amplified (*see Note 4*).

MSP: (1) PCR products should be less than 200 bp; (2) primers should contain at least one CpG site at the most 3′ end, and primer sequence should span more than one CpG site; and (3) primers amplifying methylated or unmethylated DNA should contain the same number of CpG sites [14].

3.5 PCR Amplification of Bisulfite-Converted HBV cccDNA

BSP:

1. Set up a PCR in a total of 50 μl , as follows (or using other DNA polymerase such as AmpliTaq Gold 360 Master Mix or ZymoTaq DNA Polymerase)

Table 1
Primers used in the bisulfite-converted cccDNA amplification

BSP primers:		
Target	Primers, 5'-3'	Position (bp)
<i>CpG1</i>		53–283
Nested PCR options: ① f2 + down f2 + r2 ② f2 + down 1' f2 + r2 ③ ViveF + down 1' viveF + viveR		
CpG1-BSP-f2	TATTTTTTTTGGTGGTGGTTTTAGTTT	54–77
CpG1-BSP-r2	TAAAAAATTAATAAAAAATCCACCA	286–266
CpG1-BSP-seminested downstream	ACAAAAAATAAAACATAACAACAAAATA	439–411
CpG1-BSP-semidown 1'	AACAACATACCTTAATAATCCAA	470–448
Primers from reference [16]:		
Vivekanandan-CpG1-F	TTTGTGGTGGTTTTAGTTTAGGA	58–81
Vivekanandan-CpG1-R	TCCCCCTAAAAAATTAATAAAAAATC	288–264
<i>CpG2</i>		1195–1666/ 1389– 1666
Nested PCR options: ① f2 + down f2 + r2 ② f2 + down CF2 + CR2 ③ CF2 + CR2 f3 + r2		
CpG2-BSP-f2	GTAATTTTTTATTGGWTGGGGTTTTGGT	1048–1069
CpG2-BSP-r2	ATCCTCTTATAYAAAAACCTTAAACAA	1680–1655
CpG2-BSP-seminested downstream	TTATACCTACAACCTCCTAATACAAAA	1794–1768
CpG2-CF2	TTTTATGGTTGTTAGGDTGTGTTGTT	1052–1077
CpG2-CR2	TAACCTAAHCTCCTCCCCAAC	1762–1741
CpG2-BSP-f3	TGTGTTGTTAATTGGATTTTG	1389–1409
Primers from reference [13, 16, 17]:		
Vivekanandan-CpG2-F	GTAATTTTTTATTGGTTGGGGTTTG	1194–1217
Vivekanandan-CpG2-R	TCCAATTAACAACACAHCCTAACAAC	1404–1379
Kim-CpG2-F	GGGATTGATAATTTTGTGTTTTTTTT	1329–1354
Kim-CpG2-R	TCCAAAAATCCTCTTATATAAAAACCTTAA	1672–1644
Guo-CpG2-F	GTTTTTTGTTTATGTTTTGTTGG	1418–1440
Guo-CpG2-R	AAATAAAAACAAAATACACACAATCCG	1598–1573

(continued)

Table 1
(continued)

<i>BSP primers:</i>			
Target	Primers, 5'-3'	Position (bp)	
<i>CpG3</i>		2206–2461	
Nested PCR: up+r2 f2+r2			
<i>CpG3-BSP-f2</i>	GTGGTTTTATATTTTTTGTTTTAT	2208–2228	
<i>CpG3-BSP-r2</i>	AAAATACTAACATTAAAATTCCCAA	2460–2435	
<i>CpG3-BSP-seminested upstream</i>	GTTATGTTAATGTTAATATGGGT	2162–2184	
<i>MSP primers</i> (primers from reference [15]):			
<i>CpG1-MSP-F</i>	ACGTGTTTTGGTTAAAATTCGTAGTTTTTA	292–322	95 °C 5 min
<i>CpG1-MSP-R</i>	AATATAATAAACGCCGCAAACACATC	376–402	(95 °C 10s,
Probe: [6FAM] GTTTTTAAATTTGTTTTGGTTATCGTTG		348–379	55 °C 30s,
	GATG [BHQ1]		72 °C 10s) ×
			50 cycles
<i>CpG2-MSP-F</i>	TGTCGTTTCGGTCGATTAC	1502–1520	95 °C 5 min
<i>CpG2-MSP-R</i>	CACGATCCGACAAATAAAAA	1560–1579	(95 °C 10s,
SYBR Green			52 °C 30s,
			72 °C 10s) ×
			45 cycles,
			Melting curve,
			40 °C 30s
<i>CpG3-MSP-F</i>	GTGTGGATTCGTATTTTTTTC	2270–2290	95 °C 10 min
<i>CpG3-MSP-R</i>	GACGATTAACCTTCGTCT	2393–2412	(95 °C 10s,
Probe: [6FAM]AACCTACCTCGTCGCTAACAACAAT[BHQ1]		2339–2364	53 °C 30s,
			72 °C 10s) × 45
			cycles

MightyAmp DNA Polymerase	1 µl
PCR mix	25 µl
Forward primer (10 µM)	1.5 µl
Reverse primer (10 µM)	1.5 µl
Bisulfite-converted DNA	5–10 µl
Total reaction volume	50 µl

- Amplification condition: initial denaturation at 98 °C for 3 min, followed by 30 cycles of 98 °C for 15 s, 55 °C for 20 s, and 68 °C for 45 s, with a final extension of 10 min at 68 °C. 1 µl of the ten times diluted PCR product was then subjected to a second round of amplification: 98 °C for 3 min, followed by 35 cycles of 98 °C for 15 s, 55 °C for 20 s, and 68 °C for 45 s, and a final extension of 10 min at 68 °C (*see Note 5*).
- Separate the PCR products on a 1.5% agarose gel. Purify the PCR products with a Gel Extraction Kit. Elute the DNA with 25 µl of TE buffer.

4. The PCR products of the three CpG islands were cloned into T-vector (mol ratio, 3~10:1) and subject to sequencing to study the methylation status of each CpG island. More than ten clones need to be analyzed for each island to get a reliable conclusion.

MSP (Protocols from Reference)

1. Amplification condition: 95 °C 10 min (95 °C 10s, 53 °C 30s, 72 °C 10s)×45 cycles.
2. The quantitative analysis of HBV DNA methylation is carried out by calculating the ratio methylated HBV copies/3000copies of BS-actin DNA [15].

3.6 Data Processing

The methylation status can be obtained by comparing the original HBV CpG island sequence and the sequencing data of the BSP products. For CHB patients in China, we use the consensus sequence of B or C genotype HBV as the template sequence for each patient. The analysis can be conducted manually by using software such as VectorNTI, or using the methylation analyzing software such as BiQ Analyzer, which can transform the methylation status into a visualized graph:

1. Import the original HBV CpG island sequence (unconverted sequence) and then all the sequencing files.
2. Perform the sequence alignment; the conversion rate of each sequence will be calculated by the software, and the sequence with conversion rate lower than 90% will be excluded. The inverted sequence needs the reverse and complementary transformation (*see* Notes 6 and 7).
3. The results of analysis will be presented in two ways: (A) Lollipop diagram: The black and white circles represent methylated and unmethylated CpG dinucleotides, respectively. The vertical line indicates dinucleotides other than CpG at the corresponding site. (B) Box diagram: The vertical box indicates all HBV DNA clones from patients at corresponding CpG position. The blue and yellow regions represent the proportion of unmethylated and methylated clones, respectively. The gray color refers to the absence of CG dinucleotide due to single nucleotide polymorphism. The number of unmethylated and methylated clones is listed under the corresponding dinucleotides (Fig. 2).

4 Notes

1. Make sure the tissue ruptor is placed below the liquid surface of cell lysis buffer to avoid the production of foam, which will cause sample loss and make it difficult in adding the following reagent.

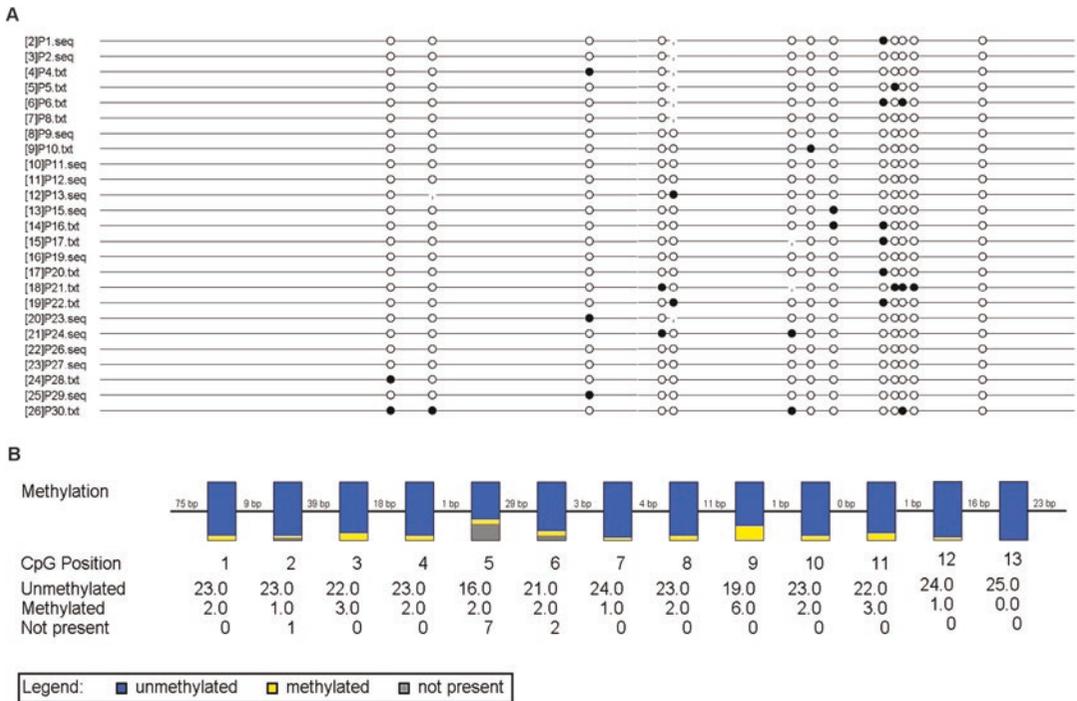


Fig. 2 BiQ Analyzer output of the methylation status of HBV cccDNA CpG island III. **(a)** Lollipop diagram: The *black* and *white circles* represent methylated and unmethylated CpG dinucleotides, respectively. **(b)** Box diagram: The *vertical box* indicates all HBV DNA clones from patients at corresponding CpG position. The *blue* and *yellow* regions represent the proportion of unmethylated and methylated clones, respectively. The *gray* color refers to the absence of CG dinucleotide due to single nucleotide polymorphism. The number of unmethylated and methylated clones is listed under the corresponding dinucleotides

- To maximize the bisulfite conversion rate of DNA samples, it is critical to linearize cccDNA, which is a supercoiled double-stranded DNA before starting the bisulfite treatment. Or during the subsequent PCR, the unconverted cccDNA may also be amplified, and the presence of unconverted “C” makes it difficult for data analysis.
- The bisulfite mix needs to be freshly prepared, and the bisulfite-converted DNA samples need to be aliquoted in the thin-wall plastic PCR tubes and stored at -80°C . Avoid multiple freeze-thaw cycles.
- The restriction endonuclease used in the linearization of cccDNA should not be chosen in the region of CpG islands.
- Choosing DNA polymerase which can perform well in the AT-rich or GC-rich DNA sample amplification. The annealing temperature for each island amplification and the amount of the DNA template used in the second round of PCR need to be tested and optimized.

6. The software BiQ Analyzer will exclude the sequence with high nucleotide sequence homology (sequences are equal in all of the genomic sequence's aligned C positions) to make sure that the data are not the repeat sequencing results from the same clone. However, we choose to do the clone sequencing which have ensured that each sequencing result comes from one separate clone, so each sequencing result needs to be involved for a reliable analysis.
7. The most accurate alignment should include the original CpG island sequences, at least from the dominant stain of the patient. Aligning using the original sequence as template can minimize the impact of the sequence polymorphism on the methylation analysis. Such as the nucleotide in template is "AG," while the sequencing shows a "CG" in the corresponding position, the methylated "C" will be missed, which will lead to the false negativity.

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A T7 Endonuclease I Assay to Detect Talen-Mediated Targeted Mutation of HBV cccDNA

Kristie Bloom, Abdullah Ely, and Patrick Arbuthnot

Abstract

Gene editing using designer nucleases is now widely used in many fields of molecular biology. The technology is being developed for the treatment of viral infections such as persistent hepatitis B virus (HBV). The replication intermediate of HBV comprising covalently closed circular DNA (cccDNA) is stable and resistant to available licensed antiviral agents. Advancing gene editing as a means of introducing targeted mutations into cccDNA thus potentially offers the means to cure infection by the virus. Essentially, targeted mutations are initiated by intracellular DNA cleavage, then error-prone nonhomologous end joining results in insertions and deletions (indels) at intended sites. Characterization of these mutations is crucial to confirm activity of potentially therapeutic nucleases. A convenient tool for evaluation of the efficiency of target cleavage is the single strand-specific endonuclease, T7EI. Assays employing this enzyme entail initial amplification of DNA encompassing the targeted region. Thereafter the amplicons are denatured and reannealed to allow hybridization between indel-containing and wild-type sequences. Heteroduplexes that contain mismatched regions are susceptible to action by T7EI and cleavage of the hybrid amplicons may be used as an indicator of efficiency of designer nucleases. The protocol described here provides a method of isolating cccDNA from transfected HepG2.2.15 cells and evaluation of the efficiency of mutation by a transcription activator-like effector nuclease that targets the *surface* open reading frame of HBV.

Key words HBV, TALEN, cccDNA, T7EI, Indels

1 Introduction

Designer nucleases have become popular tools for generating permanent modifications of DNA in living cells. This gene editing technology has now been widely applied in a variety of organisms [1]. The engineered nucleases are typically designed to bind discrete DNA sequences where they introduce double strand breaks (DSBs) at an intended target site. Subsequent activation of intrinsic error-prone nonhomologous end joining (NHEJ) results in insertion or deletion of nucleotide pairs (indels) at the site of the DSB [2]. When used in conjunction with donor sequences that have arms that are complementary to the sites flanking a DSB, homology-directed repair (HDR) may occur. Sequences may thus

be introduced at the site of nuclease-mediated cleavage and the mechanism has successfully been employed to disrupt, repair or knock in sequences at defined loci. To date, four major classes of designer nucleases have been developed for gene editing. These are meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and RNA-guided endonucleases (RGENs). Meganucleases and the RGEN Cas9 protein have inherent endonuclease activity, while the enzymatic function of ZFNs and TALENs is engineered by artificially fusing DNA binding and endonuclease domains. In the field of gene therapy, utility of nucleases has most commonly been developed for treatment of genetic diseases. Exploring the antiviral potential of these engineered agents has been a more recent development [3]. The approach is particularly useful against viruses such as hepatitis B virus (HBV), which cause chronic infection and replicate using a DNA intermediate [4–7].

Chronic HBV infection is associated with persistence of episomal covalently closed circular DNA (cccDNA) in the nucleus of infected hepatocytes [8]. cccDNA is stable and establishes itself as an epigenetically regulated minichromosome [9–11]. This replication intermediate evades effects of licensed anti-HBV drugs, such as nucleoside and nucleotide analogs, which act as inhibitors of viral reverse transcription [12, 13]. Effectively disabling cccDNA is thus a priority of HBV therapy, and this has generated enthusiasm for investigating the potential of advancing use of gene editing as a mode of treating chronic infection with HBV [4–7].

Verification of nuclease-mediated cleavage at the intended targets is crucial to confirm activity of potentially therapeutic nucleases. Protocols employing next generation sequencing are ideal for detailed characterization of targeted mutagenesis. However the methods are typically costly, demanding of bioinformatic resources and not suited to the screening of efficacy of gene editing. Mismatch-sensitive endonucleases, such as T7 endonuclease I (T7EI) [14] and members of the CEL family of endonucleases, may be conveniently employed to identify targeted mutations. The assays initially entail amplification of the targeted region using conventional PCR. Thereafter the amplicons are denatured and reannealed to allow hybridization between indel-containing and wild type sequences. Resultant heteroduplexes contain single stranded mismatches at the sites of errors introduced at the nuclease-generated DSBs, which are in turn susceptible to mismatch-sensitive endonucleases. Cleavage of the amplicons may therefore be used as an indicator of efficiency of target cleavage by a designer nuclease (Fig. 1). In this chapter, we describe a revised method for the isolation of cccDNA, which is coupled with the detection of targeted disruption of the viral DNA. The procedure employs T7EI endonuclease and is carried out on extracts of cultured human liver-derived cells. The method of isolating cccDNA efficiently removes other viral DNA molecules, such as rcDNA and

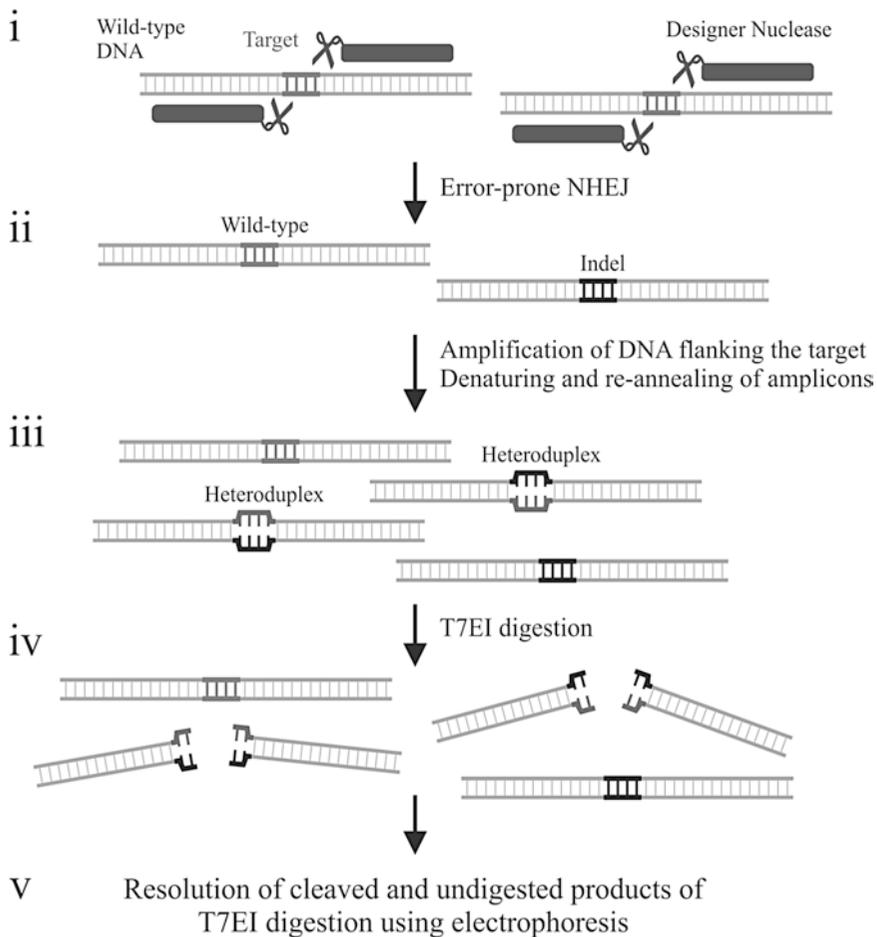


Fig. 1 Schematic illustration of the assay using T7EI to characterize targeted mutations by designer nucleases. Intracellular digestion of target DNA by designer nucleases (*i*) is followed by repair using the error-prone nonhomologous end joining (NHEJ) pathway (*ii*). Mutations (indels) may thus be introduced into target DNA at the intended sites. Extracted DNA is subjected to amplification using primers that flank the intended target (*iii*). Denaturation, then reannealing, leads to the formation of homoduplexes and heteroduplexes comprising strands of the wild type and mutated DNA. Mismatched regions are susceptible to digestion by T7EI endonuclease, while the perfectly matched homoduplexes are resistant to cleavage by the endonuclease (*iv*). Products of the cleavage reaction are then resolved electrophoretically to enable quantification of the efficiency of the designer nuclease (*v*)

linear viral DNA. The previously described anti-HBV *S*TALEN pair [4] is used as an example in this protocol, but the approach is generally applicable to designer nucleases that target HBV cccDNA.

2 Materials

2.1 Cell Cultures

1. Complete Dulbecco's Modified Eagle Medium (DMEM): 10% fetal calf serum (FCS), 100,000 U/mL penicillin, 100,000 µg/mL streptomycin.
2. HepG2.2.15 cells [15].

3. 1× trypsin–EDTA.
4. 10× (1 mg/mL) Polyethylenimine (PEI), pH 7.0.
5. 150 mM NaCl.
6. S TALEN left and right plasmid DNA.
7. GFP expressing plasmid DNA (pCMV-GFP).
8. Control plasmid expressing neither nuclease nor GFP (e.g., pUC118).
9. Humidified incubator: 37 °C, 5% CO₂.
10. Humidified incubator: 30 °C, 5% CO₂.

2.2 Extraction of cccDNA

1. EconoSpin® All-In-One Silica Membrane Mini Spin Column and plasmid extraction protocol (Epoch Life Sciences, TX, USA).
2. Plasmid-safe™ ATP-dependent DNase (Epicentre Biotechnologies, WI, USA).

2.3 Agarose Gel Electrophoresis

1. 5× Orange G loading dye: 0.5 M EDTA (pH 8.0), 0.2% (w/v) Orange G, 30% (v/v) glycerol.
2. 1× TAE buffer: 0.04 M Tris base, 20 mM glacial acetic acid, 1 mM EDTA, pH 8.2–8.4. Prepare 50× stock solutions, and store at room temperature.
3. Agarose (molecular biology grade).
4. O'GeneRuler™ DNA Ladder Mix 100–10,000 bp (Thermo Scientific, MA, USA).
5. UV transillumination imaging system.
6. ImageJ (Version 1.46) [16].

2.4 T7EI Assay

1. KAPA2G™ Robust HotStart ReadyMix (Kapa Biosystems, MA, USA).
2. Primer set: S Fw 5' CCT AGG ACC CCT TCT CGT GT 3'; S Rv 5' ACT GAG CCA GGA GAA ACG GG 3'.
3. Gradient thermocycler.
4. EconoSpin® All-In-One Silica Membrane Mini Spin Column and PCR clean-up protocol (Epoch Life Sciences, TX, USA).
5. NanoDrop™ 2000 spectrophotometer (Thermo Scientific, MA, USA).
6. T7 endonuclease I (T7EI) (New England BioLabs, MA, USA).
7. NEB buffer 2 (New England BioLabs, MA, USA).

3 Methods

3.1 Transfection of HepG2.2.15 Cells with Plasmids Expressing TALENs

1. Seed HepG2.2.15 cells in six-well plates at 140,000 cells per well in a final volume of 1.8 mL growth media 1 day prior to transfection.
2. Prepare nuclease transfection solutions by initially combining 0.4 ng pCMV-GFP and 2.3 ng of each of the left and right S TALEN plasmids with 150 mM NaCl in a total volume of 100 μ L (*see Note 1*). In addition, prepare a no nuclease control transfection solution which contains 0.4 ng pCMV-GFP and 4.6 ng of pUC118 (*see Note 2*).
3. Dilute PEI (pH 7.0) to 1 \times (0.1 mg/mL) in 150 mM NaCl and add 100 μ L to each transfection solution and incubate at room temperature for 8 min.
4. Gently add the solution dropwise to the cells. Mix by gently rocking the plate from side to side.
5. Incubate the cells at 30 °C and 5% CO₂ (mild hypothermic conditions) (*see Note 3*).
6. Replace growth medium at days 2 and 3 after transfection.
7. On day 5, harvest the cells by gentle trypsinization and re-seed a fifth of the cells in a total of 1.8 mL of pre-warmed growth media in one well of a new six-well plate. Store the remaining cells in 50 μ L of 1% PBS at -70 °C. Incubate the re-seeded cells at 37 °C and 5% CO₂ for 1 day. Repeat the abovementioned transfection procedure twice (*see Notes 4 and 5*).

3.2 Isolation of cccDNA

1. Thaw frozen TALEN-treated and untreated HepG2.2.15 cells (stored after the final transfection).
2. For the extraction of HBV cccDNA, perform a modified plasmid DNA extraction [17] using the EconoSpin® All-In-One Silica Membrane Mini Spin Column and plasmid extraction protocol according to the manufacturer's instructions with minor modifications (*see Note 6*).
3. Perform a Plasmid-safe™ ATP-dependent DNase treatment of the cccDNA extraction according to the manufacturer's instructions.

3.3 Amplification of Sequences Encompassing the TALEN Cleavage Site

1. Amplify the sequences encompassing the nuclease binding site by combining 12.5 μ L of KAPA2G™ Robust HotStart ReadyMix, 10 pmol of each S Fw and S Rv primer (*see Note 7*), 5 μ L of cccDNA template (TALEN-treated or untreated) and ddH₂O to a final volume of 25 μ L in a 0.2 mL PCR tube.
2. Perform PCR reactions in a gradient thermocycler under the following conditions: initial denaturation at 95 °C for 2 min;

30 cycles of 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 15 s; final elongation at 72 °C for 5 min and cooling to 4 °C.

3. Prepare a 1.5 % TAE agarose gel (0.0025 % Ethidium Bromide) and 1×TAE running buffer to fill the electrophoresis tank.
4. Once the PCR cycle is complete, mix 3 µL of the PCR product with 3 µL of 5× Orange G loading dye. Load the samples and 1.5 µL of O'GeneRuler DNA Ladder Mix in separate wells of the 1.5 % agarose gel and run at 120 V for 30 min.
5. Confirm that PCR products resolve as a single band under UV transillumination (*see Note 8*).
6. Purify the remaining 22 µL PCR product using the EconoSpin® All-In-One Silica Membrane Mini Spin Column and PCR clean-up protocol according to the manufacturer's instructions. Elute the clean PCR product in 35 µL of ddH₂O (*see Note 9*).
7. Quantify the DNA concentration of the PCR product using a NanoDrop™ 2000 spectrophotometer or similar device.

3.4 T7 Endonuclease I Assay

1. Prepare a 2% TAE agarose gel (0.0025% Ethidium Bromide) with a small gel comb for electrophoresis (*see Note 10*). In addition prepare 1× TAE running buffer to fill the electrophoresis tank.
2. Prepare 150 ng of each PCR product, in duplicate, in a 0.2 mL PCR tube as follows: 150 ng PCR product with 2 µL NEB buffer 2 (10×) and ddH₂O in a final volume of 12 µL (*see Note 11*).
3. Program a thermocycler with the following parameters:
 - (a) Heat to 95 °C.
 - (b) Cool to 35 °C at a rate of -0.1 °C/s and hold at 35 °C for 2 min.
 - (c) Cool to 4 °C and hold at 4 °C for 10 min.
 - (d) Heat to 37 °C and incubate at 37 °C for 15 min.
4. For heteroduplex formation, place the samples in the thermocycler and run the program described above in **step 3** (*also see Note 12*).
5. Following the 2 min hold at 35 °C, remove the samples from the thermocycler (without stopping the program) and place them on ice for 5 min.
6. Add 3 U of T7EI to one of the duplicate samples (+T7 EI) and 0.3 µL of ddH₂O to the other (-T7 EI).
7. Immediately place the samples back in the thermocycler for the 37 °C incubation step (*see Note 13*).
8. Add 4 µL of 5× Orange G loading dye to each sample and resolve on the 2 % agarose gel (*see Note 14*). Include 1.5 µL of O'GeneRuler DNA Ladder Mix in a separate well as a molecular weight marker (*see Note 15*).

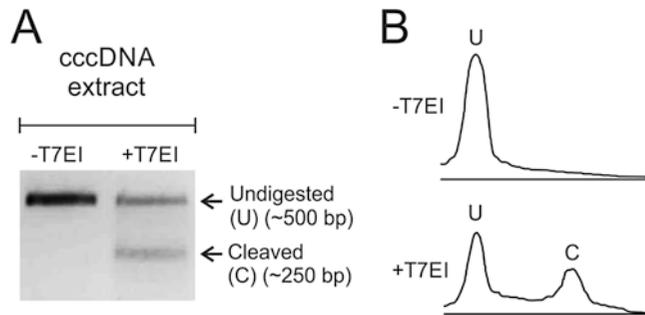


Fig. 2 Resolution and quantification of targeted disruption using the T7EI assay. **(a)** Resolution of cccDNA extracted from S TALEN triple transfected cells following treatment in the absence (–T7EI) or presence (+T7EI) of the T7EIIenzyme. The *arrows* indicate the undigested (U) and cleaved (C) PCR products corresponding to ~500 and ~250 bp respectively. **(b)** Representation of T7EI products as histograms following scanning. The peaks corresponding to the band intensities of the U products are identified in both the –T7EI and +T7EI samples. The distinct peak of the C product is only identified in the +T7EI sample. The difference between the profiles of the histograms of the –T7 EI and +T7 EI samples can be used to calculate the percentage of nuclease-mediated targeted disruption

9. After 20 min of electrophoresis at 120 V, assess band migration patterns using a UV transillumination system (*see* **Notes 16** and **17**). If cleavage bands have successfully been resolved (Fig. 2a), invert and save the gel image, preferably as a TIFF, GIF, JPEG, BMP, or PGM.
10. Open the gel image using ImageJ 1.47v software [16] (*see* **Note 18**). Use the rectangular tool to map the first lane of the gel. To designate this as the first lane use the “ctrl1” shortcut.
11. Without altering the size of the rectangle, drag and superimpose the rectangle from lane 1 onto lane 2. Use the “ctrl2” shortcut to designate this as the second lane for analysis. Repeat this step for all additional lanes.
12. Once all lanes have been identified, use the “ctrl3” shortcut to plot individual histograms for each lane selected (Fig. 2b). The peaks of these histograms represent the band intensities.
13. The percentage of targeted disruption may then be calculated using the method described by Guschin et al. [18].

4 Notes

1. This is the optimal concentration for the TALEN pair described in this protocol. However, it may be necessary to identify the ideal conditions when using other nucleases. The cleavage efficacy of TALENs is often initially determined using plasmids containing reporter genes with target binding sites.

These plasmids may then be used to assess efficacy after transfection of easy-to-transfect cell lines such as HEK293T.

2. The “no nuclease transfection” is an essential internal control for the T7EI enzyme digestion and should be included for all transfections.
3. Incubating the cells under mildly hypothermic conditions improves discrimination of nuclease cleavage activity in certain cell lines. The effect is likely to be a result of more pronounced slowing of DNA replication with little inhibition of nuclease activity [19].
4. HepG2.2.15 cell cultures are notoriously difficult to transfect, with reported efficiencies of only 20% [20]. In our hands, maximum transfection efficiencies of 30% have been detected following a single PEI transfection. High transfection efficiencies are essential to achieve good nuclease cleavage. For this reason HepG2.2.15 cells are triple transfected. Alternatively, if for instance a GFP reporter gene is included, successfully transfected cells may be sorted using flow cytometry and then expanded.
5. Transfection may be substituted with either nucleofection or electroporation. If either of these procedures is preferred, the TALEN encoding sequence may be administered as RNA or DNA.
6. This modified extraction protocol is based on the traditional alkaline lysis-based method of plasmid DNA extraction. Not all plasmid DNA extraction kits are compatible for this step since some of the buffer systems or column membranes have been modified. If unsure about the compatibility of a plasmid DNA kit, a transient transfection may be performed in an easy-to-transfect cell line followed by recovery of the plasmid after 2–3 days.
7. Primer design is an integral part of the T7EI assay. Amplification products between 400 and 800 bp are ideal, since the likelihood of introducing unwanted polymerase-associated mutations is lower. Additionally the position of the primer binding sites in relation to the nuclease cleavage site is important. The S Fw and S Rv primers are designed to bind approximately 250 bp upstream and downstream of the nuclease target site, leading to a PCR product of approximately 500 bp and bands resulting from T7EI digestion of approximately 250 bp. Primers may be designed to generate two different sized cleavage bands, however it is recommended that the smallest cleavage product should be >100 bp.
8. It is very important that only a single amplicon is detectable after the PCR. Any additional bands may be a source of mismatched heteroduplexes, which often cause false positive

results of the T7EI assay. If additional bands are observed, the PCR conditions should be further optimized.

9. Do not use the gel extraction method to purify the residual PCR product since the DNA recovery using this method is low. Any PCR cleanup kit can be used to purify the product, however small final elution volumes are important to maintain an optimal DNA concentration for the T7EI assay.
10. Many factors may affect the resolution of the T7EI assay. Two important considerations are the percentage of agarose in the gel and the size of the gel comb. A high percentage gel will improve visualization, especially when multiple cleavage bands are expected. The size and shape of the gel comb can affect the resolution of the cleaved products. We recommend using a gel comb that forms wells with narrow width.
11. Ideally the volume limit of 12 μ L should not be exceeded. Working with smaller volumes tends to result in better T7EI cleavage. If the concentration of the DNA is too low, the volume may be increased but the resolution of the bands is often compromised. If this is problematic, the PCR should be repeated and optimized in an attempt to improve the yield of DNA.
12. A heating block may be used as an alternative to a thermal cycler. Incubate the samples at 95 °C for 5 min, then switch the heating block off and allow it to cool for at least 2 h. Check the temperature of the block, and if it is below 35 °C remove the samples and place them on ice. Proceed to **step 5**.
13. Alternatively use a heating block set to 37 °C for the incubation.
14. It is important to use an Orange G-based loading dye since the dye front does not interfere with resolution of the cleaved DNA.
15. Use only a small amount of DNA ladder. If the concentration of the DNA ladder is too high identification of cleaved DNA may be difficult, which is especially the case when the cleavage efficiency is low.
16. It is better to perform an initial analysis after 20 min of electrophoresis to determine whether the T7EI assay has been successful. A longer time of electrophoresis may be carried out if the bands have not resolved. Importantly, short T7EI cleavage products may become faint and difficult to quantify if the gel is run for too long.
17. A polyacrylamide gel may also be used to resolve products of a T7EI assay. More recently the Qiagen QIAxcel capillary electrophoresis system has been used to resolve and quantify DNA produced from T7EI assays [21].

18. ImageJ is freely available from <http://imagej.net/Welcome>. Some UV transillumination instruments have preinstalled software programs that permit lane quantification of band intensities. If these programs are already available, they may be used to plot the band intensity peaks for each of the samples.

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Detection of Hepatocyte Clones Containing Integrated Hepatitis B Virus DNA Using Inverse Nested PCR

Thomas Tu and Allison R. Jilbert

Abstract

Chronic hepatitis B virus (HBV) infection is a major cause of liver cirrhosis and hepatocellular carcinoma (HCC), leading to ~600,000 deaths per year worldwide. Many of the steps that occur during progression from the normal liver to cirrhosis and/or HCC are unknown. Integration of HBV DNA into random sites in the host cell genome occurs as a by-product of the HBV replication cycle and forms a unique junction between virus and cellular DNA. Analyses of integrated HBV DNA have revealed that HCCs are clonal and imply that they develop from the transformation of hepatocytes, the only liver cell known to be infected by HBV. Integrated HBV DNA has also been shown, at least in some tumors, to cause insertional mutagenesis in cancer driver genes, which may facilitate the development of HCC. Studies of HBV DNA integration in the histologically normal liver have provided additional insight into HBV-associated liver disease, suggesting that hepatocytes with a survival or growth advantage undergo high levels of clonal expansion even in the absence of oncogenic transformation. Here we describe inverse nested PCR (invPCR), a highly sensitive method that allows detection, sequencing, and enumeration of virus–cell DNA junctions formed by the integration of HBV DNA. The invPCR protocol is composed of two major steps: inversion of the virus–cell DNA junction and single-molecule nested PCR. The invPCR method is highly specific and inexpensive and can be tailored to DNA extracted from large or small amounts of liver. This procedure also allows detection of genome-wide random integration of any known DNA sequence and is therefore a useful technique for molecular biology, virology, and genetic research.

Key words Integrated DNA, Inverse nested PCR, Virus–cell DNA junction, Clonal expansion, Insertional mutagenesis

1 Introduction

Hepatitis B virus (HBV) is a blood-borne virus that causes significant morbidity and mortality worldwide. Chronic HBV infection is generally lifelong and confers risk of liver cirrhosis and hepatocellular carcinoma (HCC) [1–3].

HBV is a small, enveloped, double-stranded DNA virus with a genome size of 3.2 kbp encoding four overlapping open reading frames (ORF), from which seven different proteins are translated. HBV enters hepatocytes using the cellular transporter protein

sodium taurocholate cotransporting peptide as an entry receptor [4, 5]. Within hepatocytes, the virion relaxed circular DNA (rcDNA) genome is converted by cellular DNA repair enzymes into episomal covalently closed circular DNA (cccDNA), which acts as the transcriptional template for viral mRNAs and pregenomic RNA (pgRNA) [6]. After transcription, pgRNA, complexed near its 5' end by the viral DNA polymerase and cellular heat-shock proteins, is packaged into nucleocapsids within an outer shell of HBV core protein. The HBV polymerase then reverse transcribes pgRNA into either an rcDNA genome (present in 90% of virions) or a double-stranded linear DNA (dslDNA) genome [7, 8]. The HBV DNA-containing nucleocapsids are enveloped and exported from the hepatocyte or are transported to the nucleus, where rcDNA is converted into more cccDNA [6, 9]. DslDNA can also be converted to cccDNA, but the cccDNA, formed by non-homologous end joining, is usually defective. At low frequencies, dslDNA molecules can also integrate into the host cell genome at random sites by nonhomologous end joining, resulting in replication-incompetent forms of HBV DNA [10, 11]. Each integration event forms a unique virus–cell DNA junction (VCJ). Integrated HBV DNA has been subject to investigation as a driver of hepatocarcinogenesis and as a tool for tracing the fate of hepatocytes in situ.

Firstly, integration of HBV DNA into cancer driver genes and their disruption by insertional mutagenesis has long been a focus of HCC research. In the woodchuck model of HBV-associated HCC, integration of woodchuck hepatitis virus (WHV) DNA into or near the oncogene *N-myc2* is commonly observed in tumor tissues [12–14]. Furthermore, in humans, HBV DNA integration close to tumor suppressor genes fibronectin 1, mixed-lineage leukemia 4, and hTERT has been found in a minor fraction of HCCs [15–18]. Recent studies have also suggested that integration of HBV DNA into long interspersed nuclear elements produces HBV–cell fusion transcripts that have pro-oncogenic functions [19], though the significance of fusion transcripts as a driver in the majority of HCC tumors has been disputed [20].

Secondly, since HBV integration occurs at random sites in the hepatocyte genome, VCJs can be used as molecular markers for lineages of infected hepatocytes. This relies on the fact that all progeny cells of a hepatocyte containing a particular VCJ will contain the same VCJ. It is important because hepatocytes constitute a self-renewing population, with little if any contribution from hepatic stem/progenitor cells [21, 22]. Thus, clonal expansion mirrors genetic narrowing of the hepatocyte population, which may be a cancer-risk factor. However, to detect and quantify VCJ, highly sensitive methods are needed, since the integration rate is low and progeny of any hepatocyte will seldom compromise a major fraction of even a small liver tissue sample. For instance, in the duck hepatitis B virus (DHBV) model, only 1/10,000 hepatocytes was found to contain integrated DHBV DNA at 6 days post-infection [10].

In the healthy uninfected liver, hepatocyte death and compensatory division are thought to be random events, with virtually all hepatocytes having the ability to contribute to liver regeneration. However, past studies have suggested that death and/or replacement does not occur in a strictly stochastic fashion in the HBV-infected liver [23–30]. Instead, the size of some of the observed hepatocyte clones seems to require that they had either a survival or growth advantage. The existence of very large hepatocyte clones in both tumor and cirrhotic liver tissues has been deduced using restriction fragment analysis of total DNA extracts coupled with Southern blot hybridization [23–27]. A number of studies have also found that clonal expansion of hepatocyte subpopulations occurs at all stages of chronic HBV infection in humans and chimpanzees [28–30]. Clonal expansion of hepatocytes in cirrhotic nodules probably reflects selection of rare hepatocytes that are able to survive in this environment. Quantification of VCJ in samples of non-cirrhotic liver has also revealed hepatocyte subclones that were too large to have been produced by random turnover, but likely represented subclones of hepatocytes with a survival or growth advantage [29, 30].

Various methods have been used to detect integrated HBV DNA, including:

Southern blot hybridization—Using a DNA probe specific for HBV DNA, restriction fragment analysis coupled with Southern blot hybridization has been used to show that HBV DNA exists not only as replicative intermediates but also as high-molecular-weight bands representing integrated forms of HBV DNA in total DNA extracts of HCC and the surrounding HBV-infected tissue [23–27]. While this technique is relatively cheap and simple to carry out, it cannot identify the sequence of the VCJ or quantify, except in rare cases, the copy number of individual integrants. Southern blot hybridization is also insensitive, with a lower detection limit of 1–10 pg of HBV DNA [31, 32], so that clones of ~300,000–3,000,000 hepatocytes would be required for detection of clonally integrated HBV DNA assuming the DNA sample was entirely from clonally expanded hepatocytes.

Alu-PCR—PCR using primers specific for HBV and Alu elements have been used to amplify virus–cell DNA junctions in both HCC and the liver tissue of patients with HBV infection [33, 34]. Alu elements are common short interspersed nuclear elements (SINEs) present in primate genomes and number ~1,000,000 copies per human genome [35]. In these studies, HBV integration sites were observed close to the ORF of genes associated with the progression of HCC. However, Alu-PCR may be inherently biased as Alu elements in the human genome are concentrated near ORFs [36].

Next-generation sequencing (NGS)—Two NGS strategies have been used to detect HBV integration events. Firstly, whole-genome sequencing (WGS) has been used to detect HBV DNA integration

sites, as well as other genomic alterations, in HBV-associated HCC and the surrounding non-tumor tissue [18, 37–40]. WGS is a broad technique that detects HBV DNA integration over the entire mappable genome. However, as >99% of reads do not align to integrated HBV DNA (due to its comparative rarity) and the requirement of large amounts of starting DNA (~1 µg or ~10⁶ cells), WGS has poor sensitivity for low-frequency VCJ. Further, the expense of sequencing a whole genome at >100-fold coverage limits the number of samples that can be analyzed. Also, most VCJs are detected only by extrapolation due to relatively short read lengths (<400 nt), occurring when one end of a paired-end sequence aligns to a cell-specific sequence and the other a HBV-specific sequence. That is, the cellular DNA sequence directly at the VCJ is not known for most VCJ detected by WGS. Finally, due to the non-linear amplification of DNA libraries during preparation for NGS, absolute quantification of VCJ can be difficult. While these drawbacks could be circumvented by novel isolation methods (e.g. single-cell WGS), the relative rarity of HBV integration events (~1 per 10,000 cells) make these approaches challenging and expensive.

Secondly, a more targeted, high-throughput sequencing approach called massive anchored parallel sequencing (MAPS) has been designed to enrich for DNA fragments containing VCJ using a selective PCR strategy during library preparation [17]. However, while improving the efficiency and quantification of WGS, MAPS is subject to the same drawbacks in cost and VCJ identification as other NGS techniques.

Inverse nested PCR—Our group and others have used the invPCR technique to detect VCJ in studies of DHBV, WHV, and human HBV-infected liver [10, 28–30, 41, 42, 43]. As it is a highly selective and sensitive technique, invPCR can identify the exact VCJ sequence and quantify multiple VCJs in absolute numbers, both crucial requirements to detect and quantitate hepatocyte clones. Furthermore, invPCR brings together an ideal balance of cost, simplicity, and relatively unbiased VCJ detection.

This chapter describes the invPCR protocol performed using DNA extracted from snap-frozen liver tissue. We have performed invPCR on DNA extracted from ethanol-fixed paraffin-embedded tissue sections and hepatocytes isolated from tissue sections by laser microdissection, and these approaches only differ only in the DNA extraction method and decreased DNA input into the invPCR reaction.

InvPCR design (Fig. 1; see **Note 1**) has been hampered by the availability of restriction enzymes (RE) and compatible buffers. Using principles from the original description of invPCR which was used to detect VCJ in WHV-infected woodchucks [41], two invPCR designs have been successfully used in multiple HBV genotypes [29, 30] (see Tables 1 and 2). With the advent of designable RE (via TALEN and CRISPR/Cas9 systems) and more standardized RE buffers, new designs are possible but have not yet been

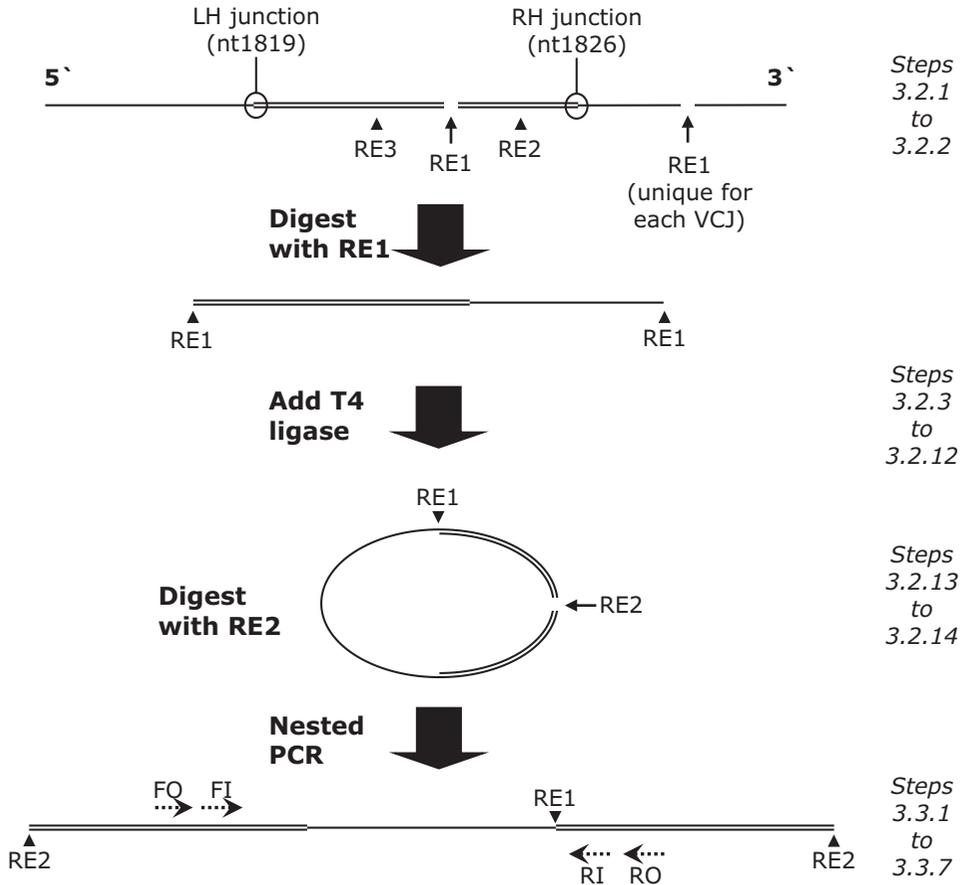


Fig. 1 Design of invPCR. The *right-hand* VJC formed by the integration of HBV DNA (*double line*) into cellular DNA (*single line*) is excised by digestion with RE1. The excised product is then added to a T4 DNA ligase-mediated ligation reaction in a large volume to promote intra-molecular DNA circularisation (as opposed to inter-molecular joining of DNA fragments). The circularized product is subsequently cleaved with RE2 to form an inverted product, with viral sequences flanking unknown cellular sequences. The inverted products are also cleaved with RE3 to reduce aberrant amplification of HBV cccDNA (explained in detail in Fig. 2). Inverted products are then amplified using nested PCR primers (*dashed arrows*). RE sites are shown as *triangles*; RE digestions during the steps are indicated as *arrows*. The relevant steps in the invPCR protocol are shown on the *right*

Table 1
Restriction enzymes (RE) used in invPCR designs

Design	RE1	RE1 site ^a	RE2	RE2 site ^a	RE3	RE3 site ^a	Refs
1	<i>NcoI</i>	1374	<i>BsrHKA1</i>	1585	<i>SphI</i>	1235	[29, 30]
2	<i>DpnII</i>	1420	<i>BsrHKA1</i>	1585	<i>NcoI</i>	1374	[30]

^aRE sites were predicted based on GenBank Accession #AB241115

Table 2
PCR primers used in invPCR Design 1 and Design 2, reproduced from [30]

InvPCR design	Forward primers			Reverse primers			
	Outer or inner	Name	Sequence (5' → 3') ^a	Position on HBV ^b	Name	Sequence (5' → 3') ^a	Position on HBV ^b
1	Outer	FO-1A	TTCGCTTACCTCTGCACG	1603–1621	RO-1A	AAAGGACGTCCC GCGCAG	1422–1405
		FO-1B	TGGAGACCACCGTGAACG	1626–1643	RO-1B	AAAGGACGTCCC GCGGAAG	1422–1405
	Inner	FI-1A	CGCATGGAGACCCCGTGA	1623–1641	RI-1A	AGTACAGCCTAGCAGCCAT	1388–1370
		FI-1B	CGCATGGAGACCCCGTGA	1623–1641	RI-1B	CACACCCCTAGCAGCCATGG	1390–1372
2	Outer	FO-2A	TTCGCTTACCTCTGCACG	1603–1621	RO-2A	GGGACGGTAGAGTCCC AAAAC	1515–1497
		FO-2B	TTCGCTTACCTCTGCACG	1603–1621	RO-2B	GGAGAAAGCGGGCGGTAGAGC	1516–1496
	Inner	FI-2A	CGCATGGAGACCCCGTGA	1623–1641	RI-2A	GGGACGAGAGAGTCCC AAG	1516–1497
		FI-2B	CGCATGGAGACCCCGTGA	1623–1641	RI-2B	AAAGGACGTCCC GCGCAG	1422–1405

^aThese outer and inner PCR primer sets were previously used [30] to amplify VCJ by nested PCR. A single forward primer could be used with one of multiple reverse primers. For example, primer FO-1A can be used with primer RO-1A or RO-1B. The specific primer set used for any given patient was based on compatibility of primer sequence with the DNA sequence of the infecting HBV DNA strain

^bPrimer positions were based on numbering of the HBV DNA nucleotide sequence from GenBank Accession #AB241115

evaluated. Below we describe the steps required for invPCR using HBV-infected liver tissue, providing comments throughout that highlight caveats and pertinent issues. A series of linked Notes are provided at the end of the Chapter.

2 Materials

2.1 Extraction of DNA

1. Sterile petri dish and scalpel blade (single use only).
2. 0.5% sodium hypochlorite for decontamination.
3. Digestion solution: 100-mM NaCl, 0.5% w/v SDS, 50-mM Tris-HCl pH 7.5, 10-mM EDTA, 2-mg/mL proteinase K (Roche).
4. Thermomixer comfort (Eppendorf).
5. Ultrapure phenol (Life Technologies).
6. Ultrapure phenol/chloroform/isoamyl alcohol (25:24:1) (Life Technologies).
7. 3-M sodium acetate, pH 4.6.
8. Ethanol (analytical reagent grade).
9. DNase-free water or 100-mM Tris-HCl pH 7.5.
10. Qubit® fluometric dsDNA quantification kit and fluorometer (Thermo Fisher Scientific).

2.2 Inversion of DNA

1. Restriction enzyme (RE) kits (New England Biosciences, NEB) (Figs. 1 and 2; *see Note 1*).
2. T4 DNA ligase kit (NEB).
3. 10% SDS.
4. 5-M sodium chloride.
5. 10-mg/mL dextran (Sigma Aldrich).
6. Ethanol (analytical reagent grade).

2.3 Nested PCR

1. 96-well PCR plates (Axygen).
2. Thermowell® reusable silicone rubber sealing mats (Corning).
3. PCR primers (Fig. 3; *see Table 2*).
4. AmpliTaq Gold (Applied Biosystems) and GoTaq Flexi (Promega) PCR mixes: 2.5-U Taq polymerase, 2.5-mM MgCl₂, and 0.5 mM of each PCR primer (*see Note 2*).
5. Bunsen burner.
6. Nunc 96-pin replicator (Thermo Scientific).

2.4 PCR Product Gel Extraction and Sequencing

1. Agarose for gel electrophoresis (Bio-Rad).
2. GelRed dye (Biotium).
3. UV gel imager with wavelength ~250–350 nm.

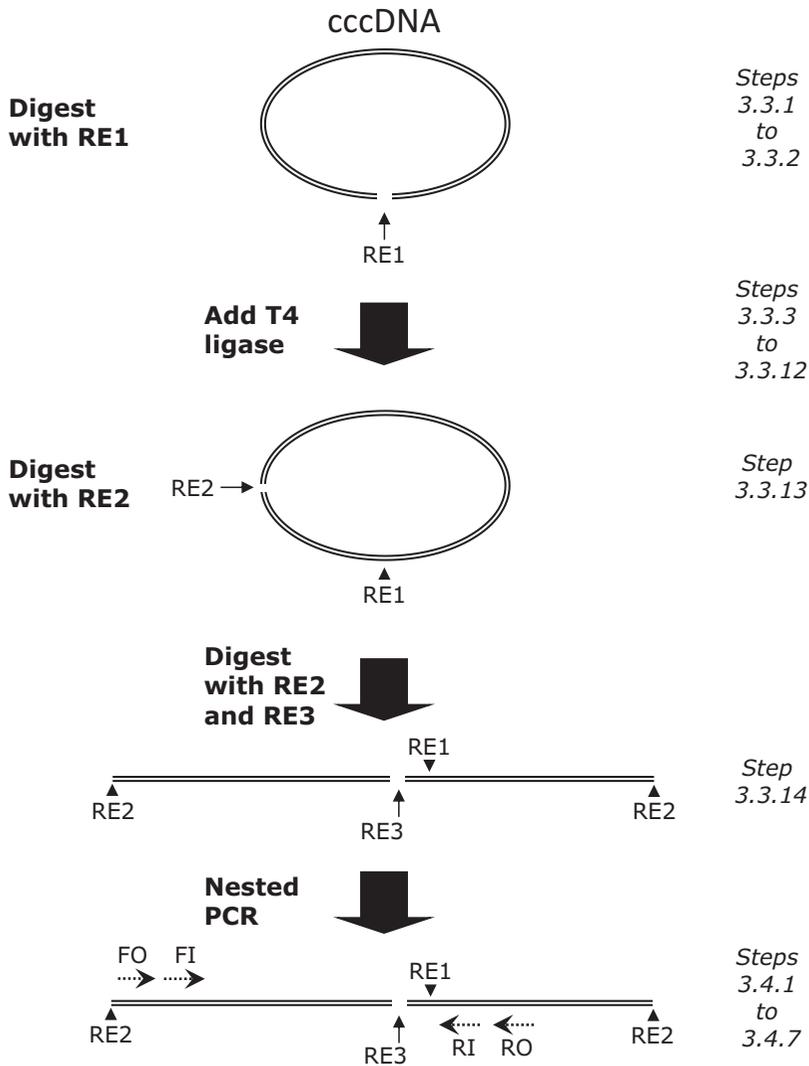


Fig. 2 Minimizing PCR amplification of fragments of HBV cccDNA. HBV cccDNA (*black double lines*) can generate amplifiable fragments during the inversion reaction. If sites generated by RE1 are ligated, then products could potentially be amplified by nested PCR. However, if RE3 is used to digest inverted products, then nested primers (*dashed arrows*) are unable to amplify the cccDNA-derived inversion product. RE sites are shown as *triangles*; RE digestions during the steps are indicated as *arrows*. The relevant steps in the invPCR protocol are shown on the *right*

4. Small (~5 mm diameter) drinking straws.
5. Thermowell® 96-well thick-skirted PCR plates (Costar).
6. QIAEX II Gel Extraction Kit (Qiagen): contains QX1 Buffer, QiaexII glass beads, PE buffer, and elution buffer.
7. 10% Triton X-100.

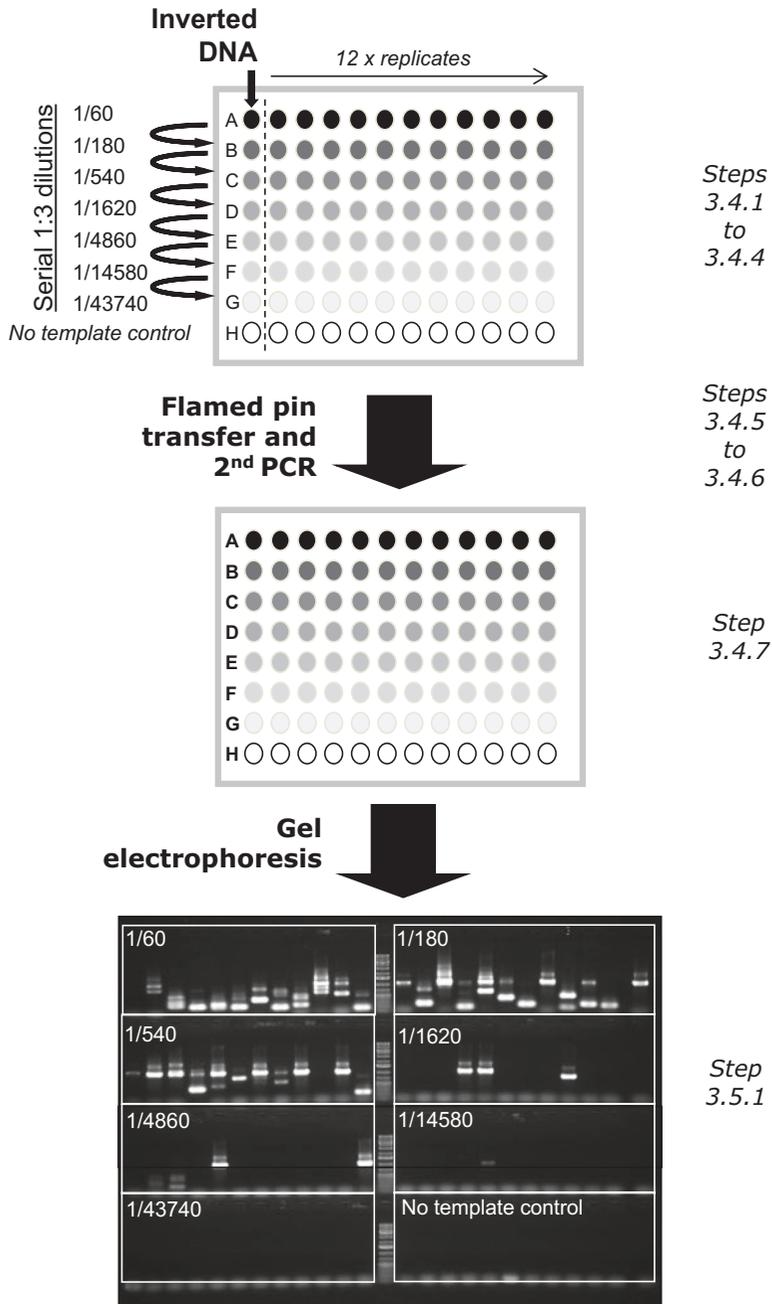


Fig. 3 Nested PCR of inverted DNA. Inverted DNA is serially 1:3 diluted down from well A1 to well G1 of a 96-well plate in AmpliTaq Gold PCR mix containing the forward outer (FO) and reverse outer (RO) primers. The numbers on the left represent the dilution with respect to the original DNA extract (e.g., 1/60 means that 1/60 of the original DNA extract is in the 120- μ L PCR mix in well A1). The mixture is distributed along each row producing 12 replicates per dilution and is then subjected to PCR. A flame-sterilized 96-pin replicator is used to transfer products of the first PCR to another 96-well plate with each well containing GoTaq PCR mix and forward inner (FI) and reverse inner (RI) primers, and a second PCR is performed. Products are visualized by gel electrophoresis, isolated, and sequenced. Repeated unique VCJs represent clones of hepatocytes, the size of which is determined by multiplying the number of wells containing a particular VCJ at the greatest dilution by the dilution factor. The relevant steps in the invPCR protocol are shown on the *right*

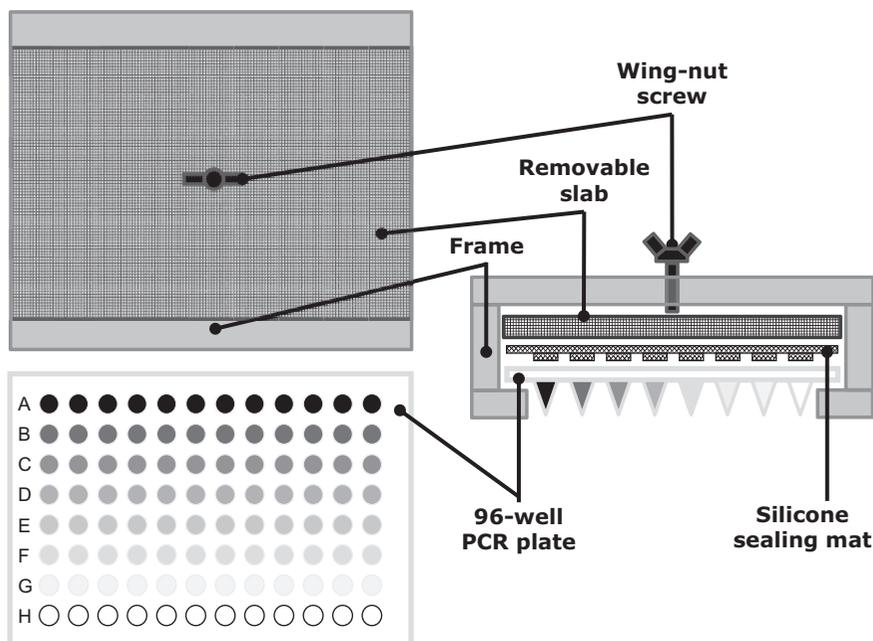


Fig. 4 A custom-designed clamp. Top-down and end-on views of the custom-designed clamp (modified from an original design by Dr. Jesse Summers, University of New Mexico, Albuquerque) are shown. All reusable items (frame, removable slab and silicon sealing mat) should be decontaminated before and after use with 0.5 % hypochlorite as described in Subheading 3.1, step 4. To operate, the silicone sealing mat (*diamond-filled*) is first pressed onto the 96-well PCR plate (*light gray*). The removable slab is placed on top of the sealed plate (*cross-hatched*) and then the plate is placed into the frame (*dark gray*). The wing-nut screw (*black*) is then tightened to finger-tight tension, clamping the sealing mat securely onto the 96-well PCR plate with an evenly distributed force

8. 3-M sodium acetate, pH 4.6.
9. Big Dye terminator v3.1 cycle sequencing kit (Life Technologies).
10. 96-well tray holder (Ambion).
11. Thermowell® reusable silicone rubber sealing mats (Corning).
12. Custom-designed frame and clamp for holding the silicone sealing mats onto the PCR tray (Fig. 4; see Note 3).
13. Qubit® fluometric dsDNA quantification kit and fluorometer (Thermo Fisher Scientific).
14. Agencourt CleanSEQ magnetic bead kit (Beckman Coulter).
15. Agencourt 96-ring SPRI plate magnetic base (Beckman Coulter).
16. Ethanol (analytical reagent grade).
17. 0.1-mM EDTA pH 7.5.

3 Methods

3.1 General Strategies

All steps were carried out at room temperature (RT) unless otherwise indicated. As this is a highly sensitive technique that amplifies down to single copies of DNA template and uses the same PCR primers for separate samples, limiting DNA contamination is a major issue. General strategies to limit PCR contamination include:

1. Identify and establish four physically separate areas including (from “more dirty” to “less dirty”):
 - (a) Tissue DNA extraction area (carried out in a biosafety cabinet with a UV lamp for decontamination).
 - (b) DNA extraction (pre- and post-PCR), inversion, and sequencing reaction setup area.
 - (c) Template addition to PCR and flamed-pin transfer area (we have used PCR hoods with a decontaminating UV lamp with good results).
 - (d) Stock solution and PCR master-mix setup area to be used to prepare fresh dedicated reagents for carrying out the inversion reaction, PCR, and DNA extractions. Make up buffers from new powdered stocks.
2. Have separate lab gowns (which are changed often) for all four areas (areas a-d listed above) and change gloves when moving from “more dirty” to “less dirty” areas.
3. Limit cross-current air flow within the lab, especially in the template addition to PCR area. Cross-contamination of wells at this point will lead to inaccurate quantification of VCJ. To locate an area of the lab with the least cross-currents, we hold up a piece of tissue and measure the amount of air movement by the eye. PCR hoods can also be used to limit these currents. Use negative control wells in the 96-well PCR to test for cross-contamination.
4. Decontaminate all work surfaces with 0.5 % sodium hypochlorite solution. Wipe off with a damp paper towel, and then dry with a clean paper towel. Decontaminate all reusable items (frames, removable slabs and silicon sealing mats) before and after use by soaking in 0.5 % hypochlorite for >1 h, then rinsing with distilled water.
5. Never decontaminate with 70 % ethanol for the purpose of removing DNA contaminants. Ethanol will fix any DNA to the surface, making it even harder to remove.

3.2 Extraction of DNA

1. Within a biosafety cabinet, use a sterile plastic petri dish and a sterile scalpel blade to excise a ~5-mg fragment from a snap-frozen sample of the biopsy or autopsy liver tissue (*see Note 4*).

2. Place the tissue immediately in a 2-mL screw-cap Eppendorf tube with 400 μL of digestion solution (*see Note 5*).
3. Incubate in a thermomixer rotating at 3000 rpm at 55 $^{\circ}\text{C}$ for >2 h (extendable to overnight) until no obvious pieces of liver parenchyma are visible. Some pieces of the fibrotic tissue or fat may still be observed.
4. In a fume cupboard, add 400 μL of ultrapure phenol and mix by multiple, vigorous inversions or by vortexing for a few seconds, until the phases are thoroughly mixed and the solution appears milky.
5. Centrifuge at 14,000 $\times g$ for 10 min and transfer the top aqueous layer to a clean 2-mL screw-cap Eppendorf tube, taking care to avoid any milky or cloudy interface.
6. Repeat **steps 4** and **5** twice with 400 μL of 25:24:1 ultrapure phenol/chloroform/isoamyl alcohol in the place of phenol.
7. Add 35 μL of 3-M sodium acetate pH 4.6 to the extracted DNA, followed by 800 μL of ethanol.
8. Incubate at -20°C overnight or at -80°C for 4 hr to precipitate the DNA.
9. Pellet the precipitated DNA by centrifugation at 14,000 $\times g$.
10. Wash the pellet with 1 mL of 70% ethanol.
11. Repeat Subheading **3.2**, **step 10**.
12. Vacuum-dry for 20 min and redissolve in 50 μL of DNase-free water or 100-mM Tris-HCl pH 7.5.
13. Determine the concentration of DNA using Qubit[®] fluometric quantification or another DNA-specific quantification method.

3.3 Inversion of DNA

An overview of the DNA inversion process and the relative positions of the RE sites (RE1, RE2, and RE3) is shown in Fig. 1. The two inversion designs used in previous studies are shown in Table 1. When possible, use high-fidelity REs to minimize star activity (i.e., cleavage at sites similar but not identical to the nominal RE target sequence). This will reduce PCR amplification of products derived from cccDNA (*see Note 6*).

Each invPCR design requires careful selection of RE and nested PCR primers based on the sequence of the infecting HBV genotype. If the prevalent HBV DNA sequence is unknown for any liver sample, it must first be determined by PCR amplification of the HBV genome and sequencing (as described in Subheading **3.4**, **step 4**, and Subheading **3.6**, **steps 1–8**). In previous published studies [30], we used three sets of primers based on highly conserved HBV DNA regions (*see Table 3*) to amplify overlapping HBV DNA fragments upstream, spanning, and downstream from the expected HBV DNA integration junctions near nt 1832 at the downstream end of dsDNA (GenBank accession #AB241115).

Table 3
Primers used for amplifying and sequencing HBV DNA for invPCR design

HBV DNA fragment	Forward primer sequence (5' → 3')	Position on HBV genome ^a	Reverse primer sequence (5' → 3')	Position on HBV genome ^a
Upstream	CCCCTGTYTGGCTT TCAGT	717–735	CAATTTATGCCTACAGC CTCCT	1796–1775
Spanning	CATAAGAGGACTCT TGGACT	1653–1672	TAAGGCTTCTCGATAACA GAGC	2026–2006
Downstream	ATGCAACTTTTTTCA CCTCTGC	1814–1834	TCGGGAAAGAATCCC AGAG	2927–2909

^aPrimer positions were based on numbering of the HBV DNA nucleotide sequence from GenBank Accession #AB241115

The HBV DNA sequence can be used to ensure that the RE and the PCR primers used for the invPCR design are compatible with your samples (suggested criteria outlined in **Note 1**).

1. Digest 1/10 of the total nucleic acid extract from Subheading **3.2, step 12** (~1–2 µg of DNA), in a 40-µL reaction volume (containing 1× of the appropriate RE buffer and 5 U of RE1) at the appropriate temperature for 1 h (*see Note 7*).
2. Heat-inactivate the enzyme at the temperature recommended by the manufacturer for 20 min.
3. Add 450 µL of 1× T4 DNA ligase buffer containing 500 U of T4 DNA ligase to circularize the DNA fragments produced by the RE digestion (*see Note 8*).
4. Incubate the ligation reaction at room temperature (RT) for 2 h.
5. Heat-inactivate the T4 ligase at 70 °C for 20 min.
6. Denature the T4 ligase with 10 µL of 10% SDS.
7. Add 10 µL of 5-M NaCl and 2 µL of 20-mg/mL dextran as carrier to increase the efficiency of ethanol precipitation.
8. After pulse vortexing, add 1 mL of 100% ethanol and mix by multiple inversions or brief vortexing.
9. Precipitate the DNA at –20 °C overnight.
10. Pellet the precipitated DNA by centrifugation at 14,000×g.
11. Wash the pellet with 1 mL of 70% ethanol.
12. Dry the DNA pellet under vacuum at RT for 20 min.
13. Redissolve the pellet in 40 µL of an RE reaction mix (containing 1× of the appropriate RE buffer and 5 U of RE2) and incubate at the appropriate temperature for 1 h (*see Note 9*).

14. Digest with 5 U of RE3 to minimize the amplification of fragments generated from wild-type HBV cccDNA (Fig. 2). Incubate at the temperature recommended by the manufacturer for 1 h.

3.4 Nested PCR

An overview of the steps during the nested PCR process is shown in Fig. 3 (*see Note 10*). Primers used in the nested PCR are designed based on the prevalent HBV DNA sequence in each patient [30] (*see Table 2*):

1. Add 10 μL of the inverted DNA from Subheading 3.3, **step 14**, to a 170- μL 1 \times AmpliTaq Gold PCR mix containing the forward outer (FO) and reverse outer (RO) primers at a concentration of 0.5 μM in well A1 of a 96-well PCR plate as shown in Fig. 3.
2. Separately add 120 μL of the same 1 \times AmpliTaq Gold PCR mix containing the FO and RO primers to wells B1–H1, also as shown.
3. Serially transfer 60 μL from well A1 down the column into wells B1 to G1 (resulting in serial 1:3 dilutions with final volumes of 120 μL), leaving 120 μL of PCR mix in well H1 as a no-template control (Fig. 3; *see Note 11*).
4. Aliquot 10- μL samples of the mixture from wells A1–H1 into wells A2–H2, A3–H3, etc. through to wells A12–H12 of the 96-well plate. Use a multichannel pipetter for this step.
5. Seal the plate (*see Note 12*) and perform a PCR reaction using the following conditions: initial 10-min step at 95 $^{\circ}\text{C}$ to activate hot-start polymerase; 35 cycles of 15-s denaturation at 95 $^{\circ}\text{C}$, 15-s annealing at 54 $^{\circ}\text{C}$, and 3-min elongation at 72 $^{\circ}\text{C}$; then a holding step at 15 $^{\circ}\text{C}$.
6. Carefully remove the seal to avoid cross-contamination.
7. Transfer products of this PCR to a second 96-well plate (each well containing 10 μL of GoTaq Flexi Green PCR mix and the forward inner (FI) and reverse inner (RI) primers at 0.5 μM) using a cooled 96-pin replicator, with pins previously heated until red hot with a Bunsen burner (*see Note 13*).
8. The nested PCR is carried out using the same conditions as Subheading 3.4, **step 4**, except that the initial denaturation step at 95 $^{\circ}\text{C}$ is for 2 min.

3.5 PCR Product Gel Extraction

1. Analyze the PCR products by 1.3% agarose gel electrophoresis on a 96-well gel. Post-stain the gel using GelRed dye and visualize using a UV gel imager (*see Note 14*).
2. Excise the DNA bands from agarose gels using disposable drinking straws (*see Note 15*). For each PCR product, the straw and agarose gel plug are placed into a single Thermowell[®]

96-well thick-skirted PCR plate and then trimmed to size. At this point, the whole tray can be frozen at $-20\text{ }^{\circ}\text{C}$ for later extraction. Freezing the tray also facilitates freeing the agarose gel from the segment of straw.

3. Squeeze each straw to liberate the frozen agarose plug into each well.
4. To each well, add 100 μL of QX1 buffer (Qiagen), 5 μL of QiaexII glass beads (Qiagen), 1 μL of 10% Triton X-100, and 10 μL of 3-M sodium acetate pH 4.6.
5. Seal the plate with a silicone rubber mat (*see Note 12*) and dissolve the agarose at $55\text{ }^{\circ}\text{C}$ for 30 min with occasional vortexing. We use custom-designed frames to hold the seals onto the tray (Fig. 4, *see Note 3*).
6. Place the plate in a 96-well tray holder (Ambion) and pellet the glass beads by centrifugation at $1850\times g$ for 3 min.
7. Wash the beads two times with 50 μL of QX1 buffer containing 0.1% Triton X-100, each time resealing the plate, vortexing until the bead pellet is suspended followed by centrifugation at $1850\times g$ for 3 min.
8. Wash the beads three times with 100 μL of PE buffer (Qiagen), again with sealing, vortexing, and centrifugation as above.
9. Vacuum-dry the pelleted beads for 20 min at RT.
10. Elute DNA from the beads with 30 μL of elution buffer (Qiagen).
11. Vortex the tray and incubate for 5 min at RT.
12. Pellet the beads by centrifugation at $1850\times g$ for 3 min and remove the DNA-containing supernatant for sequencing.
13. Determine the concentration of DNA using Qubit[®] fluorometric quantification or any other DNA-specific quantification method.

3.6 PCR Product Sequencing

1. Add 3 μL ($\sim 100\text{ ng}$) of purified DNA to a sequencing reaction with the final volume of 10 μL (1- μL BigDye terminator mix, $1\times$ sequencing buffer, 160 nM of the FI primer used in the nested PCR).
2. Run a sequencing reaction with the following conditions: initial 2-min denaturation step at $95\text{ }^{\circ}\text{C}$, 25 cycles of 10-s denaturation step at $95\text{ }^{\circ}\text{C}$, 10-s annealing step at $55\text{ }^{\circ}\text{C}$, and 4-min elongation step at $60\text{ }^{\circ}\text{C}$, and then a holding step at $15\text{ }^{\circ}\text{C}$.
3. Remove unincorporated dye terminator nucleotides using a CleanSEQ magnetic bead kit: add 42 μL of 85% ethanol and 10 μL of magnetic beads to each well of the 96-well plate and vortex.
4. Incubate the plate on a 96-ring SPRI plate magnetic base for 10 min.

5. While leaving the plate on the magnetic base, wash the magnetic beads twice by addition of 100 μ L of 85% ethanol, waiting until the magnetic beads reattach to the sides of the well, and then removing the ethanol by aspiration.
6. Vacuum-dry the magnetic beads for 30 min.
7. Elute the sequencing products by addition of 80 μ L 0.1-mM EDTA, pH 7.5.
8. Submit 40 μ L of the elute for capillary separation.

3.7 Sequence Analysis

1. Analyze chromatograms using software of choice—we use the freely available Chromas Lite software (Technelysium).
2. Align the sequence to the HBV genome using BLAST and trim away the HBV sequence.
3. If no cellular DNA sequence remains, the fragment was probably generated by HBV cccDNA recombination during the inversion process.
4. If unknown sequence remains, align the sequence to the human genome using BLAST to confirm integration into the host genome. With this confirmation, the VCJ sequence can then be used as a marker for a hepatocyte clone.
5. Estimate the size of individual hepatocyte clones by multiplying the number of wells (>1) containing a particular VCJ at the greatest dilution by the dilution factor (Fig. 3). For example, if three identical VCJs were detected in separate wells of row C (1/540 dilution of the original DNA extract from 5 mg of the liver tissue in this protocol), then the hepatocyte clone size would have an estimated size of 3×540 or 1620 cells.

4 Notes

1. To design a new inversion protocol, our suggested criteria for invPCR designs are as follows:
 - (a) As a guideline RE1, RE2, and RE3 restriction sites should occur within ~ 1 kbp of the expected right-hand VCJ site on the HBV genome at or upstream of \sim nt 1832 [7, 10] to limit the size and so improve circularization and PCR amplification (*see* Note 7 and Note 8) of the excised fragments.
 - (b) Note that sequences may be lost from the right-hand end of HBV dsDNA during integration, so the invPCR design must allow >40 nt between the RE2 restriction site and the expected VCJ site to allow nested primer design. In both invPCR designs, the distance between the RE2 restriction site and the expected VCJ site was 241 nt, and the forward inner primers mapped between 203 and 191 nt upstream of nt 1832 (Table 2).

- (c) Similarly, the design must allow >40 nt between the RE1 and RE2 sites on the HBV genome for nested primer design.
 - (d) RE1 site should occur as frequently as possible in the host genome, but occur very rarely (preferably uniquely) in the HBV DNA genome to limit formation of amplifiable cccDNA-derived fragments (Fig. 2).
 - (e) RE1 should be able to be heat-inactivated to simplify transition to the ligation step.
 - (f) RE2 and particularly RE3 should occur as frequently as possible in the HBV genome to limit formation of amplifiable cccDNA-derived fragments, but occur rarely in the human genome to avoid cleavage between the VCJ and a downstream RE1 site, resulting in an unamplifiable fragment. Further, RE2 and RE3 should be active in the same digestion buffer.
2. We use AmpliTaq Gold for the first PCR and GoTaq Flexi for the nested PCR. GoTaq Flexi also comes with a gel loading-ready buffer, which simplifies the agarose gel electrophoresis.
 3. We use a custom-made clamp to hold the silicone rubber sealing mat onto the thick-skirted plate during vortexing (Fig. 4). This allows greater speeds for resuspension of the glass beads after settling or centrifugation, which is necessary for efficient binding and washing.
 4. To keep the tissue cool, we place an inverted plastic petri dish or lid on top of dry ice. We then put the frozen tissue in the inverted lid and break into ~5-mg fragments (we generally analyze five 5-mg liver fragments per patient), changing dishes and scalpels for each patient. If forceps are used during the extraction procedure, immerse in 0.5% sodium hypochlorite solution for at least 15 min between samples, rinse in water, and dry on paper towels.
 5. Keep the digestion solution at RT or above to prevent the precipitation of SDS at low temperatures.
 6. Using the inversion and PCR amplification conditions described, full-length cccDNA should not amplify to detectable levels. However, it may still be necessary to purify high MW DNA to reduce cccDNA contamination, since residual amplification may result due to (1) naturally occurring deletion mutants of cccDNA or (2) low level star activity of RE1 and circularization of cccDNA fragments. In each case, the DNA molecules generated are small enough to be amplified to detectable levels. An additional gel purification step using 1% low-melting temperature agarose (Bio-Rad) can be performed prior to any digestion in the inversion step (Subheading 3.3, steps 1–14). This has successfully been used in previous studies

to isolate high-molecular-weight cellular DNA from low (<3.2 kb)-molecular-weight intermediates [28]. It is important to quantify the DNA before and after this step to account for any lost DNA yield during the purification in order to calculate VCJ number in the original DNA sample.

7. As a lower concentration of DNA should improve the inversion efficiency (*see Note 8*), less DNA can be added in this step. The amount of DNA can be determined by probability calculations of DNA fragment self-ligation as described in **Note 8**.
8. The large 400- μ L ligation reaction volume favors self-ligation and circularization of DNA fragments as opposed to intramolecular ligation. The probability of circularization of linear DNA during ligation is governed by the ratio of factors j and i [44], where j is the effective concentration of one end of a DNA molecule in the neighborhood of the other end of the same molecule in ends/mL and i is the total concentration of DNA ends within a given solution in ends/mL. The probability of circularization can be shown as simply j/i . When $j/i=1$, equal products of circular and linear ligations are expected. When $j/i>1$, self-ligated circular forms are favored and when $j/i<1$, intermolecular linear ligation is preferred. Covalently closed circular structures of DNA can be found only when $j/i<2-3$ [44]. Using the assumptions that:
 - (a) RE1-cleaved VCJ-containing fragments are 1000 bp long.
 - (b) 1.75 μ g of DNA in the ligation reaction (overestimate).
 - (c) Cellular DNA has a random distribution of bases.
 - (d) RE1 has a four-base recognition sequence.
 - (e) 400 μ L ligation volume.
9. Subheading 3.3, steps 13, and 14, can be combined in a single reaction if RE2 and RE3 incubation temperatures are compatible.
10. In some instances when DNA concentrations are low (e.g., extracts from laser-microdissected tissue samples), we do not carry out the serial dilution step; instead we add the inverted DNA mixture directly to 1 mL of AmpliTaq Gold PCR mix, vortex briefly, and distribute 10 μ L of PCR mix to each well, after which we proceed directly to the PCR step (Subheading 3.4, step 4).
11. Serial dilutions should be mixed by slowly pipetting up and down ten times (approximately once per 2 s) using a 1.0-mL pipette set at \sim 100 μ L. This will limit bubbles and cross-contamination of PCR products.

12. We use reusable silicone sealing mats to seal the PCR plates. After use, these are soaked in 0.5% sodium hypochlorite for >1 h to denature any residual DNA, then rinsed thoroughly with distilled water.
13. Heat the 96-pin replicator until the pins are red hot. Note that the heated 96-pin replicator will cause convection air currents, so cool for 5–10 min at RT before transferring the templates from the first PCR tray to the second. The replicator will transfer only ~1 μ L. Some carbon flecks may also be transferred, but are sterile and should not affect the PCR.
14. Take care to limit exposure of DNA to short wavelength UV (usually ~250–350 nm in gel imaging systems) to minimize the level of DNA damage and to prevent subsequent errors in sequencing.
15. Minor cross-contamination during the PCR product purification steps (Subheading 3.5, steps 2–12) is not a problem since no additional PCRs will be carried out downstream in the protocol.

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Highly Sensitive Detection of HBV RNA in Liver Tissue by In Situ Hybridization

Diego Calabrese and Stefan F. Wieland

Abstract

As soon as HBV was identified, HBV localization at the cellular level became instrumental in studying HBV infection. Multiple methodologies for detection of viral antigens and nucleic acids at the cellular level, not only in patient derived liver biopsy samples, but also in many other experimental systems, have been developed over the years. Recently, the development of highly sensitive and specific in situ hybridization systems enabled detection of cellular mRNAs at the single copy level. Adaptation of such a system (ViewRNA ISH Tissue Assay; Affymetrix, Santa Clara, CA, USA) for the detection of viral RNAs allowed us to reliably identify hepatitis C virus RNA positive cells in the liver of HCV infected patients. Similarly, this protocol enabled detection of very rare HBV positive cells in liver biopsy tissue. Here, we now describe the specifics of the protocol for detection of HBV RNA using the ViewRNA ISH system. The protocol focuses on probe set design, sample preparation and data interpretation for accurate HBV RNA detection in liver tissue samples. The methodology is straightforward and will be very useful in the study of basic HBV virology as well as in clinical applications.

Key words Multiplex in situ hybridization, Viral RNA detection, Needle biopsy, Liver, Chromogenic probe detection

1 Introduction

It was early on recognized that analysis of HBV infection in the liver is crucial for advancing our understanding of HBV pathogenesis. Not surprisingly, immunohistological demonstration of HBV infection has become the method of choice for demonstrating HBV infection in the liver, independently of the presence or absence of serological markers of infection [1]. Nevertheless, there have been many efforts to establish more sensitive methods of HBV detection in the liver and this was achieved, for example, by HBV DNA-specific in situ hybridization [2] or in situ polymerase chain reaction (PCR) containing protocols [3]. While in situ PCR based methods (i.e., target amplification) are very sensitive, they are prone to false positive signals due to contamination and/or insufficient primer specificity. Furthermore, differences in

efficiency of in situ target amplification between samples can preclude accurate target quantification.

Branched DNA (bDNA) signal amplification [4] addresses these problems as both target detection and signal amplification can be optimized and standardized, thus drastically reducing sample to sample variability (reviewed in ref. [5]). Furthermore, the nature of bDNA technology allows for simultaneous detection of multiple targets enabling, for example, multiplex gene expression profiling [6]. While these bDNA technologies are mostly used for nucleic acid detection in solution, they also served as the basis for the development of multiplex branched DNA in situ hybridization systems, that are now available from different manufacturers. We recently used one of these systems (ViewRNA in situ hybridization, Affymetrix, Santa Clara CA) to develop a very sensitive and specific assay for the detection of hepatitis virus RNA in liver biopsy tissue. This methodology allowed for reproducible detection of weakly positive and/or rare hepatitis C virus (HCV) [7] or HBV positive cells [8] in liver biopsy tissue. Furthermore, the multiplex capability of this system allowed for investigating the spatial and quantitative relationship of viral RNA and host gene expression [7, 8].

The following is a detailed description of probe design (*see Note 1*), biopsy tissue processing and in situ hybridization. Finally, we also include a short section summarizing general guidelines for data interpretation (*see Note 15*).

2 Materials

2.1 Equipment, Buffers, and Disposables

1. ThermoBrite StatSpin oven (Abbott Laboratories, IL, USA).
2. Temperature controlled water bath.
3. Hot plate stirrer (max setting ≥ 200 °C).
4. Glass Coplin jar.
5. 1000 ml Pyrex glass beaker.
6. Vertical metal slide rack fitting into the Pyrex glass beaker.
7. Plastic slide box.
8. StainTray slide staining system.
9. Tap water.
10. Water (Bi-distilled).
11. 10× PBS (molecular biology grade, nuclease free).
12. 1× PBS (molecular biology grade, nuclease free).
13. Fixation buffer: 4% formaldehyde in 1× PBS, freshly prepared from 37% formaldehyde; store at 4 °C.
14. Ethanol 100%.

15. Ethanol 50%, 75% (200 ml each, V/V in water), prepared from 100% ethanol.
16. Xylene.
17. Gill's hematoxylin I.
18. Water based, DAPI containing mounting media.
19. Hydrophobic barrier pen (Vector Laboratories, Burlingame, CA, USA).
20. Superfrost Plus Gold glass slides (Thermo Scientific, Waltham, MA, USA).
21. 24×50 mm nr.1 (0.13–0.16 mm) coverslip glass (MEDITE GmbH, Burgdorf, Germany).
22. Transparent nail polish.
23. ViewRNA ISH Tissue Assay Kit (2-plex) (Affymetrix, Santa Clara, CA, USA).
24. Appropriate type 1 and type 6 probe sets (Affymetrix, Santa Clara, CA, USA).
25. Pre-warmed (90°–95 °C) 1× pretreatment solution prepared according to the manufacturer's instructions (Affymetrix, Santa Clara, CA, USA).
26. 1× working protease solution prepared according to the manufacturer's instructions (Affymetrix, Santa Clara, CA, USA).
27. Pre-warmed (40 °C) Probe Set Diluent QT (Affymetrix, Santa Clara, CA, USA).
28. Pre-warmed (40 °C) PreAmplifier Mix QT (Affymetrix, Santa Clara, CA, USA).
29. Pre-warmed (40 °C) Amplifier Mix QT (Affymetrix, Santa Clara, CA, USA).
30. Pre-warmed (40 °C) Label Probe Diluent QF (Affymetrix, Santa Clara, CA, USA).

2.2 Preparing and Processing Frozen Tissue Embedded in OCT

1. Benchtop liquid nitrogen container (Thermo Scientific).
2. Plastic beaker fitting inside the liquid nitrogen container.
3. Liquid nitrogen.
4. Methylbutane (106056; Merck).
5. Optimal cutting temperature compound (OCT; Sakura Tissue-Tek, Sakura Finetek, Torrance, CA, USA).
6. Disposable plastic molds for tissue embedding (Sakura Tissue-Tek, Sakura Finetek).
7. Dry ice.
8. Cryostat (Leica, Wetzlar, Germany).
9. Carbon blades C35 for cryosectioning (FEATHER Safety Razor Co., Ltd., Osaka, Japan).

2.3 Processing Formalin-Fixed and Paraffin- Embedded Tissue

1. Formalin-fixed and paraffin-embedded liver tissue.
2. Ice or cooling plate.
3. Microtome.
4. Stainless steel blades S35 for sectioning (FEATHER Safety Razor Co., Ltd., Osaka, Japan).
5. General purpose lab oven set at 50 °C.

3 Methods

The following protocol outlines the protocol for duplex in situ hybridization (ISH) for localization of hepatitis B virus RNA and human albumin mRNA in liver tissue sections. This protocol has been tested and verified for human and mouse specimens derived from needle biopsies or necropsy, respectively.

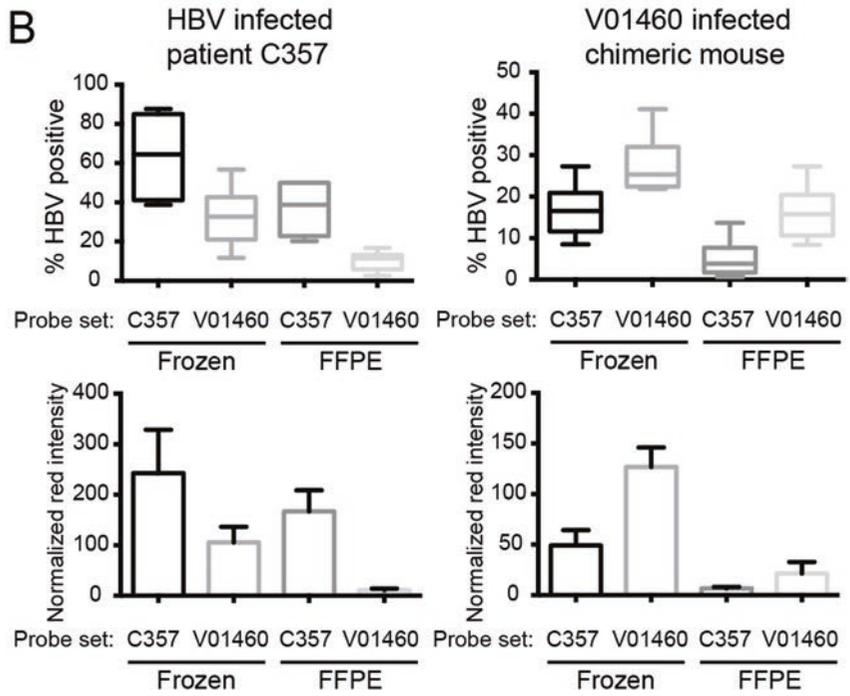
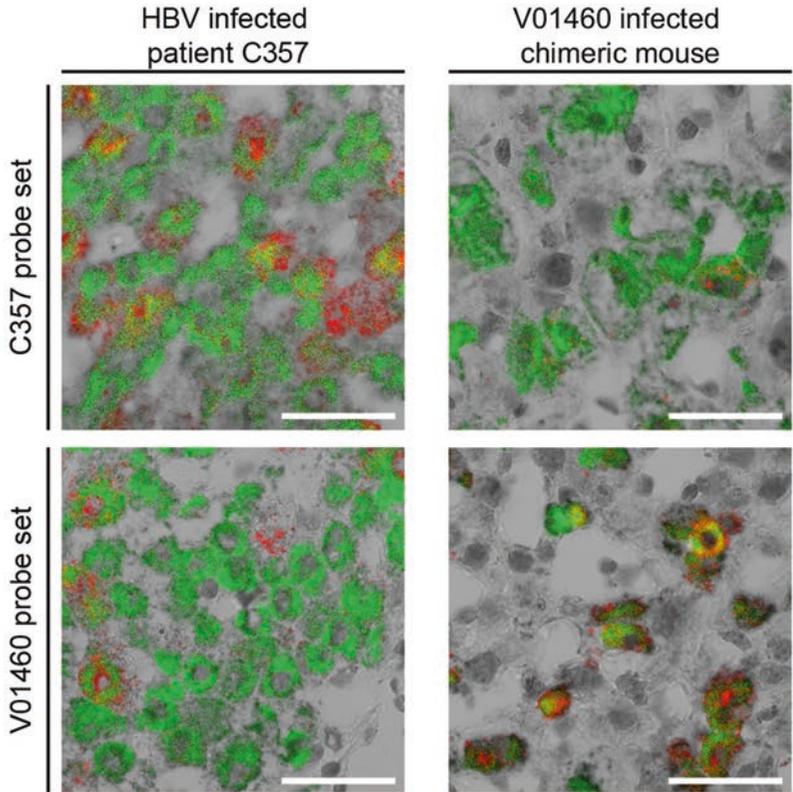
The method is adapted from the ViewRNA ISH (Affymetrix, Santa Clara, CA) Tissue Assay system. Modifications were introduced to the manufacturer's instructions to achieve optimal viral RNA visualization while maintaining sensitivity and specificity of host mRNA detection.

The ISH method is suitable for both formalin-fixed, paraffin-embedded (FFPE) and frozen tissue (OCT embedded). However, as described in Wieland et al. [7, 8] and shown in Fig. 1b, optimally harvested and processed frozen tissue is superior for quantitative detection of RNA. Thus, the protocol below also includes a detailed description of collection, embedding, freezing, and cryo-sectioning of frozen liver tissue samples.

FFPE tissue is normally processed in pathology departments. Fixation, dehydration and paraffin embedding are performed using protocols and equipment that are different in each laboratory. We therefore omit description of tissue harvesting and processing, and focus on describing optimal sectioning and ISH procedures using FFPE specimens.

Fig. 1 In situ hybridization on frozen liver sections of OCT-embedded HBV positive human needle biopsy (C357) and HBV (V01460) positive human liver chimeric mouse tissue samples obtained at necropsy. Sections were hybridized with patient C357 and V01460 HBV RNA-specific type 1 probe sets (*red*) and a human albumin mRNA-specific type 6 probe set (*green*). **(a)** Representative images of the HBV (*red*) and albumin (*green*) fluorescence signals overlaid onto a brightfield image of the liver tissue. Scale bar = 50 μm. **(b)** Results of unsupervised image analysis showing the frequency of HBV positive cells within the human albumin positive cell population (*top panels*) and quantitative HBV signals normalized to human albumin positive cells (*lower panels*). Each panel shows the results obtained using frozen sections (Frozen) or formalin-fixed and paraffin-embedded sections (FFPE). Ten random images were used for image processing and data analysis

A HBV positive frozen liver tissue



**3.1 Tissue Collection
and ISH Procedure
for Frozen Tissue
Specimens**

1. Prepare a benchtop liquid nitrogen container halfway filled with liquid nitrogen.
2. Pour 250 ml of methylbutane in a plastic beaker that closely fits into the liquid nitrogen tank.
3. Chill the methylbutane by floating the beaker on the liquid nitrogen.
4. Wash freshly harvested tissue twice in sterile saline or 1× PBS to remove excess blood (*see Note 2*).
5. Cut 1 cm long sections from a needle liver biopsy cylinder or blocks of 0.5 cm diameter from resection tissue, place them on the bottom of disposable plastic molds, immediately cover the tissue and completely fill the mold with OCT avoiding any bubble formation.
6. Dip the filled mold into the chilled methylbutane (use long forceps) and wait until the OCT turns completely white (a few minutes). Transport the molds containing the frozen OCT blocks on dry ice and store samples at -80°C .
7. Set the cryostat chamber to -16°C , the sample holder to -14°C and let the temperature equilibrate for 30 min before sectioning. If dual temperature control is not available, set the cryostat chamber to $-15^{\circ}\text{C} \pm 2^{\circ}\text{C}$.
8. Set the blade holder inclination in the range of 2° – 10° .
9. Use C35 disposable blades for cryosectioning (do not use S35 blades).
10. Set up the cryostat for single motorized cutting of $10\ \mu\text{m}$ sections.
11. Carefully clean the blade holder and cryostat chamber with 80% ethanol and let them air-dry.
12. Remove the OCT block from the disposable plastic mold inside the cryostat chamber and keep the mold for re-embedding in Subheading 3.1, step 18 (*see Note 3*).
13. Add a small amount of fresh OCT on the cryostat's removable specimen holder and put the frozen OCT block on it with the tissue facing up. Distribute the fresh OCT between frozen OCT block and specimen holder by carefully pressing on the frozen OCT block. Transfer the holder and the block to the cryostat's freezing station and wait until the fresh OCT turns completely white.
14. Lock the specimen holder, fitted with the tissue containing OCT block, into the cryostat specimen head and wait 10 min to allow the sample to reach the correct cutting temperature.
15. Start sectioning.
16. Mount the sections onto Superfrost Plus Gold glass slides.

17. Immediately transfer slides into a prechilled slide storage box. Keep the box in the cryostat chamber during sectioning. When finished, transfer the slide boxes on dry ice and store them at -80°C until use (*see Note 4*).
18. After sectioning, add a few drops of fresh OCT into the corresponding mold, re-embed the sectioned OCT block in the mold and place it on the cryostat freezing station. After a few minutes, remove the OCT block from the metal specimen holder, using the specific function of the cryostat or by heating the back surface of the specimen holder with your palm of hand, until the OCT block and specimen holder can be separated. Store re-embedded OCT blocks at -80°C (*see Note 5*).
19. Clean the cryostat with 80% ethanol and run a long UV-light decontamination cycle if possible (*see Note 6*).

3.2 Fixation, Pretreatment and Hybridization

1. Cool down 80 ml of fixation buffer to 4°C in a Coplin Jar (≥ 1 h).
2. Directly submerge the frozen slides in the chilled fixation buffer and incubate over night at 4°C (16–18 h).
3. Turn on the ThermoBrite and let equilibrate to 37°C .
4. Insert two water soaked humidifier strips into the ThermoBrite lid.
5. Heat 400 ml pretreatment solution on a hot plate stirrer to between 85 and 95°C .
6. Pre-warm the following reagents in a water bath set to 40°C :
 - (a) 40 ml of $1\times$ PBS buffer.
 - (b) Probe Set Diluent QT.
 - (c) PreAmplifier Mix QT.
 - (d) Amplifier Mix QT.
 - (e) Label Probe Diluent QF.
7. Thaw probe sets on ice.
8. Spin down Label Probe 6-AP and Label Probe 1-AP and keep on ice.
9. Bring Naphthol Buffer, AP Enhancer solution, and Blue Buffer to room temperature. Keep Fast Red Tablets and Fast Blue reagent on ice.
10. Equilibrate fixation buffer containing slides to room temperature (10–15 min), decant fixation buffer and keep it for later.
11. Wash the slides twice for 1–5 min in $1\times$ PBS (shake vigorously).
12. Sequentially soak slides for 10 min in 50, 75, and 100% ethanol.

13. Bake the slides in the open ThermoBrite oven for 5 min at 37 °C.
14. Draw hydrophobic barrier around tissue section with the hydrophobic barrier pen.
15. Bake slides for an additional 5 min at 37 °C.
16. Remove slides from the ThermoBrite oven and let them cool to room temperature.
17. Start HYB program on ThermoBrite oven (i.e., constant 40 °C) and close lid for good humidification.
18. Place slides in a vertical metal rack and submerge them in the preheated (85–95 °C) pretreatment solution for 1 min.
19. Transfer slides to a Coplin jar and briefly wash twice in 1× PBS.
20. Prepare working protease solution by diluting protease QF stock solution 1:100 in pre-warmed 1× PBS.
21. Remove the slides from PBS, tap them to remove the excess fluid, place them on the ThermoBrite oven, add 400 µl working protease solution and incubate for 10 min at 40 °C (*see Note 7*).
22. Decant protease solution and wash slides once with water, followed by two washes in 1× PBS in a Coplin jar.
23. Transfer slides into fixation buffer and incubate for 3 min at room temperature (*see Note 8*).
24. Wash slides twice with 1× PBS.
25. Prepare working hybridization mix by diluting probe sets 1:40 in pre-warmed (40 °C) Probe Set Diluent QT per the manufacturer's instructions (*see Note 9*).
26. Hybridize sections with 400 µl working hybridization mix for 2.5 h at 40 °C in the humidified ThermoBrite oven.
27. Decant hybridization solution, transfer slides in a vertical rack in a Coplin jar containing 1× Wash Buffer, wash slides three times (2 min per wash) under vigorous agitation.

3.3 Two-Plex Target Detection

1. Incubate sections with 400 µl of PreAmplifier Mix QT for 25 min at 40 °C in the humidified ThermoBrite oven.
2. Decant PreAmplifier Mix QT solution, transfer slides in a vertical rack in a Coplin jar containing 1× Wash Buffer and wash slides three times (2 min per wash) with vigorous agitation.
3. Incubate sections with 400 µl of Amplifier Mix QT for 15 min at 40 °C in the humidified ThermoBrite oven.
4. Decant Amplifier Mix QT solution, transfer slides in a vertical rack in a Coplin jar containing 1× Wash Buffer and wash slides three times (2 min per wash) under vigorous agitation.
5. Prepare working label probe mix by diluting Label Probe 6-AP, 1:1000 in pre-warmed (40 °C) Label Probe Diluent QF per the manufacturer's instructions (*see Note 10*).

6. Incubate sections with 400 μ l of label probe mix for 15 min at 40 °C in the humidified ThermoBrite oven.
7. Decant label probe mix, transfer slides in a vertical rack in a Coplin jar containing 1 \times Wash Buffer, wash slides three times (2 min per wash) with vigorous agitation.
8. Prepare Fast Blue Substrate by adding 105 μ l Blue Reagent 1–5 ml Blue Buffer and vortex. Then add 105 μ l Blue Reagent 2 and vortex. Finally, add 105 μ l Blue Reagent 3 and vortex again (*see Note 11*).
9. Incubate sections with 400 μ l Fast Blue Substrate mix for 30 min at room temperature in a horizontal slide holder (e.g., StainTray slide staining system).
10. Decant Fast Blue Substrate mix, transfer slides in a vertical rack in a Coplin jar containing 1 \times Wash Buffer and wash slides three times (2 min per wash) with vigorous agitation.
11. Incubate sections with 400 μ l of AP Stop Buffer for 30 min at room temperature in a horizontal slide holder (*see Note 12*).
12. Decant AP Stop Buffer, transfer slides in a vertical rack in a Coplin jar containing 1 \times Wash Buffer and wash slides three times (2 min per wash) with vigorous agitation.
13. Prepare type 1 working label probe mix by diluting Label Probe 1-AP 1:1000 in pre-warmed (40 °C) Label Probe Diluent QF per the manufacturer's instructions.
14. Incubate sections with 400 μ l of label probe mix for 15 min at 40 °C in the humidified ThermoBrite oven.
15. Decant label probe mix, transfer slides in a vertical rack in a Coplin jar containing 1 \times Wash Buffer and wash slides three times (2 min per wash) with vigorous agitation.
16. Incubate sections with 400 μ l of AP Enhancer Solution for 5 min at room temperature in a horizontal slide holder.
17. Prepare Fast Red Substrate by dissolving one Fast Red tablet in 5 ml of Naphthol Buffer (*see Note 13*).
18. Decant the AP Enhancer Solution and incubate sections with 400 μ l of Fast Red Substrate mix for 30 min at 40 °C in the humidified ThermoBrite oven.
19. Decant Fast Red Substrate mix, transfer slides in a vertical rack in a Coplin jar containing 1 \times PBS solution and wash slides two times for 1–2 min.

3.4 Counterstaining and Slide Mounting

1. Remove slides from PBS and incubate them in fixation buffer for 5 min at room temperature.
2. Briefly wash slides two times in 1 \times PBS.
3. Add 500 μ l Gill's hematoxylin to each slide and incubate for 5 min at room temperature.

4. Wash slides with water in a Coplin jar.
5. Incubate the slides with running tap water for 1 min to reveal the hematoxylin.
6. Wash slides twice with water.
7. Mount slides with water based mounting media containing DAPI and cover with Nr. 1 glass coverslip.
8. Drain excess mounting media and let the slide air-dry for 15 min.
9. Seal the coverslip with transparent nail polish and store at 4 °C (*see Note 14*).
10. Acquire images using a brightfield and/or fluorescence microscope (*see Note 15*).
11. Analyze images with image analysis software (e.g., CellProfiler [9]) (*see Note 16*).

3.5 Processing of Formalin-Fixed, Paraffin-Embedded Liver Tissue

This section describes the tissue processing and ISH steps specific for FFPE tissue specimens.

1. Cool FFPE blocks to –20 °C for at least 2 h before sectioning. Alternatively cool down the blocks to –5 °C on a cooling plate for at least 2 h.
2. Set the blade inclination in the range of 5°–10° (e.g., on the HM340 microtome we use 6°).
3. Use S35 disposable blades for sectioning (do not use C35 blades) (*see Note 17*).
4. Set microtome for 5 µm sections.
5. Cut sections and transfer them to a water bath pre-warmed to 40 °C (*see Note 18*).
6. Keep sections floating on water for a few minutes to allow for complete tissue/paraffin unfolding.
7. Transfer sections onto Superfrost Plus Gold glass slides.
8. Place slides vertically in a slide holder and let drain off excess water.
9. Transfer the slide holder to a lab oven pre-warmed to 50 °C.
10. Incubate slides at 50 °C for 2 h.
11. Transfer slides into a slide storage box and store at –20 °C until use (*see Note 19*).
12. Pre-warm the ThermoBrite oven to 80 °C without humidifier strips.
13. Heat 400 ml pretreatment solution on a hot plate stirrer to between 85 and 95 °C.
14. Pre-warm the following reagents in a water bath set to 40 °C:

- (a) 40 ml 1× PBS.
 - (b) Probe Set Diluent QT.
 - (c) PreAmplifier Mix QT.
 - (d) Amplifier Mix QT.
 - (e) Label Probe Diluent QF.
15. Thaw probe sets on ice.
 16. Spin down Label Probe 6-AP and Label Probe 1-AP and store on ice.
 17. Bring Naphthol Buffer, AP Enhancer solution, and Blue Buffer to room temperature.
 18. Keep Fast Red and Fast Blue reagents on ice.

3.6 Pretreatment and Hybridization

1. Warm up slides to 80 °C in the ThermoBrite oven for 15 min.
 2. Quickly transfer slides in a Coplin jar containing xylene for paraffin removal. Incubate for 15 min at room temperature. Transfer slides into fresh xylene and incubate a second time for 30 min at room temperature.
 3. Set the ThermoBrite oven to 37 °C during the time of xylene treatment.
 4. Wash slides twice for 2 min in 100% ethanol.
 5. Bake slides in the ThermoBrite oven for 5 min at 37 °C.
 6. Draw hydrophobic barrier around tissue sections.
 7. Bake slides in the ThermoBrite oven for 5 min at 37 °C.
 8. Transfer slides in a vertical metal rack and submerge slides in 85°–95 °C Pretreatment solution and incubate for 10–20 min (*see Note 20*).
- During pretreatment: insert two water soaked humidifier strips into the ThermoBrite lid and start the HYB program (i.e. constant 40 °C).
9. Quickly transfer slides into 1× PBS in a Coplin jar and briefly wash twice with 1× PBS.
 10. Prepare working protease solution by diluting protease QF 1:100 in pre-warmed (40 °C) 1× PBS.
 11. Add 400 µl working Protease QF to tissue sections and incubate for 10–20 min at 40 °C in the ThermoBrite oven (*see Note 21*).
 12. Wash slides once with water, then twice with 1× PBS in a Coplin jar.
 13. Transfer slides to a Coplin jar containing fixation buffer and incubate for 3 min at room temperature.
 14. Wash slides twice with 1× PBS in a Coplin jar.

15. Prepare working hybridization mix by diluting the probe sets 1:40 in pre-warmed (40 °C) Probe Set Diluent QT.
16. Add 400 µl working hybridization mix to tissue sections and incubate for 2.5 h at 40 °C in the humidified ThermoBrite oven.
17. Decant hybridization solution, transfer slides in a Coplin jar containing 1× Wash Buffer solution and wash slides three times (2 min per wash) with vigorous agitation.

3.7 Target Detection, Counterstaining, and Slide Mounting

1. For pre-amplification, amplification, and target detection follow the steps outlined in Subheading 3.3. For counterstaining and slide mounting follow steps outlined in Subheading 3.4.

4 Notes

1. **Probe Set Design.** The extremely high signal to noise ratio that is a prerequisite for detection of low level viral and/or cellular RNAs is achieved by simultaneous binding of multiple (≥ 20) adjacent probe set pairs to the target RNA. The target recognition size of each probe is around 20 nucleotides in length. Thus, optimal target recognition requires a perfect match of each probe set to the target. Unfortunately, neither the HCV nor HBV genome contains long enough regions that are sufficiently conserved between or within viral genotypes suitable for probe set design. As we have shown previously, probe set design to patient-specific consensus sequence information is required for successful HCV RNA detection [7]. Using liver needle biopsy tissue obtained from an HBV infected patient (C357, HBV genotype E) and necropsy derived liver tissue from a human liver chimeric mouse that was infected with a monoclonal HBV serum (GenBank V01460), we show in Fig. 1 that specimen-specific probe design is also important for HBV detection. Probe sets were designed and synthesized based on consensus sequence information of a ~1 kb fragment spanning the envelope region of the HBV genome. As shown in Fig. 1a for frozen tissue sections, probe set cross-reactivity among HBV genotypes is not optimal. Figure 1b summarizes the data from 5 to 10 random images taken from the samples shown in Fig. 1a and also from FFPE sections of the same tissues. The results in Fig. 1b demonstrate that probe set cross-reactivity is affected both at the level of frequency of HBV positive cells (i.e., % of HBV positive cells) as well as the overall signal intensity. Furthermore, data analysis showed that while cross-reactivity ratios are similar, overall ISH sensitivity is considerably lower using FFPE tissue compared to the signals obtained with frozen sections. ISH signals for albumin mRNA

were similarly decreased in FFPE tissue as HBV signals (data not shown). Based on these data and preliminary HBV sequencing, showing that intra-genotypic sequence variations among patients can be as high as the inter-genotypic ones, it is well advised to design patient-specific HBV probe sets and use frozen tissue specimens whenever possible.

2. Minimize the time from tissue harvest to freezing (Subheading 3.1, steps 4–6) to prevent RNA degradation.
3. Perform Subheading 3.1, steps 12–17 inside the cryostat chamber to avoid any exposure of the OCT block to room temperature.
4. Use slides within 2 weeks. For longer-term storage, add some dried silica gel crystals into the slide storage boxes as desiccant.
5. Re-embedding improves the quality of tissue preservation.
6. Discard used blades; do not reuse them to avoid suboptimal blade performance and cross-contamination.
7. Although the manufacturer's instructions typically suggest to add 400 μ l of solution (protease, hybridization reagents, etc.) to each section on a slide, in our experience 200–300 μ l are sufficient without affecting the assay performance.
8. Incubation in fixation buffer for more than 3 min (Subheading 3.2, step 23) may result in reduced hybridization efficiency.
9. Subheading 3.2, steps 25–27 and Subheading 3.3, steps 1–19 are per the manufacturer's instructions.
10. For single target detection, incubate sections with only the Label Probe AP specific for the probe set used and only perform the corresponding target development (i.e., Subheading 3.3, step 5–10 for type 6 probe sets or Subheading 3.3, step 13–19 for type 1 probe sets).
11. The Fast Blue Substrate mix decays quickly. Prepare it not more than 5–10 min before usage for optimal results.
12. Complete quenching of alkaline phosphatase at this point is crucial to avoid additional reaction of Label Probe 6-AP with the Fast Red Substrate in Subheading 3.3, step 18.
13. The Fast Red Substrate mix decays quickly. Prepare it not more than 5–10 min before usage for optimal results.
14. For best results, acquire images within a few days of performing the ISH assay.
15. **Image acquisition.** The View RNA ISH system makes use of alkaline phosphatase to transform fast red and fast blue substrates into red and blue chromogenic precipitates, respectively. Accordingly, visual observation of red and blue signals

using bright field microscopy represents the primary readout of the View RNA ISH assay. However, as described below, both chromogenic precipitates can also be visualized by fluorescence, but acquiring fluorescence images should always be guided by bright field analysis.

In addition, the use of two consecutive chromogen precipitation steps imposes some basic considerations for optimal target detection. For instance, visibility of the blue precipitate is influenced by the degree of hematoxylin counterstaining, while recognition of the red substrate is not. Accordingly, the manufacturer suggests using the type 1 probe set for detection of the most important (or least abundant) target and reserve the type 6 probe set for more abundant targets (e.g., housekeeping genes). However, if RNA targeted by the type 6 probe set is in large excess over the other one, the blue precipitate might partially block the second RNA-probe-set complex and thus, interfere with detection of the second target RNA. In this situation, the manufacturer suggests using a type 1 probe set for detection of the more abundant transcript and a type 6 probe set for the less abundant one. Ultimately, the best combination of probe sets and targets has to be determined by the experimenter.

In an effort to keep the benefits of using a type 1 probe set for detection of the less abundant RNA, we have reduced the amount of the type 6 probe set in our assays, in order to minimize the masking effect described above. However, this approach requires careful determination of the correct amount of type 6 probe set in the hybridization reaction as to best reduce signal intensity per cell, without underestimating the frequency of ISH positive cells.

As described in the manufacturer's instructions, fast red and fast blue precipitates can also be visualized by fluorescence microscopy: fast red's excitation and emission maxima are 530 ± 20 nm and 590 ± 20 nm, respectively. Thus, fluorescence acquisition for this chromogen can be performed with standard Cy3/TRITC filter sets.

The excitation and emission maxima of fast blue are 630 ± 20 nm and 775 ± 25 nm, respectively. Fluorescence acquisition for this chromogen requires a custom filter set as described in the manufacturer's instructions.

Because the fast red emission spectrum partially overlaps with the fast blue excitation spectrum (cross-talking) and because of the specifics of fast blue detection, we use a Zeiss laser scanning confocal microscope (Zeiss, Jena, Germany) for image acquisition. This microscope allows to precisely adjust the emission windows for each fluorophore, thus enabling optimal fast blue fluorescence acquisition and complete prevention of any inference between the fast red and fast blue channels.

Fluorescent image acquisition is optimal for downstream image processing, but great care has to be taken to avoid cap-

turing artifactual fluorescence signals due to inappropriate signal amplification. Accordingly, we set digital gain and threshold during image acquisition so that the captured fluorescence signals reflect only the chromogenic precipitates observed by bright field inspection. While this approach might lead to loss of some very low level signals because fluorescence detection is more sensitive than inspection by eye, it yields much more consistent results especially in a multi-user environment.

Generation of robust data sets requires acquisition of multiple random fields in each section. Typically, using the Zeiss Zen software, we acquire 10 images per section, each at a resolution of 2048×2048 pixels, having fluorescence signals and a bright field image assigned to the corresponding channels and saved as “.lsm” images. Subsequently, the “.lsm” files are split into their color channels (red = type 1 probe set; green = type 6 probe set; blue = nuclei and grey = brightfield) and individually saved as Tiff-images using ImageJ (freely available at <http://imagej.nih.gov/ij/index.html>).

16. We used CellProfiler [9, 10] software to establish an image processing pipeline for unsupervised image analysis, including image segmentation for single cell identification and signal quantification.

Briefly, we make use of the relatively uniform polygonal-round shape of hepatocytes for single cell identification. Specifically, we apply the distance function of CellProfiler to approximate the cell boundaries between adjacent nuclei. The correct cell segmentation can then be confirmed by overlaying the segmentation image on the bright field image.

Next, ISH images containing the red and green signals (dots) are overlaid onto the segmentation image to associate the ISH signals with individual cells.

Finally, the number of red and/or green positive and negative cells, signal intensity, and number of dots per cell and/or field are quantified and exported for further processing.

17. Discard used blades at the end of each cutting session; do not reuse them to avoid suboptimal blade performance and cross-contamination.
18. To maintain optimal cutting performance, don't cut more than 4 sections before re-cooling the block.
19. Do not store slides longer than 2 weeks.
20. Optimal time of pretreatment depends on the particular tissue fixation procedure used and has to be determined experimentally.
21. Optimal time for protease treatment depends on the particular tissue fixation procedure used and has to be determined experimentally.

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Immunofluorescent Staining for the Detection of the Hepatitis B Core Antigen in Frozen Liver Sections of Human Liver Chimeric Mice

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Abstract

The hepatitis B virus (HBV) is the causative agent for chronic hepatitis B infection, which affects an estimate of 240 million people worldwide and puts them at risk of developing terminal liver disease. The life cycle of the virus and its interactions with the host immune system are still incompletely understood, and currently available treatment options rarely achieve a cure. Therefore, basic research and new drug development are needed. One parameter for measuring the intrahepatic activity of the virus is monitoring the production of the HBV core antigen (HBcAg), which not only serves as the main structural protein of its nucleocapsid but is also recruited to the covalently closed circular DNA (cccDNA), the nuclear HBV genome responsible for infection persistence. Here, we report a sensitive immunofluorescence staining method to detect HBcAg in cryopreserved liver sections. The method combines conventional immunofluorescence staining procedures with the Tyramide Signal Amplification (TSA) system.

Key words Hepatitis B virus (HBV), Hepatitis B core antigen (HBcAg), Immunofluorescence, Cryopreserved liver sections, Tyramide Signal Amplification technology

1 Introduction

Monitoring the intrahepatic activity of the hepatitis B virus (HBV) infection will provide insights into the pathophysiology of the infection and will be useful to optimize treatment regimens with existing antiviral drugs or test for the efficacy and mode of action of newly developed antivirals. The viral core protein, the hepatitis B core antigen (HBcAg), consists of 183 AA and after translation in the cytoplasm of infected hepatocytes forms homodimers that self-assemble into the icosahedral viral nucleocapsid containing either 90 or 120 core dimers [1, 2]. However, it can also be localized in the nucleus and cell membrane of infected hepatocytes [3, 4] which is in line with its pleiotropic functions in the viral life cycle. HBcAg has been shown to associate with the covalently closed circular DNA (cccDNA) [5], the viral minichromosome

and template of transcription residing in the nucleus of infected hepatocytes, and affect its epigenetic regulation [6–9]. By interacting with the pregenomic RNA, the core protein initiates reverse transcription inside the nucleocapsid and signals its completion in order to interact with the viral surface antigens to be secreted into the bloodstream [10]. The core protein also interacts with various host proteins to facilitate its transport and regulation by phosphorylation [11]. The detection of HBcAg in hepatocytes is generally associated with an active HBV replication and thus can be used to monitor the intrahepatic activity of HBV. It is also useful in dissecting the effects on the production of viral components elicited with different therapeutic agents. For instance, human chimeric mice studies have shown that pegylated interferon alpha can suppress the production of viral DNA as well as RNA resulting in reduced levels of HBV antigens including HBcAg, which was not observed in mice treated with nucleoside analogs [12]. Moreover, sensitive detection of HBcAg might prove particularly useful in testing a new class of anti-HBV drugs, the core inhibitors [1], to determine the effect of treatment on the quantity and localization of core proteins.

We report here a highly sensitive immunofluorescent staining method for the detection of HBcAg in cryopreserved liver sections alone or in combination with additional targets [13–15]. To increase the sensitivity of standard immunofluorescent methods, we take advantage of the Tyramide Signal Amplification (TSA™) technology which can be included in a standard staining protocol involving a primary anti-HBcAg antibody and a secondary antibody coupled with horseradish peroxidase. To amplify the signal, the peroxidase activity converts fluorescein-coupled tyramides into highly reactive, short-lived tyramide radicals that bind to electron-rich regions of adjacent proteins (mostly tyrosine residues) which in turn generates a stable and strongly enhanced labeling of the target with a low diffusion-related loss of the signal resolution [16, 17]. In human liver chimeric mice infected with HBV [18], we combine this amplification technique for HBcAg with a conventional immunofluorescent staining of a marker for human hepatocytes.

2 Materials

2.1 Preparation of Cryopreserved Liver Sections

1. Isopentane.
2. Cryotome.
3. Embedding media, i.e., Tissue-Tek OTC compound, Sakura, catalog number 4583.
4. Microtome blades, i.e., Feather C35 type.
5. Superfrost microscope slides (75 × 25 mm).

2.2 HBcAg Staining

1. Staining jars.
2. Acetone.
3. Wash buffer: 0.1 M Tris-HCl, 0.15 M NaCl, pH 7.5. To prepare a 10× stock solution dissolve 121.14 g Tris base and 87.66 g NaCl in deionized water, adjust pH with HCl and fill up to 1 l. Dilute 1:10 with water before use. Store at RT.
4. PBS and H₂O₂ for the peroxidase quenching buffer.
5. Humidifying chamber, i.e., a covered box with wet paper towels.
6. Lap wipes.
7. Blocking buffer: 0.5% blocking reagent (supplied in the TSA kit) in 1× washing buffer. Dissolve the blocking reagent by warming to 60 °C and continuous stirring. Aliquot and store at -20 °C.
8. Primary antibodies: polyclonal rabbit anti-HBcAg antibody, Dako, catalog number B0586 and monoclonal mouse anti-cytokeratin 18 antibody (clone DC-10), Santa Cruz, catalog number sc-6259.
9. Secondary antibodies: horseradish peroxidase-conjugated goat anti-rabbit antibody, i.e., Jackson, catalog number 111-035-003 and Alexa Fluor 546-conjugated goat anti-mouse antibody, i.e., Life Technologies, catalog number A11035.
10. Tyramide Signal Amplification (TSA™) Fluorescence Systems (PerkinElmer, catalog number NEL701A001KT).
11. Dimethyl sulfoxide (DMSO).
12. Hoechst 33258, 10 mg/ml in H₂O.
13. Fluorescent mounting medium.
14. Glass cover slips (24 × 50 mm).
15. Fluorescence microscope including the appropriate filters: Fluorescein, excitation 494 nm, emission 517 nm, common filter set FITC, and Alexa Fluor 546, peak excitation 556 nm, peak emission 573 nm, common filter set TRITC. Alternatively, a confocal microscope with the appropriate laser lines.

3 Methods

3.1 Preparation of Cryopreserved Liver Sections

1. Sacrifice the mouse, excise the liver, and cut the lobes into small pieces of around 1 × 0.5 × 0.5 cm size. Snap freeze the single pieces in prechilled isopentane (fill a glass beaker with isopentane and keep on dry ice), and store at -80 °C.
2. Place a piece of liver in embedding medium to attach it to the object holder of a cryotome set at -20 °C. Cut 12 μm thick sections. Mount two sections on each microscope slide.

Let the sections dry for 10 min at room temperature and store them at -80°C . Allow the slides to stay at -80°C for at least one night before using them.

3.2 HBcAg Staining

1. Use appropriate controls to test for the validity of the signal. Use a section of a highly infected chimeric mouse as a positive control and one of a noninfected mouse as a negative control. In addition to these biological controls, it is advisable to also use technical control slides such as a negative control slide (eliminating the primary antibody but including the TSA Fluorescence Systems reagents) and one unamplified control slide (including all reagents except the TSA reagents).
2. All steps are carried out at room temperature. Make sure the section does not dry out during the entire procedure.
3. Fill a staining jar with acetone (*see Note 1*). Remove the slides from -80°C and immerse them immediately in the fixative. Fix for 10 min.
4. Transfer the slides to a new jar filled with $1\times$ wash buffer. Wash three times for 5 min (*see Note 2*).
5. Prepare a 0.4% H_2O_2 /PBS solution in a new staining jar, transfer the slides, and let incubate for 1 h to quench endogenous peroxidase activity (*see Note 3*).
6. Wash the slides as indicated above.
7. Block unspecific protein binding sites on the section with the blocking buffer. Remove the slides from the wash buffer and put $200\ \mu\text{l}$ of blocking buffer directly on the sections (*see Note 4*). Incubate in a humidifying chamber for 30 min.
8. Without washing, continue to the incubation with the primary antibodies. Remove the blocking buffer and add the antibody solution. The rabbit anti-HBcAg antibody should be diluted 1:2000 in wash buffer (*see Note 5*), and the mouse anti-cytokeratin 18 antibody should be diluted 1:400, which is used to distinguish between human and murine hepatocytes when staining human chimeric mice (*see Note 6*). Use around $200\ \mu\text{l}$ antibody solution per slide. When using antibodies different from the ones used here, the optimal dilutions have to be determined empirically. Incubate the slides in a humidifying chamber for 1 h (at room temperature) or at 4°C overnight. Overnight incubation usually results in enhanced staining and less unspecific background staining.
9. Wash the slides as indicated above.
10. Incubate the slides with the secondary antibodies. Dilute the HRP-conjugated anti-rabbit antibody 1:200 and the Alexa Fluor 564-conjugated anti-mouse antibody 1:800 in wash buffer. When using antibodies different from the ones used here,

the optimal dilutions have to be determined empirically. Add 200 μ l of the antibody solution to each slide and incubate in a humidifying chamber for 1 h. From now on, slides should be kept in the dark.

11. Wash the slides as indicated above.
12. Proceed to the TSA step. The fluorophore tyramide amplification reagent can be prepared ahead of time by dissolving the fluorescein tyramide in DMSO as indicated in the TSA manual. Freshly prepare a 1:50 working solution of the fluorophore tyramide amplification reagent using the amplification diluent provided in the kit. Remove the wash buffer and pipet 100 μ l of the working solution on every slide. Incubate for 5 min in a humidifying chamber in the dark (*see Note 7*).
13. Wash the slides as indicated above.
14. Perform nuclear staining by diluting Hoechst 1:20,000 in a staining jar. Immerse the slides and incubate for 2 min.
15. Remove the slides from the staining jar one by one, remove any liquid, apply one drop of fluorescent mounting medium, and cover with a glass cover slip. Avoid trapping air bubbles under the cover slip. Store the slides in the dark, flat and cool.
16. HBcAg staining can now be assessed with a fluorescence light microscope or confocal laser scanning microscope to quantify the number of HBV-infected cells or compare staining patterns and intensities between experimental groups of mice. Figure 1 shows an example of a human chimeric mouse in the spreading phase of HBV infection showing scattered HBcAg-positive hepatocytes and one fully HBV-infected mouse where all human hepatocytes stain positive for HBcAg.

4 Notes

1. Fixation needs to be performed with acetone. Using other fixatives such as methanol or paraformaldehyde will lead to loss of HBcAg.
2. It is not recommended to use detergents such as Tween or Triton for the washing steps or dilution of the antibodies as HBcAg is extremely sensitive to detergents and will be washed out of the tissue.
3. Standard quenching procedures commonly include a shorter incubation time with a higher concentration of H_2O_2 diluted in methanol. However, methanol cannot be used for the preparation of the peroxidase quenching buffer when staining HBcAg (*see Note 1*), and therefore PBS should be used instead. Since high H_2O_2 concentrations in PBS may lead to

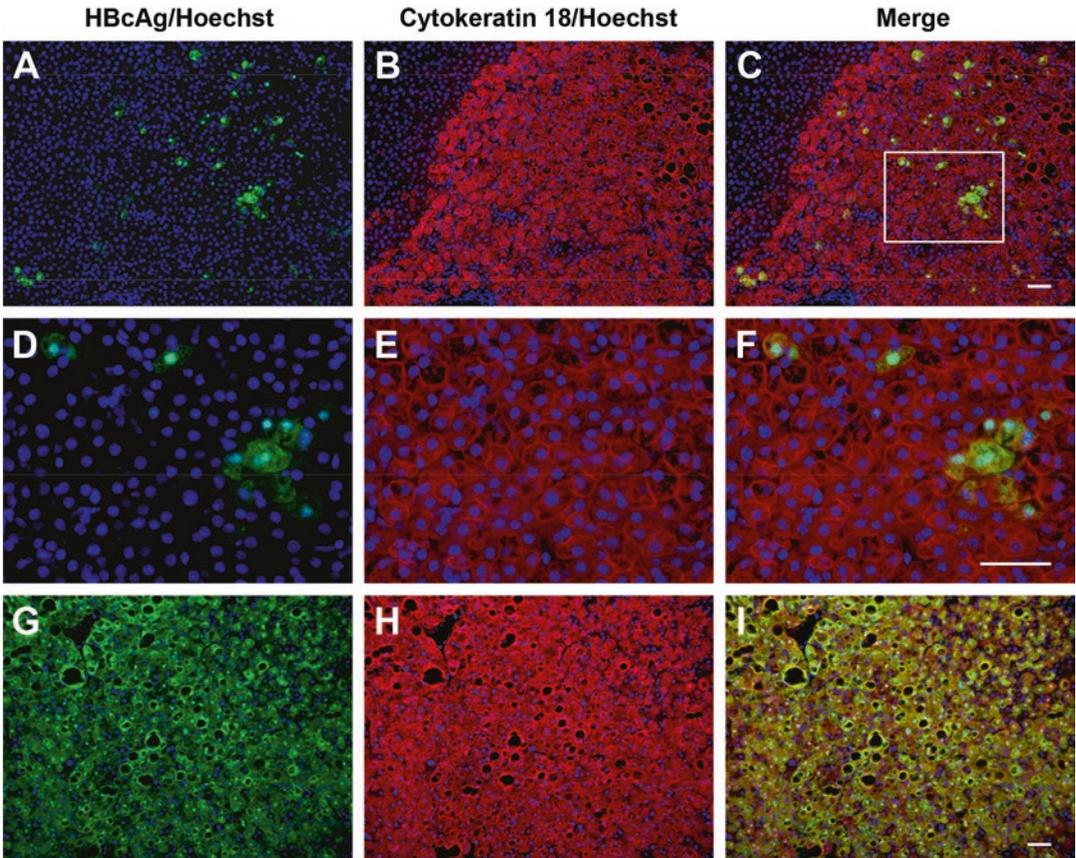


Fig. 1 HBcAg staining in HBV-infected human chimeric mice (uPA/SCID/beige). (a–c) A uPA/SCID/beige mouse in the spreading phase of the infection shows scattered HBcAg-positive cells within clusters of human hepatocytes. Stained sections were analyzed by fluorescence microscopy (Biorevo BZ-9000, Keyence, Osaka, Japan) using a 20x/0.75 NA objective. (d–f) Merged z stack image, 5 μm in total thickness, of the area in the white box in (c) captured with fluorescence microscopy (Biorevo BZ-9000, Keyence, Osaka, Japan) using a 60x/1.40 NA oil objective. (g–i) A chronically HBV-infected uPA/SCID/beige mouse was analyzed as in (a)–(c) revealing HBcAg positivity in almost all human hepatocytes. In the photographs in the left column (a, d, g) HBcAg is shown in *green*, in the middle column (b, e, h) Cytokeratin 18, a marker for human hepatocytes, is shown in *red*, and in the right column (c, f, i) a merged picture is shown. Nuclei are counterstained with Hoechst (*blue*). The scale bar represents 50 μm

the detachment of tissue sections from the slides, we ended up using a low concentration of H_2O_2 with an increased incubation period.

4. When applying a solution directly on the slide, remove excess liquid and carefully wipe around the sections always leaving the same small amount of liquid on the sections. Then add the new solution and pipet up and down on the slide to mix the liquid. Make sure that the sections are entirely covered and that the rim of the liquid is beyond the rim of the tissue section in order avoid artifacts at the edges of the section. In our hands, the use

of a hydrophobic barrier pens for circling the sections was not found to be beneficial but rather increased unspecific background staining when not handled with exhaustive care.

5. When the amount of HBcAg-positive cells is expected to be low, the anti-HBcAg antibody can further be diluted down to 1:5000. A high concentration of primary antibody together with a small number of positive cells might otherwise lead to considerable background staining.
6. The protocol can also be combined with other primary antibodies. For instance, the delta antigen of the hepatitis delta virus (HDV) can be co-stained in an HBV/HDV-infected mouse [19], or a proliferation marker such as KI-67 might be used to co-stain dividing cells. Moreover, depending on the species and the cellular localization of the other antibodies used in one particular staining, instead of cytokeratin 18, other human-specific hepatocyte markers such as CANX, SPI100, or NUP98 can be used.
7. When the aim of the HBcAg staining is a quantitative comparison of staining intensities in different experimental groups, special care has to be taken to ensure conformity in the staining procedure. For instance, the incubation step with the fluorophore tyramide amplification reagent should last for exactly 5 min as the time of incubation influences the staining intensity. It is also advisable to run all samples which are to be compared in parallel as the age of the TSA kit, i.e., the number of times the fluorophore tyramide amplification reagent was used and exposed to light, may likewise influence the staining intensity. In addition, a confocal microscope should be used for the analysis.

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Measuring Changes in Cytosolic Calcium Levels in HBV- and HBx-Expressing Cultured Primary Hepatocytes

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Abstract

Chronic infection with hepatitis B virus (HBV) remains a major worldwide health concern and is the leading cause of hepatocellular carcinoma (HCC). The HBV X protein (HBx) is the only regulatory protein encoded in the HBV genome; HBx stimulates HBV replication *in vivo* and *in vitro*. HBx also regulates cytosolic Ca^{2+} signaling, and altered Ca^{2+} signaling is associated with the development of many diseases, including HCC. Importantly, many HBx functions, including HBx modulation of cell proliferation, apoptosis, and transcription pathways, have been linked to changes in cytosolic Ca^{2+} signaling. Additionally, several stages of HBV replication, including capsid formation and activation of the HBV polymerase, are dependent on intracellular Ca^{2+} . Consequently, defining the molecular mechanism that underlies HBV and HBx modulation of cytosolic Ca^{2+} levels is important for understanding HBV pathogenesis and the role of HBx in HBV replication. Here, we describe a single-cell Ca^{2+} -imaging protocol that we use to investigate HBV and HBx effects on the level of cytosolic Ca^{2+} . We specifically outline two methods that we use to evaluate HBV and HBx regulation of cytosolic Ca^{2+} levels in cultured primary hepatocytes. This protocol can also be adapted for use in liver cell lines.

Key words HBV, HBx, Calcium, Fura-4F, Hepatocyte

1 Introduction

Chronic infection with hepatitis B virus (HBV) is the leading cause of several life threatening liver diseases, including cirrhosis and hepatocellular carcinoma (HCC) [1, 2]. Despite the availability of an HBV vaccine, more than 350 million individuals are chronically infected with HBV worldwide [2, 3]. The molecular mechanisms underlying HBV pathogenesis remain incompletely understood. The HBV X protein (HBx) is the smallest viral protein encoded in the HBV genome and the only HBV protein that exhibits regulatory functions. One critical HBx function is stimulation of HBV replication, which occurs both *in vitro* and *in vivo* [4]. HBx is believed to play a central role in the development of HBV-associated diseases. Interestingly, HBx modulates intracellular

calcium (Ca^{2+}) signaling [5–7], and altered Ca^{2+} signaling is associated with the development of many diseases, including cancer [8–10]. Specifically, altered Ca^{2+} levels and altered expression of Ca^{2+} signaling proteins have been linked to the proliferation of human hepatoma cells and the development of HCC [11, 12].

Cumulatively, these observations support the notion that HBx-induced alterations in cytosolic Ca^{2+} signaling influence the development of HBV-associated HCC.

Cytosolic Ca^{2+} is required for several stages of HBV replication, including HBV core particle assembly and activation of the HBV polymerase [5, 13–15]. Studies that have assessed roles of cytosolic Ca^{2+} in HBV replication have often used the cell permeable Ca^{2+} chelator 1,2-*Bis*(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetrakis-acetoxymethyl ester (BAPTA-AM) to analyze effects on HBV replication. Although these types of studies have been important for defining Ca^{2+} -dependent processes that affect HBV replication, and have provided a starting point for understanding the relationship between HBV and cytosolic Ca^{2+} signaling, these types of studies do not define the molecular mechanism(s) underlying specific Ca^{2+} effects on HBV replication. The results of various studies have linked HBx expression to modulation of cytosolic Ca^{2+} levels [6, 7, 16]. Moreover, several studies have shown that BAPTA-AM can block HBx-mediated activities, including HBx regulation of cell proliferation, apoptosis, and transcription pathways and HBx stimulation of HBV replication [5, 13, 14, 16–19]. Importantly, HBx expression has also been directly linked to elevation of the overall basal cytosolic Ca^{2+} level in various cell lines [6, 7, 16]. The results of these various studies suggest that HBx elevation of cytosolic Ca^{2+} levels likely activates downstream signaling pathways that alter the cellular environment to facilitate HBV replication. In turn, this altered cellular environment may favor disease progression. Therefore, defining the molecular mechanism underlying HBx regulation of cytosolic Ca^{2+} levels and signaling can provide important insights into processes that regulate HBV replication and HBV pathogenesis.

Ca^{2+} is a universal and versatile second messenger, and Ca^{2+} signaling impacts a large number of hepatocyte functions, including hepatocyte proliferation, hepatocyte apoptosis, and hepatocyte transcription [8, 20]. In most cells, basal cytosolic Ca^{2+} concentrations are maintained at around 100 nM [21]. Changes in the cytosolic Ca^{2+} level are sensed by Ca^{2+} signaling modulator and effector proteins; these Ca^{2+} signaling modulator and effector proteins then stimulate a variety of downstream signaling cascades [22]. In hepatocytes, there are two main sources of free intracellular Ca^{2+} . The first source is the endoplasmic reticulum (ER), the main intracellular Ca^{2+} store. Activation of inositol triphosphate (IP_3) receptors (IP_3R) located on the ER causes rapid Ca^{2+} release from the ER into the cytosol. The second source of free intracellular Ca^{2+} in

hepatocytes is Ca^{2+} influx from outside the cell through store-operated Ca^{2+} (SOC) channels located on the plasma membrane (PM). These channels are activated upon ER Ca^{2+} depletion [20, 23, 24]. Excess cytosolic Ca^{2+} can be detrimental to the cell and is rapidly cleared by ATPase pumps; these pumps include the family of PM Ca^{2+} -ATPases (PMCA), which pump Ca^{2+} from the cytosol into the extracellular matrix, and the family of sarcoplasmic reticulum Ca^{2+} -ATPases (SERCA), which pump Ca^{2+} from the cytosol into the ER [20].

The use of fluorescent Ca^{2+} indicators to study changes in intracellular Ca^{2+} responses is widely popular. Ca^{2+} signaling studies in indicator-loaded cells are usually performed following the addition of a Ca^{2+} -inducing agonist, and measurement of the subsequent Ca^{2+} response in cells is indicated by changes in the fluorescence emission or excitation of the indicator. We have used this technique to compare the intracellular Ca^{2+} response between HBV- or HBx-expressing and control cells. Here, we describe a protocol that can be used to detect HBV- and HBx-related alterations in cytosolic Ca^{2+} . We have used this protocol, which was modified from standard Ca^{2+} signaling protocols [25], for studies in cultured primary rat and human hepatocytes, and, with slight modifications, in human hepatoblastoma HepG2 cells [7]. The protocol that is outlined utilizes Fura-4F, a ratiometric fluorescent cytosolic Ca^{2+} indicator. Ca^{2+} binding to Fura-4F causes a shift in the excitation wavelength from 380 nm (Ca^{2+} -free Fura-4F) to 340 nm (Ca^{2+} -bound Fura-4F). By calculating the ratio of Ca^{2+} -bound Fura-4F to Ca^{2+} -free Fura-4F (340/380), changes in the cytosolic Ca^{2+} level in a single cell can be determined [26, 27]. We also describe two methods to initiate a Ca^{2+} response: the first method involves the induction of an IP_3 -linked cytosolic Ca^{2+} response using either ATP, a P2Y purinergic receptor agonist, or vasopressin (Vp), a Vp receptor V1a agonist; the second method involves activation of SOC channels using the SERCA inhibitor thapsigargin (TG).

2 Materials

2.1 Plating Cells

1. 12 mm glass coverslips.
2. 70% ethanol.
3. Collagen Type I, Rat Tail (3–4 mg/ml).
4. 0.02 N acetic acid.
5. Sterile 1× phosphate-buffered saline (PBS).
6. Primary hepatocyte medium: Williams E medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 4 $\mu\text{g}/\text{ml}$ insulin-transferrin-selenium, 5 $\mu\text{g}/\text{ml}$ hydrocortisone, and 5 ng/ml epidermal growth factor.

2.2 Transfecting Cells

1. Plasmids: HBV- or HBx-expressing plasmid, control plasmid, dsRED-expressing plasmid (*see Note 1*).
2. Lipofectamine 2000 (Invitrogen) (*see Note 2*).

2.3 Loading Cells with Fura-4F-AM

1. HEPES-buffered balanced salt solution (HBSS): 25 mM HEPES, 121 mM NaCl, 4.7 mM KCL, 1.2 mM MgSO₄, 1.2 mM KPO₄, 5 mM NaHCO₃, 2.0 mM CaCl₂, 10 mM Glucose, pH solution to 7.4.
2. 25 % (w/v) dialyzed bovine serum albumin (BSA), Fraction V stock.
3. 50 mM bromsulfalein (BSP) stock.
4. 10 % (w/v) pluronic acid stock.
5. 1 mM Fura-4F-AM stock (*see Note 3*).

2.4 Stimulating and Measuring an IP₃-Mediated Ca²⁺ Response

1. HBSS supplemented with BSA and BSP.
2. 100 mM ATP stock or 100 μM Vp stock.
3. Ca²⁺-free HEPES-buffered balanced salt solution (HBSS): 25 mM HEPES, 121 mM NaCl, 4.7 mM KCL, 1.2 mM MgSO₄, 1.2 mM KPO₄, 5 mM NaHCO₃, 10 mM glucose, pH solution to 7.4 (plus BSA and BSP).
4. 1 mM ionomycin stock.
5. 5.9 M MnCl₂ stock.

2.5 Stimulating and Measuring SOCE

1. Ca²⁺-free HBSS supplemented with BSA and BSP.
2. 1 mM TG stock.
3. 200 mM CaCl₂ stock.
4. 1 mM ionomycin stock.
5. 5.9 M MnCl₂ stock.

3 Methods**3.1 Plating Cells**

1. Wash glass coverslips with 70% ethanol for 15 min. Allow coverslips to air-dry in a cell culture (laminar flow) hood to ensure maintenance of sterility.
2. Dilute sterile collagen with sterile 0.02 N acetic acid. Transfer each coverslip to a well of a 12-well plate and coat coverslips with collagen for at least 2 h. Shake plates every 15 min to ensure complete coverage.
3. Aspirate any remaining collagen mix and sterilize in a laminar flow hood with an ultraviolet light. The collagen can be allowed to dry on the coverslip.
4. Immediately prior to adding cells, wash the coverslips with sterile 1× PBS.

5. Plate ~0.5–0.75 million hepatocytes/well in primary hepatocyte medium.
6. Change medium following 30 min or when the hepatocytes settle on the slide. Gently swirl medium prior to aspirating to dislodge any dead or unattached cells.

3.2 Transfecting Cells

1. Within 24 h of plating, transfect primary hepatocytes (*see Note 4*). We typically transfect primary rat hepatocytes with 1 μg of HBV- or HBx-expressing or control plasmid and 0.2 μg of dsRED-expressing plasmid (*see Note 5*). Hepatocytes are transfected using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

3.3 Loading Cells with Fura-4F-AM

1. Supplement HBSS with BSA (0.25 % final) (*see Note 6*) and BSP (200 μM final) (*see Note 7*). Warm HBSS containing BSA and BSP in 37 °C water bath.
2. Add 2 μl of the 10% pluronic acid stock (*see Note 8*) to an empty well of a 12-well plate.
3. Add 5 μl of the 1 mM Fura-4F-AM stock (*see Notes 9 and 10*) directly onto the pluronic acid. Pipette up and down to mix.
4. Add 1 ml HBSS containing BSA and BSP to the Fura-4F-AM/pluronic acid mix. Pipette up and down to mix well.
5. Aspirate medium from one well containing hepatocytes plated on a coverslip. Wash the coverslip twice with HBSS containing BSA and BSP. Transfer the coverslip to the well of the 12-well plate containing the Fura-4F-AM mix.
6. Incubate on a platform shaker for 40 min (37 °C, 100 rpm) (*see Note 11*).
7. Aspirate loading mix. Wash twice with HBSS containing BSA and BSP.
8. Add 1 ml HBSS containing BSA and BSP to coverslip.

3.4 Stimulating and Measuring an IP_3 -Linked Ca^{2+} Response

1. Transfer slide containing Fura-4F-loaded hepatocytes (see Subheading 3.3) to imaging chamber (*see Note 12*). Add 1 ml HBSS containing BSA and BSP to coverslip in the imaging chamber and transfer to the imaging stage of a fluorescent microscope that is connected to a camera and proper imaging software (*see Notes 13 and 14*). Figure 1 shows our imaging set up.
2. Start imaging program (*see Note 14*).
3. Using a Texas Red filter, focus on and select dsRED-expressing hepatocytes.
4. Switch to a Fura filter and check to be sure all selected hepatocytes are alive and loaded with Fura-4F (*see Note 15*). To confirm that hepatocytes are loaded with Fura-4F, alternately

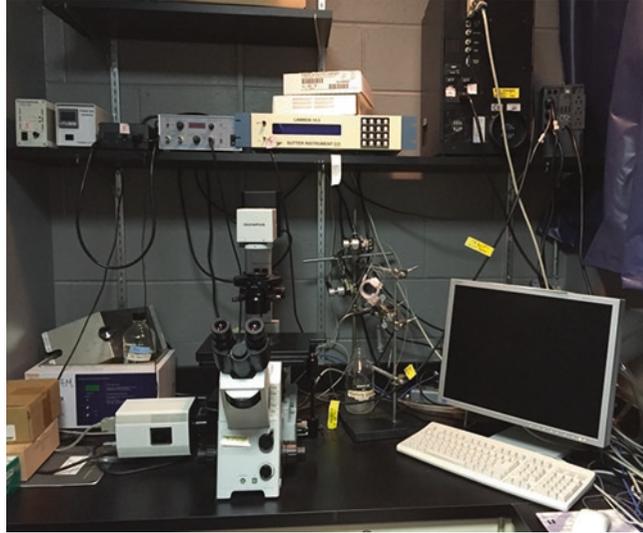


Fig. 1 Imaging setup. Includes an Olympus 1X71 inverted microscope equipped with a 20× objective used for Fura imaging, a cooled charged-coupled-device camera, a photometrics coolsnap ES camera driver, a Lambda 10-2 25 mm filter wheel (no shutter), a 1X71 emission adapter, and a DG4 Galvo-wavelength switcher

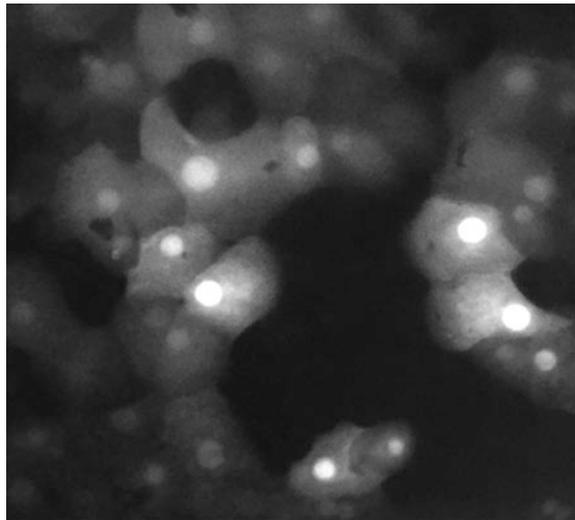


Fig. 2 Fura-4F-loaded primary rat hepatocytes, excited at 380 nm

focus on cells when exciting the dye at both 340 and 380 nm. Hepatocytes with optimal loading will have a brightly fluorescent nucleus at 380 nm and a dark nucleus at 340 nm. Figure 2 shows an image of Fura4F-loaded primary rat hepatocytes.

5. Begin imaging cells. Fura-4F 340/380 nm ratios can be recorded every 2 s.
6. After ~60 s (*see Note 16*), stimulate cells with 100 μ M ATP or 100 nM Vp. Both ATP and Vp will activate the IP₃R and

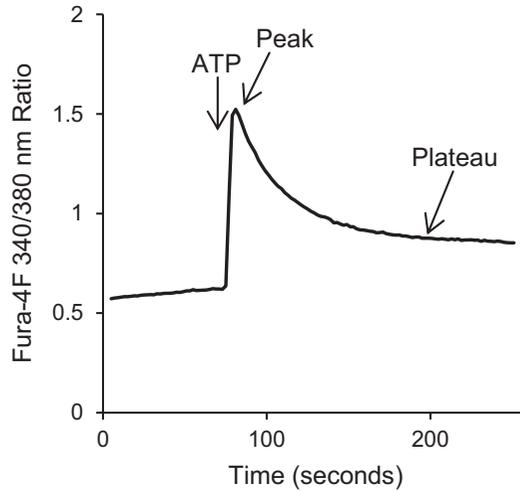


Fig. 3 Typical IP_3 -linked Ca^{2+} response in a single primary rat hepatocyte

stimulate a biphasic Ca^{2+} response. The first phase, the release phase, encompasses a rapid release of Ca^{2+} from the ER upon ATP or V_p addition. The second phase, the recovery phase, reflects simultaneous removal of excess cytosolic Ca^{2+} through PMCA or SERCA pumps and entry of Ca^{2+} through SOC channels to refill the ER Ca^{2+} store. The recovery phase results in an enhanced cytosolic Ca^{2+} level, termed the plateau. Upon ATP or V_p addition, there should be an immediate increase in the Fura-4F 340/380 nm ratio. Figure 3 shows an example of a typical Ca^{2+} response to ATP in primary hepatocytes.

7. Continue to image cells until the Fura-4F 340/380 nm ratios have reached a steady plateau (~250 s) following ATP or V_p stimulation.
8. Remove HBSS used during imaging and add back 500 μl Ca^{2+} -free HBSS containing 10 μM ionomycin (*see Note 17*).
9. After ionomycin permeates the cell membrane (~10 s), add 500 μl Ca^{2+} -free HBSS containing 20 mM MnCl_2 (*see Note 18*).
10. Stop imaging once the 340 and 380 nm values have reached a stable baseline.

3.5 Stimulating and Measuring SOCE

1. Transfer slide containing Fura-4F-loaded hepatocytes (*see Subheading 3.3*) to imaging chamber (*see Note 12*). Add 1 ml Ca^{2+} -free HBSS containing BSA and BSP to coverslip in the imaging chamber and transfer to the imaging stage of a fluorescent microscope that is connected to a camera and proper imaging software (*see Notes 13 and 14*).
2. Start imaging program (*see Note 14*).
3. Using a Texas Red filter, focus on and select dsRED-expressing hepatocytes.

4. Switch to a Fura filter and check to be sure all selected hepatocytes are alive and loaded with Fura-4F (*see Note 15*). To confirm that hepatocytes are loaded with Fura-4F, alternately focus on cells when exciting the dye at both 340 and 380 nm. Hepatocytes with good loading will have a brightly fluorescent nucleus at 380 nm and a dark nucleus at 340 nm. Figure 2 shows an image of Fura4F-loaded primary rat hepatocytes.
5. Begin imaging cells. Fura-4F 340/380 nm ratios can be recorded every 2 s.
6. After ~60 s (*see Note 16*), stimulate cells with 2 μM TG to inhibit SERCA, the ATPase pump on the ER membrane. The ER is naturally leaky and the addition of TG will slowly cause the ER to lose and eventually be drained of the Ca^{2+} , thereby activating SOCE (*see Note 19*). Upon TG addition, there should be a steady, moderate increase in the Fura-4F 340/380 nm ratio, reflective of Ca^{2+} draining from the ER. This increase will peak and Fura-4F 340/380 nm ratios will eventually return back to the baseline value.
7. Once the ER is completely drained and SOCE is fully activated (~10 min), add 1 mM CaCl_2 . There should be an immediate increase in the Fura-4F 340/380 nm ratios, reflective of Ca^{2+} entering into the cell through the SOC channel. This increase will eventually level off and form a plateau (~after 5–10 min). An example of a graph depicting a typical SOCE imaging experiment is shown in Fig. 4.
8. Remove HBSS used during imaging and add back 500 μl Ca^{2+} -free HBSS containing 10 μM ionomycin (*see Note 17*).
9. After ionomycin permeates the cell membrane (~10 s), add 500 μl Ca^{2+} -free HBSS containing 20 mM MnCl_2 (*see Note 18*).
10. Stop imaging once the 340 and 380 nm values have reached a stable baseline.

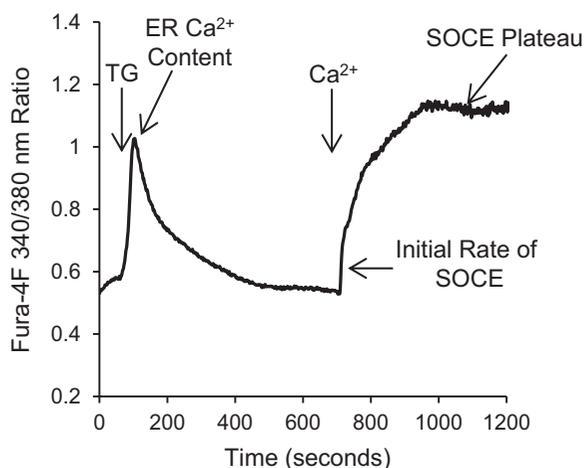


Fig. 4 Typical SOCE imaging experiment in a single primary ray hepatocytes

3.6 Calculations

1. The addition of Mn^{2+} at the end of an imaging experiment will quench the Fura signal at both the 340 and 380 nm wavelengths. Any remaining fluorescence emission detected at 510 nm will stem from cell autofluorescence or intraorganellar trapping of Fura. It is very important to subtract the remaining (background) fluorescent values at both wavelengths from all 340 and 380 nm values (510 nm emission values at these excitation wavelength) when calculating the 340/380 nm Fura-4F ratio for each hepatocyte. To do this, take the last 340 and 380 nm value and subtract that value from all preceding 340 and 380 nm values, respectively. Use the newly corrected 340 and 380 nm values to calculate the 340/380 nm ratio. Use the corrected ratio when plotting a graph. There is typically a significant difference between a graph depicting non-corrected Fura ratios and a graph depicting corrected Fura ratios. Figure 5 shows an example of the difference between the two.
2. To calculate HBV- or HBx-mediated changes in an IP_3 -linked Ca^{2+} response, there are two main parameters that can be compared. The first is the peak Ca^{2+} level following ATP or V_p addition. This indicates the amount of Ca^{2+} released from the ER upon IP_3R stimulation. The peak can be calculated as the change in the peak Fura-4F 340/380 nm ratio following ATP or V_p stimulation and the basal Fura-4F 340/380 nm ratio prior to ATP or V_p stimulation ($R_{peak} - R_{basal}$). The second parameter is the plateau Ca^{2+} level, which can be calculated as a percentage of the peak $[(R_{plateau} - R_{basal}) / (R_{peak} - R_{basal})]$ for each cell.

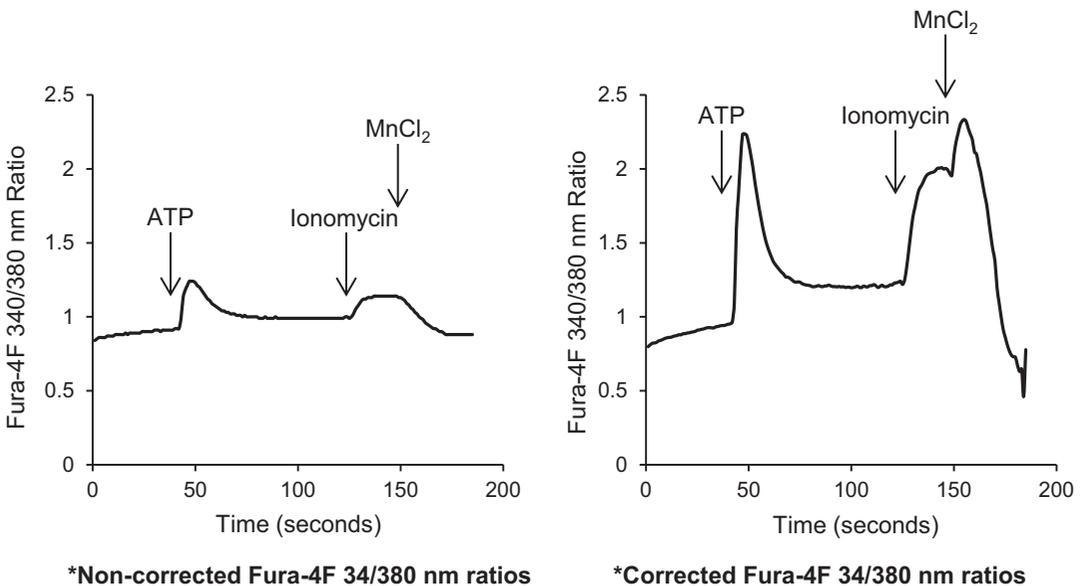


Fig. 5 Typical graphs of an IP_3 -linked Ca^{2+} response in a single primary rat hepatocyte before correcting 340/380 nm Fura-4F ratio values (*left panel*) and after correcting 340/380 nm Fura-4F ratio values (*right panel*)

3. To calculate HBV- or HBx-mediated changes in SOCE, there are three main parameters that can be compared. The first is the peak Ca^{2+} level following TG addition. This peak is reflective of the ER Ca^{2+} level and can be calculated as the difference in the peak Fura-4F 340/380 nm ratio following TG treatment and the basal Fura-4F 340/380 nm ratio ($R_{\text{TG peak}} - R_{\text{basal}}$). The second parameter is the initial rate of SOCE, which can be calculated as the slope representing Ca^{2+} influx during the initial 15 s following Ca^{2+} addition. The SOCE plateau, which represents the total Ca^{2+} accumulation in the cytosol during SOCE, can also be compared and calculated as the difference in the plateau Fura-4F 340/380 nm ratio following Ca^{2+} addition and the baseline Fura-4F 340/380 nm ratio prior to Ca^{2+} addition ($R_{\text{plateau}} - R_{\text{baseline}}$).

4 Notes

1. We use expression of the red fluorescent protein dsRED to identify and select transfected cells. It is preferable to use dsRED, rather than GFP, because the fluorescent wavelength of GFP can overlap with that of Fura. Although we express dsRED from a separate vector than HBx or HBV, dsRED could also be expressed from the same vector.
2. We have optimized transfection protocols for using Lipofectamine 2000 (Invitrogen) to transfect cultured primary rat hepatocytes. Other transfection reagents could also be used; the selection of a transfection reagent will be dependent upon the cell type used and will require the optimization of transfection protocols with that reagent.
3. Resuspension of Fura-4F-AM should be done with anhydrous DMSO. This is critical, as the addition of the AM group (*see Note 9*), causes the indicator to be highly lipophilic.
4. Due to extremely low transfection efficiencies in primary human hepatocytes (less than 10%), we deliver HBV and HBx expression to primary human hepatocytes via recombinant adenoviruses that co-express GFP. Although there is some overlap with the fluorescence emission of Fura and GFP, we have still been able to calculate changes in hepatocyte cytosolic Ca^{2+} levels. Adenoviral delivery ensures almost 100% of primary human hepatocytes express HBV or HBx. GFP expression is used to identify and select HBV- or HBx-expressing or control hepatocytes. Primary human hepatocytes are infected within 24 h of plating on coverslips and imaged 24 h following infection. Protocols for Fura-4F loading and imaging are identical in cultured primary rat and human hepatocytes.

5. Transfection efficiencies in primary rat hepatocytes are approximately 30–40%. Cells are transfected with five times as much HBV- or HBx-expressing plasmid as dsRED-expressing plasmid to ensure that dsRED-expressing cells also expressed HBV or HBx.
6. BSA is important to help disperse Fura-4F.
7. BSP is an organic anion transport inhibitor. Inclusion of this compound is critical, as Fura can be pumped back across the cell membrane and out of the cell; BSP prevents this and helps retain Fura in the cytosol. Sulfinpyrazone can also be used; however, BSP is more water soluble and easier to dissolve in solution.
8. Pluronic acid is a detergent and inclusion of this compound prevents the formation of indicator micelles that could form during loading.
9. In its Ca^{2+} -sensitive form, Fura is hydrophilic and cannot readily pass through lipid membranes. The addition of the AM (acetoxymethyl) group to Fura masks the charge with esterification, allowing the dye to cross the cell membrane. Once inside the cell, cellular esterases cleave the AM group from Fura, leaving Fura in its active, Ca^{2+} -sensitive form.
10. Fura-2-AM can also be used for Ca^{2+} imaging studies. Primary hepatocytes have a high basal intracellular Ca^{2+} concentration; Fura-4F has a higher K_D than Fura-2 (770 nM vs 140 nM, respectively) and as most Ca^{2+} imaging studies involve cytosolic Ca^{2+} levels in the 100 nM–1 μM range, it is often advantageous to use an indicator with a greater K_D than Fura-2 [27].
11. This time will vary slightly depending upon cell type and optimal loading times and will need to be defined prior to starting experiments. 35–60 min is a typical loading duration. Ideally, cells will have a bright nucleus, when excited at 380 nm, and minimal intraorganellar compartmentalization of Fura. If intraorganellar compartmentalization of Fura is an issue, consider shortening the loading time.
12. Our imaging chamber is composed of two parts: a small apparatus that holds the slide and 1 ml of buffer and a larger stage adaptor that holds the apparatus in place above the objective (Harvard Apparatus, RC-25F and SA-OLY/2, respectively).
13. Imaging can be performed on any fluorescent microscope that is properly equipped with a Fura filter set. We use an Olympus IX71 inverted microscope equipped with a 20 \times objective used for Fura imaging, a cooled charged-coupled-device camera, a photometrics coolsnap ES camera driver, a Lambda 10-2 25 mm filter wheel (no shutter), a IX71 emission adapter, and a DG4 Galvo-wavelength switcher. Figure 1 shows our imaging set up.

14. The imaging program must be able to alternately excite Fura dye at 340 and 380 nm and record emissions at 510 nm. The program must also allow the user to focus on and select individual cells. We use the MetaFluor/MetaMorph Premier Combo Imaging System fluorescence ratio imaging software and computer (Universal Imaging Corporation, Molecular Devices, Downingtown, PA).
15. Primary hepatocytes can have high levels of autofluorescence, and it is important to confirm that the cells imaged are both alive and loaded with Fura-4F prior to proceeding with imaging.
16. In order to stimulate an optimal Ca^{2+} response in hepatocytes, it is important that the basal Fura-4F 340/380 nm ratios are stable prior to the addition of a Ca^{2+} -inducing agonist. It is therefore suggested that cells be imaged for ~60 s prior to stimulation with an agonist to ensure cells are healthy and behaving normally. If Fura-4F 340/380 ratios are steadily increasing in the absence of an agonist, this suggests that Fura-4F may be getting pumped out of the cell and the concentration of BSP may need to be adjusted. If Fura-4F 340/380 ratios are fluctuating in the absence of an agonist, this suggests that the cells may not be healthy and completion of the experiment should be reevaluated.
17. The addition of ionomycin will permeabilize the plasma membrane, which will allow the MnCl_2 to enter the cell.
18. The addition of MnCl_2 will quench the Fura-4F signal so that any remaining fluorescence will stem from autofluorescence or intraorganellar compartmentalization of the Fura-4F dye.
19. Other SERCA inhibitors, such as cyclopiazonic acid, or low concentrations of ionomycin can also be used to drain the ER Ca^{2+} store and activate SOCE.

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In Vitro Assays for RNA Binding and Protein Priming of Hepatitis B Virus Polymerase

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Abstract

The hepatitis B virus (HBV) polymerase synthesizes the viral DNA genome from the pre-genomic RNA (pgRNA) template through reverse transcription. Initiation of viral DNA synthesis is accomplished via a novel protein priming mechanism, so named because the polymerase itself acts as a primer, whereby the initiating nucleotide becomes covalently linked to a tyrosine residue on the viral polymerase. Protein priming, in turn, depends on specific recognition of the packaging signal on pgRNA called epsilon. These early events in viral DNA synthesis can now be dissected in vitro as described here.

The polymerase is expressed in mammalian cells and purified by immunoprecipitation. The purified protein is associated with host cell factors, is enzymatically active, and its priming activity is epsilon dependent. A minimal epsilon RNA construct from pgRNA is co-expressed with the polymerase in cells. This RNA binds to and co-immunoprecipitates with the polymerase. Modifications can be made to either the epsilon RNA or the polymerase protein by manipulating the expression plasmids. Also, the priming reaction itself can be modified to assay for the initiation or subsequent DNA synthesis during protein priming, the susceptibility of the polymerase to chemical inhibitors, and the precise identification of the DNA products upon their release from the polymerase. The identity of associated host factors can also be evaluated. This protocol closely mirrors our current understanding of the RNA binding and protein priming steps of the HBV replication cycle, and it is amenable to modification. It should therefore facilitate both basic research and drug discovery.

Key words Hepatitis B virus, Polymerase, Reverse transcriptase, Protein priming, RNA binding

1 Introduction

Hepatitis B virus encodes one enzymatic protein, a multifunctional polymerase (called variously P, HP, Pol, and RT). The hepatitis B virus polymerase consists of four domains: the terminal protein (TP) domain at the N-terminus, a spacer region, the reverse transcriptase (RT) domain, and the C-terminal RNase H domain. During its replication cycle, HBV polymerase synthesizes DNA from both RNA and DNA templates, and has the interesting characteristic of acting as its own primer. The TP domain contains a tyrosine residue at position 63 (Y63) which covalently attaches to the nascent DNA through a phosphotyrosyl bond [1–3].

The polymerase is encoded on the viral pre-genomic RNA (pgRNA), which is transcribed by RNA Pol II in host cell nuclei from viral DNA [4]. The pgRNA serves as messenger RNA for translation of viral proteins, and also acts as the template for reverse transcription to produce progeny viral DNA. The pgRNA contains a packaging signal near the 5' end, which is a 61-nucleotide stem-loop structure called epsilon (ϵ RNA) to which the RT binds. This ϵ RNA contains an internal bulge on the hairpin with the sequence UUC [5]. This is copied by the polymerase, first to the initial dGMP, and then to dAMP nucleotides at the 5' end of the viral minus-strand DNA. During viral replication, this small dGAA oligonucleotide then moves to the 3' end of the RNA, facilitating a switch of templates so a nearly full-length DNA copy can be made from the pgRNA. This RNA is degraded during minus-strand DNA synthesis, and the plus-strand DNA is then copied. The result is a partially double-stranded circular DNA that is not covalently closed, but is covalently attached to the polymerase on the 5' end of the minus strand. This relaxed circular DNA (rcDNA) is what is found in infectious viral particles of HBV [6, 7].

The priming assay described in this protocol can be used to test the early steps of DNA synthesis—both priming initiation and downstream synthesis of the nascent minus-strand of DNA [8–10]. Since dGMP is the obligatory initiating nucleotide, it can be used alone in a priming reaction to test initiation. Other nucleotides can be added to test subsequent steps in synthesis. This simple setup can facilitate drug screening. The inclusion of a nucleoside or non-nucleoside inhibitor in the reaction can reveal if the drug affects these early steps of viral DNA synthesis. For example, the guanidine analog RT inhibitor, entecavir, was shown to act competitively (with respect to dGTP) to block priming in a competitive manner and is incorporated into the DNA product [9]. This guanidine analog thus blocks the initiation step of priming, which normally requires dGTP. Interestingly, clevudine—a thymidine analog—was also able to inhibit priming initiation as well as elongation, which requires dATPs. It was further shown that clevudine triphosphate is not incorporated into the DNA product, but instead acts as a non-nucleoside inhibitor, unlike any other nucleoside analog inhibitors [9]. In addition to these findings, the priming assay described herein may help reveal the mechanisms of the next generation of HBV RT inhibitors.

One interesting function of the polymerase is what has been described as a novel protein-primed terminal transferase activity [11]. When manganese instead of magnesium is used as the enzyme cofactor, there is a loss of template dependence. The polymerase will synthesize DNA by random incorporation of nucleotides, but favors TTP incorporation for making DNA strands. It is not known how relevant this function is in vivo during an infection.

An important step during viral replication is the removal of the RT from the rcDNA genome. The protein must be removed in order to close the circular DNA covalently [12–14]. The covalently closed circular DNA (cccDNA) is the root source of virus production, since the cell uses it to make all viral RNAs. It is unknown how the polymerase is removed from rcDNA, but one candidate is the human enzyme tyrosyl DNA phosphodiesterase 2 (Tdp2) [15, 16]. Whether or not it is used during actual infections, it can be used *in vitro* and is employed here to evaluate the synthesized DNA by cleaving the DNA from the HBV RT enzyme [8–11].

The current protocol is basically a two component system: the HBV polymerase and an epsilon-containing RNA construct. Both can be mutagenized by manipulating the plasmids used to express them. Once co-transfected in mammalian cell culture, the HBV polymerase and ϵ RNA will associate. During HBV replication, both protein priming and the subsequent DNA synthesis occur inside the viral nucleocapsid. However, in this reduced system which expresses no capsid protein, only RNA binding and limited amounts of protein priming occur while inside the transfected cells. Upon purification from the cells, the polymerase is competent to carry out protein-primed DNA synthesis *in vitro* using the ϵ RNA bound to it. Also, *in vitro* transcribed ϵ RNA can be added to HBV polymerase which is purified alone (no ϵ RNA expressed with it in cell culture) to determine the level of RNA binding activity. This *in vitro* RNA binding assay is another way to evaluate this critical association.

The entire pgRNA is not expressed, in part because pgRNA contains two copies of the epsilon sequence, however only the 5' copy is functional as a packaging signal *in vivo*. The construct used here produces an mRNA that is 330 bases in length (not counting the poly(A) tail which would be added by the cell), as opposed to the 3.5 kb full length pgRNA.

The purified HBV polymerase in this system is associated with host cell factors, some of which are chaperone proteins which facilitate polymerase function [17–19]. A lack of necessary host factors may be the reason that no *in vitro* translated human HBV RT has been shown to be active enzymatically in protein priming. These facilitating host factors have been characterized in part, but other host factors may be found and studied using the current protocol.

For years the ability to measure the amount of HBV DNA has been available in a DNA synthesis assay, which consists of purifying capsids and detecting the DNA contained in these nucleocapsids, usually by Southern blot. Similarly, an RNA packaging (encapsidation) assay is a similar procedure, but detects viral RNA, usually by northern blot. Although these protocols accurately reflect the activity of the viral polymerase, they cannot evaluate the initial steps in DNA synthesis. The current protocol satisfies a need to molecularly dissect these early DNA synthesis steps which are

critical to the viral replication cycle and may be inhibited by drug compounds.

The use of the solid-phase agarose beads facilitates purification and buffer exchange, and relatively large amounts of purified protein can be obtained. Modifications can be made to the RT or the ϵ RNA to examine critical features of these partners and how these features affect their interaction. The ability to test several enzymatic roles of the HBV polymerase, and test their inhibition through drug screening, makes this procedure an important and novel method to study this important virus.

2 Materials

2.1 HBV Polymerase

Expression and Purification (See Note 1)

1. HEK 293T cells.
2. DMEM/F12 (1:1) medium. Complete medium is supplemented with 10% FBS, 100 U/mL penicillin and 10 μ g/mL streptomycin.
3. pcDNA-3FHP: Expresses a triple FLAG-tagged HBV polymerase (HP) under the human cytomegalovirus (CMV) promoter in the pcDNA3 (Invitrogen) backbone. The plasmid was constructed by fusing in-frame three copies of the FLAG epitope tag to the N-terminus of the HBV polymerase coding sequence [8]. Strain ayw (GenBank accession number X59795.1) was used, which is phylogenetically HBV genotype D as in pCMV-HBV [20].
4. pCMV-HE: For ϵ RNA expression in human cells, pCMV-HE was produced by substituting a 0.5 kb fragment of the CMV promoter plus HBV sequence from 1801–1993 from pCMV-HBV (NdeI to XbaI) for the CMV and T7 promoter in pcDNA3 (NdeI to XbaI fragment) [8]. pCMV-HE will produce, upon RNA Pol II transcription in transfected mammalian cells, a capped and polyadenylated HBV RNA initiating at the authentic pgRNA initiation site (1814) and containing the 5' DR1 (1822–1832) and ϵ RNA sequences (1845–1905) (see Note 2).
5. Calcium phosphate transfection kit (Clontech).
6. Anti-FLAG M2 antibody (Sigma).
7. Protein A/G agarose beads.
8. Low retention pipet tips.
9. Diethylpyrocarbonate (DEPC) (see Note 3).
10. RNaseZap or similar RNase removal product (Ambion).
11. Dithiothreitol (DTT), 1 M, make fresh for each use, keep RNase-free (see Note 4).
12. β -mercaptoethanol, 12.8 M, keep RNase-free (see Note 5).

13. Complete EDTA-free protease inhibitor, 25× concentration, store prepared solution for up to 3 months at $-20\text{ }^{\circ}\text{C}$, keep RNase-free (Roche).
14. Complete protease inhibitor, 25× concentration, store prepared solution for up to 3 months at $-20\text{ }^{\circ}\text{C}$, keep RNase-free (Roche).
15. E-64 protease inhibitor, 2 mM, store prepared solution for up to 9 months at $-20\text{ }^{\circ}\text{C}$, keep RNase-free.
16. Leupeptin protease inhibitor, 1 mg/mL, store prepared solution for up to 6 months at $-20\text{ }^{\circ}\text{C}$, keep RNase-free.
17. Phenylmethanesulfonyl fluoride (PMSF) protease inhibitor, 200 mM in isopropanol, store at room temperature for up to 9 months, keep RNase-free (*see Note 6*).
18. RNasin Plus (Promega) or RNaseOUT (Thermo Fisher) RNase inhibitors.
19. RNase-free Tris-HCl, 1 M, pH 7.0 (Ambion).
20. RNase-free NaCl 5 M (Ambion).
21. KCl, 1 M, keep RNase-free.
22. Glycerol, 80%, keep RNase-free.
23. NP-40, 10%, keep RNase-free.
24. EDTA, 0.5 M, pH 8.0, store at room temperature, keep RNase-free.
25. TN buffer: 50 mM Tris-HCl pH 7.0, 100 mM NaCl. For 50 mL, combine 2.5 mL of 1 M Tris-HCl pH 7.0, 1 mL of 5 M NaCl, and 46.5 mL of DEPC-treated water (*see Note 7*). Store at room temperature.
26. 10× RNase-free PBS (Ambion). Prepare 1× PBS with DEPC-treated water, keep RNase-free.
27. 1× PBS with protease inhibitors: 28 μM E-64, 5 $\mu\text{g}/\text{mL}$ leupeptin, 1 mM PMSF. To 50 mL of 1× PBS, add 350 μL of 2 mM E-64, 125 μL of 1 mg/mL leupeptin, and 125 μL of 200 mM PMSF. Prepare just before use and do not store, keep RNase-free.
28. FLAG lysis buffer: 50 mM Tris-HCl pH 7.0, 100 mM NaCl, 50 mM KCl, 10% glycerol, 1% NP-40, 1 mM EDTA pH 8.0. For 50 mL, combine 2.5 mL of 1 M Tris-HCl pH 7.0, 1 mL of 5 M NaCl, 2.5 mL of 1 M KCl, 6.25 mL of 80% glycerol, 5 mL of 10% NP-40, 100 μL of 0.5 M EDTA pH 8.0, and 32.65 mL of DEPC-treated water. Store at room temperature, keep RNase-free.
29. FLAG lysis buffer with inhibitors: FLAG lysis buffer plus 1× Complete protease inhibitor cocktail, 10 mM β -mercaptoethanol, 2 mM DTT, 1 mM PMSF, 250 U/mL RNase inhibitor. To 1 mL of FLAG lysis buffer, add 40 μL of

25× Complete protease inhibitor cocktail, 0.78 μL of 12.8 M β -mercaptoethanol, 2 μL of 1 M DTT, 5 μL of 200 mM PMSF, and 6.25 μL of 40 U/ μL RNase inhibitor. Prepare just before use, use on ice, do not store, keep RNase-free.

30. FLAG wash buffer with inhibitors: FLAG lysis buffer plus 28 μM E-64, 5 $\mu\text{g}/\text{mL}$ leupeptin, 1 mM PMSF, 10 mM β -mercaptoethanol, 2 mM DTT, 10 U/ μL RNase inhibitor. To 1 mL of FLAG lysis buffer, add 14 μL of 2 mM E-64, 5 μL of 1 mg/mL leupeptin, 5 μL of 200 mM PMSF, 0.78 μL of 12.8 M β -mercaptoethanol, 2 μL of 1 M DTT, and 0.25 μL of 40 U/ μL RNase inhibitor. Prepare just before use, use on ice, do not store, keep RNase-free.
31. 2× SDS lysis buffer: 125 mM Tris-HCl pH 6.8, 20% glycerol, 4.6% SDS, 0.1% bromophenol blue. Store at room temperature. For 50 mL, combine 6.94 mL of 1 M Tris-HCl pH 6.8, 11.1 mL of glycerol, 25.56 mL of 10% SDS, 1.1 mL of 5% bromophenol blue, and 5.28 mL of distilled water. Before use, add one-tenth volume 12.8 M β -mercaptoethanol.

2.2 *In Vitro* and *In Vivo* RNA Binding (See Note 8)

1. MAXIscript SP6 kit (Ambion).
2. [α - ^{32}P] UTP (10 mCi/mL, 3000 Ci/mmol) (PerkinElmer).
3. Quick Spin columns for radiolabeled RNA purification (Sephadex G-25, fine) (Roche).
4. RIPA buffer: 50 mM Tris-HCl pH 7.0, 150 mM NaCl, 1 mM EDTA pH 8.0, 0.05% NP-40. For 50 mL, combine 2.5 mL of 1 M Tris-HCl pH 7.0, 1.5 mL of 5 M NaCl, 100 μL of 0.5 M EDTA pH 8.0, 250 μL of 10% NP-40, and 45.65 mL of DEPC-treated water. Store at room temperature, keep RNase-free.
5. RIPA buffer with inhibitors: RIPA buffer plus 2 mM DTT, 1 mM PMSF, 1× Complete protease inhibitor cocktail, 1 U/ μL RNase inhibitor. To 1 mL of RIPA buffer add 2 μL of 1 M DTT, 5 μL of 200 mM PMSF, 40 μL of 25× Complete protease inhibitor cocktail, and 25 μL of 40 U/mL RNase inhibitor. Prepare just before use, use on ice, do not store, keep RNase-free.
6. RIPA wash buffer: RIPA buffer plus 2 mM DTT, 1 mM PMSF, 28 μM E-64, 5 $\mu\text{g}/\text{mL}$ leupeptin, and 10 U/mL RNase inhibitor. To 1 mL of RIPA buffer add 2 μL of 1 M DTT, 5 μL of 200 mM PMSF, 14 μL of 2 mM E-64, 5 μL of 1 mg/mL leupeptin, and 0.25 μL of 40 U/mL RNase inhibitor. Prepare just before use, use on ice, do not store, keep RNase-free.
7. Formamide gel loading buffer II (Ambion).
8. 10× TBE: 0.89 M Tris, 0.89 M boric acid, 20 mM EDTA. For 1 L, add 108 g of Tris base, 55 g of boric acid, and 40 mL of 0.5 M EDTA pH 8.0. Fill to 1 L with water.

9. 1× TBE: 89 mM Tris, 89 mM boric acid, 2 mM EDTA. For 1 L, add 100 mL of 10× TBE and 900 mL of water.
10. ULTRAhyb solution (Ambion).
11. 20× SSC pH 7.0: 3 M NaCl, 0.3 M sodium citrate. For 1 L, add 175.3 g NaCl and 88.2 g sodium citrate. Adjust pH and fill to 1 L with water.
12. Low stringency wash buffer: 2× SSC, 0.1% SDS. For 500 mL, add 50 mL of 20× SSC, 2.5 mL of 20% SDS, and 447.5 mL of water.
13. High stringency wash buffer: 0.1× SSC, 0.1% SDS. For 500 mL, add 2.5 mL of 20× SSC, 2.5 mL of 20% SDS, and 495 mL of water.

2.3 In Vitro Protein Priming, Transferase Activity, and Tdp2 Cleavage (See Note 8)

1. [α -³²P] dATP (10 mCi/mL, 3000 Ci/mmol) (PerkinElmer).
2. [α -³²P] dCTP (10 mCi/mL, 3000 Ci/mmol) (PerkinElmer).
3. [α -³²P] dGTP (10 mCi/mL, 3000 Ci/mmol) (PerkinElmer).
4. [α -³²P] TTP (10 mCi/mL, 3000 Ci/mmol) (PerkinElmer).
5. 100 mM deoxynucleotide triphosphate set (dNTPs) (Invitrogen).
6. 1 M MgCl₂, store at -20 °C, keep RNase-free.
7. 1 M MnCl₂, store at -20 °C, keep RNase-free (*see Note 9*).
8. 10× TMgNK buffer: 200 mM Tris-HCl pH 7.0, 150 mM NaCl, 100 mM KCl, 40 mM MgCl₂. For 500 μ L, combine 315 μ L DEPC-treated water, 100 μ L of 1 M Tris-HCl pH 7.0, 15 μ L of 5 M NaCl, 50 μ L of 1 M KCl, and 20 μ L of 1 M MgCl₂. Store at -20 °C, keep RNase-free.
9. TMgNK priming buffer: 1× TMgNK, 1× Complete EDTA-free protease inhibitor cocktail, 4 mM DTT, 1 U/ μ L RNase inhibitor, 1 mM PMSF. For 100 μ L, combine 82.6 μ L DEPC-treated water, 10 μ L of 10× TMgNK, 4 μ L of 25× Complete EDTA-free protease inhibitor cocktail, 0.4 μ L of 1 M DTT, 2.5 μ L of 40 U/ μ L RNase inhibitor and 0.5 μ L of 200 mM PMSF. Prepare just before use, use on ice, do not store, keep RNase-free.
10. 10× TMnNK buffer: 200 mM Tris-HCl pH 7.0, 150 mM NaCl, 100 mM KCl, 20 mM MnCl₂. For 500 μ L, combine 325 μ L DEPC-treated water, 100 μ L of 1 M Tris-HCl pH 7.0, 15 μ L of 5 M NaCl, 50 μ L of 1 M KCl, and 10 μ L of 1 M MnCl₂. Store at -20 °C, keep RNase-free.
11. TMnNK priming buffer: 1× TMnNK, 1× Complete EDTA-free protease inhibitor cocktail, 4 mM DTT, 1 U/ μ L RNase inhibitor, 1 mM PMSF. For 100 μ L, combine 82.6 μ L DEPC-treated water, 10 μ L of 10× TMnNK, 4 μ L of 25× Complete EDTA-free protease inhibitor cocktail, 0.4 μ L of 1 M DTT,

- 2.5 μL of 40 U/ μL RNase inhibitor, and 0.5 μL of 200 mM PMSF. Prepare just before use, use on ice, do not store, keep RNase-free.
12. TNK buffer: 20 mM Tris-HCl pH 7.0, 15 mM NaCl, 10 mM KCl. For 50 mL, combine 1 mL of 1 M Tris-HCl pH 7.0, 150 μL of 5 M NaCl, 500 μL of 1 M KCl, and 48.35 mL of DEPC-treated water, store at room temperature, keep RNase-free.
 13. TNK wash buffer: TNK buffer plus 28 μM E-64, 1 mM PMSF, 5 $\mu\text{g}/\text{mL}$ leupeptin, and 10 mM β -mercaptoethanol. To 50 mL of TNK buffer, add 700 μL of 2 mM E-64, 250 μL of 200 mM PMSF, 250 μL of 1 mg/mL leupeptin, and 39.06 μL of 12.8 M β -mercaptoethanol. Prepare just before use, use on ice, do not store, keep RNase-free.
 14. Tyrosyl DNA phosphodiesterase 2 (Tdp2/TTRAP) (Abnova). Enzyme concentration was 0.23 $\mu\text{g}/\mu\text{L}$ but may vary by lot, suspended in 50 mM Tris-HCl pH 8.0 and 10 mM reduced glutathione. Store at -80°C .
 15. Tdp2 mock buffer: 50 mM Tris-HCl pH 8.0 and 10 mM reduced glutathione. Store at -20°C .
 16. 2 \times Tdp2 buffer: 50 mM Tris-HCl pH 8, 260 mM KCl, 2 mM DTT, 20 mM MgCl_2 . For 1 mL, combine 50 μL of 1 M Tris-HCl pH 8.0, 260 μL of 1 M KCl, 2 μL of 1 M DTT, 20 μL of 1 M MgCl_2 , and 668 μL of DEPC-treated water (*see Note 7*). Make fresh and do not store.
 17. 1 \times Tdp2 wash buffer: 1 \times Tdp2 buffer, 28 μM E-64, 1 mM PMSF, 5 $\mu\text{g}/\text{mL}$ leupeptin. For 1 mL, combine 500 μL of 2 \times Tdp2 buffer, 476 μL of DEPC-treated water, 14 μL of 2 mM E-64, 5 μL of 200 mM PMSF, and 5 μL of 1 mg/mL leupeptin. Make fresh, use on ice, and do not store.
 18. 1 \times Tdp2 reaction mix: 1 \times Tdp2 buffer, 1 \times Complete EDTA-free protease cocktail inhibitor, with Tdp2 enzyme or mock buffer. For 100 μL , combine 50 μL of 2 \times Tdp2 buffer, 4 μL of 25 \times Complete EDTA-free protease inhibitor cocktail, 26 μL of DEPC-treated water, and 20 μL Tdp2 enzyme (or Tdp2 mock buffer). Make fresh and do not store.

3 Methods

3.1 Cell Culture and Transfection

1. Human embryonic kidney (HEK) HEK293T cells are maintained in complete DMEM/F12 (1:1) media in a humidified cell culture incubator at 37°C , 5% CO_2 (*see Notes 10 and 11*).
2. Passage cells 1 day before transfection, plating approximately 1.25×10^6 cells per 6 cm dish, or an amount that yields 60–90% confluence the next day (*see Note 12*).

3. Change medium 2–3 h before transfection.
4. Transfect each plate with half pCDNA-3FHP and half pCMV-HE (by weight) using calcium phosphate transfection (*see Note 13*). Use 10 μg total weight of DNA for 6 cm dishes, 20 μg for 10 cm dishes, and 50 μg for 15 cm dishes. Include any desired controls (*see Notes 14 and 15*).
5. Calculate how much sterile water would be needed to have a total volume of 500 μL total for a 6 cm dish, 1 mL total for a 10 cm dish, or 2.5 mL for a 15 cm dish (when combining water, DNA, the calcium solution, and the 2 \times HBS phosphate solution).
6. Add in the following order: sterile water, DNA, and calcium chloride (calcium chloride volume is 31 μL for 6 cm, 62 μL for 10 cm, or 155 μL for 15 cm dishes).
7. To the DNA-calcium tube, add 2 \times HBS dropwise (250 μL for 6 cm, 500 μL for 10 cm, or 1.25 μL for 15 cm dishes) while agitating the receiving tube by flicking or agitating on a vortexer which is set low enough that no splashing occurs.
8. After 5–20 min, apply the transfection reagent dropwise onto labeled plates.
9. Incubate at 37 $^{\circ}\text{C}$, 5% CO_2 for 8 h to overnight with transfection reagent. Wash cells once with 1 \times PBS and apply fresh medium.
10. Allow cells to grow for 2 days, at which point the cells are lysed according to the procedure below. Alternatively the cells can be frozen at -80°C (*see Note 16*).

3.2 Protein and RNA Purification

Two days after transfection, the anti-FLAG antibody is bound to agarose beads, the cells are lysed, and then the lysate is combined with the beads overnight to immunoprecipitate FLAG-tagged HBV polymerase and any associated RNA or host factors (Fig. 1a).

The purified protein, expressed with or without ϵ RNA, is the basis for all subsequent protocols. The beads, which contain the immunoprecipitated HBV polymerase and all associated proteins and RNA, are the starting material for the in vitro and in vivo RNA binding assays, the protein priming assay, the transferase activity, and release of the DNA product by the Tdp2 cleavage assay (Fig. 1).

Wash steps are performed by adding the indicated wash buffer, resuspending the beads by inverting the tube, centrifugation at $350\times g$ at 4 $^{\circ}\text{C}$ for 2 min, and removing the wash buffer. Any RNase-free filtered tips can be used for wash steps; however, perform all pipet transfers of the beads themselves with low-retention tips to reduce bead adherence to the pipet tip.

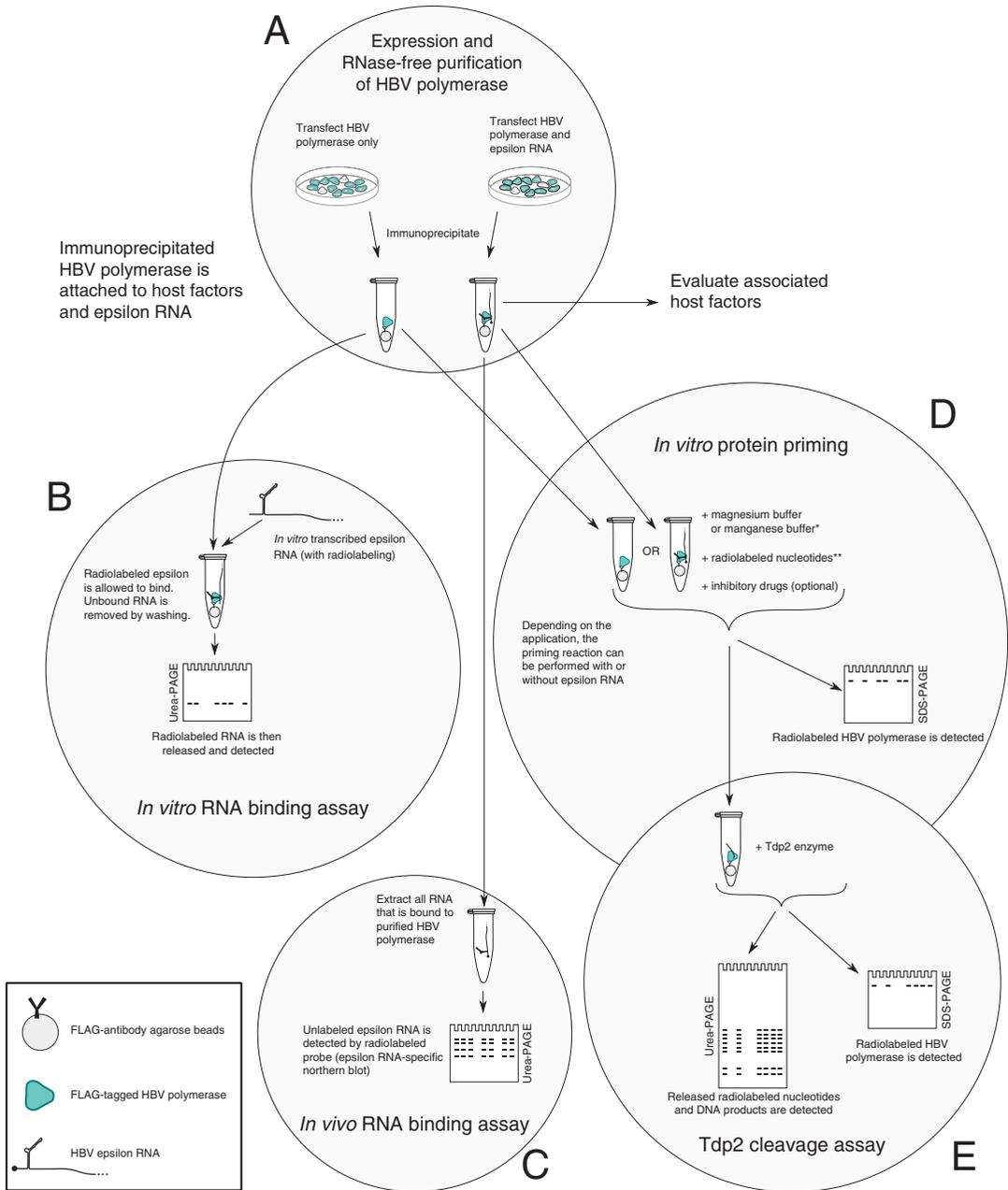


Fig. 1 Protein priming and RNA binding protocol diagram. (a) The HBV polymerase can be purified (with or without epsilon RNA) by binding the FLAG-tagged polymerase to agarose beads, which are pre-bound with anti-FLAG antibodies. (b) An *in vitro* synthesized RNA is added to HBV polymerase (purified without epsilon) to perform the *in vitro* RNA binding assay. (c) The epsilon RNA which is co-purified with the polymerase can be evaluated for the *in vivo* RNA binding assay. (d) The purified polymerase, which is enzymatically active, can synthesize new DNA by adding nucleotides and buffer in the *in vitro* priming assay. Due to the protein priming activity, the polymerase itself is covalently labeled with the nucleotides and can be detected by autoradiography. (e) Alternatively, the DNA product from the priming reaction can be released from the polymerase by Tdp2 (the Tdp2 cleavage assay). *The priming assay, when using the manganese buffer, can also evaluate HBV polymerase's terminal transferase activity. This novel activity is epsilon independent but also protein-primed. **The epsilon RNA template is copied beginning with an initial dGAA oligonucleotide. Therefore the initiation is dependent on dGTP, whereas the continued polymerization is dependent on dATP, and then other nucleotides

3.2.1 Prepare FLAG Ab-Bound Beads

1. Resuspend the immobilized protein A/G beads by inverting the bottle several times. Transfer 20 μL of the bead suspension per 6 cm plate (50 μL per 10 cm plate, 125 μL per 15 cm plate) into a single tube, which will be split into bead groups later.
2. Pellet the beads by centrifugation and remove the storage buffer. Wash the beads three times with TN buffer. Wash by adding the buffer to resuspend the beads, then centrifuge and remove the buffer.
3. After washing, resuspend the beads in TN buffer at half the original bead volume but at least 200 μL .
4. Bind anti-FLAG IgG Ab onto the washed beads by adding 2.8 μL of anti-FLAG antibody per each 6 cm dish (7 μL per 10 cm dish, or 17.5 μL per 15 cm dish).
5. Rotate at room temperature for 3–4 h. Proceed to cell lysis during incubation.
6. After the anti-FLAG antibody is bound to the beads, spin and remove the unbound antibody (*see Note 17*). Wash the beads three times with 500 μL FLAG lysis buffer (without inhibitors) (*see Note 18*). When adding the wash buffer the final time, resuspend the beads with the low retention tips, and divide equal volumes into separate tubes for each condition used in the transfection. Place the tubes on ice.

3.2.2 RNase-Free Cell Lysis

1. Wash cells once with 2 mL 1 \times PBS per 6 cm plate (4 mL per 10 cm plate, or 10 mL per 15 cm plate) (*see Note 19*).
2. Wash cells once with 2 mL cold 1 \times PBX with protease inhibitors per 6 cm plate (4 mL per 10 cm plate, or 10 mL per 15 cm plate) (*see Note 20*).
3. To each 6 cm plate, add 0.4 mL cold FLAG lysis buffer with inhibitors (1 mL to 10 cm plates, or 2.5 mL to 15 cm plates). Free cells from the dish by scraping with a cell scraper or spraying with the buffer from a pipet tip.
4. Collect cells from the same treatment condition into a single chilled tube, and rotate for 20 min at 4 $^{\circ}\text{C}$.
5. Spin lysate at 4 $^{\circ}\text{C}$ for 10 min at maximum speed in a microcentrifuge $\sim 18,000\times g$ (*see Note 21*).
6. Transfer the supernatant to a chilled tube of prepared Ab-bound beads (*see Note 22*).
7. Rotate the Ab-bound beads and cell lysate supernatant at 4 $^{\circ}\text{C}$ overnight to allow the immunoprecipitation to occur.
8. The next day, spin beads at 4 $^{\circ}\text{C}$ for 2 min at 350 $\times g$. Remove unbound supernatant (*see Note 23*).
9. Wash the beads five times with 500 μL FLAG wash buffer. When adding the wash buffer the final time, resuspend the beads with the low retention tips, and divide equal volumes

into separate tubes for each assay to be performed (*see* **Notes 24** and **25**).

10. Store bead aliquots at $-80\text{ }^{\circ}\text{C}$ (*see* **Note 26**). Approximate bead volume is $10\text{ }\mu\text{L}$ per tube (*see* **Note 27**).

3.2.3 Downstream Analysis of Immunopurified HBV Polymerase Complexes

1. For western blotting or protein staining (*see* **Note 28**) such as Coomassie blue or silver stain: Keeping the samples on ice throughout, remove the wash buffer, and add $20\text{ }\mu\text{L}$ $2\times$ SDS lysis buffer to the $\sim 10\text{ }\mu\text{L}$ beads.
2. Boil for 5 min, vortex, boil another 5 min, and place the samples back on ice. Spin briefly ($\sim 4000\times g$ for one second) to remove condensed liquid.
3. Mix the samples and load $20\text{ }\mu\text{L}$ on 9% SDS-PAGE gel (*see* **Note 29**).
4. If analyzing/troubleshooting each step, load lysate supernatant ($30\text{ }\mu\text{L}$ lysate + $30\text{ }\mu\text{L}$ $2\times$ SDS lysis buffer, load $30\text{ }\mu\text{L}$) and load insoluble pellet (add $50\text{ }\mu\text{L}$ TE, vortex to resuspend pellet, then add $200\text{ }\mu\text{L}$ $2\times$ SDS lysis buffer, mix, boil, load $50\text{ }\mu\text{L}$).
5. At this point, the gel can be used for staining or transferred to a membrane for western blotting (*see* **Note 30**).

3.3 In Vitro and In Vivo RNA Binding Assays

3.3.1 In Vitro Transcription of ^{32}P -Radiolabeled ϵ RNA and In Vitro RNA Binding

The purified HBV polymerase can be tested for its capacity to bind in vitro transcribed ϵ RNA. Any unbound RNA is removed by washing and the bound RNA is detected by autoradiography (Fig. 1b).

1. Radiolabeled ϵ RNA is transcribed using the SP6 MAXIscript kit components (*see* **Note 31**). For a $20\text{ }\mu\text{L}$ reaction add $4\text{ }\mu\text{L}$ of DEPC-treated water; $1\text{ }\mu\text{L}$ each of 10 mM ATP, CTP, and GTP; $2\text{ }\mu\text{L}$ of 0.5 mM UTP (diluted from 10 mM stock 1:20 with DEPC-treated water); $1\text{ }\mu\text{L}$ of $1\text{ }\mu\text{g}/\mu\text{L}$ template DNA; $3\text{ }\mu\text{L}$ of SP6 enzyme; and $5\text{ }\mu\text{L}$ of $[\alpha\text{-}^{32}\text{P}]$ UTP (*see* **Note 32**).
2. Incubate at $37\text{ }^{\circ}\text{C}$ for 5 h total. Halfway through the incubation, spin briefly ($\sim 4000\times g$ for one second) to collect condensate.
3. After incubation, add $1\text{ }\mu\text{L}$ DNase and incubate at $37\text{ }^{\circ}\text{C}$ for 30 min.
4. Inactivate the DNase by incubating at $70\text{ }^{\circ}\text{C}$ for 15 min.
5. Labeled samples can be stored at $-80\text{ }^{\circ}\text{C}$ (*see* **Note 33**).
6. Combine each aliquot of immunoprecipitated HBV polymerase-bound beads (*see* **Note 34**) with $0.5\text{ }\mu\text{g}$ in vitro-transcribed ^{32}P -labeled ϵ RNA (approximately $1\text{--}3\text{ }\mu\text{L}$) and $20\text{ }\mu\text{L}$ RIPA buffer with inhibitors [19] (*see* **Note 35**).
7. Incubate for 3 h at room temperature with shaking to allow HBV polymerase to bind the ϵ RNA (*see* **Note 36**).

8. Save the supernatant, which contains any unbound components at -80°C .
9. Wash the beads five times with 200 μL RIPA wash buffer. Samples may be stored at -80°C for later processing (*see Note 37*).
10. After removing the final wash buffer, elute bound materials by adding 60 μL of $2\times$ SDS lysis buffer and boiling 5 min. Vortex and boil 5 more minutes. Load 30 μL of sample onto a 15% SDS-PAGE gel (*see Note 38*).
11. Cut the gel horizontally at approximately 50 kDa. The top portion of the gel which contains the HBV polymerase protein can be visualized by western blotting using the anti-FLAG antibody. The bottom portion of the gel containing the ^{32}P -labeled ϵ RNA can be dried and directly exposed to film to detect the labeled ϵ RNA, which was bound to (and then disassociated from) the purified RT.

3.3.2 In Vivo RNA Binding Assay

Any RNA associated with the purified HBV polymerase is extracted from the bead sample. An ϵ RNA-specific probe is used to detect the amount of binding (Fig. 1c).

1. Using immunoprecipitated HBV polymerase (*see Note 34*), perform a TRIzol extraction of 20 μL lysate (*see Note 22*) and the ~ 10 μL bead aliquot (*see Note 39*). Resuspend the extracted RNA samples in a final volume of 20 μL DEPC-treated water. Add 20 μL of formamide gel loading buffer II to each sample and heat to 95°C for 5 min by boiling or in a thermal cycler. Load the hot samples onto a 6% acrylamide 8 M urea-PAGE gel in TBE. Run the gel at 300 V until bromophenol blue dye front comes off (approximate time is 2 h).
2. Rinse gel for 20 min in $1\times$ TBE, rinse a nylon membrane in water for 5 min then in $1\times$ TBE for 5 min.
3. For electrophoretic transfer, run below 300 mA (6–7 V) for 1 h.
4. While the transfer is running, preheat ULTRAhyb solution to 68°C to dissolve precipitated material.
5. Cross-link the RNA to the membrane by exposing to UV transilluminator.
6. Prehybridize for at least 30 min at 68°C . Use 10 mL ULTRAhyb buffer per 100 cm^2 of membrane.
7. Add prepared ϵ RNA-specific radiolabeled probe (*see Note 40*) to the ULTRAhyb and allow the probe to hybridize 2 h to overnight.
8. Make sure wash buffers have no precipitate, if so, heat to 37°C . Preheat high stringency wash to 68°C .
9. Wash blot twice in room temperature low stringency wash solution (20 mL per 100 cm^2 blot) with agitation for 5 min, dispose of washes in ^{32}P waste.

10. Wash twice for 15 min at 68 °C with high stringency wash solution with agitation. Dispose of washes in ³²P waste.
11. Seal radiolabeled blot in plastic wrap and expose to film or a phosphorimager screen.

3.4 *In Vitro* Protein Priming Assay

For priming, the buffer and labeled nucleotides are added to the beads, and priming and/or DNA synthesis can then occur. Due to the use of HBV polymerase itself as the primer, it becomes a radio-labeled protein, which can be visualized by autoradiography. The labeled RT is run on 9% SDS-PAGE gel, and the gel is dried and exposed to film (Fig. 1d).

1. To a bead aliquot (approximately 10 μL beads and 10 μL residual buffer), add 19 μL of either the TMgNK (*see* **Note 41**) or TMnNK (*see* **Notes 42** and **43**) priming buffer.
2. Add 1 μL of radiolabeled nucleotides or 1 μL of a 100 μM solution of unlabeled nucleotides (*see* **Note 44**). Additionally, you may include nucleoside analogs or other RT inhibitors (*see* **Note 45**).
3. Shake at room temperature for 4 h (*see* **Notes 36** and **46**).
4. Pellet the beads at 350×*g* for 1 min. Remove supernatant to radioactive waste (*see* **Note 47**).
5. Wash each sample once with 500 μL of TNK wash buffer. Remove the supernatant to radioactive waste.
6. Add 20 μL 2× SDS lysis buffer. Boil for 5 min, vortex, and boil for another 5 min. Spin briefly to collect condensate (~4000×*g* for 1 s).
7. Load 20 μL of each sample on a 9% SDS-PAGE gel.
8. Place the gel onto a piece of filter paper and cover with plastic wrap. Dry for 3 h on a vacuum gel drying apparatus at 75 °C (*see* **Note 48**).
9. Expose the dried gel to a film or phosphorimager screen.

3.5 *Tdp2* Cleavage Assay

Tdp2 mediates the release of DNA which is covalently linked to HBV polymerase, and the released nucleotides and labeled polymerase are detected by autoradiography (Fig. 1e).

1. Perform the priming assay as described above (*see* **Note 42**). Complete a priming assay with sufficient aliquots of protein so that each condition can be mock treated and *Tdp2* treated (*see* **Note 49**).
2. Wash the priming reaction three times with 500 μL TNK wash buffer.
3. Wash twice with 100 μL 1× *Tdp2* wash buffer.
4. Add 10 μL of *Tdp2* reaction mix (which includes the *Tdp2* enzyme or mock buffer) to each sample.

5. Incubate at 37 °C for 1 h and with shaking (*see Note 50*).
6. After the incubation, spin for 1 min at 350 × *g*.
7. Separate the supernatant (released nucleotides) into a new tube and save the bead pellet (protein). Samples may be stored at −80 °C until testing.
8. For testing the supernatant (released nucleotides): Add 5 μL 2× formamide gel loading buffer II to a 5 μL aliquot. Heat to 95 °C for 5 min by boiling or with a thermal cycler. Load all 10 μL of the samples while still hot onto a 20% acrylamide 8 M urea-PAGE gel in 1× TBE (*see Note 51*). Expose the gel to film or a phosphorimager screen.
9. For testing the bead pellet (protein): Add 20 μL of 2× SDS lysis buffer, boil 5 min, vortex, and boil another 5 min. Spin briefly to collect condensate (~4000 × *g* for 1 s), and load 10 μL of each sample on a 9% SDS-PAGE gel. Expose the gel to a film or phosphorimager screen.

4 Notes

1. All autoclavable buffers that come into contact with HBV polymerase during experimentation should be autoclaved to avoid polymerase degradation (the polymerase tends to be very sensitive to degradation). After 2 months of storage and use of autoclaved reagents, re-autoclave the buffers.
2. Polyadenylation of this ε RNA can occur at the strong bovine growth hormone (BGH) poly(A) site on pcDNA3, 223 nucleotides downstream from the native HBV poly(A) site. The native site (1915–1919) is used infrequently since it is inherently weak due to its proximity to the 5' cap. Both RNA species can be seen on RNA gels.
3. DEPC-treated water or other molecular biology grade nuclease-free water should be used.
4. When using powdered chemicals under RNase-free conditions, standard (not certified RNase-free) weigh boats and disposable spatulas may be used, but a new package should be used which is kept separate and only for RNA work. When possible, weighing should be done in the tube and not on a weigh boat. If desired, certified RNase-free boats or spatulas are available.
5. When using commercially available liquids under RNase-free conditions, an unopened bottle is labeled for RNA work and then only used for such. RNA work should be done in a dedicated area, or an area cleaned by an RNase-removal product. Clean pipets with 70% ethanol or RNase removal products before beginning work. Use dedicated tip boxes, and keep samples in storage boxes set apart for RNA work.

6. PMSF is always added last, and never placed on ice with other components, since it will precipitate out of solution at colder temperatures.
7. Some buffers need not be RNase-free for the purpose of their use, but as a matter of continuity and good practice they are made with DEPC-treated water and kept RNase-free.
8. Non-isotopic labeling was not tested, but could be a viable option.
9. Manganese solutions are slightly pink. Freeze thaw cycles tend to turn the solutions a dark brown. This coloration appears to have little effect on the priming reaction.
10. Although all experiments were performed using this cell line, any human or mammalian cell line that supports transfection could potentially work.
11. Passage number appeared to play an important role in HBV polymerase expression. After reviving a batch of HEK293T cells, the cells were passaged for at least 3 weeks before transfection. Cells were then frozen down at this stage for later revival. If cells were transfected a week after reviving, HBV polymerase expression tended to be lower. HEK293T cells were passaged and used for transfection to express HBV polymerase for 3 months after reviving. If cells were used for transfection after this period, RT expression decreases dramatically.
12. The number and size of plates can be determined empirically and according to need. A good starting point may be single 10 cm dishes (yield is five bead aliquots), three 6 cm dishes (all transfected with the same plasmids and pooled together when lysed, yield is six bead aliquots), or larger plates when an increased amount of purified protein is desired. The lysis protocol is written for 6, 10, and 15 cm dishes, but could be scaled. An advantage of pooling together smaller plates is to reduce variability of transfection efficiency. A good practice is to plate several extra plates in preparation for transfection, and choose the best plates to transfect, discarding any plates that are not at the same confluency, etc. as the other plates.
13. Any transfection method could potentially work. Transfection efficiency can approach 90% with HEK 293T cells using the calcium phosphate method.
14. The ability to use wild-type or mutant plasmids is an advantage of this protocol. Both the HBV polymerase and/or the HBV epsilon RNA construct can be altered by introducing mutations on the plasmids.
15. A separate plate transfected in parallel with a GFP-expressing plasmid may be included. This GFP plate can be used to gauge

the transfection efficiency. It can also be lysed with the other plates to control for nonspecific binding to the beads.

16. When freezing the plates, perform the PBS wash as shown in **step 1** of the lysis procedure. Leave the plates tilted for 1 min and remove all PBS. Freeze the plates in a single layer (not stacked) in a -80°C freezer, and once chilled they can be stacked. When beginning with **step 2**, place the plates on ice to continue the lysis procedure.
17. If desired, the unbound antibody can be saved and used in anti-FLAG western blotting to determine HBV polymerase levels in the transfected cell lysates.
18. The wash steps may be performed with a wash buffer that includes protease and RNase inhibitors; however, since these antibody-bound beads are only exposed to samples that already contain these inhibitors, it is unnecessary.
19. HEK 293T cells may exhibit weak attachment to cell culture plates. Wash by spraying the liquid slowly onto the side, not the bottom of the plate, being very careful not to detach the cells during the wash steps. If cells do become detached, they can be recovered by pelleting the suspended cells—centrifuge at $1000\times g$ at 4°C for 5 min.
20. Transfer cells out of cell culture hood before or after the first wash. Note that this and all subsequent steps are on ice, and are performed under RNase-free conditions.
21. This NP-40-based lysis contains mainly cytoplasm in the supernatant and nuclei in the pellet.
22. Before transferring all of the supernatant to the antibody-bound beads, it is very helpful to save $\sim 100\ \mu\text{L}$ of supernatant and the insoluble pellet at -80°C . These can be stored in a single or separate tubes. The pellet and/or supernatant can be used if additional analysis and troubleshooting are needed. Analysis of the lysate supernatant is recommended for the ϵ RNA northern blot (the *in vivo* RNA binding assay), where it is useful to run RNA extracted from both lysate supernatant and beads on the same gel.
23. The unbound supernatant can be saved at -80°C for troubleshooting the immunoprecipitation.
24. During the centrifugation steps, prepare and label tubes for bead aliquots and store the empty tubes on ice for a cold transfer. The number of bead aliquot tubes needed may vary, but include at a minimum one tube for a western blot to verify RT expression, and one for a northern blot to evaluate the amount of ϵ RNA bound to the beads. Other tubes may be used for magnesium priming, manganese priming, the Tdp2 assay, or other assays.

25. After the final wash, it is not necessary to remove the wash buffer before freezing. However, before any downstream assay remove as much wash buffer as you are comfortable removing without disturbing the beads in a manner that leaves the same volume in all samples, approximately 10 μ L beads and 10–20 μ L wash buffer.
26. Purified HBV polymerase is stable at -80°C for years, although it is recommended that samples be used promptly after expression.
27. The expression of HBV polymerase varies from experiment to experiment, ranging from approximately 20–100 ng per 6 cm dish.
28. HBV polymerase is bound to host proteins which can be detected by staining or western blot. Expression of HBV polymerase itself can be verified by an anti-FLAG western blot.
29. The acrylamide percentage can be adjusted depending on what proteins are to be detected; 9% is apt for the large RT protein, which is approximately 94 kDa.
30. The same anti-FLAG antibody used in the immunoprecipitation should be used for western blotting to evaluate HBV polymerase levels in the transfected cells. A suggested amount is a 1:2000 dilution of primary antibody, and a 1:20,000 dilution of anti-mouse secondary antibody.
31. The template used is a DNA oligonucleotide coding for the ϵ RNA sequence annealed to an SP6 promoter [19]. Other constructs such as plasmid-based expression constructs should work as well.
32. Negative controls may include immunoprecipitated HBV polymerase that is deficient in RNA binding or RNA constructs that cannot bind HBV polymerase [5, 10].
33. If desired, transcribed labeled RNA can be visually verified on a 1.5% agarose gel in $1\times$ TAE under RNase-free conditions. Use an appropriate RNA ladder to evaluate size.
34. The RNA binding assay, when performed in an *in vitro* manner, would use beads from a single-plasmid transfection of HBV polymerase alone (and not a co-transfection of polymerase and ϵ RNA). The *in vivo* RNA binding assay uses co-transfected HBV polymerase and ϵ RNA.
35. Inhibitory compounds can be tested with this assay. They should be added prior to addition of ^{32}P -labeled ϵ RNA.
36. Place a vortexer behind a shield, set between 2 and 3. The beads should be moving and not sitting at the bottom of the tube. Another method is to shake by hand every 5–10 min to keep the beads suspended.

37. As controls for the washing procedure, you may desire to save the first and fifth wash to run alongside the washed samples.
38. Controls may include the input ϵ RNA (before binding) at a 1:100 dilution, the unbound ϵ RNA at a 1:100 dilution, and the washes (*see* **Note 37**). To prepare these controls, add 30 μ L of 2 \times SDS lysis buffer to 30 μ L of the indicated sample. Boil 5 min, vortex, and boil 5 more minutes. Load 30 μ L onto a 15% SDS-PAGE gel.
39. This will reveal the amount of ϵ RNA co-immunoprecipitated with the HBV polymerase. This is referred to as an *in vivo* RNA binding assay. It is useful to run both beads and lysate supernatant on the same gel to reveal the amount of ϵ RNA produced in transfected cells compared to the amount bound by the RT.
40. The same pCMV-HE plasmid used to transfect can be used to make an ϵ RNA-specific antisense probe. The probe is a 1389 bp fragment purified from an NcoI digest under RNase-free conditions. This purified fragment is used in an SP6 *in vitro* transcription using radiolabeled [α -³²P] UTP.
41. Priming occurs in a template-directed manner when using magnesium as the enzyme cofactor. The template on the ϵ RNA is UUC, which is copied as a dGAA trinucleotide. Therefore, if using a single nucleotide with magnesium priming, it should be dGTP.
42. For manganese-dependent transferase activity, the HBV RT prefers TTP, and this radiolabeled nucleotide will give the strongest signal. TTP is also ideal for the Tdp2 cleavage assay.
43. When including manganese as the enzyme cofactor, the RT can perform a transferase reaction, which is random DNA synthesis that is not necessarily template directed. The manganese-dependent transferase reaction can thus be performed with beads from HBV polymerase alone (from a single-plasmid transfection), as opposed to beads from a co-transfection expressing both HBV polymerase and ϵ RNA. When performing transferase activity, HBV polymerase will synthesize homo- and hetero-oligomeric or polymeric DNA strands up to several hundred nucleotides in length.
44. These should be the triphosphate form of the nucleotide or drug compound. When inhibitors are in the triphosphate form, they can be evaluated for their ability to terminate polymerization. Termination can also be tested by using dideoxynucleotide triphosphates (ddNTPs) which lack a 3' -OH.
45. Any inhibitory compound should be added before addition of nucleotides.

46. A series of two reactions can be performed instead of a single 4 h reaction. When performing two reactions, incubate the first reaction for 2 h, then spin and remove supernatant. Wash twice with TNK wash buffer, and apply the second round of nucleotides or other compounds in fresh TMgNK or TMnNK buffer. Incubate a further 2 h and continue the protocol. For example, to study the addition of the dAMP in the synthesis of the dGAA oligomer, add unlabeled dGTP in the first reaction, then [α - 32 P] dATP—this will evaluate if polymerization occurs after the initiation step. In another example, nucleoside inhibitors can be added in the first round, then [α - 32 P] dATP—this will evaluate if the incorporated drug can inhibit polymerization.
47. At this point, the priming products may be visualized directly as the labeled polymerase protein or alternatively, the labeled nucleotides or DNA oligomers can be released from the polymerase by Tdp2 cleavage and subsequently visualized.
48. The drying step could potentially be skipped if a gel drying apparatus is not available. In this case, keep the gel well sealed to avoid drying, such that the gel does not shrink and move relative to the film or phosphorimager screen during exposure.
49. Testing the protein is only useful in the context of the mock-treated sample. There should be a decrease in signal in the Tdp2-treated protein when compared to the mock-treated protein. This is due to the radiolabeled nucleotides being cleaved from the RT.
50. Place a vortexer behind a shield in a 37 °C incubator, set between 2 and 3. The beads should be moving and not sitting at the bottom. Another method is to incubate in a water bath and shake by hand every 5–10 min to keep the beads suspended.
51. Pre-run gels for 30 min and flush out wells before loading. For a sequencing-style gel (40×16 cm with 1 mm spacers), run at 2500 V for approximately 2 h, or until the bromophenol blue dye front has migrated approximately 2/3 the length of the gel. For a smaller gel (16×17 cm with 2 mm spacers), run at 300 V for approximately 2 h, or until the bromophenol blue dye front has migrated approximately 2/3 the length of the gel.

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In Vitro Enzymatic and Cell Culture-Based Assays for Measuring Activity of HBV RNaseH Inhibitors

Elena Lomonosova and John E. Tavis

Abstract

HBV is a small, enveloped DNA virus that replicates by reverse transcription via an RNA intermediate. Current anti-HBV treatment regimens that include interferon α and nucleos(t)ide analogs have insufficient efficiency, are of long duration and can be accompanied by systemic side effects. Though HBV RNaseH is essential for viral replication, it is unexploited as a drug target against HBV. RNaseH inhibitors that actively block viral replication would represent an important addition to the potential new drugs for treating HBV infection. Here we describe two methods to measure the activity of RNaseH inhibitors. DNA oligonucleotide-directed RNA cleavage assay allows low-throughput screening of compounds for potential anti-HBV RNaseH activity in vitro. Analysis of preferential inhibition of plus-polarity DNA strand synthesis by HBV RNaseH inhibitors in a cell culture model of HBV replication can be used to validate the efficiency of these compounds to block viral replication.

Key words Hepatitis B virus, Ribonuclease H, Inhibitors, DNA-oligonucleotide directed RNA cleavage assay, HBV core particle DNA, Quantitative PCR

1 Introduction

The hepatitis B virus (HBV) is small, enveloped DNA virus that replicates by reverse transcription via RNA intermediate [1–3]. The encapsidated viral genome consists of a 3.2 kb partially double-stranded relaxed circular DNA (rcDNA). HBV is hepatotropic virus and upon hepatocyte entry the DNA-containing core particle is translocated into the nucleus where the rcDNA is converted into a covalently closed circular DNA (cccDNA). The cccDNA is the template for viral mRNA and pre-genomic RNA (pgRNA) synthesis [4] and is responsible for HBV persistence [5]. It is believed that persistence of cccDNA in infected cells is the major mechanism of HBV chronicity [6, 7].

HBV infection remains a major public health problem despite the availability of a prophylactic vaccine [8–10]. More than 250 million people worldwide are chronically infected with HBV [11]

and are at an increased risk for developing end-stage liver disease and hepatocellular carcinoma [12].

Treatment of HBV infection includes the use of IFN- α and nucleos(t)ide analog chain terminators such as lamivudine, adefovir, tenofovir disoproxil fumarate, and entecavir [13, 14]. While these drugs can slow progression of HBV-induced disease, they are only partially effective and only very rarely lead to a full elimination of viral infection [15–17]. For example, the first direct-acting anti-HBV drug, lamivudine, is very effective and well tolerated [18]. However, after 5 years of therapy 75% of the treated patients develop resistant HBV strains [19]. Moreover, due to the overlapping polymerase and HBsAg open reading frames some of the resistance mutations also led to mutations to the HBsAg that can cause reduced binding to anti-HBs antibody [20]. Clearly, new drugs with different targets and mechanisms for anti-HBV therapy are urgently needed.

Promising therapeutic approaches that directly target the virus-infected cells, as well as immunotherapeutic strategies that activate the HBV-specific adaptive immune response or innate intrahepatic immunity are being developed [9, 21–27]. The HBV polymerase, the only protein with enzymatic activity encoded by the virus, is a multifunctional enzyme. The reverse transcriptase (RT) domain possesses nucleotidyl transfer activity and synthesizes viral DNA during reverse transcription of single-stranded pre-genomic RNA to rcDNA. The RNaseH domain of the HBV polymerase hydrolyzes the RNA strand of RNA–DNA hybrids that are generated during reverse transcription to enable synthesis of double-stranded DNA. Both activities are necessary for viral replication; however, the current clinically available direct-acting anti-HBV drugs—the nucleos(t)ide analogs—target HBV RT, whereas RNaseH inhibitors are yet to be developed. Therefore, RNaseH is an attractive target for new anti-HBV drugs that might provide new approach to treat patients resistant to currently available anti-HBV therapies as well as may be used in combination with current drugs to increase effectiveness of treatment and prevent the development of resistance [28]. Recently we developed a low throughput screening strategy using recombinant HBV RNaseH and identified several promising compounds that effectively inhibit both HBV RNaseH activity and viral replication [29–33].

This chapter describes two protocols for measuring activity of HBV RNaseH inhibitors. The first is used for initial screening of compounds for anti-HBV RNaseH activity *in vitro* in a DNA oligonucleotide-directed RNA cleavage assay [29, 33]. In this assay, the activity of recombinant HBV RNaseH is determined by its ability to cleave a ^{32}P -labeled RNA substrate in the form of an RNA–DNA heteroduplex. The RNA fragment products of the reaction are then resolved by urea polyacrylamide gel electrophoresis and detected by autoradiography (Fig. 1a). Inhibition of RNaseH

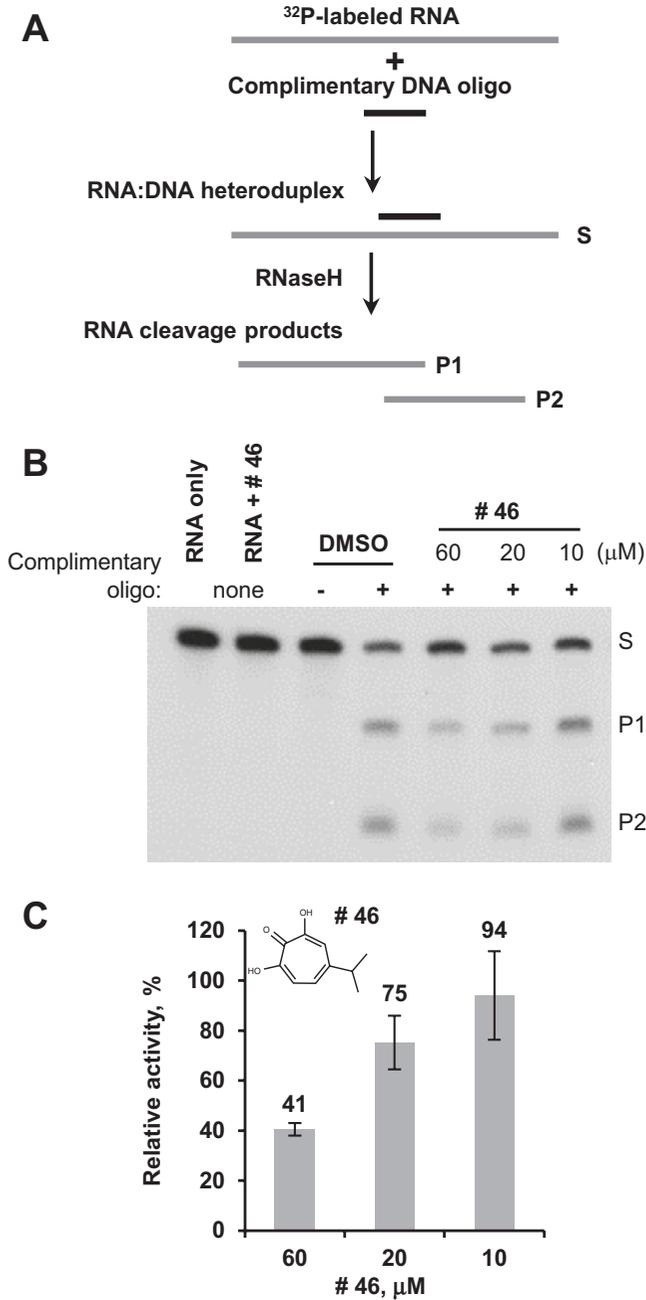


Fig. 1 Oligonucleotide-directed RNA cleavage assay. (a) Schematic representation of oligonucleotide-directed RNA cleavage assay. Internally radiolabeled RNA is bound to a complementary DNA oligonucleotide to form the RNA–DNA heteroduplex substrate for RNaseH. The RNaseH cleaves the RNA within the RNA–DNA heteroduplexes. RNA, *grey line*; DNA, *black line*; S, substrate; P1 and P2, RNaseH cleavage products of RNA. (b) Example of primary compound screening assay for HBV RNaseH inhibition by an oligonucleotide-directed RNA cleavage assay. Activity of HBV RNaseH is assessed in the presence of different concentration of compound of interest (compound # 46, β-thujaplicinol is shown) or in the presence of DMSO as a vehicle control. Reaction products are resolved by denaturing polyacrylamide electrophoresis and detected by autoradiography. S, substrate, ³²P-labeled 264 nt RNA derived from the Duck Hepatitis B Virus genome (DRF+ RNA); P1, cleavage product 1;

activity is assessed by quantifying the intensity of cleaved RNA bands after compound addition to the reaction mixture and comparing to the activity of vehicle-treated enzyme (Fig. 1b and c). The second protocol is used to measure inhibition of HBV replication in cell culture by RNaseH inhibitors [33]. This method includes isolation of DNA from HBV core particles followed by measurement of preferential inhibition of plus-polarity DNA strand synthesis by quantitative PCR (Fig. 2). This is because during HBV replication inside of viral capsid particles the plus-polarity DNA can only be made when the RNaseH removes the pgRNA from newly synthesized minus-polarity DNA strand and generates the short RNA primer for synthesis of plus-polarity DNA strand [34–37]. Thus, the amount of plus-polarity DNA is strictly dependent on HBV RNaseH activity. In contrast, synthesis of the minus-polarity DNA strand is largely unaffected by RNaseH inhibitors [29–32, 38]. Antiviral activity of RNaseH inhibitors is tested by treating cells replicating HBV (e.g., HepDES19 cells, which contain a tetracycline-repressible expression cassette for a replication-competent HBV genotype D genome [39]) with the compounds, and determining the amount of HBV minus- and plus-polarity DNA strands in core particles [31, 32].

2 Materials

1. 10% dimethyl sulfoxide (DMSO).
2. Diethylpyrocarbonate (DEPC) H₂O (*see Note 1*).
3. 10× RH buffer: 500 mM Tris–HCl, pH 7.5, 50 mM MgCl₂, 1000 mM NaCl, 20 mM DTT (*see Note 2*).
4. 0.5% octylphenoxy poly(ethyleneoxy)ethanol (NP-40).
5. RNaseOut™ Recombinant Ribonuclease Inhibitor (Life Technologies).
6. 5× TBE buffer: 1.1 M Tris–HCl, 900 mM boric acid, 25 mM EDTA, pH 8.3.
7. In vitro-transcribed using MEGAscript® Kit (Ambion) ³²P labeled RNA (DRF+) [40], sequence:

Fig. 1 (continued) P2, cleavage product 2; “–”, a noncomplementary DNA oligonucleotide as a specificity control; “+”, complementary DNA oligonucleotide; RNA only, includes RNA but no enzyme as a control for RNA quality; RNA+ #46, control for compound solution purity and the lack of compound-induced nonspecific RNA cleavage, no enzyme present. **(c)** Example of quantitation of compound anti-HBV RNaseH activity. The intensity of P2 band was quantified using ImageJ. Nonspecific background values were determined from the incorrect oligonucleotide negative control lane and subtracted from all experimental values. Relative activity of HBV RNaseH is presented as the percentage of intensity of the P2 band with compound present in the reaction relative to the intensity of P2 band in the control vehicle-containing reaction. Data are mean values ± one standard deviation from three independent experiments. Insert, structure of compound #46, β-thujaplicinol

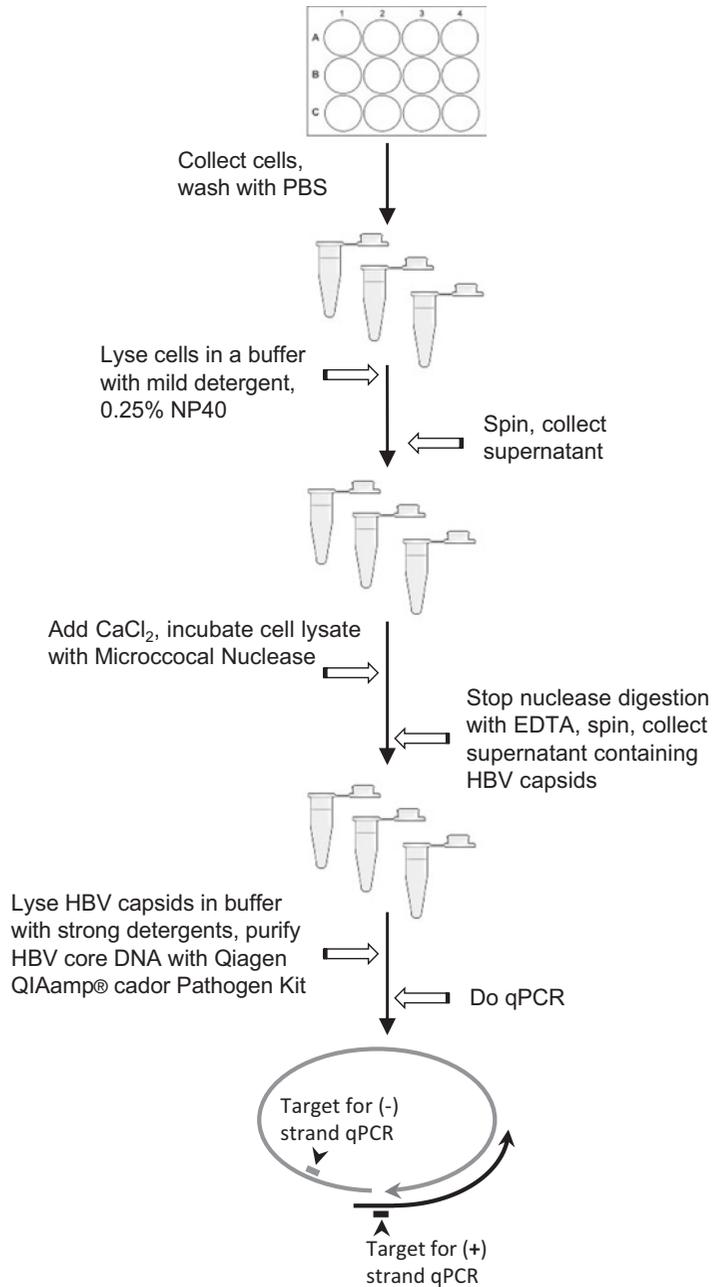


Fig. 2 Replication inhibition assay. Plus-polarity DNA is measured by amplifying HBV DNA across the gap in the minus-polarity DNA strand. Minus-polarity DNA strands are measured by amplifying sequences downstream of the 3' end of the vast majority of plus-polarity DNA strands in viral capsids

5'GGGAACAAAAGCUUGCAUGCCUGCAGGU
CGACUCUAGAGGAUCCCCACUUUGUCCCGAGCA
AAUAUAAUCCUGCUGACGGCCCAUCCAGGCACAGA
CCGCCUGAUUGGACGGCUUUUCCAUAACACCC

CUCUCUCGAAAGCAAUAUAUAUUCAC
 AUAGGCUAUGUGGAACUUAAGAAUACACCC
 CUCUCCUUCGGAGCUGCUUGCCAAGGUAUCU
 UUACGUCUACAUUGCUGUUGUCGUGUG
 UGACUGUGGGUACCGAGCUCG' (*see Note 3*).

8. Complementary DNA oligo, D2507: 5'GTTCCACATAG CCTATGTGG3'; noncomplementary control DNA oligo, D2526: 5'CCACATAGGCTATGTGGAAC3'; 1 µg/µL (*see Note 4*).
9. 6% Sequencing Acrylamide solution, for 100 mL: 5× TBE, 20 mL, 5.7 g acrylamide, 0.3 g *N,N'*-methylenebisacrylamide, 48 g urea, H₂O to 100 mL (*see Note 5*).
10. Ammonium persulfate (APS): 10% solution in water (*see Note 6*).
11. *N,N,N',N'*-tetramethylethylenediamine (TEMED).
12. Sequencing loading buffer: 98% formamide, 10 mM EDTA, 0.025% xylene cyanol, 0.025% bromophenol blue (*see Note 6*).
13. 1 M Tris-HCl, pH 7.5.
14. 0.5 M ethylenediamine tetraacetic acid (EDTA), pH 8.0.
15. 5 M NaCl.
16. 1 M CaCl₂.
17. Core preparation lysis buffer: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.25% octylphenoxy poly(ethyleneoxy)ethanol (NP-40), 50 mM NaCl, and 8% sucrose.
18. Micrococcal nuclease (MN) (2000 U/µL, NEB #M0247S), MN buffer (NEB #B0247S).
19. QIAamp® *cad*® Pathogen Mini Kit (Qiagen, Cat. No. 54104).
20. TaqMan® Fast Advanced Master Mix (Applied Biosystems, Cat. No. 4444556).
21. Primers and probe (IDT Inc.) for the plus-polarity HBV DNA strand: 5'CATGAACAAGAGATGATTAGGCAGAG3'; 5'GGAGGCTGTAGGCATAAATTGG3'; 5'/56-FAM/CTGCGCACC/ZEN/AGCACCATGCA/3IABkFQ.
22. Primers and probe for the minus-polarity HBV DNA strand: 5'GCAGATGAGAAGGCACAGA3'; 5'CTTCTCCGTCTGCCGTT3'; 5'/56-FAM/AGTCCGCGT/ZEN/AAAGAGA GGTGCG/3IABkFQ.

3 Methods

3.1 Oligonucleotide-Directed RNA Cleavage Assay

1. Thaw frozen compound solutions. Vortex, then briefly centrifuge. Dilute test compound to 10× desired concentration in 10% DMSO in DEPC H₂O.

2. Prepare Master Mix. Calculate the total volume required for each component: Volume for one reaction \times the total number of reactions.

Reaction mixture for one reaction:

3.5 μL DEPC H_2O .

2 μL 0.5% NP-40.

2 μL 10 \times RH buffer.

0.5 μL RNaseOut.

3 μL DNA oligo, 1 $\mu\text{g}/\mu\text{L}$.

2 μL Test compound or 10% DMSO.

6 μL HBV RNaseH.

1 μL RNA, 0.02 $\mu\text{g}/\mu\text{L}$.

Assemble Master Mix in the following order: H_2O , NP-40, RH buffer, RNaseOut. Add all components to 1.5 mL microcentrifuge tube, vortex briefly to mix, then centrifuge the tubes briefly to spin down the contents and eliminate air bubbles.

3. Assemble reactions on ice. Aliquot 8 μL Master Mix to each reaction tube. Add 3 μL of the appropriate DNA oligo to each reaction tube. Add 2 μL test compound or DMSO to each reaction tube. Add 6 μL HBV RNaseH protein to each reaction tube (*see Note 7*). Vortex tubes to mix, then briefly centrifuge the tubes. Initiate the reaction by adding 1 μL of ^{32}P -radiolabeled RNA diluted 10 \times with DEPC H_2O immediately before the experiment to each reaction tube, so that the final concentration of RNA in the reaction is 0.001 $\mu\text{g}/\mu\text{L}$. Vortex briefly to mix, then centrifuge the tubes briefly to spin down the contents and eliminate air bubbles (*see Note 8*).
4. Incubate reactions at 42 $^\circ\text{C}$ for 90 min.
5. Stop the 20 μL RNaseH reactions with 80 μL 1 \times formamide loading buffer per reaction.

3.2 Denaturing Polyacrylamide Gel Electrophoresis

1. Warm the 6% Sequencing Acrylamide solution (6% acrylamide/bis-acrylamide [19:1]/6 M urea in 1 \times TBE) to near room temperature and check to be sure the urea has not crystallized before pouring a gel.
2. Set up a vertical mini-gel. Prepare 6% Sequencing Acrylamide mix—for each 10 cm \times 11 cm mini-gel mix 20 mL 6% Sequencing Acrylamide solution, 150 μL 10% APS and 20 μL TEMED. Pour a single phase gel and insert the comb. Let the gel solidify at room temperature, the gel will be ready to use in approximately 40 min.
3. Remove casting clamps, rinse the plates carefully with dH_2O and remove the comb. Mount the gel in a mini-gel rig, using 1 \times TBE as the running buffer. Immediately rinse the residual unpolymerized acrylamide and urea from the wells using a syringe and needle.

4. Pre-run the gel for 5 min at 40 mA (220–230 V).
5. Heat samples to >90 °C for 3–5 min and chill them immediately on ice. Rinse the wells in the gel again with a syringe and needle to remove urea that has diffused into the wells. Promptly load 50 μ L of sample per well.
6. Electrophorese at 40 mA (220–230 V) until bromophenol blue is near the bottom of the gel. Cut the bromophenol blue band from the bottom of the gel and discard it in the radioactive waste.
7. Soak gel in dH₂O with shaking for 30 min to remove the urea. Change the water two or three times during the soaking phase.
8. Dry the gel at 80 °C under vacuum for 1 h.
9. Detect radiolabeled RNA by standard autoradiography. Analyze film images and quantitate using ImageJ software (<http://rsb.info.nih.gov/ij/>).
10. Calculate inhibition activity of compounds as the percentage of intensity of P2 band (Fig. 1b) with compound present in the reaction relative to the intensity of P2 band in the control vehicle containing reaction as follows: % Inhibition = $100\% - [(relative\ intensity\ of\ P2\ band\ in\ the\ presence\ of\ inhibitor / relative\ intensity\ of\ P2\ band\ in\ control\ vehicle\ treated\ sample) \times 100\%]$ (*see* **Note 9**).

3.3 Cell Culture and Compound Treatment

1. *Day 0*. Plate 0.3×10^6 HepDES19 (tetracycline-inducible, tet-off) cells [39] per well in 12-well plates in 2 mL of DMEM/F12 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin plus 1 μ g/mL tissue-culture grade tetracycline.
2. *Day 1*. 24 h later replace medium with 2 mL fresh medium without tetracycline to induce HBV replication.
3. *Day 3*. 48 h later replace medium with 1 mL medium without tetracycline containing the desired concentration of the test compounds. Balance the dimethyl sulfoxide (DMSO) concentration for all samples and limit DMSO to $\leq 1\%$ v/v. Also use DMSO as vehicle control, final concentration $\leq 1\%$.
4. *Day 4*. Repeat compound treatment. Change the medium 24 h later with medium containing the test compounds without tetracycline (*see* **Note 10**).
5. *Day 5*. Repeat compound treatment. Change the medium 24 h later with medium containing the test compounds without tetracycline.
6. *Day 6*. 24 h later harvest the cells. Aspirate the medium from the wells. Wash cells two times with phosphate-buffered saline (PBS), add 0.5 mL of trypsin (0.05%), incubate 5 min at 37 °C, and collect cells into 1.5 mL tubes. Spin cells at room

temperature, 2 min at $2000\times g$. Carefully aspirate supernatant, wash cell pellets with 1 mL PBS. Again, spin cells at room temperature, 2 min at $2000\times g$. Carefully aspirate supernatant, and loosen the pellet by vortexing.

3.4 Extraction of HBV Core Particles DNA

1. Add 0.2 mL core preparation lysis buffer to the cell suspensions to lyse the cells, vortex thoroughly, and incubate at $37\text{ }^{\circ}\text{C}$ for 10 min (*see Note 11*).
2. Add 2 μL of 1 M CaCl_2 to each tube (*see Note 12*).
3. Centrifuge at $21,000\times g$ for 5 min at room temperature. Transfer supernatants into new tubes.
4. Prepare 80 U/ μL solution of micrococcal nuclease in MN buffer. Add 5 μL of micrococcal nuclease solution to each tube. Incubate at $37\text{ }^{\circ}\text{C}$ for 60 min (*see Note 13*).
5. Centrifuge at $21,000\times g$ for 5 min at room temperature. Transfer supernatants into new tubes.
6. Terminate nuclease digestion by adding 20 μL of 0.5 M EDTA per tube. Mix carefully (*see Note 14*).
7. Extract nucleic acid from core particles using the QIAamp[®] *cador*[®] Pathogen Mini Kit according to the manufacturer's instructions with modifications described below in **steps 8–12**.
8. Add 20 μL proteinase K into each tube with core particles, vortex, and then briefly centrifuge.
9. Add 100 μL Buffer VXL with Carrier RNA in it (1 μg Carrier RNA per 100 μL Buffer VXL) into each tube, vortex.
10. Incubate overnight (16 h) at $37\text{ }^{\circ}\text{C}$ (*see Note 15*).
11. Proceed according to the protocol from QIAamp[®] *cador*[®] Pathogen Mini Handbook, p. 21 (<https://www.qiagen.com/us/resources/resourcedetail?id=dd291a2f-f2ff-4768-ad97-8e2462fe58c0&lang=en>), starting at **step 6** of the manufacturer's protocol.
12. Elute core-associated nucleic acids with 50 μL buffer AVE. Store at $-75\text{ }^{\circ}\text{C}$.

3.5 Real-Time qPCR

1. Thaw frozen HBV core DNA samples on ice. Vortex, then briefly centrifuge.
2. Thoroughly mix the TaqMan[®] Fast Advanced Master Mix.
3. Calculate the total volume required for each component: volume for one reaction \times the total number of reactions.
Reaction mixture for one reaction:
10 μL TaqMan Universal PCR Master Mix.
0.2 μL Forward Primer, 20 μM .

- 0.2 μL Reverse Primer, 20 μM .
 0.4 μL TaqMan probe, 2 μM .
 5.2 μL H_2O .
 4 μL sample DNA.
4. Prepare two PCR Master Mix solutions (one for measuring (+) polarity strand of HBV core particles DNA and the second one for measuring (-) polarity DNA). Add all components, except for sample DNA, to 1.5 mL microcentrifuge tubes, vortex briefly to mix, then centrifuge the tubes briefly to spin down the contents and eliminate air bubbles.
 5. Add the appropriate volume of PCR Master Mix (16 μL) to each well of an optical reaction plate (MicroAmp Fast Optical 96-well reaction plate, Applied Biosystems, Cat. No. 4346906).
 6. Add 4 μL of HBV core DNA samples or standard HBV DNA (*see* **Note 16**) to each well, cover the reaction plate with Optical Adhesive Covers (Applied Biosystems, Cat. No. 4360954), then centrifuge the plate briefly to spin down the contents and eliminate air bubbles.
 7. Run the reaction. For the Applied Biosystems Real-Time PCR 7500 Fast system instrument, use the following parameters: 95 °C for 10', 40 cycles at 95 °C for 15", 60 °C for 1', and detection at 60 °C for 1" after each cycle.
 8. Calculate the amount (+) and (-) polarity strands DNA as the percentage relative to the quantity of DNA in DMSO-treated cells to estimate inhibitory activity of compounds (**Fig. 3**).

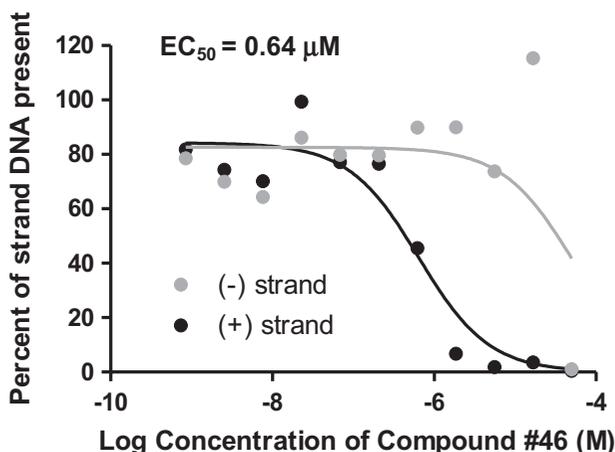


Fig. 3 Inhibition of HBV replication by RNaseH inhibitors. Inhibition of replication by compound #46 was measured against an HBV genotype D isolate in HepDES19 cells. The EC₅₀ value was calculated based on the decline of the plus-polarity DNA strand with GraphPad Prism using three-parameter log(inhibitor) vs. response nonlinear curve

4 Notes

1. DEPC treatment is the most commonly used method for eliminating RNase contamination from water and other solutions. DEPC destroys enzymatic activity by modifying -NH, -SH, and -OH groups in RNases and other proteins. To prepare DEPC water, add 1 mL of DEPC to 1 L of H₂O. Shake well to disperse the DEPC through the H₂O. Incubate at room temperature for at least 12 h, then autoclave on liquid cycle for 20 min to inactivate the remaining DEPC.
2. Prepare 10× RH buffer in relatively large batches and freeze aliquots at -20 °C.
3. Use the standard protocol provided by manufacturer for in vitro transcription and radiolabel RNA with [³²P] UTP. Please refer to the reference [40] for further information. Purify in vitro transcribed radiolabeled RNA using RNeasy MinElute™ Cleanup Kit (Qiagen). Determine concentration of RNA fluorimetrically using QuantiFluor® RNA System (Promega) and dilute to 0.2 µg/µL. Check quality by agarose electrophoresis. Store radiolabeled RNA at -75 °C in small aliquots.
4. You may use any other RNA and complementary DNA oligonucleotide. A noncomplementary oligonucleotide is included in the assays to determine specificity of RNaseH reaction for heteroduplexes and exclude the potential contribution of contaminating nucleases to the RNA cleavage reaction.
5. Unpolymerized acrylamide is a neurotoxin. Always wear personal protective equipment when handling acrylamide, avoid skin contact. Filter the acrylamide-urea solution before storage. Store at 4 °C protected from light. Urea can precipitate from the solution at 4 °C, warm the solution to dissolve urea crystals before preparing the gel. For more details about urea-PAGE *see* [41].
6. Store frozen at -20 °C in small aliquots.
7. We expressed the HBV RNaseH in *E. coli* as a C-terminally hexahistidine-tagged protein [29] or as a maltose-binding protein fusion with a hexahistidine tag at its C-terminus [33]. The HBV RNaseH is purified by nickel-affinity chromatography [29], dialyzed in buffer containing 50 mM Hepes, pH 7.3, 0.3 M NaCl, 20% glycerol, and 5 mM DTT, and stored under liquid nitrogen in small aliquots.
8. We recommend including a control reaction without HBV RNaseH into each experiment to monitor the quality of RNA and any unspecific cleavage that might occur due to contamination.

9. Quantitation of P1 band intensity is unreliable due to a 3'-5' exonuclease activity of RNaseH.
10. RNaseH inhibitors can be added only once at day 3 if desired. In this case cells are incubated for 2 more days without changing the media, and are collected as described at day 6. However, false negative results can arise from instability of a compound at 37 °C.
11. Core preparation lysis buffer is very mild cell lysis buffer that contain 0.25% of NP-40 and will only lyse cellular membrane but not affect HBV nucleocapsids that are resistant to NP-40 detergent [42] or disrupt the nucleus. Consequently, low speed centrifugation will separate the lysates from nucleus fraction and unbroken cells.
12. CaCl₂ is needed for subsequent treatment with micrococcal nuclease which activity is dependent on Ca²⁺ [43].
13. Micrococcal nuclease is a relatively nonspecific endo-exonuclease that digests double-stranded, single-stranded, circular and linear RNA and DNA substrates. The enzyme is active in the pH range of 7.0–10.0 as long as salt concentration is less than 100 mM [43]. Micrococcal nuclease treatment is necessary to remove any non-encapsidated RNA and DNA from the lysate that might later interfere with qPCR. HBV core DNA which is protected from micrococcal nuclease digestion by the viral capsid.
14. Addition of excess EDTA is necessary to inactivate micrococcal nuclease.
15. Buffer VXL contains strong detergents that will lyse HBV core particles and release rcDNA. Proteinase K will degrade proteins in the lysate as it exhibits broad substrate specificity and is active in the presence of detergents, and is routinely used to digest unwanted proteins in DNA preparations [44]. We use a long incubation time with Proteinase K to ensure the cleavage of HBV polymerase protein which is covalently attached to the (–) polarity strand HBV DNA. If not removed by digestion with protease, the HBV polymerase that is covalently bound to DNA causes binding to purification column and hence incomplete DNA elution [45].
16. Double-stranded HBV DNA template for a standard curve is prepared by isolating total DNA from HepDES19 cells using QIAamp DNA Mini Kit (Qiagen, Cat. No. 51304) and amplifying a 1388 base pair genomic region of HBV DNA with primers H2900+ (5'-CCGCTTGGGACTCTCTCGTCCC-3') and HPE180STOP- (5'-TCACCATGGGAAGCTTAC TCTTGTTCCCAAGAATATGG-3').

Acknowledgments

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Detection of Hepatitis B Virus Particles Released from Cultured Cells by Particle Gel Assay

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Abstract

The culture fluid of HBV replicating cells contains a mixture of viral particles with different structural and genetic components, including enveloped infectious virions, genome-free virion, envelope-only subviral particles, and nonenveloped naked capsids. Based on their different physical and chemical properties, the enveloped and nonenveloped particles can be separated by the native agarose gel electrophoresis and transferred onto a positively charged microporous membrane, then the virus particle-associated protein components and nucleic acid content can be detected by antibody-based enzyme immunoassay (EIA) and hybridization, respectively. Such convenient experimental procedure is called HBV particle assay and described in detail in this chapter. The particle gel assay can be used to study viral and host regulations of HBV virus morphogenesis and egress, and for antiviral assessment of HBV inhibitors as well.

Key words HBV particles, Particle gel assay

1 Introduction

Hepatitis B virus (HBV) is an enveloped DNA virus belonging to the *Hepadnaviridae* family [1]. The infectious HBV virion is comprised of a single copy of approximately 3.2 kb genomic relaxed circular DNA (rcDNA) within an icosahedral capsid (core) which is enveloped by a lipid bilayer shell studded with complexes of viral glycoproteins including “large” (L), “medium” (M), and “small” (S) surface proteins [2, 3]. The three HBV surface proteins are all synthesized from the same open reading frame of the viral genome by using different starting codons. They share the common S domain at the C-terminus. The M and L proteins contain S plus pre-S2 and S plus pre-S2 and pre-S1 domains, respectively. S protein is the major viral surface protein. An HBV virion contains approximately 100 copies of S protein for every five copies of M and one copy of L protein [2].

Besides infectious virion particles, two additional forms of HBV particles are routinely found in hepatitis B patient blood,

which are, the envelope-only particles (also called subviral particles) and the DNA-free empty virions (enveloped empty capsid) [4]. Several groups have demonstrated that HBV virion and subviral particles egress the host cell by different routes. The budding of 42-nm-diameter virion particles takes place at intracellular membranes through the multivesicular bodies (MVB) pathway, a process dependent on the endosomal sorting complex required for transport (ESCRT) system that consists of the ESCRT-0, -I, and -III complexes together with the Vps4 ATPase and other associated proteins [5–7]. The production of virions is always accompanied by the formation of subviral particles, which are 20-nm-diameter spherical and filamentous particles consisting only of lipid and HBV envelope proteins, predominantly S protein [8, 9]. HBV subviral particles are assembled on the ER membrane, and secreted through the constitutive ER-Golgi secretory pathway [10]. Normally, a HBV infected cell secretes excess amounts of empty virions over infectious virions (usually ≥ 100 -fold) [11], and hundreds to thousands of subviral particles for every virion [12]. Both DNA-free enveloped particles are known as hepatitis B surface antigen (HBsAg) and likely assumed to serve as immunological decoy to mask the bona fide infectious virions from host immune surveillance and attack [11]. In addition to enveloped particles, nonenveloped capsids (naked capsids) have also been found to be noncytolytically released into the culture fluid of HBV-replicating cells usually at a large excess to virions, though they are not detected in the blood of infected patients [4, 13–15]. Although a number of host proteins have been implicated to regulate naked capsid egress, the secretion pathway for naked capsids and their biological significance remains elusive [16–18].

In order to study the morphogenesis and secretion of hepadnavirus particles, Lenhoff and Summers invented a native agarose gel based particle assay for duck hepatitis B virus (DHBV) [19], which was later adapted for the study of HBV and woodchuck hepatitis virus (WHV) [4, 13, 14, 18, 20]. The principle of particle gel assay is built upon the different migration rate of HBV particles in native agarose gel electrophoresis due to their different composition, particle size, and electric charge, which permits separation of enveloped particles from naked capsids. The virus particles are then transferred to a membrane for enzyme immunoassay (EIA) with antibodies against HBsAg or core. Furthermore, viral DNA within the nucleocapsids can be released in situ and detected by DNA hybridization. Currently, quantitative PCR (qPCR) is widely used to measure the amount of infectious virions (or virus genome equivalent, or vge) secreted from HBV-replicating cells, especially in antiviral assessment assays and preparation of HBV inoculum for infection assays. However, because it does not discriminate between virion particles and genome-containing secreted naked capsids, the qPCR will overestimate the levels of infectious vge.

Therefore, the particle gel assay also provides a convenient and reliable method for measuring the ratio between virion DNA and capsid DNA [21]. We describe herein a protocol for HBV particle gel assay in detail.

2 Materials

2.1 Precipitation of HBV Particle from Cell Cultured Media

1. 35 % PEG8000 in 1.5 M NaCl.
2. TNE buffer: 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1 mM EDTA.

2.2 Agarose Gel Electrophoresis

1. 10× DNA gel loading buffer: 10 mM EDTA (pH 8.0), 50 % (V/V) glycerol, 0.25 % (W/V) bromophenol blue.
2. 1× TAE buffer: 0.04 M tris base, 0.04 M glacial acetic acid, 1 mM EDTA (pH 8.2–8.4). Prepare 30× stock solution, store at room temperature.
3. Agarose (molecular biology grade).

2.3 Particle Gel Transfer

1. TNE buffer: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl and 1 mM EDTA.
2. Protran BA85 Nitrocellulose (NC) Blotting Membrane, 0.45 μm (GE Healthcare).
3. 3MM Chromatography (Chr) paper (GE Healthcare).

2.4 Enzyme Immunoassay

1. Polyclonal rabbit anti-HBV core antigen (Dako).
2. Monoclonal mouse anti-HBV surface antigen (Dako).
3. IRDye 800CW goat anti-rabbit IgG (H+L) (LI-COR Biosciences).
4. IRDye 800CW goat anti-mouse IgG (H+L) (LI-COR Biosciences).
5. WesternBreeze Blocker/Diluent (Part A and B) (Life Technologies).
6. WesternBreeze Wash Solution (Life Technologies).
7. Phosphate buffered saline (PBS).
8. Formaldehyde.
9. Methanol.
10. Odyssey Imager (LI-COR Biosciences).

2.5 HBV DNA Riboprobe

1. pGEM-3Z vector (Promega) with an insertion of genome-length HBV DNA fragment (EcoRI linearized), in which the transcription of HBV sense RNA is under the control of prokaryotic SP6 promoter.

2. 800 Ci/mmol [α - 32 P] UTP.
3. Riboprobe in vitro transcription system-Sp6 (Promega).
4. 5 M NH_4OAc .
5. 10 mg/mL yeast RNA (Ambion).
6. Isopropanol.
7. Formamide.

2.6 Southern Blotting and DNA Hybridization

1. Denaturing buffer: 0.5 M NaOH, 1.5 M NaCl.
2. Neutralization buffer: 1.5 M NaCl, 1 M Tris-HCl (pH 7.4).
3. UV cross-linker.
4. EKONO hybridization buffer (G-Biosciences).
5. Hybridization wash buffer: 0.1% SDS, 0.1 \times SSC.
6. Phosphorimager system (GE Healthcare).

3 Methods

3.1 Cell Cultures

1. Culture HBV-producing cells, such as transiently transfected HepG2 or Huh7 cells, or stable cell lines like HepG2.2.15 or tetracycline-inducible (tet-off) HepAD38 or HepDE19 cells, in collagen coated cell culture flask or plate at approximately 100% confluence with appropriate culture medium.
2. 24–48 h post transfection or seeding, change the culture medium with fresh medium. For tetracycline inducible cell lines, remove tetracycline from culture medium to induce HBV replication.
3. Change and harvest the culture medium at designed time interval for specific experiment. Clarify the harvested supernatant by centrifugation at 1000 $\times g$ for 10 min at 4 °C. Transfer the supernatant to a new tube, store at –80 °C if not used immediately.

3.2 HBV Particle Precipitation from Cell Cultured Media

1. Transfer 1 mL of harvested cell culture supernatant to a 1.7 mL Eppendorf tube. Add 400 μL of 35% PEG8000 to reach final concentration of 10%, and rotate the tube on a rotisserie for at least 2 h at 4 °C.
2. Centrifuge at 10,000 $\times g$ for 5 min at 4 °C to precipitate particles (*see Note 1*).
3. Discard the supernatant completely. Submerge the pellet in 20 μL TNE buffer with 1 \times DNA loading buffer to dissolve the virus particles. Let the tube sit at 4 °C overnight (*see Note 2*).

3.3 Agarose Gel Electrophoresis

1. Prepare 150 mL of 1% agarose gel by mixing 1.5 g agarose, 5 mL of 30× TAE buffer, and 145 mL of distilled water in a microwavable glass bottle. Microwave the mixture until the agarose is thoroughly melted and dissolved in the solution. Let it cool down to about 50 °C at room temperature. Pour the liquid gel into the gel box with an appropriate comb inserted. Let the gel solidify at room temperature for at least 2 h before removing the comb.
2. Prepare 600 mL of gel running buffer by diluting 20 mL of 30× TAE buffer in 580 mL of deionized water.
3. Assemble gel running apparatus by correctly positioning the gel box, adding running buffer into the chamber.
4. Pipette up and down very gently with an open mouth tip to mix the dissolved virus particle samples, and load each sample into a separate well (*see Note 3*). Connect the electrophoresis unit to a power supply and run the gel at 23 V constantly overnight.

3.4 Particle Gel Transfer

1. Disconnect the gel running unit and take out the gel carefully. Trim the gel to appropriate size.
2. Prepare the NC membrane with the same size of the gel. Submerge the membrane in a tray with deionized water for 5 min at room temperature. Replace the deionized water with TNE buffer and continue to agitate for 15 min.
3. To transfer particles from the gel to the NC membrane, set up the transfer cassette as following: Layer two sheets of 8" × 11" Whatman 3MM Chr paper in the gel transfer tray and pour TNE buffer on top to completely wet the Chr papers. Smooth out the bubbles in between the Chr papers and the tray gently with a plastic roller, and get rid of the excess amount of the buffer. Place the gel on top of the Chr papers with the loading well side facing down, make sure there is no air bubble between the papers and the gel. Place the presoaked NC membrane on top of the gel, and use a roller to get rid of the air bubbles between the gel and the membrane. Layer two sheets of TNE buffer pre-wet Chr papers that are cut to the gel size on top of the membrane, and use a roller to remove bubbles in between. Place a stack of dry paper towels (about 4 in.) pre-cut to the gel size on top of the Chr papers. Seal the whole transfer tray with a big piece of plastic wrap to prevent buffer evaporation during transfer. Put some weight (approx. 500 g such as metal plate, casserole dish) on top of the transfer apparatus (*Fig. 1*). Let the assembled transfer apparatus sit still on a flat surface and transfer for 24 h (*see Note 4*).
4. After gel transfer, take out the membrane with the gel still attached, and mark the position of the loading wells on the membrane by a pencil (*see Note 5*). Wrap up the gel and paper towels by plastic wrap and discard.

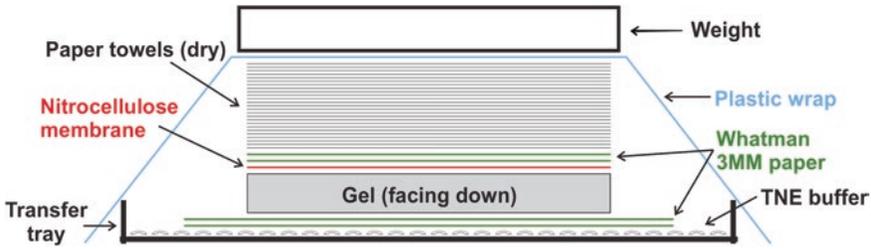


Fig. 1 Assembly of HBV particle gel transfer apparatus. See Subheading 3.3 for details

3.5 Enzyme Immunoassay

1. Prepare WesternBreeze blocking buffer with the ratio of H₂O–part A–part B as 7:2:1 (*see Note 6*).
2. Soak membrane in PBS buffer containing 2.5% formaldehyde at room temperature for 10 min (*see Note 7*).
3. Rinse the membrane with deionized water.
4. Fix the membrane with 50% methanol at room temperature for 30 min.
5. Wash the membrane three times with deionized water.
6. Incubate the membrane in blocking buffer for 1 h at room temperature.
7. Dilute anti-hepatitis B virus core antigen or anti-hepatitis B virus surface antigen in blocking buffer (*see Note 6*). Incubate with blot for 1–3 h.
8. Wash the membrane four times for 10 min in WesternBreeze wash solution (*see Note 6*).
9. Dilute IRDye 800CW secondary antibody in blocking buffer (1:10,000). Incubate the membrane for 1 h.
10. Wash four times for 10 min in WesternBreeze wash solution.
11. Dry the membrane by using a filter paper, then scan the membrane with Odyssey imager system.
12. The typical image of HBV particle surface and core EIA is shown in Fig. 2a and b, respectively.

3.6 Release of Particle-Associated DNA on Membrane

1. Submerge the particle bonded membrane in a tray containing denaturing buffer, and agitate gently for 15 min at room temperature (*see Note 8*).
2. Rinse the membrane three times with distilled water.
3. Submerge the membrane in a tray of neutralization buffer, and agitate gently for 5 min at room temperature.
4. Dry the membrane briefly in air at room temperature.
5. Cross-link the NC membrane in a UV cross-linker with UV energy dosage at 120 mJ/cm². The membrane can be directly subject to hybridization, or sandwiched between two sheets of filter papers and stored at –20 °C.

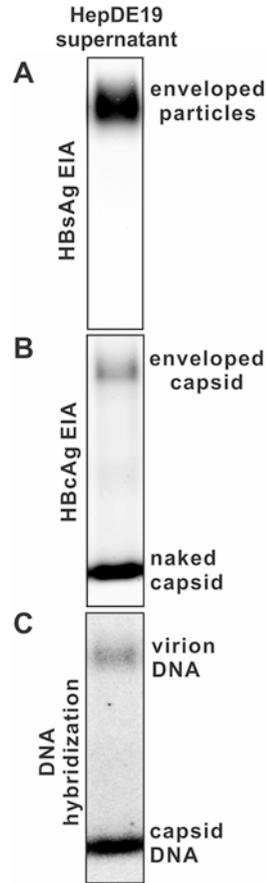


Fig. 2 Detection of HBV particles released into cell culture fluid by particle gel assay. HBV virus particles in the supernatant of HepDE19 cells cultured in tetracycline-free medium were precipitated down and analyzed by particle gel assay described in this chapter. (a) HBsAg EIA of enveloped HBV particles, including virions and subviral particles. (b) HBcAg EIA of Virion-containing capsids and nonenveloped naked capsids. (c) In situ hybridization of HBV DNA in virions and naked capsids. Reproduced from [14] with permission from American Society for Microbiology

3.7 Riboprobe Preparation for HBV DNA Detection (See Note 9)

1. Prepare rNTP solution by mixing 50 μL of 10 mM ATP, 50 μL of each 10 mM rGTP, 10 mM rCTP, and 0.2 mM UTP.
2. Add 4 μL of 5 \times reaction buffer, 4 μL of NTP solution from the previous step, 2 μL of 100 mM DTT, 0.25 μg of Sal I-linearized pGEM-HBV DNA template, 1 μL of RNasin, and 1 μL of Sp6 polymerase into a nuclease free tube. Adjust the volume to 14 μL with nuclease free water before adding 6 μL of [α - ^{32}P] UTP into the mixture. Incubate the reaction at 37 $^{\circ}\text{C}$ for 1 h.
3. To digest the DNA template, add 1 μL of RNase-free RQ1 DNase to the reaction and incubate at 37 $^{\circ}\text{C}$ for 15 min.

4. Add 15 μL of 5 M NH_4OAc to stop the reaction. To precipitate the RNA probe, add 113 μL of nuclease free water, 2 μL of yeast RNA, and 150 μL of isopropanol, gently mix and incubate at room temperature for 10 min.
5. Centrifuge the mixture at $12,000\times g$ for 30 min at 4 °C, and discard the supernatant carefully without disturbing the pellet. Dissolve the probe in 400 μL of deionized formamide, followed by measurement of the counts per minute (CPM) of acid-insoluble ^{32}P with scintillation counter (PerkinElmer). Store the probes at -20 °C.

3.8 DNA Hybridization

1. Place the cross-linked membrane in a hybridization tube with the DNA-binding side facing the center of the tube. Add 5 mL of EKONO hybridization buffer, and pre-hybridize the membrane by rotating the hybridization tube at 65 °C for 1 h in a hybridization oven (*see Note 10*).
2. Replace the pre-hybridization buffer with 5 mL of fresh hybridization buffer, and add HBV riboprobes with 1×10^7 CPM. Rotate the hybridization tube at 65 °C overnight.
3. Discard the hybridization solution on the following day, and wash the hybridization membrane with approximately half a tube of wash buffer. Rotate at 65 °C for 30 min.
4. Discard the previous wash buffer, and replace with half a tube of fresh wash buffer. Continue to wash at 65 °C for 1 h.
5. After the second wash, take out the membrane and dry it with paper towels. Seal the membrane with plastic wrap. Expose the membrane to the phosphorimager screen in a closed cassette for overnight.
6. Scan the phosphorimager screen with Typhoon phosphorimager system (GE Healthcare) for quantitation of signal intensity of virion and capsid DNA (*see Note 11*). A typical result of HBV particle DNA hybridization is shown in Fig. 2c.

4 Notes

1. The precipitated particles will be difficult to dissolve if the centrifugation speed is higher than $10,000\times g$ or the time of centrifugation is more than 5 min.
2. Do not try to dissolve the pellet by pipetting up and down, which may physically damage the particles. The pellet will be slowly dissolved in TNE buffer at 4 °C.
3. There may be a small piece of gel-like pellet that cannot be dissolved at all. Do not load the undissolved pellet into the gel well.

4. When assembling the transfer apparatus, avoid possible short circuiting of capillary liquid flow. Use Parafilm strips to seal the edges of the gel.
5. After transfer, the gel becomes thinner. Use a pencil tip to penetrate the loading wells and mark their positions on the membrane. This procedure is used to label the particle binding side of the membrane and the gel lanes.
6. Each lab may use their preferred Western blot blocking buffer, antibody dilution buffer, and washing buffer.
7. Formaldehyde is used to disinfect HBV virions and cross-link the proteins to membrane.
8. The same particle gel membrane can be subjected to DNA hybridization after EIA, or prepare a duplicate for DNA detection only.
9. Radiolabeled DNA probes can also be used for hybridization.
10. If other types of hybridization buffers are used, follow the pre-hybridization and hybridization conditions specified by the manufacturers.
11. When preparing HBV virus stocks from cell cultures for infection assays, the ratio of virion DNA–capsid DNA is needed to calculate the actual infectious vge of the inoculum [21].

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Microtiter-Format Assays for HBV Antigen Quantitation in Nonclinical Applications

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Abstract

Measurement of secreted HBV antigens in cell culture is an important endpoint in many experimental settings. Here we describe convenient and inexpensive protocols for 96-well format sandwich ELISA assays for this purpose, in which there are many options for customization of antibodies used and other parameters. These protocols can be adapted to use for animal serum samples, compound library screening, and other purposes.

Key words HBsAg, HBeAg, HBV antigen, ELISA, Screening, Z-factor

1 Introduction

In the context of a natural chronic infection, production and secretion of HBV surface and e antigens (HBsAg and HBeAg, respectively) is generally believed to be important in the maintenance of immune tolerance of infection. The three versions of surface antigen (L, M, and S) that are collectively referred to as HBsAg are secreted from HBV-infected or HBV-replicating cells as the primary components of the virus envelope. However, the vast majority of HBsAg is secreted in the form of noninfectious subviral particles (SVPs) at a large excess to the infectious virions [1, 2]. High levels of HBsAg are present in the serum of patients in a condition often referred to as antigenemia, which is implicated in the neutralization of antibodies, attenuation of cell mediated immunity, and possible pro-inflammatory effects in the liver [3–5]. HBeAg is secreted as a monomer generated as a result of endoplasmic insertion, proteolytic processing and secretion of the HBV precore protein, which shares most of its coding region with the core protein that comprises the capsid [1, 2]. Patients

who are positive for HBeAg exhibit the highest levels of serum and intrahepatic HBV DNA, and are at higher risk for cirrhosis than those who are HBeAg negative [6]. Precore protein itself has been linked to suppression of Toll-like receptor signaling and cytokine production in hepatocytes [7, 8]. The therapeutic reduction of HBsAg and HbeAg has thus become a topic of interest in antiviral research and drug discovery, with a resultant need for assay systems to quantitate large numbers of small volume samples. The presence of these antigens is also a useful marker for specific events in the virus life cycle, such as successful infection and cccDNA expression in NTCP-expressing cell lines, primary hepatocytes, and the HepaRG in vitro infection systems [9, 10]. HBeAg expression is also used as an indirect reporter for cccDNA formation and establishment in specific stably HBV-transfected inducible cell systems (DE19, AD38) that express pregenomic RNA from an integrated transgene, but in which precore RNA and HBeAg are expressed only from cccDNA [11–13]. Along with HBsAg-producing cell lines like HepG2.2.15, microtiter antigen assays have permitted screening of compound libraries and identification of inhibitors of HBsAg secretion and cccDNA formation [11, 14, 15].

Here we outline the procedures for detection of non-denatured HBsAg and HBeAg in cell culture supernatants by basic sandwich ELISA. The same procedures are easily adaptable to detection in clarified animal serum and other types of non-particulate samples containing soluble HBsAg and HBeAg. Although we have limited experience in assaying of intracellular samples, soluble cell fractions from lysates made without detergents (for example, by Dounce homogenization) will also work, but we have found that detection HBsAg and HBeAg in such samples may be somewhat limited by the antigens being present largely inside the lumen of closed membranous vesicles that rise from disruption of the endoplasmic reticulum, ERGIC, Golgi and other secretory organelles; this problem can be alleviated by sonication of the lysate to disrupt the membrane. It is also likely that disruption of the cells with a mild non-ionic detergent (Nonidet P-40, CHAPS, etc.) will enhance detection of intracellular non-denatured antigen, but the effects of the detergent conditions on the ELISA assay should be systematically determined by the end user.

Although many options exist for commercial kits in 96-well format, the protocols outlined below for HBsAg and HBeAg allow customization of the methods for detection in 96 well and smaller formats, the use of different antibodies for different purposes (conformation specificity, epitope tags, etc.), and lastly, are much more economical, with average costs of set up at 10–20% of the cost of commercial nonclinical kits.

2 Materials

2.1 General Materials and Tools

1. Antibody binding buffer (0.17% Na₂CO₃, 0.29% NaHCO₃, pH 9.6 with 5 M NaOH) (*see Note 1*).
2. PBS-Tween (PBST): (1× PBS, 0.5% Tween 20).
3. Blocking buffer: 2.0% bovine serum albumin (≥98% pure, heat shock fractionation purified), 1× PBST.
4. BM Blue POD substrate, soluble (Roche Life Sciences and Sigma-Aldrich).
5. H₂SO₄ (1.0 M, for optional use in stopping reaction).
6. 96-well plates, sterile, tissue culture coated, polystyrene (*see Note 2*).
7. Parafilm and/or plate sealing films.
8. 12-channel 96-well multipipettors, 20–200 μL and 100–1200 μL (we prefer electronic programmable models), compatible 20–200 and 100–1200 μL sterile pipette tips, and wash/sample reservoirs (*see Note 3*).
9. Optional: orbiting plate shaker.
10. 96-well plate-compatible plate reader, set up for colorimetric detection at 650 nm with reference wavelength 490 nm, and 450 nm with reference wavelength at 690 nm.

2.2 Materials Specific to HBsAg ELISA

1. Capture Antibody (Ab): Anti-HBsAg mouse monoclonal (Fitzgerald Industries) (*see Note 4*).
2. Detection Antibody: Anti-HBsAg rabbit polyclonal conjugated to horseradish peroxidase (HRP) (Fitzgerald Industries).
3. Pure recombinant HBsAg, serotype to match antibody specificity (*ayw* serotype for the clones mentioned in Notes) (Fitzgerald).

2.3 Materials Specific to HBeAg ELISA

1. Capture antibody: Anti-HBeAg mouse monoclonal (Fitzgerald Industries) (*see Note 5*).
2. Detection antibody: Anti HBeAg mouse monoclonal conjugated to horseradish peroxidase (HRP) (Fitzgerald Industries) (*see Note 6*).
3. Pure recombinant HBeAg, genotype to match antibody specificity (genotype D for the clones mentioned in Notes) (Fitzgerald).

3 Methods

3.1 Pre-coating of Plate with Capture Antibody, and Blocking

1. Decide whether to include minus Ab control wells, and design plate layout accordingly. For a 96-well plate, we normally reserve columns 1 and 11 for control samples.
2. Dilute capture Ab to 0.9 mg/ml (approximately 1:100) in Ab binding buffer or PBS as noted, calculating total volume required for 50 μ L/well, and increasing by 10% for pipetting error. Use Ab binding buffer or PBS for minus Ab negative control wells (*see Note 7*).
3. Dispense 50 μ L/well of appropriate Ab or control solution. Tap side of plate and inspect visually to make sure liquid has been distributed evenly over bottom of each well. Cover with plate lid of upper plate in a stack.
4. Incubate at 4 °C overnight. May incubate up to 48 h, but should use sealing film or wrap plate stack in Parafilm to minimize evaporation.
5. Remove capture Ab solution by aspiration. Alternatively, invert plate by hand to shake out solution and blot plate top dry on paper towels. To ensure even and thorough removal, turn the plate around and repeat blotting on paper towels.
6. Wash wells once by dispensing 150 μ L of PBST into each well, and rest plate for 5 min. Optional step: Shake plates for 4 min at 600 rpm (*see Note 8*).
7. Empty wells as above and add 150 μ L of blocking buffer per well. Incubate at 37 °C for a minimum of 1 h, or overnight at 4 °C (*see Note 9*).
8. Empty wells and wash once with 150 μ L of PBST. Rest plate for 5 min, or shake 4 min at 600 rpm. Do not let plates dry out prior to sample addition in next step.

3.2 Sample Preparation and ELISA

1. Standard Curve. For absolute measurements of antigen, prepare 300 μ L samples of the recombinant purified HBeAg or HBsAg in cell culture media or other matrix, at the following concentrations (ng/ml): 10, 3.18, 1.0, 0.318, 0.1, 0.032, 0.01, 0.003. Process standard curve samples in parallel with experimental samples.
2. Experimental samples. As determined by user, samples may need processing prior to assay. Low speed centrifugation steps can be executed on the sample plates themselves in a table top centrifuge with swinging bucket rotor and plate adapters. Intermediate dilution steps can be done in a separate plate containing PBS, media, or other matrix. For testing of inhibitor compounds in cell culture, the undiluted supernatant samples can be assayed directly for HBeAg and HBsAg (*see Note 10*).

3. Transfer 100 μL of samples to each well by multichannel pipetter or liquid handler.
4. Incubate overnight at 4 $^{\circ}\text{C}$. Longer incubation times (up to 3 days) seem to increase sensitivity by twofold.
5. Remove sample solution by aspiration. Alternatively, invert plate by hand to shake out solution and blot plate top dry on paper towels. To ensure even and thorough removal, turn the plate around, repeat.
6. Wash wells 2 \times with 150 μL PBST as outlined above. It is important to change pipette tips to prevent carryover of sample. Do not let plates dry out between washes and prior to sample addition.
7. Wash well 2 \times with 150 μL PBST as outlined above. Do not let plates dry out between washes or after removal of second wash.
8. Dilute the HRP-conjugated detection Ab 1:5000 in blocking buffer, calculating total volume required for 25 μL /well, and increasing by 10% for pipetting error.
9. Dispense 25 μL /well. Tap side of plate and inspect visually to make sure liquid has been distributed evenly over bottom of each well. Cover with plate lid of upper plate in a stack. Incubate at 37 $^{\circ}\text{C}$ for a minimum of 1 h.
10. Wash wells 2 \times with 150 μL PBST as outlined above. It is extremely important to prevent carryover of detection Ab between washes; change pipette tips after each step, and make sure not to touch the side of the wells when adding the wash buffer. Do not let plates dry out between washes and prior to substrate addition.
11. Empty wells and blot plate top dry on paper towels. Add 50 μL of BM Blue POD substrate, and incubate at room temperature for 30 min. Color change to blue will be linear out to 60 min, and at 30 min the plates should be read promptly at 650 nm with reference wavelength 490 nm. Alternatively, the reaction can be stopped (color will change to yellow) with addition of 20 μL of 1.0 M H_2SO_4 , and plates are read at 450 nm with reference wavelength at 690 nm (*see Note 11*).

3.3 Evaluating Assay Performance and Sensitivity

The signal to noise ratio (S:N) would be defined as the ratio of the signal from positive controls (reference samples known to contain antigens) versus negative controls (samples in the same matrix, like culture media, which should not contain the antigen). For example: (1) for measuring HBsAg produced in HepG2.2.15 cells, samples from the parental HepG2 cell line would be used as a negative control (we normally see 10:1); (2) when measuring the levels of cccDNA-dependent HBeAg in DE19 or AD38 cells, samples from the same cells in the induced state but treated to prevent

cccDNA formation would be an appropriate negative control (*see Note 5*; this is highly dependent on the choice of detection antibody, and normally ranges from 3:1 to 6:1).

Plotting of the standard curve assay results vs the known sample concentrations will permit evaluation of the assay's sensitivity, after subtraction of the noise.

For screening applications, users may also wish to calculate the Z-factor of the assay.

4 Notes

1. In lieu of this buffer, PBS pH 7.4 may also work with some specific antibody preparations but not all; user may try both if desired.
2. We have actually found that plate types marketed for "ELISA" applications do not work as well tissue culture plates.
3. Access to liquid handling and plate washing equipment is very desirable for the processing of large numbers of plates.
4. M701007 has been our favorite clone, but most other clones or polyclonal preparations we have tried have been very suitable.
5. Clone M2110146 has been our favorite. Specificity of anti-HBeAg antibodies is famously plagued by cross-reactivity with core protein. This clone has been the most specific we have found, but may be difficult to source as it has been discontinued by Fitzgerald. We have tried several other clones and all exhibit moderate levels of cross-reactivity. Alternate clones, or even polyclonal Abs may be tested by the user by setting up appropriate controls with supernatants from HBV expression-induced AD38 or DE19 cells that have also been treated with lamivudine or entecavir, resulting in core production but no cccDNA-dependent HBeAg. We do not recommend the use of recombinant purified core and HBeAg for determining specificity as there are antigenic differences between these and the proteins present from cell culture or in vivo sera.
6. Clone M10071922 has been our favorite. Core-protein cross-reactivity of this HBeAg antibody is of less concern, since detection specificity is determined by the capture antibody.
7. This concentration is a guideline, for the minimal amount required for linear detection, and works well for HBV antigens from cell culture supernatants. Final concentrations to be used for different applications should be determined by end user in titration experiments.
8. This shaking step seems to aid in signal specificity, but is not essential.

9. Volumes of wash and blocking buffers should always be higher than the volumes of antibody solution and samples, to prevent nonspecific signal detection from exposure of sample to unblocked and/or unwashed areas of the well sides.
10. Dilution schemes of the test samples may be carried out as desired by the user, and may have to be determined experimentally so that the typical signal obtained is within the linear range of the assay's detection capacity.
11. We have found increased sensitivity of the assay without the color change, and so we normally do not employ the reaction stop.

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Deep Sequencing of the Hepatitis B Virus Genome: Analysis of Multiple Samples by Implementation of the Illumina Platform

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Abstract

The quasispecies variation of hepatitis B virus (HBV) was believed to be a viral response to antiviral treatment and host immune pressure. Sanger sequencing was previously the classic approach for quasispecies analysis, but this method was also time-consuming and laborious. Ultra-deep sequencing has been widely used in viral quasispecies research, especially for low-frequency mutation detection. Here we present a multiple samples deep sequencing method employing the Illumina platform to detect HBV quasispecies variation in patient-derived samples.

Key words Hepatitis B virus, Illumina sequencing

1 Introduction

Hepatitis B Virus (HBV) is a DNA virus which has chronically infected more than 350 million persons worldwide, predisposing them to hepatocellular carcinoma (HCC) and cirrhosis [1]. The extremely high replication rate of HBV, coupled with the proof-reading deficiency of the viral polymerase yields the potential to rapidly generate mutations within the entire genome of HBV. The generated variants with genetic heterogeneity are described as viral quasispecies [2, 3]. More and more studies suggest that viral quasispecies are an important factor in disease progression, pathogenesis, immune escape, and the development of drug resistance [4]. Cloning and sequencing the genome DNA of variants is a classic method for quasispecies assessment, however this method is time consuming and laborious, and has difficulty in detecting quasispecies with a proportion less than 3% [5, 6].

Ultra-deep sequencing has been widely used in viral quasispecies research, including the detection of the low-abundance resistance mutations of HIV that arise during antiviral treatment [7, 8],

the analysis of transmission characteristics of HIV and HCV [9–11], and the study of the dynamics of HBV drug resistant mutants [3, 12, 13]. The Illumina Genome analyzer and Roche 454 pyrosequencing [14, 15] are the most commonly used next-generation sequence platforms for viral quasispecies estimation. The former reaches higher coverage of variants in a quasispecies population with lower cost, and the latter produces longer reads. Although shorter length reads result in less mutation linkage in a genome, the sequence length obtained per run with the Illumina platform is considerably larger and thus enables a deeper analysis of a DNA sample. This favors site mutation or epitope variation analysis. Here we introduce a deep sequencing procedure for mutant spectrum profiling of the HBV genome based on Illumina platform; the method permits analysis of multiple samples, and is currently being used to detect HBV quasispecies variation within mother-to-child transmission of chronic hepatitis B.

2 Material

1. Commercial kit: DNeasy Blood & Tissue Kit (Qiagen), High Pure PCR Product Purification kit (Roche), Nextera DNA Sample Preparation Kit (Illumina).
2. Software: Fastqc (<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/>).
3. Equipment: Eppendorf mastercycler nexus.

3 Method

3.1 HBV DNA Extraction and PCR Amplification

1. HBV DNA is extracted from 50 μ L serum using DNeasy Blood & Tissue Kit (Qiagen) according to the instruction of the manufacturer. The final elution volume is 50 μ L and the total HBV genomic DNA should above 50 ng (*see Note 1*).
2. Amplify HBV genome DNA using extracted HBV DNA sample as template. This amplification usually works well when the viral titer is above 1.0×10^4 copies/mL (*see Note 2*). As a negative control, the serum of a healthy HBV DNA-negative volunteer is processed in parallel.

PCR system:

HBV DNA	1 μ L
F 1821 (10 μ M)	1 μ L
R 1798 (10 μ M)	1 μ L
2 \times PrimeSTAR HS premix	25 μ L

ddH ₂ O	23 μ L
Total volume	50 μ L

PCR was performed with an Eppendorf mastercycler nexus.

PCR parameters:

95°C 5min

95°C 15s

68°C 15s -1°C/cycle

72°C 3min

} 8 cycles

95°C 15s

60°C 15s

72°C 3min

} 32 cycles

72°C 5min

3. A hepatitis C virus (HCV) 1A whole genome cloned into the plasmid HFL/1 was amplified and used as a positive control in for further deep sequencing.
4. PCR products were analyzed on 0.8% agarose gels stained with ethidium bromide to confirm the size and purity, and then purified by the High Pure PCR Product Purification kit (Roche) for the further deep sequencing.

3.2 Tagmentation of Genomic DNA

Prepare the library by using a Nextera DNA Sample Preparation Kit (Illumina) according to the manufacturer's manual (*see Note 3*).

3.3 Sequence Data Analysis

1. Download raw reads files from Illumina platform, employing the Fastqc (<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/>) to evaluate the quality of the reads.
2. Remove 5' adaptor sequence from raw reads. If only barcode sequences, rather than adaptors, were attached to raw reads, this step can be omitted.
3. Remove the low quality reads from the reads pool. A low quality read was defined as one meeting one of the following criteria: (1) A read with average quality lower than 30; (2) A read containing more than 3 ambiguous bases; (3) A read more than 90% of which is composed of one specific base (Poly N). The filtered reads pool is considered as "a clean reads pool".

4. Reads are further separated according to their barcode, and sorted into different patient pools.
5. A BLASTN comparison analysis is performed between clean reads and the HBV genomic reference sequence, to eliminate non-HBV sequences (anything with similarity < 70%). After this filtering step, the average coverage of HBV sequence site should be above 2600× (Fig. 1).
6. Shan entropy and Relative entropy calculations are carried out.
 - (a) *Shannon entropy*. The complexity of each site of the HBV genome is evaluated by the Shannon entropy (SE) (Fig. 2), which is based on the frequencies of different bases present in the position, by using the following formula:

$$SE = \sum_{i=1}^n f_i \log(f_i)$$

where n is the number of different bases of each site, and f_i is the frequency of a base at a specific site.

- (b) *Relative entropy*. Relative entropy ($RE\lambda$) can be considered as the most likely common source distribution of both mother (m) and child (c), which is based on Jensen–Shannon (JS) divergence between probability distributions (Fig. 3). In each site, bases distribution of m and c , for every $0 \leq \lambda \leq 1$, the $RE\lambda$ is defined as:

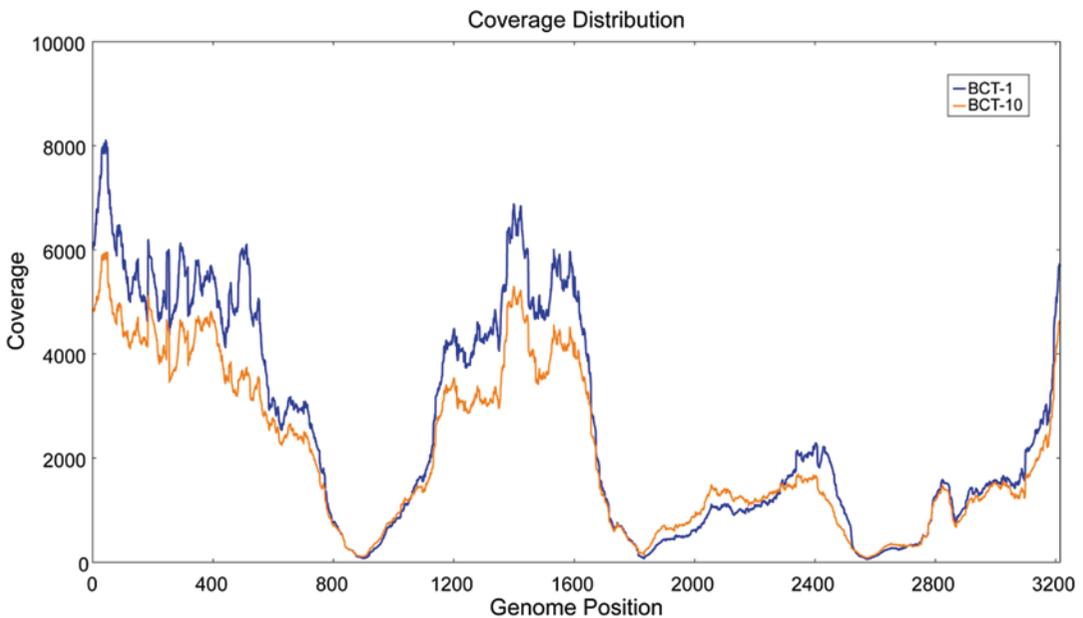


Fig. 1 The sequence coverage distribution of whole HBV genome sequence

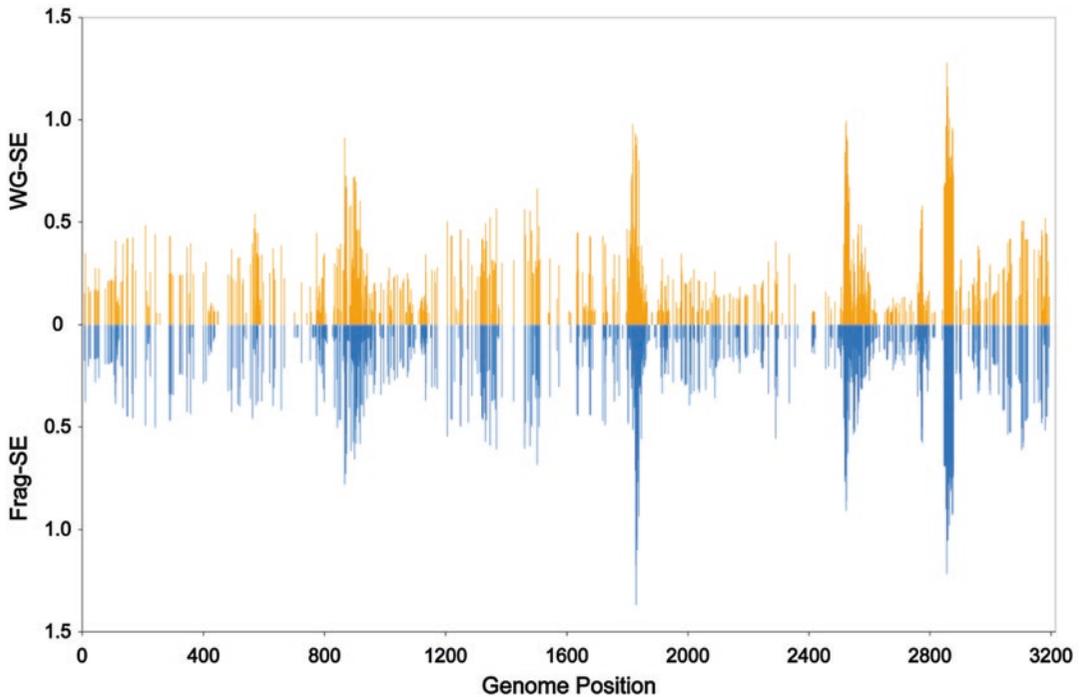


Fig. 2 The complexity of HBV genome sequence represented by Shannon entropy. WG-SE: Shannon entropy of sequences from HBV whole genome amplification, Frag-SE: Shannon entropy of sequences from HBV genome fragment amplification. This result also showed different PCR amplifications had no significant difference in coverage distribution and Shannon entropy

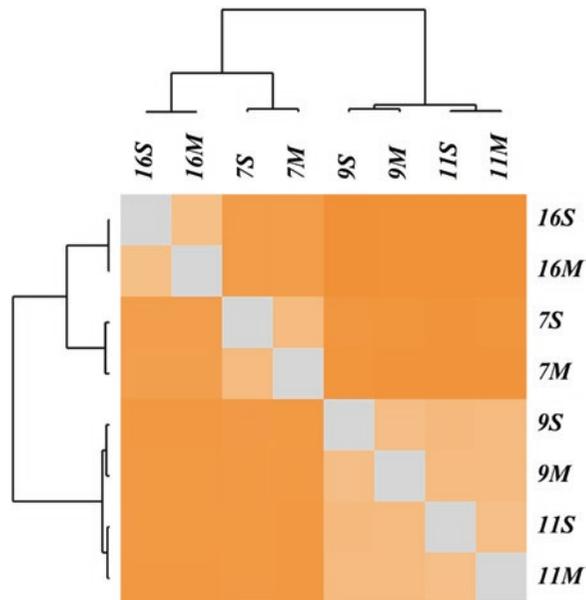


Fig. 3 Cluster analysis of HBV population between mothers and children using profile-profile comparison with Relative entropy

$$RE_{\lambda}(m | c) = \lambda \times D(m | r) + (1 - \lambda) \times D(c | r)$$

where:

$$D(m | r) = \sum_{i=1}^n f_i | \log_2 \left(\frac{f_i}{r_i} \right)$$

$$r = \lambda \times m + (1 - \lambda) \times c$$

RE λ can be considered as the most likely common source distribution of both m and c , with λ as a prior weight. A natural $\lambda = 0.5$ was chosen in this study ($\lambda = 0.5$ is a natural parameter of TRE, it means there is no bias of two groups). Total relative entropy (TRE) means the cumulative RE value of the 3215 sites.

4 Notes

1. Make sure the quantity of total genomic DNA is above 50 ng. If **step 2** of Subheading **3.1** PCR amplification fails because of low viral titer, amplify two overlap fragments covering the whole genome as following.

Table 1
PCR condition for the generation of HBV genomes^a

Primers for whole HBV genome amplification	
F1821	CCGGAAAGCTTATGCTCTTCTTTTTTCACCTCTGCCTARTCATCTC
R1788	CATGCTCTTCAAAAAGTTGCATGGTGCTGGTGAACASACCAATTTATG
Primers for HBV genome fragment amplification	
F381	GTCTGCGGCGTTTTATC
R1804	TCATGCTCTTCAAAAAGTTGCATGGTGCTGGTG
F1821	TTATGCTCTTCTTTTTTCACCTCTGCCTARTCATC
R709	ACAGTGGGGGAAAGC
Primers for HCV genome amplification	
HCV-F	CGGATCCCATATGAGCACGAATCCTAAACCTCAAAGAAAACCAAACGTAAC
HCR-R	TAAAGCTTCAGCAACCTCCACC

^aEach PCR started with an initial denaturing step for 5 min at 95 °C. A touchdown step was performed with a denaturation at 98 °C for 10s, an annealing temperatures decrement of 1 °C per cycles as indicated and an extension at 72 °C for 3 min

PCR system for fragment 1 (nt381–3215/1–1804) and fragment 2 (nt1821–3215/1–709) amplification were the same as that in **step 2** of Subheading 3.1, except that primers F381+R1804 and F1821+R709 were used respectively (Table 1). PCR parameters are the same as that in **step 2** of Subheading 3.1.

2. This amplification strategy can work when the viral titer above 1.0×10^4 copies/mL.
3. The enzyme amount used for genomic DNA tagmentation can be reduced to half of standard usage. In details, for a sample well containing 20 μ L of genomic DNA at 2.5 ng/ μ L (50 ng total), 2.5 μ L TDE1 was enough for HBV genomic DNA tagmentation.

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Generation of Replication-Competent Hepatitis B Virus Genome from Blood Samples for Functional Characterization

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Abstract

Hepatitis B virus (HBV) infection can be associated with a spectrum of clinical outcomes. Transient transfection of the clinical HBV isolates in human hepatoma cell lines can establish their biological properties to shed light on their different pathogenic potentials, yet very few clinical HBV isolates have been functionally characterized so far. The technical challenges include faithful amplification of the full-length HBV genome from clinical samples and conversion into a replication-competent form. We have improved a published method to amplify the full-length HBV genome from blood samples. Two alternative approaches are used to render the cloned HBV genome replication competent: release and circularization of the 3.2-kb HBV genome prior to each transfection experiment or conversion of the monomeric clone into a tandem dimer version.

Key words HBV clinical isolates, Tandem dimer, Transfection

1 Introduction

1.1 Established Method to Amplify Full-Length HBV Genome from Blood Samples

So far at least eight hepatitis B virus (HBV) genotypes with nucleotide sequence divergence of 8% or greater have been identified. They display distinct geographic distributions and clinical manifestations [1]. For example, infection with genotype C is associated with delayed seroconversion from hepatitis B e antigen (HBeAg) to anti-HBe [2] and increased risk to develop liver cirrhosis and hepatocellular carcinoma than genotype B infection [3]. Genotype C patients also respond less favorably to interferon therapy. In addition, the immune clearance phase of chronic infection selects for mutations in the precore, core promoter, and preS/S regions [4]. Comparison of the biological properties of different HBV mutants and genotypes requires the cloning of the entire viral genome, ideally from a large number of clinical samples to avoid bias [5, 6]. In this regard, the

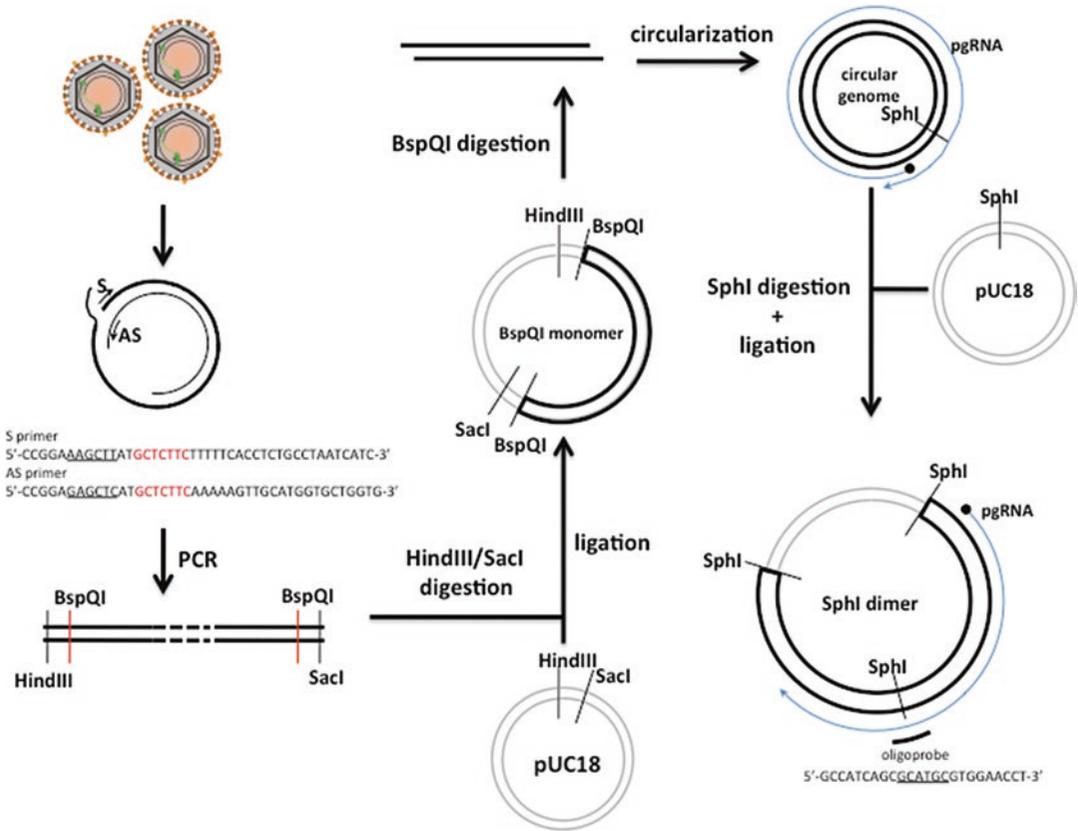


Fig. 1 Flow chart for the generation of replication-competent HBV genomes from blood samples. Virion-associated HBV DNA has the minus strand DNA (*thick line*) complete. The sense (S) primer anneals to its 3' end to generate full-length plus strand, which will serve as the template for the antisense (AS) primer to generate more minus strand DNA. The HindIII and SacI sites introduced to the sense and antisense primers, respectively, will allow efficient cloning of the PCR product to pUC18 vector, whereas the internal BspQI sites allow subsequent precise release of the HBV genome. Such a linear HBV genome can be ligated in vitro to make it replication competent (capable of producing the terminally redundant pg RNA), or the ligated DNA is further digested with SphI and ligated with SphI cut, dephosphorylated pUC18 DNA. Bacterial colonies harboring tandem SphI dimer can be screened by hybridization with an oligoprobe spanning the SphI site. The 3.5-kb pg RNA can be produced from such a tandem dimer construct

virion-associated HBV genome is relaxed circular and partially double stranded. Only the minus strand DNA is full length with the precore region present at both the 5' and 3' ends (Fig. 1). Twenty years ago, Gunther and colleagues developed a method to amplify the full-length HBV genome from blood samples by polymerase chain reaction (PCR) [7]. Both the sense and antisense primers target the precore region, which happens to be highly conserved among diverse HBV isolates.

1.2 HBV Genome Replication Requires Production of 3.5-kb Terminally Redundant Pregenomic RNA, Which Can Be Achieved by Circularization of the Full-Length HBV Genome

Upon infection of hepatocytes, virion-associated relaxed circular DNA is converted to covalently closed circular (ccc) DNA. The cccDNA in the nucleus serves as the template for transcription of coterminal 3.5-, 2.4-, 2.1-, and 0.7-kb RNAs. The 3.5-kb RNA is terminally redundant and has a heterogeneous 5' end, with the shorter version (pregenomic RNA or pg RNA) responsible for genome replication. First, it serves as the mRNA for translating both polymerase (P) and core proteins. Second, it is packaged into capsid particle assembled from core protein, where it is converted by co-packaged P protein into partially double-stranded DNA. In this regard, the full-length HBV genome amplified via the precore region is a linear molecule unable to produce the terminally redundant pg RNA. Gunther and colleagues solved this problem by adding at the 5' ends of both primers a recognition site for SapI, a class II-S restriction enzyme that cleaves downstream of the recognition site (Fig. 1) [7]. SapI-digested PCR product can be joined correctly by T4 DNA ligase to preserve the functionality of the circular HBV genome. They found replication of the SapI-digested PCR product in Huh7 human hepatoma cells even in the absence of *in vitro* ligation [7], possibly due to activity of a cellular ligase.

1.3 Use of Cloned Full-Length HBV Genome for Transfection: Problems and Solutions

The lack of a cloning step in the Gunther protocol makes the transfection work expensive and difficult to repeat. Besides, DNA circularization inside transfected cells is less efficient compared with *in vitro* ligation [8]. We added HindIII and SacI restriction sites (absent on the HBV genome) to the sense and antisense primers, respectively, to facilitate efficient cloning of the PCR product (Fig. 1) [5]. However, the use of cloned HBV genome for transient transfection poses novel problems. First, the mutation rate of the High Fidelity^{plus} DNA polymerase (Roche) used for PCR amplification [7] is only six times lower than that of *Taq* DNA polymerase. When it was employed to re-amplify the full-length HBV genome from a replication-competent HBV clone, five of nine PCR clones turned out to be defective in genome replication, protein expression, or both [8]. In contrast, the Phusion DNA polymerase (New England Biolabs) has 50 times lower mutation rate than *Taq* DNA polymerase, and all the ten PCR clones displayed comparable genome replication and protein expression [8]. Second, virion DNA from the same blood sample can display sequence heterogeneity, especially if collected at the immune clearance phase of chronic HBV infection. Two approaches can be used to overcome this problem. First, DNA from several PCR clones can be pooled for SapI digestion and self-ligation. Second, after ligation of the HindIII/SacI double-digested PCR product with pUC18 DNA, the entire transformation product can be grown directly in liquid culture to obtain plasmid DNA of the clone pool [8].

1.4 The 3.5-kb pg RNA Can Be Transcribed from Two Tandem Copies of the HBV Genome Cloned via the SphI Site in the Viral Genome

The need for SapI digestion of cloned HBV DNA followed by genome circularization introduces experimental variability. In this regard, the 3.5-kb terminally redundant pg RNA (around position 1818–1921) can be transcribed from two tandem copies of the HBV genome cloned to a vector via certain unique restriction sites on the viral genome, such as SphI (cleavage site at position 1238) (Fig. 1). Therefore, such tandem dimers can be used directly for transfection experiments without the need for enzymatic manipulation. A protocol to convert cloned monomeric HBV DNA into a replication-competent tandem dimer version is provided.

2 Materials

2.1 Viremic Serum Samples

Freshly obtained serum samples from HBV carriers are stored at 4 °C for several days prior to DNA extraction. Alternatively, they are stored at –80 °C for prolonged period of time.

2.2 Reagents

Chloroform.

Isoamyl alcohol.

Tris-saturated phenol.

QIAamp DNA Blood Mini Kit.

QIAquick PCR Purification Kit.

QIAquick Gel Extraction Kit.

QIAprep Miniprep Kit.

HiSpeed Plasmid Midi Kit.

dNTP.

Enzymes: BspQI, HindIII, SacI, ScaI, SphI, alkaline phosphatase, and Q5 DNA polymerase (New England Biolabs).

T4 DNA Ligase and DNA 3'-End Labeling Kit (Roche).

DH5α competent cells.

[α-³²P] dCTP.

PCR primers for amplification of the full-length HBV genome are sense 5'-CCGGAAAGCTTATGCTCTTCTTTTCACCTC TGCCTAATCA TC-3' (*Hind*III site underlined) and anti-sense 5'-CCGGAGAGCTCATGCTCTTCAAAAAGTTGCA TGGTGCTGGTG-3' (*Sac*I site underlined).

Oligonucleotide probe for screening SphI dimer has the sequence 5'-GCCATCAGCGCATGCGTGGAACCT-3' [9].

3 Methods

3.1 DNA Extraction

1. Mix 200 μl serum sample with 20 μl of protease and 200 μl of Buffer AL, and incubate at 56 °C for 10 min.

2. Add 200 μl of ethanol to precipitate DNA and pass the solution through QIAamp Mini spin column by centrifugation. Wash the column successively with buffer AW1 and AW2. After extra steps of spinning to remove residual liquid (*see Note 1*), elute retained DNA with 50 μl of distilled water.

3.2 PCR Amplification of the Full-Length HBV Genome

1. In a specialized PCR hood or cell culture hood, set up a 50- μl PCR reaction containing 0.5 mM each of the sense and anti-sense primers, 200 μM of dNTP, 1 \times Q5 DNA polymerase buffer, 1 u of Q5 DNA polymerase (*see Note 2*), and 1–5 μl of template DNA (*see Note 3*). Include a tube of PCR reaction lacking template DNA to serve as a negative control.
2. The PCR conditions are initial denaturation at 98 $^{\circ}\text{C}$ for 30 s, followed by 35–40 cycles of 98 $^{\circ}\text{C}$ for 10 s and 72 $^{\circ}\text{C}$ for 30 s, and final extension at 72 $^{\circ}\text{C}$ for 10 min. Run an aliquot of the PCR product (5 μl) in 1% agarose gel to verify successful amplification of the 3.2-kb HBV DNA (*see Note 4*).

3.3 Cloning of the PCR Product to Generate Monomeric HBV Construct

1. Run the remainder of the PCR product in 1% agarose gel to separate the 3.2-kb HBV DNA from primer dimers. Cut out the 3.2-kb band, and extract DNA from gel slice using QIAquick DNA extraction kit. Elute DNA in 50 μl of water.
2. Digest eluted HBV DNA and 2 μg of pUC18 plasmid DNA at 37 $^{\circ}\text{C}$ for >2 h with 20 u each of HindIII and SacI, and purify both HBV and pUC18 DNA through QIAquick PCR purification column according to manufacturer's manual. Elute DNA in 30–50 μl TE (pH 8.0) buffer. Measure DNA concentration using Nanodrop 2000c spectrophotometer.
3. Ligate HBV DNA with pUC18 DNA in a 10- μl volume using 3:1 molar ratio (about 65 ng of HBV DNA with 20 ng pUC18 DNA), at 14 $^{\circ}\text{C}$ overnight. Set up a tube of pUC18 DNA self-ligation to serve as a negative control.
4. Incubate 5 μl of the ligation product with 50 μl of competent DH5 α cells on ice for 30 min, followed by heat shock at 37 $^{\circ}\text{C}$ for 45 s. Let stand on ice for 2 min and add 500 μl of LB medium. Shake at 37 $^{\circ}\text{C}$ for 30 min and spread 50 and 200 μl each onto an LB plate containing 100 $\mu\text{g}/\text{ml}$ ampicillin. Incubate the plates at 37 $^{\circ}\text{C}$ overnight (*see Note 5*).
5. Pick up five or more well-separated single colonies from the plates and grow them overnight in 5 ml of LB medium supplemented with 100 $\mu\text{g}/\text{ml}$ of ampicillin. Extract plasmid DNA using QIAprep Miniprep Kit, followed by measurement of DNA concentration. Double digest 0.5–1 μg of plasmid DNA with HindIII and SacI, followed by gel electrophoresis. Recombinant plasmids should contain a 3.2-kb band in addition to a band of 2.7 kb (pUC18).

6. If necessary, determine the nucleotide sequence of the cloned HBV DNA by customer sequencing. About 5–6 overlapping sequencing reactions are needed to cover the entire HBV genome.

3.4 Preparation of Circularized HBV Genome for Transfection Experiments

1. Digest recombinant plasmid at 37 °C for at least 4 h with BspQI (*see Note 6*) and ScaI (*see Note 7*). Run the DNA digest in 1% agarose gel and cut out the 3.2-kb band. Extract DNA using QIAquick DNA extraction kit and elute DNA in 50 µl TE buffer.
2. Measure DNA concentration using Nanodrop spectrophotometer. Ligate at 14 °C overnight the full-length HBV genome with T4 DNA ligase at a low concentration of 0.5 ng/µl to promote intramolecular ligation. Extract the ligation product sequentially with equal volumes of Tris–HCl (pH 8.0) saturated phenol and chloroform/isoamyl alcohol (24:1), and precipitate DNA with two volumes of ethanol in the presence of 300 mM sodium acetate, pH 5.2. Store the Eppendorf tubes at –20 °C overnight.
3. Centrifuge the Eppendorf tubes in the cold room at 14,000 × g for 30 min. Wash the DNA pellet with cold 70% ethanol followed by another wash with cold pure ethanol. Let air dry, and dissolve the purified HBV DNA in TE buffer (pH 8.0) at 0.2–0.5 µg/µl for transfection experiment.

3.5 Conversion of Monomeric HBV DNA Construct into SphI Dimer

1. Digest the circularized full-length HBV DNA described above with SphI. Also digest 2 µg of pUC18 DNA with SphI to completion, followed by treatment with alkaline phosphatase to prevent self-ligation.
2. Ligate the SphI cut HBV DNA with SphI cut, dephosphorylated pUC18 DNA at 10:1 molar ratio [9] (*see Note 8*). Perform pUC18 self-ligation to serve as a negative control. Transform competent DH5α cells with the ligation products and spread out *E. coli* on LB/ampicillin plates.
3. Pick up individual colonies from the pUC18 + HBV plates and regrow them on gridded nylon membrane placed on top of LB/ampicillin plates. After overnight growth, make a membrane lift (use a semi-wet membrane) to transfer a fraction of bacteria to another disc of nylon membrane (*see Note 9*). Place the disc on top of three layers of Whatman paper wetted with denaturation solution (0.5 N NaOH/1.5 M NaCl) and let stand for 5 min. Next, transfer the disc on top of neutralization solution (0.5 M Tris–HCl, pH 7.5/1.5 M NaCl). Let float for 10 min and then shake several times to let it submerge into the solution. Rinse briefly in 2×SSC solution and let air dry.
4. Prehybridize the lift at 60 °C for 2 h in a solution containing 6×SSC/0.1% SDS, and 100 µg/ml sheared and denatured

salmon sperm DNA. Meanwhile, label 20 pmol of the oligonucleotide 5'-GCCATCAGCGCATGCGTGGAAACCT-3' with 5 μ l of [α - 32 P] dCTP (10 mCi/ml) using a DNA 3'-End Labeling Kit (Boehringer Mannheim), at 37 °C for 1 h. Replace with fresh prehybridization solution, and add 32 P labeled oligonucleotide probe at 5–10 \times 10⁵/ml. Hybridize at 60 °C for 2 h. Wash the membrane sequentially with 6 \times SSC/0.1% SDS and 2 \times SSC/0.1% SDS, at 60 °C for 10 min. Expose to an X-ray film.

5. Pick up several hybridization positive colonies from the master plate and grow them into 5-ml liquid culture. Perform plasmid preparation and digest the miniprep DNA with a panel of restriction enzymes including HindIII, SphI, and BglII (or RsrII, XbaI), followed by electrophoresis in 0.8% agarose gel. HindIII has a single cleavage site on pUC18 but none on HBV and will generate a single band of 9.1 kb for a dimer. SphI should generate two bands: 2.7 and 3.2 kb, with the 3.2-kb band twice stronger than that of the 2.7-kb band for a dimer. Both BglII and RsrII have a single cleavage site on the HBV genome but none on the pUC18 vector and will generate a 3.2-kb band and a 5.9-kb band if the dimer is tandem (tail to head). A tandem SphI dimer will also generate a 3.2-kb band by XbaI digestion, although the remaining DNA is cleaved into two fragments due to the presence of an XbaI site on the polycloning site of pUC18.

4 Notes

1. Make sure that liquid is completely spun out of the column before elution. Otherwise residual ethanol will inhibit subsequent PCR reaction.
2. Q5 DNA polymerase has 100 times lower mutation rate than Taq DNA polymerase and thus has higher fidelity than Phusion DNA polymerase.
3. Observe all precautions to minimize contamination, including UV treatment of the hood, cotton tips, adding template DNA at the very last step, and strict separation of the pre- and post-PCR areas and equipments.
4. Successful amplification of the full-length HBV genome from blood samples is dependent on high viremia titer. For low viremic samples, the HBV genome could be amplified as two overlapping DNA fragments (as shorter DNA fragments are much easier to amplify than the full-length genome), followed by their joining by overlap extension PCR. A concern is that the full-length genomes thus generated may not derive from the same parental molecules.
5. Alternatively, the transformation product is transferred to 20 ml of LB medium supplemented with 100 μ g/ml of ampicillin and

shaken at 37 °C overnight. The plasmid DNA thus extracted will represent the clone pool.

6. BspQI is an isozyme of SapI, but about half the price.
7. ScaI converts the 2.7-kb pUC18 DNA into smaller 1.8- and 0.5-kb fragments, thus facilitating unambiguous recovery of the 3.2-kb HBV DNA.
8. A high insert/vector ratio is needed to promote joining of more than one copies of the HBV genome to the vector. The yield of dimer could be increased by ligating HBV DNA alone for 5–10 min prior to addition of pUC18 DNA. In case this approach fails to generate tandem dimer, the SphI monomer clones thus obtained can be used to obtain large amount of HBV DNA linearized at the SphI site for dimer construction. This will facilitate dimer construction.
9. Alternatively, spike the bacteria into duplicate petri dishes and use the membrane from one dish for hybridization experiment.

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Hydrodynamic HBV Transfection Mouse Model

Li-Ling Wu, Hurng-Yi Wang, and Pei-Jer Chen

Abstract

Hydrodynamic HBV transfection mouse model is an established method of the last decade where macromolecules, non-normally permeable to cell membrane, are delivered intracellular. The basic principle is that a large volume of solution, containing HBV plasmid construct, is infused rapidly in circulation to permit the preferential entrance of these macromolecules to liver parenchymal cells. The aim of this chapter is to describe the basic principles of the hydrodynamic HBV transfection in mouse models.

Key words Hydrodynamic injection, Gene transfer, In vivo transfection, HBV persistence, Liver tolerance

1 Introduction

Hydrodynamic gene delivery was established in the late 1990s [1, 2]. It uses a hydrodynamic force generated by pressurized injection of a large volume of DNA solution into the blood vessel to permeabilize the capillary endothelium and generate “pores” in the plasma membrane of the surrounding parenchymal cells through which DNA or other macromolecules of interest can reach the cell interior [3, 4]. This approach has been successfully and widely applied to delivery gene into murine hepatocytes. The standard procedure involves a rapid tail vein injection into a mouse using a relatively large quantity of DNA solution, usually 8–10% of mouse body weight, which induces transient cardiac congestion and results in an elevated hydrodynamic pressure in the inferior vena cava that drives DNA solution back to the liver and kidneys through hepatic and renal vein, respectively [4, 5]. This pressure widens the fenestration and space between the endothelial cells and hepatocytes (Disse space) and subsequently causes hepatocyte membrane defects, resulting in intra-hepatocyte DNA transfer [5–7]. Animals survive well from hydrodynamic tail vein injection. The blood composition of mice return to normal range 12–72 h after injection [8]. The functions and structures of liver can be restored

within 1–2 days after injection [9]. Because it is highly efficient, relatively simple, safe, and versatile, hydrodynamic delivery has also been explored for gene delivery into various tissues and animals, as well as in basic and translational researches (*see* [10] for review). This method was used to establish a mouse model of hepatitis B virus persistence by transfecting hepatocytes *in vivo* with HBV genome expressing viral antigens and replicative intermediates, resulting in production of viral particles [11]. Transfection in an engineered, replication-competent HBV DNA, pAAV/HBV1.2, into mouse hepatocytes by hydrodynamic injection showed that 40% of injected young C57BL/6 mice were still surface antigen positive for >6 months [12], which mimics chronic HBV infection in humans and enables us to study responses against the virus in immunocompetent hosts. Using the hydrodynamic transfection method, we demonstrated that host genetics and age, and their interaction, are crucial in the generation of strong, diverse immune responses against HBV [13, 14]. This age- and genetic-related tolerance model is valuable to the study of liver tolerance and enables the investigation of the mechanisms involved in effective control of HBV.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Unless otherwise notice, prepare and keep all reagents at room temperature.

2.1 Components for Preparation of pAAV/HBV1.2 Plasmid

1. **LB medium:** Weigh 10 g tryptone, 5 g yeast extract, and 10 g NaCl, and transfer to 2 L flask containing 900 mL of water (*see Note 1*). Mix powder well to bring into solution, and transfer the solution to a measuring cylinder and add further water to make a total of 1 L. Sterilize at 121 °C for 30 min (*see Note 2*). After cooling (to <50 °C), add ampicillin (stock solution: 50 mg/mL) to the medium making a final concentration of 50 µg ampicillin per mL medium.
2. **LB agar:** Weigh 5 g tryptone, 2.5 g yeast extract, 5 g NaCl, and 7.5 g agar and transfer to a graduated cylinder containing 250 mL of water (*see Note 1*). Mix powder well to bring into solution, add water to total volume of 500 mL, and transfer to 1 L flask. Sterilize at 121 °C for 20 min (making sure to loosen top). Let agar cool to ~55 °C and add 1 mL ampicillin (stock solution: 50 mg/mL). Pour a thin layer (5 mm) of LB agar (~10 mL) into each plate being careful to not lift the cover off excessively (you should be able to just open up enough to pour). Swirl plate in a circular motion to distribute agar on bottom completely. Let each plate cool until it is solid (~20 min) and then flip so as to avoid condensation on the agar. Store plates in plastic bags in fridge.

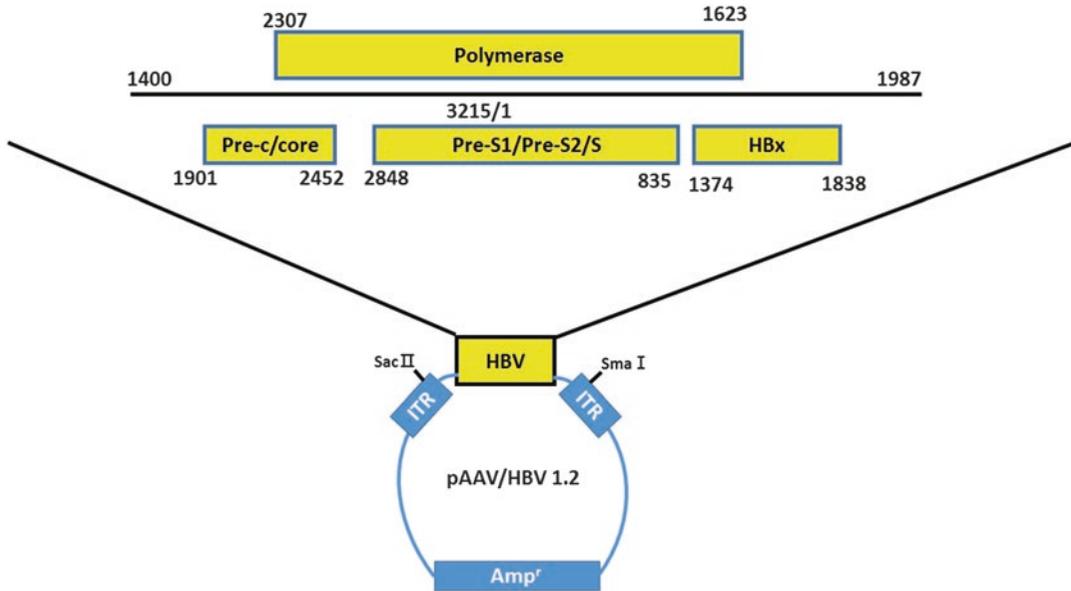


Fig. 1 Map of HBV construct. The pAAV/HBV1.2 plasmid was constructed by Huang [12]. An overlength HBV whole-genome DNA was cloned into a pAAV vector. The plasmid is able to express HBV transcripts and proteins in mouse hepatocytes and to secrete HBsAg, HBeAg, and HBcAg

3. **HBV plasmid, pAAV/HBV1.2**, which contains the HBV fragment of genotype A spanning nucleotides 1400-3182/1-1987 by inverted terminal repeats of AAV (Fig. 1), was constructed by previous study [12].
4. ECOS™ 101 Competent Cells (stored in -80°C).
5. Restriction enzyme: SmaI and SacII.
6. High-Speed Plasmid Mini Kit (Geneaid, Taiwan).
7. EndoFree® Plasmid Maxi kit (QIAGEN, USA).

2.2 Hydrodynamic Injection

1. 10× phosphate-buffered saline buffer, pH 7.4 (10× PBS buffer; containing NaCl 1.37 M, KCl 27 mM, KH_2PO_4 18 mM, Na_2HPO_4 100 mM).
2. 0.22 μm pore size hydrophilic PVDF membrane (Millex®-GS, Merck Millipore Ltd., USA).
3. BD Ultra-Fine™ II Short Needle Insulin Syringe 1 mL 31 G×8 mm (5/16 in.).
4. 27 gauge needle and 3 mL syringe.
5. Ketamine (Imalgene 1000, Merial France).
6. Xylazine (Rompun 2%, Bayer German).
7. Mouse restraint device.
8. Heat source (e.g., heat lamp with 120 W bulb).

9. Alcohol swabs.
10. Goldenrod™ Animal Lancet 4 mm.
11. Eppendorf.

3 Methods

3.1 HBV Plasmid Preparation

1. HBV plasmid preparation can start with a competent cell transformation or a glycerol stock (*see Note 3*). Proceed to **step 3**, if a glycerol stock is used.
2. Transform ECOS™ 101 Competent Cells with 5 μ L HBV plasmid according to manufacturer's procedure (*see Note 4*). Incubate LB plate at 37 °C overnight.
3. Inoculate the tube containing 5 mL LB with a single colony using a pipette tip or with a bacterial stock (*see Note 5*). Incubate with 250 rpm shaking overnight at 37 °C. The tube should be very cloudy as a result of bacteria.
4. Extract HBV plasmid from 1.5 mL culture medium by High-Speed Plasmid Mini Kit according to manufacturer's procedure (*see Note 6*).
5. Check the identity of extracted plasmid by SmaI and SacII, separately. Digestion with SmaI will cut HBV plasmid into two 4 Kb bands. Digestion with SacII will result in a 3.2 Kb and a 4.7 Kb bands (Fig. 2).
6. Transfer the rest 3 mL culture medium into 1 L LB medium. Incubate the medium with 250 rpm shaking at 37 °C for approximately 16 h.
7. Harvest the cells by centrifuging the medium with 6000 $\times g$ for 15 min at 4 °C (*see Note 7*) and extract plasmid by using QIAGEN EndoFree® Plasmid Maxi kit.
8. Check DNA quality and concentration by UV spectrometer (*see Note 8*). A good quality of plasmid DNA should have A260/A280 ratio ≥ 1.8 .

3.2 Hydrodynamic Injection

1. Measure body weight of the mouse.
2. Prepare 10 μ g of HBV plasmid DNA dissolved in a volume of PBS (*see Note 9*) equivalent to 8% of the mouse body weight (*see Note 10*).
3. Dilate the tail vessels prior to injection by warming the mouse tail with a safe and effective heat source (e.g., heat lamp (120 W bulb)) for 3–5 min (*see Note 11*). This step facilitates tail vein visualization and ensures optimal injection. As the mouse tail warms up, the vein should dilate and become more visible. Keep the mouse warm before hydrodynamic injection.

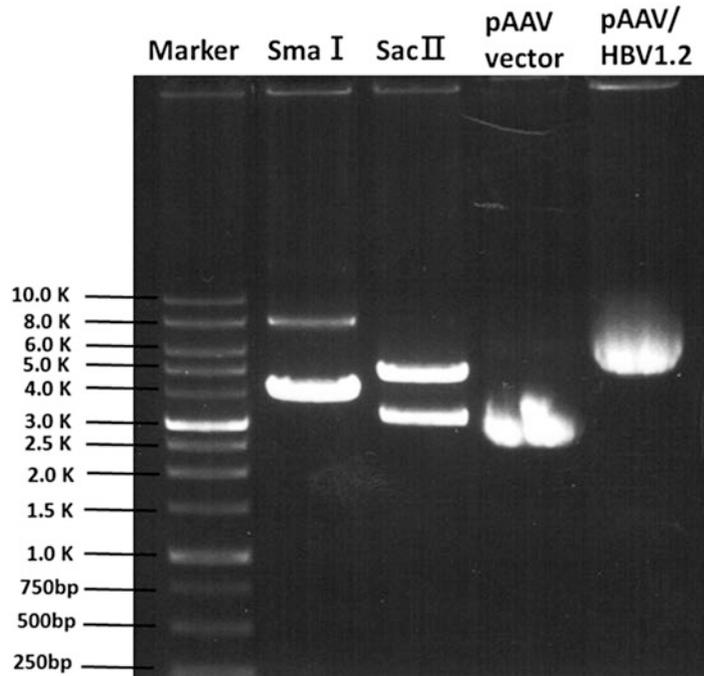


Fig. 2 Restriction digestion of HBV plasmid. The digestion products were analyzed in a 1% agarose gel. *Lane 1*, DNA size markers; *lane 2*, the result of SmaI digestion; *lane 3*, the result of SacII digestion; *lane 4*, uncut pAAV vector; *lane 5*, uncut pAAV/HBV1.2

- Anesthetize the mouse using Imalgene (ketamine, 60 mg/kg) and Rompun™ (xylazine, 12 mg/kg) administered by intramuscular injection.
- Secure the mouse with a restraint device before hydrodynamic injection.
- While working under a light source, locate the dilated vein on the ventral side of the mouse tail, preferably near the distal end (tip) of the tail.
- Swab the area with an alcohol pad and allow it air dry to further increase vein visibility and sterilize the site of injection.
- Connect the needle to the syringe and fill with the entire injection solution, ensuring that there is no air bubble in the needle or syringe (*see Note 12*).
- Place the syringe needle nearly parallel to the tail with the bevel down (toward the tail). Insert the needle into the tail vein (*see Note 13*). If the needle is inserted correctly, the vein should begin to be clear of blood (*see Note 14*).
- Dispense the complete volume of the solution into the mouse tail vein within 5–7 s at a constant rate (*see Note 15*). A good

injection is characterized by a constant resistance that does not increase during the procedure.

11. Release the mouse from the restraint device.
12. Mouse usually tolerates the hydrodynamic injection well, but immediately after injection, it may remain immobile and manifest labored breathing that persists for ~5 min. The observed apnea is probably due to a vasovagal response from the large, rapidly administered bolus of HBV DNA solution. Gentle massage to the chest of the mouse is sufficient to stimulate breathing and facilitate recovery (*see Note 16*).
13. Collect serum on day 2, 7, 10, and weekly after hydrodynamic injection from facial vein until the end of experiment (*see Note 17*).
14. Centrifuge the sample for 3 min at $16,000 \times g$ at room temperature (*see Note 18*).
15. Transfer the supernatant into a new Eppendorf tube and repeat **step 14** to obtain serum. The serum is ready for analysis. If necessary, store at $-20\text{ }^{\circ}\text{C}$.
16. Measure HBsAg and anti-HBs using an Abbott ARCHITECT HbsAg and anti-HBs kits (ARCHITECT I system) (*see Notes 19 and 20*).

4 Notes

1. Having water preloaded in the beaker helps to dissolve the components relatively easily, allowing the magnetic stir bar to go to work immediately.
2. Prior to autoclaving, some adjust the pH of LB to desired pH by NaOH or 1 mol/L TRIS stock. We generally did not adjust the pH of LB.
3. A glycerol stock is made from LB culture medium. Add 500 μL of the overnight culture to 500 μL of 50% glycerol in a 2 mL screw top tube and gently mix. Store in $-80\text{ }^{\circ}\text{C}$. The 50% glycerol medium is made by diluting 100% glycerol in water. Sterilize the glycerol medium before use.
4. Take one tube of ECOSTM 101 Competent Cells out of $-80\text{ }^{\circ}\text{C}$ and thaw on ice (~20 min). Add 5 μL of HBV plasmid into competent cells. Vortex for 1 s or flick the bottom of the tube with finger a few times. Place the mixture on ice for 5 min. Heat shock the transformation tube by placing the bottom 1/2 of the tube into $42\text{ }^{\circ}\text{C}$ for 45 s. Take 100 μL of the competent cell/DNA mixture and plate the LB plate.
5. To recover bacteria from glycerol stock, open the glycerol stock tube and use a pipette tip to scrape some of the frozen

bacteria off of the top. Do not let the glycerol stock unthaw! Dip the pipette tip into LB medium.

6. High-Speed Plasmid Mini Kit usually yield 5–10 μg of HBV plasmid dissolved in 50 μL water.
7. If necessary, make a glycerol stock by acquiring 500 μL culture medium to 500 μL of 50% glycerol in a 2 mL screw top tube and gently mix. Store in -80°C .
8. 1 L LB medium usually yields 800 μg HBV plasmid dissolved in 400 μL water (2 $\mu\text{g}/\mu\text{L}$).
9. Make 1 \times PBS by diluting 10 \times PBS with water. Sterilize the PBS by autoclave and filter with a 0.22 μm pore size hydrophilic PVDF membrane before use.
10. Usually a mouse of 6-week-old weighs between 18 and 25 g, which requires 1.9–2.6 mL of injection volume per mouse.
11. Do not overheat the mouse with the heat lamp. Excessive movement and/or perspiration are indicators of overheating.
12. With the needle pointing up, finger tap the syringe a few times to move air bubbles to the needle and carefully eject the air until a small volume of solution is ejected.
13. Introduce the needle at the distal portion of the tail. This allows for better observation of the needle entering the vein. If subcutaneous hemorrhaging occurs, the needle can be moved further up (toward the proximal end) to find a new injection site.
14. If the needle is positioned properly upon injection, clearing of the vein will be apparent, and there will be no local swelling or discoloration of the tail. If there is significant resistance, the needle may not be properly inserted into the tail vein. Improper needle insertion into tail tissue is characterized by discoloration and local swelling. When this occurs, remove the needle and reposition it correctly moving further proximal on the tail.
15. In our experience, the delivering duration is critical. On the one hand, less than 5 s of delivering may increase death rate of the mouse. On the other hand, delivering duration longer than 8 s usually compromises the efficacy of HBV transfection.
16. Chest messaging decreases the rate of mouse death from roughly 30% to less than 10%. Gently push the chest of the mouse using index finger at the rate of one time per second for 1–2 min or until spontaneous breath is recovered. Usually mouse will recover to spontaneous breath after 1–2 min.
17. There are many ways to collect blood from mice, such as from the saphenous vein, top of the foot, the tail, the orbital sinus, the jugular vein, cardiac puncture, axillary vessels, or the facial vein. We use facial vein technique described as follow. Cup the

nondominant hand (the right hand for most people) over the mouse, and scruff it firmly using the thumb and index finger. Locate the hairless freckle on the side of the jaw. Pick up the lancet with your free hand. Point the lancet at the far side of the mouse's face, at the base of the far ear or at the base of the far side of the mouth. Prick the freckle with the lancet. Collect 4–7 drops of blood to a 1.5 mL Eppendorf tube (the amount depends on frequency of bleeding). Release the mouse into its cage when you have obtained your sample. Bleeding should cease immediately.

18. Leaving the blood sample untreated longer than a couple hours is not recommended.
19. Specimens with an HBsAg value exceeding 250 IU/mL are flagged with the code ">250.00 IU/mL." Nevertheless, usually serum HBsAg will reach to highest level, which is several to 10,000 IU/mL, between 2 and 7 days after hydrodynamic injection, and the value will drop gradually afterward. We will make 1:40 dilution by adding 10 μ L serum into 390 μ L PBS during the first 2 weeks after hydrodynamic injection. After that, the dilution factor will become 1:20.
20. In our experience, mice with serum HBsAg lower than 600 IU/mL at 2 days after HDI is a sign of unsuccessful transfection and will not be included for further follow-up.

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An ELISPOT-Based Assay to Measure HBV-Specific CD8⁺ T Cell Responses in Immunocompetent Mice

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Abstract

Despite some important limitations, immunocompetent mouse models of HBV replication remain an essential tool for studying cellular and humoral immunity to the virus. CD8⁺ T cells are a critical component of the immune response to HBV due to their ability to both kill virus-infected hepatocytes and produce cytokines such as IFN- γ that non-cytopathically inhibit virus replication. A number of techniques can be used to measure the magnitude, specificity, and functionality of HBV-specific CD8⁺ T cells, each having its own unique advantages. We describe here the enzyme-linked immunospot (ELISPOT)-based assay, which, compared to other methods, is sensitive, cost-effective, and rapid and requires relatively little optimization, specialized training, or equipment.

Key words T cell, ELISPOT, HBV mouse models, IFN- γ

1 Introduction

In the study of the persistence, pathogenesis, and treatment of acute or chronic HBV, many instances exist that require evaluation of the HBV-specific CD8⁺ T cell response. For example, because of the important role of CD8⁺ T cells in the control of HBV [1, 2], assessing and/or comparing the CD8⁺ T cell response to different immunomodulators may give insight into their potential as anti-HBV agents. Similarly, understanding the properties of CD8⁺ T cells during HBV infection may contribute to a more rational design of novel HBV therapeutics.

One of the major barriers to HBV research is the lack of model systems that allow for investigation of HBV infection and the immune response to the virus. Because of their tractability, the most widely used animal models for HBV have focused on mice. Mice provide a small animal system that is low cost, well defined, and easily manipulated. Additionally, mouse-specific reagents for T cell analysis are readily available, and a variety of antigenic CD8⁺ T cell epitopes in HBV proteins have been described (Table 1;

Table 1
Mouse HBV CD8 T cell epitopes

Protein	Position	Sequence	MHC	Ref.
HBV S	191–202	IPQSLDSWWTSL	L ^d	[3]
	281–289	TGPCRTCMT	D ^d	[4]
	353–360	VWLSVIWM	K ^b	[5]
	362–371	WYWGPSLYSI	K ^d	[4]
	364–372	WGPSLYSIL	D ^d	[4]
	371–378	ILSPFLPL	K ^b	[4, 6]
HBcAg	87–95	SYVNTNMGL	K ^d	[7]
	93–100	MGLKFRQL	K ^b	[8]
	131–139	AYRPPNAPI	K ^d	[9]
Pol	140–148	HYFQTRHYL	K ^d	[10]

see Note 1). Though they do not support authentic HBV infection and spread, various immunocompetent mouse models have been developed that allow for the investigation of virus replication and the immune responses to HBV. These include hydrodynamic injection [11], HBV genome transduction with adeno-associated virus [12], and HBV transgenic mice [13] (*see Note 2*).

Various methods of analyzing CD8⁺ T cell responses exist, including MHC class I tetramer staining, cell surface marker and intracellular cytokine staining, measuring CTL (cytotoxic T lymphocyte) activity, and ELISPOT analysis, and each has certain specific advantages and disadvantages. While highly specific and reproducible, tetramer staining does not provide information on function of the cells unless used in combination with intracellular cytokine staining. Intracellular staining can be tailored to address many questions related to T cell cytokine production and transcription factor phenotype but, like tetramer staining, requires the use of a flow cytometer. CTL assays are reproducible and relatively easy to perform; however, the requirement for radioactive labeling of the target cells and relatively low sensitivity of the assay are among the disadvantages of this method. Of these techniques, ELISPOT analysis is highly sensitive (being able to detect as few as 1 in 10⁵ cells under ideal conditions) and cost-effective, measures both frequency and function of the cells, involves relatively little initial optimization, and requires no special equipment or training.

The ELISPOT assay is very similar to ELISA (enzyme-linked immunosorbent assay); however, it measures cytokine production at the single cell level (Fig. 1). Briefly, wells in a 96-well plate that

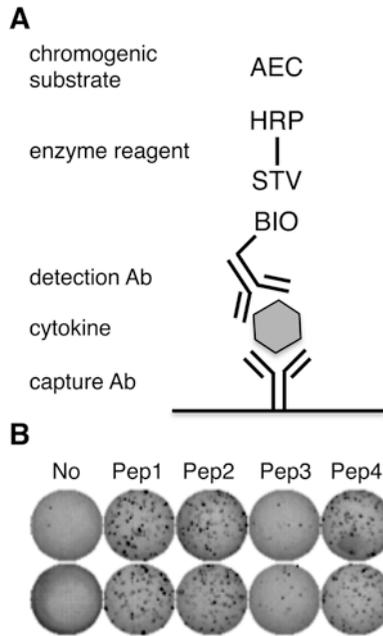


Fig. 1 (a) Principle of ELISPOT assay. Membrane-bound antibodies (Ab) capture cytokines such as IFN- γ that are secreted by the stimulated cells. Following cell removal and cytokine detection by a second antibody linked to biotin (BIO), a streptavidin (STV)-horseradish peroxidase (HRP) enzyme conjugate and chromogenic substrate (AEC) is used to generate a visible colored spot on the membrane at the location of the cells producing the cytokine. The spots are then counted to quantify the number of responding cells. **(b)** Representation of a result shown in duplicate for cells unstimulated (No) or stimulated with one of four peptides (Pep1–4)

contain a hydrophobic PVDF membrane are coated with cytokine-specific antibody, onto which cells are seeded and stimulated with desired antigen. Plate-bound antibodies in close proximity to the cells capture cytokines that are produced and secreted by the stimulated cells. Following cell removal, a second specific antibody, an enzyme conjugate, and a chromogenic substrate are sequentially added to the wells. Cells secreting the specific cytokine generate a visible colored spot on the membrane in the well. Enumeration of the spots in each well is used as a quantitative measure for the number of cytokine-producing cells.

Within the general framework of the ELISPOT assay, there are multiple applications to HBV research for which it can be employed. One of the most common techniques, which is described here, is to measure IFN- γ production after stimulation of immune cells with CD8⁺ T cell peptide epitopes [14]. However, there are other potential uses of the assay as well. For example, these include measurement of other cytokines, such as TNF- α , IL-2, or IL-17; measurement of CD4⁺ T cell responses by stimulation with whole

recombinant protein antigen [15]; and dual color staining to measure single or simultaneous production of multiple cytokines [16]. While not described in detail here, these variations may also be useful for analysis of anti-HBV T cell responses.

2 Materials

2.1 Spleen Removal

1. Dissecting scissors.
2. Dissecting forceps.
3. 70 % ethanol.
4. Hank's balanced salt solution (HBSS).

2.2 Splenocyte Preparation

1. Sterile, individually packaged 70 μ m-pore nylon strainer.
2. 1 mL syringe.
3. 15 mL conical tubes.
4. Absorbent paper or paper towels.
5. Multichannel pipette.
6. Sterile reagent/cell reservoirs.
7. HBSS.
8. ACK red blood cell lysis buffer.
9. Petri dishes.
10. Fetal bovine serum (FBS).
11. Complete media: RPMI 1640, 10% FBS, 2 mM L-glutamine, 50 μ g/mL penicillin, 50 U/mL streptomycin.

2.3 ELISPOT Assay

1. Mouse IFN- γ ELISPOT set (BD Biosciences)
 - (a) Anti-mouse IFN- γ capture antibody.
 - (b) Biotinylated anti-mouse IFN- γ detection antibody.
 - (c) Streptavidin-HRP reagent.
 - (d) 96-well ELISPOT plate.
2. 3-Amino-9-ethylcarbazole (AEC) substrate set (BD Biosciences).
3. Dulbecco's phosphate-buffered saline (PBS).
4. PBS-T: PBS, 0.05 % Tween-20.
5. PBS-FBS: PBS, 10 % FBS.
6. HBV peptide stocks (*see Note 3*).
7. Phorbol 12-myristate 13-acetate (PMA).
8. Ionomycin.
9. Dissecting microscope.

3 Methods (See Note 4)

3.1 Euthanasia and Removal of Spleen (See Note 5)

1. Euthanize mouse using an American Veterinary Medical Association-approved method of euthanasia [17]. This typically involves injection of an overdose of a barbiturate such as pentobarbital, inhalation overdose of an anesthetic such as isoflurane, or carbon dioxide asphyxiation. Care should be taken to confirm death, which can be ensured by an adjunctive physical method such as cervical dislocation. Contact your Institutional Animal Care and Use Committee for further information on approved methods.
2. Place the mouse on its back on an absorbent paper towel or dissecting board.
3. Wet the fur of the mouse with 70% ethanol (*see Note 6*).
4. Using forceps, lift the skin of the lower abdomen, and with scissors, make a lateral incision across the abdomen. Be careful to only cut the skin, and not through to the peritoneal membrane. While gripping the tail in one hand, pull the skin just above the incision toward the head in order to tear the skin and expose the peritoneum.
5. Turn the mouse on its right side. At this point, the spleen, an elongated oval-shaped organ dark red in color, should be visible through the peritoneum in the upper left abdominal quadrant (*see Note 7*).
6. To remove the spleen, cut the peritoneum near the spleen, and use forceps to gently pull the organ away from the surrounding tissues and through the incision in the peritoneum. Use scissors to remove the spleen from the attached connective tissue. Maintain sterility by not allowing the spleen to come in contact with the skin or fur of the mouse.
7. Remove any remaining connective tissue attached to the spleen and place the spleen in a 15 mL conical tube containing 5 mL HBSS (*see Note 8*).

3.2 Preparation of Splenocytes

1. For each spleen, place a 70 μm pore strainer in a petri dish (*see Note 9*).
2. Pour HBSS and spleen into the strainer, allowing the HBSS to pass through into the petri dish.
3. With the bottom of the strainer submerged in the HBSS, grind the spleen through the strainer using a plunger from a 1 mL syringe. As the connective tissue of the spleen is disrupted, the splenocytes will pass through the strainer into the HBSS in the petri dish (*see Notes 10 and 11*).
4. Collect the resulting splenocyte suspension and transfer to a 15 mL conical tube. Wash the cells off the plate by addition of 3–5 mL HBSS.

5. Centrifuge at $300\times g$ for 5 min at 4 °C to pellet the cells.
6. Remove media from tube and dissociate the pellet from the bottom of the tube by gently flicking the tube with your finger, and suspend the pellet in 2 mL of ACK lysis buffer and incubate at room temperature for 5 min.
7. Add 8 mL of HBSS to the tube and immediately centrifuge as in **step 5** (*see Notes 12–14*).
8. Decant supernatant and wash cells in 5 mL HBSS; centrifuge as in **step 5**.
9. Suspend pellet in 5 mL of complete medium and keep on ice.
10. Count cells using a hemocytometer or automated cell counter.

3.3 IFN- γ ELISPOT Assay (See Note 15)

1. On the day before the assay will be performed, dilute anti-mouse IFN- γ capture antibody according to the manufacturer's lot-specific recommendation in PBS, and add 100 μ L of diluted antibody per well to an ELISPOT plate.
2. Incubate the plate overnight at 4 °C (*see Note 16*).
3. Remove capture antibody solution, and wash wells once with complete media.
4. Block wells by adding 200 μ L/well complete media and incubating at room temperature for 2 h.
5. Prepare peptides for stimulation by diluting in complete media at a concentration of 20 μ g/mL (final concentration will be 10 μ g/mL after 1:1 dilution with cell suspension).
6. Dilute cells in complete media to 2×10^6 cells/mL (*see Notes 17 and 18*).
7. Discard blocking solution and add 100 μ L each of diluted cells (2×10^5 cells/well) and diluted peptides to the appropriate wells (*see Notes 19 and 20*).
8. As an unstimulated negative control, a well for each cell sample should also be included that contains cells but lacks peptide (add 100 μ L of complete media without peptide). As a positive control, a well can be included for each sample in which the cells are stimulated with a polyclonal T cell activator such as PMA (20 ng/mL) plus ionomycin (1 μ g/mL) (*see Note 21*).
9. Incubate ELISPOT plate overnight in a cell culture incubator (*see Note 22*).
10. Discard cells from plate, and wash wells twice with deionized or Milli-Q water, allowing wells to soak for 3–5 min during each wash.
11. Wash wells 3 \times with PBS-T (*see Note 23*).
12. Dilute biotinylated anti-mouse IFN- γ detection antibody according to the manufacturer's lot-specific recommendation

in PBS-FBS. After removal of the final wash, add 100 μ L of diluted antibody to each well.

13. Incubate plate at room temperature for 2 h.
14. Dispose of detection antibody solution, and wash wells 3 \times with PBS-T.
15. Dilute streptavidin-HRP reagent 1:100 in PBS-FBS, and add 100 μ L of the diluted enzyme conjugate to each well.
16. Incubate at room temperature for 1 h.
17. After incubation, discard streptavidin-HRP and wash wells 4 \times with PBS-T, soaking 1–2 min during each wash.
18. Wash wells an additional 2 \times with PBS.
19. Prepare substrate solution by adding 20 μ L of AEC chromogen to 1 mL of AEC substrate. Add 100 μ L of final solution to each well.
20. While monitoring spot development, incubate the plate for 5–60 min at room temperature.
21. When spots have developed sufficiently so that they are readily visible, stop the reaction by washing wells with water. Do not allow plate to overdevelop.
22. Allow the plate to dry at room temperature, removing the rubber backing to facilitate drying (*see Note 24*).

3.4 Enumeration of Response and Data Presentation

1. Using a dissecting microscope to magnify the wells, count the number of spots in each well (*see Note 25*). Spots will vary in size and intensity, and it is important to maintain well-to-well consistency in how the spots are counted. In wells with very few spots, there will be a tendency to count very small or light spots but to undercount these in wells that have many spots (*see Note 26*).
2. To quantify the specific response, subtract the corresponding “no peptide” control sample from each animal from the peptide-stimulated sample (*see Note 27*).
3. Data are often enumerated as specific IFN- γ spot-forming cells (SFC), and normalized to 10^6 cells (i.e., “specific IFN- γ SFC per 10^6 splenocytes”) (*see Note 28*).

4 Notes

1. Although mouse CD8⁺ T cell responses to many HBV epitopes have been described, the responses to some epitopes are stronger than others. The HBV S protein 191–202, 353–360, and 364–372 and HBcAg 87–95 and 93–100 epitopes are particularly immunogenic.

2. Other HBV mouse models include immunodeficient mice with humanized livers, mice with humanized livers and immune systems, and HLA transgenic mice [18]. The method described here focuses on mouse MHC-specific responses but could also be applied to human-specific responses in humanized mice or HLA transgenic mice.
3. Peptides can be provided by a number of different suppliers. Generally, peptides of 90% purity or greater are appropriate for this procedure. Most peptides are soluble in DMSO, and stock solutions of 20 mg/mL in DMSO can be prepared and aliquoted and are stable at -20°C .
4. All procedures up to Subheading 3.3, **step 10** should be performed under sterile conditions. If warranted by the biohazard potential of the experiment, use a class II biological safety cabinet for containment of infectious agents. Contact your Institutional Biological Safety Committee for further details regarding the use of biohazards.
5. Although the procedure described here uses the spleen as the source of immune cells, as an alternative, blood, lymph nodes, or intrahepatic lymphocytes can also be used with different methods of cell preparation.
6. Wetting the fur with 70% ethanol helps to sanitize the mouse but is also essential to keep disrupted fur from spreading and contaminating tissues during the dissection process.
7. If skin remains covering the left abdomen obscuring the spleen, gently separate the remaining skin away from the peritoneum using forceps until the spleen becomes visible.
8. If not proceeding directly to splenocyte preparation, place the tubes on ice to maintain cell viability.
9. The dish lid can also be used for this procedure, thereby reducing the number of dishes needed by half.
10. To make sure the cells are passing through the strainer, gently lift the strainer off the bottom of the petri dish several times during the straining process.
11. Although either end of the plunger can be used to disrupt the spleen, the circular top end works particularly well.
12. The pellet can be suspended by gently shaking the tube or by carefully pipetting up and down. Be aware that using too much mechanical force during suspension steps can damage lymphocytes and reduce cell yields.
13. Do not leave cells in ACK lysis buffer for extended periods of time. Doing so can result in lysis of lymphocytes.
14. ACK lysis buffer can also be homemade, but as the osmolarity must be exact to achieve red cell lysis without disrupting the

leukocytes, homemade solutions should be tested prior to use on important samples.

15. This procedure is adapted from the BD Biosciences protocol for catalog #551803. Procedures for other manufacturers (e.g., U-CyTech biosciences, R&D Systems, eBioscience) may vary.
16. Although overnight incubation is recommended, shorter incubations (~4 h) also seem to be sufficient.
17. Dilutions can be prepared in a reagent reservoir before transferring into the plate wells with a multichannel pipette.
18. Cells may need to be more or less diluted depending on the responses in a given experiment. It is recommended that the researcher perform a pilot experiment seeding a range of cells to determine the optimal concentration. Keep in mind that the responses obtained using different cell numbers may not be linear, as both the interactions between T cells and antigen-presenting cells and the actual number of T cells will be affected by dilution.
19. It is recommended that duplicate wells be used for each experimental condition.
20. Avoid forming bubbles when adding the cells or reagents to the wells or during the washing steps.
21. To exclude that false spots might be generated by the reagents alone, a background control well, without the cells, can also be included. Depending on the parameters of the specific experiment, other controls are also appropriate and recommended, for example, cells from a control group of mice subjected to a mock/sham experimental manipulation that are stimulated with peptide, or cells from an experimental group of mice stimulated with an irrelevant peptide (rather than no peptide).
22. To prevent formation of irregular spots, avoid disturbing the plate after addition of the cells.
23. The washing procedure is critical. Avoid touching the surface of the membrane in the wells during washing of the plate. Soak the wells in wash buffer at least 3 min before proceeding to the next step. Generally, to quickly remove cells or wash buffer, the plate can be inverted and flicked over a sink (or if appropriate, removed by vacuum suction in a biological safety cabinet) and tapped against absorbent paper to remove residual wash buffer.
24. Spots will continue to develop as the plate dries, and contrast between spots and background will improve.
25. Ideally, the number of spots will fall in the range between a minimum of 5–10 and maximum of ~100–200. Numbers

- fewer than 10 may be difficult to distinguish from background, and greater than 200 are difficult to accurately quantify.
26. There is also a tendency for different people to count spots differently, so count numbers can be confirmed by a second person, and preferably done blindly.
 27. The number of nonspecific background spots in the unstimulated and background control wells should be relatively low in comparison to the number of specific spots in the peptide-stimulated wells. A high number of spots (too many to count) in the positive control wells is indicative of a properly working assay.
 28. One limitation of the ELISPOT assay using total splenocytes is the inability to rigorously ascribe a response to a specific cell type, such as CD8⁺ T cells. Although if using well-defined peptide epitopes for stimulation that are known to not be cross-reactive for CD4⁺ T cells, there is a reasonable degree of confidence that the response is CD8⁺ T cell specific. However, specificity can be confirmed by using magnetic bead separation (Miltenyi Biotec) to enrich or deplete specific cell populations from the splenocytes prior to use in the assay.

Acknowledgments

The authors wish to dedicate this chapter to the memory of the first author, Tracy Reynolds.

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Chapter 21

Advanced Method for Isolation of Mouse Hepatocytes, Liver Sinusoidal Endothelial Cells, and Kupffer Cells

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Abstract

Separation of pure cell populations from the liver is a prerequisite to study the role of hepatic parenchymal and non-parenchymal cells in liver physiology, pathophysiology, and immunology. Traditional methods for hepatic cell separation usually purify only single cell types from liver specimens. Here, we describe an efficient method that can simultaneously purify populations of hepatocytes (HCs), liver sinusoidal endothelial cells (LSECs), and Kupffer cells (KCs) from a single mouse liver specimen. A liberase-based perfusion technique in combination with a low-speed centrifugation and magnetic-activated cell sorting (MACS) led to the isolation and purification of HCs, KCs, and LSECs with high yields and purity.

Key words Hepatocytes, Liver sinusoidal endothelial cells, Kupffer cells, Magnetic-activated cell sorting, Technology

1 Introduction

The liver lobule is formed by parenchymal cells and non-parenchymal cells (NPCs), which are the key players of the complex functions of liver [1]. Approximately 70% of the liver cells are hepatocytes (HCs), and the remaining cells are NPCs including liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs), hepatic stellate cells (HSCs), and other cells [2, 3]. For study of the precise functions of various liver cells, isolation methods are required that provide a sufficient number of cells with a high purity and viability. Significant advance in isolating liver cells was made by Seglen in 1972 through introducing a two-step isolation technique [4]. Based on it, diverse methods were developed for the isolation and cultivation of single cell types (HCs or NPC) from the liver [5–8]. In the current method, we also used the two-step perfusion technique described by Seglen with small modifications [4]. One major modification is that the Liberase™ Research Grade from Roche life science was used for the liver

digestion. The Liberase is a mixture of highly purified collagenase I and II isoforms from *Clostridium histolyticum* blended with purified neutral protease thermolysin from *Bacillus thermoproteolyticus*. These enzymes are optimized for gentle and efficient isolation of functionally active mouse liver cells. In the past, centrifugal elutriation methods and/or density gradient centrifugation methods were used for the isolation of murine and human NPCs [9–12]. However, different NPCs have similar densities which may result in contamination of the separated cell populations. Therefore, we used commercially available magnetic beads specific for KCs (F4/80) and LSECs (CD146) to increase the cell purities. This technique avoids the need for centrifugal elutriation and density gradient centrifugation.

In summary, we describe a useful method for the separation and purification of different liver cells including HCs, LSECs, and KCs from a single mouse liver specimen. The method leads to cell yields and purities that are comparable to single cell type isolation methods, allowing the application of these cells in various experimental setups.

2 Materials

2.1 Materials for Isolation

1. Peristaltic pump capable of 1–10 mL/min.
2. Water bath at 37 °C.
3. Sterilized 100-mL wide-mouth glass bottles.
4. Sterile 50-mL conical tubes.
5. 70- μ m filter, disposable mesh or reusable stainless steel.
6. 10-cm cell culture petri dishes.
7. Sterilized dissection tools; at minimum, one pair of scissors and two pairs of fine-tip forceps.
8. 27G needle.
9. 75 % ethanol and detergent.
10. Absorbent bench pads.
11. Optional: anesthetic, either injectable (pentobarb) or inhalable (isoflurane).

2.2 Reagents and Mediums for Isolation

1. Gey's balanced salt solution (GBSS) without Ca^{2+} : 0.14 mM NaCl, 5 mM KCl, 0.3 mM MgSO_4 , 1 mM Na_2HPO_4 , 3 mM NaHCO_3 , 0.2 mM KH_2PO_4 , 1 mM MgCl_2 , 5.5 mM glucose, pH 7.4.
2. Liberase TM Research Grade, Roche; storage concentration: 7 mg/mL in GBSS.
3. Liver Perfusion Medium (1 \times), Life Technologies.

4. Isolation medium: DMEM, low glucose with 1× Penicillin Streptomycin Solution (Pen-Strep) and 15 mM HEPES.
5. Washing medium: DMEM, high glucose with L-glutamine, sodium pyruvate, 10% FBS, and 1× Pen-Strep.

2.3 Materials and Reagents for Magnetic-Activated Cell Sorting

1. Mouse CD146 MicroBeads, Miltenyi Biotec.
2. Biotin-labeled anti-mouse F4/80, Miltenyi Biotec.
3. Anti-Biotin MicroBeads, Miltenyi Biotec.
4. MACS buffer: PBS with 0.5% bovine serum albumin (BSA) and 2 mM EDTA, pH 7.2.
5. MACS separator and LS Columns, Miltenyi Biotec.

2.4 Materials and Mediums for Plating and Cell Culture

1. Culture dishes/plates, collagen coated and rinsed with sterile 1× PBS before use (*see Note 1*).
2. Trypan blue, 0.4%.
3. Hemacytometer.
4. Hepatocyte culture medium: William's E medium, with 10% FCS, 1× Pen-Strep, 5 µg/mL insulin, 2% DMSO, 0.05 mM hydrocortisone hemisuccinate, 1× nonessential amino acids, 5 mM HEPES, and 200 mM L-glutamine.
5. NPC culture medium: DMEM, high glucose with L-glutamine and sodium pyruvate, with 10% FBS, 1× Pen-Strep, and 5 mM HEPES.

3 Methods

3.1 Pre-isolation Preparation

1. Prepare all necessary reagents. For each mouse, at least 15-mL liver perfusion medium and 15-mL liver digestion buffer are needed. Pre-warm liver perfusion medium and liver digestion buffer at 37 °C.
2. Place peristaltic pump under the sterile bench and rinse the pump tubing with 75% ethanol for 20 min. Remove residual ethanol from the system by flushing with autoclaved water twice. Mount a needle to the free end of the tubing. Start the pump using the liver perfusion medium. The pump system is now ready for liver perfusion.
3. Prepare a 10-cm culture dish (sterile) containing 10-mL isolation medium and place on ice for cooling the digested liver once the perfusion is complete.

3.2 Liver Perfusion and Digestion

1. Anesthetize or kill the mouse. Secure the mouse by fixing the four limbs to the working platform (*see Note 2*).
2. Quickly but thoroughly clean the abdomen region with 75% ethanol or iodine. If the animal defecates, clean it up and disinfect the area.

3. Make an incision in the upper abdomen (below the processus xiphoideus) of the mouse by using scissors and straight forceps, and puncture through the muscle layer to reveal the liver when making the incision. Continue to cut vertically until the liver, portal vein, and inferior vena cava are sufficiently exposed.
4. Insert the needle into the portal vein, start the pump, and observe the color change of the liver. If performed properly, the liver should instantly begin to blanch. Once the insertion of the needle into portal vein is successful, quickly cut the inferior vena cava to relieve pressure in the liver (*see* **Notes 3–5**).
5. Keep the flow rate of the pump to 3–5 mL/min. Allow 15-mL liver perfusion medium to rinse through the liver. During the perfusion, gently press the inferior vena cava once for 3–5 s to allow all lobes of the liver to swell (*see* **Note 6**).
6. Stop the pump flow without moving the needle in the portal vein. Change to the liver digestion buffer and continue the perfusion. During the perfusion, repeat pressing the inferior vena cava for 5–10 times to allow the liver to swell.
7. Allow 15-mL liver digestion buffer to perfuse through the liver, and then turn off the pump and remove the needle from the portal vein.

3.3 Liver Cell Suspension Preparation

1. Using a forceps to securely grip the fiber bundle of the hepatic portal, gently lift the liver up toward the operator and expose the chest cavity and the various connectives holding the liver in place. Carefully cut the tissue connections which hold the liver in place. Slowly take the liver out while cutting (*see* **Note 7**).
2. When the liver is dissociated from the abdominal cavity, immediately put it into the dish containing ice-cold isolation medium.
3. Use two pairs of forceps to tear apart the lobes of the liver. Avoid disrupting the gall bladder. If the liver has been properly digested, the medium should turn cloudy as soon as the liver is torn apart.
4. Once fully torn apart, grab the remaining part of the liver and shake gently to release residual cells. Discard any solid parts in the dish, including an intact gall bladder.
5. Gently pipette the cell suspension within the dish three times using a 25-mL serological pipette. Filter the cell suspension through a 100- μ m cell strainer and collect the filtered cells in a 50-mL conical tube (*see* **Note 8**).

3.4 Hepatocytes Purification

1. Spin the cells at $50\times g$ for 5 min at 4 °C in a swinging-arm centrifuge.
2. Aspirate and transfer the cloudy supernatant (containing liver non-parenchymal cells) to another 50-mL conical tube. Put the cells on ice during the hepatocyte purification.

3. Add 25-mL cold isolation medium to hepatocyte precipitation. Triturate gently a few times to break up the cell mass at the bottom and resuspend the hepatocytes.
4. Wash hepatocytes for a total of three times by repeating **steps 1** and **3**. Discard the supernatant after each spin. The supernatant should be clear by the third wash.
5. After the final spin, aspirate the supernatant and resuspend the cells in 25-mL cold culture medium. Remove a 100- μ L aliquot to a reaction tube and add 100 μ L 0.4% trypan blue to the cells, and pipette up and down several times to mix and count the living hepatocytes by using a hemocytometer. From a typical 8–10-week-old C57BL/6 mouse liver, the yield of hepatocytes is about $2\text{--}3 \times 10^7$ cells and the cell purity is over 95%.
6. After counting, calculate and dilute the hepatocytes to a final concentration of 5×10^6 cells/mL.
7. Plate the cells to collagen-coated plates with the following recommended plating volumes:

Plate type	Plating volume
10-cm plates	8 mL/plate
6-well plates	1.7 mL/well
12-well plates	800 μ L/well
24-well plates	360 μ L/well

8. Incubate the cells at 37 °C and allow cells to attach for 60 min. Thoroughly shake the plates forth and back every 10–15 min to suspend the hepatocytes with less viability.
9. After the cells have attached, shake the plates forth and back again and immediately change the medium. The hepatocytes are still fragile and can be easily damaged or disrupted by direct contact at this time. Therefore, pipette only down the side of the well, and never directly on top of the cells.
10. Incubate the cells overnight and wash them for three times with pre-warmed PBS. The hepatocytes are now ready for use. Hepatocyte morphology and albumin staining for cell characterization are given in Fig. 1.

3.5 LSEC Purification

1. Spin the supernatant containing non-parenchymal liver cells (Subheading 3.4, **step 2**) at $300 \times g$ for 10 min at 4 °C in a swinging-arm centrifuge.
2. Aspirate the supernatant and resuspend the cells with 50 mL cold PBS.
3. Repeat **step 1**, aspirate the supernatant, and resuspend the cells with 50-mL cold MACS buffer.

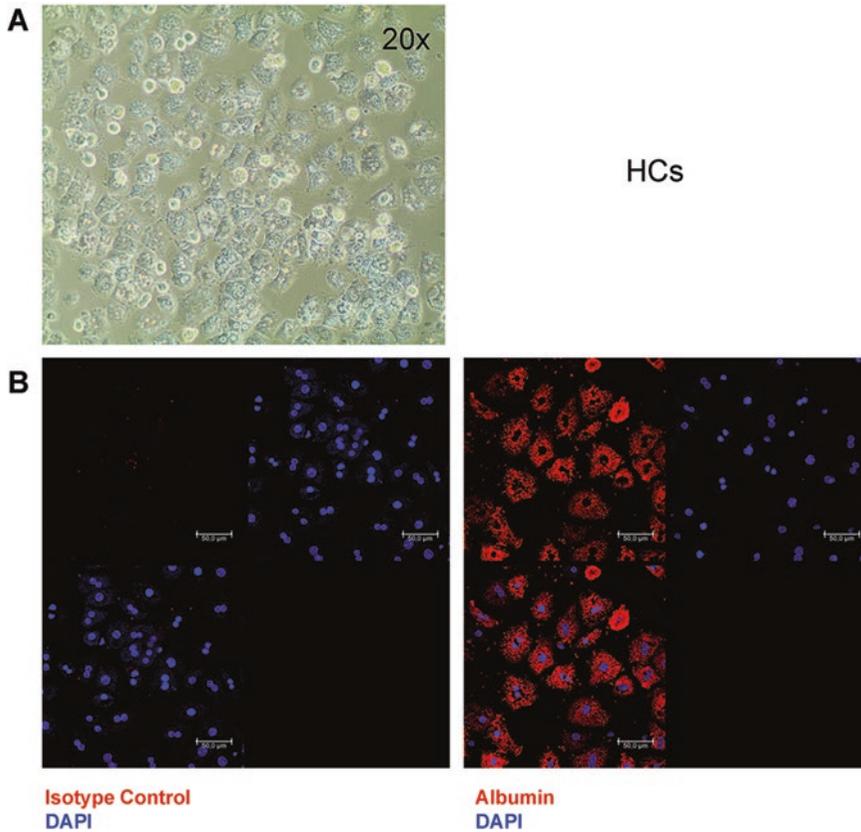


Fig. 1 Culturing and staining of HCs. HCs were isolated and cultured overnight. Cell morphology was examined by phase-contrast microscopy using EVOSTM XL Core Imaging System from AMG. The identity of isolated HCs was assessed by immunofluorescence staining of cell-specific marker—albumin. Nuclei were counterstained with DAPI (*blue*). HCs showed a binucleated polygonal shape (**a**) and a strong albumin expression (**b**, *red*). The purity of hepatocyte culture exceeded 95% as judged by fluorescence microscopy

4. Repeat **step 1** and aspirate the supernatant. Resuspend the cells with 360- μ L MACS buffer for each liver. Add 40- μ L Miltenyi CD146 MicroBeads to the cells and mix gently.
5. Refrigerate the cells for 15 min at 4–8 °C.
6. Wash cells by adding 4-mL MACS buffer and centrifuge at 300 $\times g$ for 10 min at 4 °C. Aspirate the supernatant and resuspend the cells in 500- μ L MACS buffer.
7. Proceed to magnetic separation according to the manual of Miltenyi CD146 MicroBeads.
8. Count and seed the cells to collagen-coated 24-well plates at the final density of 1×10^6 /well. From a typical 8–10-week-old C57BL/6 mouse liver, the yield of LSEC is about 5×10^6 cells and the cell purity is over 95%.
11. Culture the cells overnight and wash them with pre-warmed PBS for three times. The LSECs are now ready for use. LSEC morphology and characterization are given in Fig. 2.

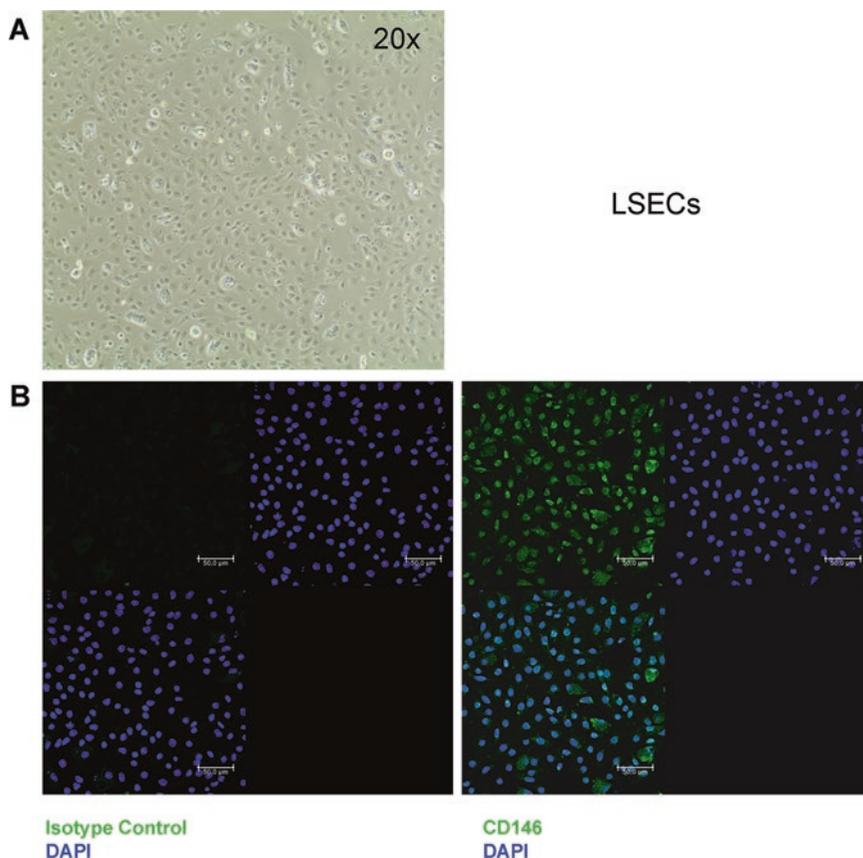


Fig. 2 Culturing and staining of LSECs. LSECs were isolated and cultured overnight. Cell morphology was examined by phase-contrast microscopy using EVOSTM XL Core Imaging System from AMG. The identity of isolated LSECs was assessed by immunofluorescence staining of cell-specific marker—CD146. Nuclei were counterstained with DAPI (*blue*). LSECs showed their unique (cobblestone) morphology (**a**) and were identified by staining of CD146 (**b**, *green*). The purity of LSEC culture exceeded 95% as judged by fluorescence microscopy

3.6 KC Purification

1. Collect the CD146-depleted cells from the LSEC purification. Centrifuge the cells at $300 \times g$ for 10 min at 4°C in a swinging-arm centrifuge.
2. Aspirate the supernatant. Resuspend the cells with 360- μL MACS buffer for each prepared liver. Add 40 μL biotin-labeled F4/80 (Miltenyi) to the cells and mix gently.
3. Refrigerate the cells for 10 min at $4\text{--}8^\circ\text{C}$.
4. Wash cells by adding 4-mL MACS buffer and centrifuge at $300 \times g$ for 10 min at 4°C . Aspirate supernatant and resuspend the cells in 180- μL MACS buffer. Add 20- μL Miltenyi Anti-Biotin MicroBeads to the cells and mix well.
5. Refrigerate the cells for 15 min at $4\text{--}8^\circ\text{C}$.

6. Wash cells by adding 2-mL MACS buffer and centrifuge at $300\times g$ for 10 min at 4 °C. Aspirate supernatant and resuspend the cells in 500- μ L MACS buffer.
7. Proceed to magnetic separation according to the manual of Miltenyi Anti-Biotin MicroBeads.
8. Count and seed the cells to 24-well plates without collagen coating (1×10^6 /well). From a typical 8–10-week-old C57BL/6 mouse liver, the yield of KC is about $2\text{--}3\times 10^6$ cells and the cell purity is over 90%.
9. Incubate the cells overnight and wash them with pre-warmed PBS for three times. The KCs are now ready for use. KC morphology and characteristics are given in Fig. 3.

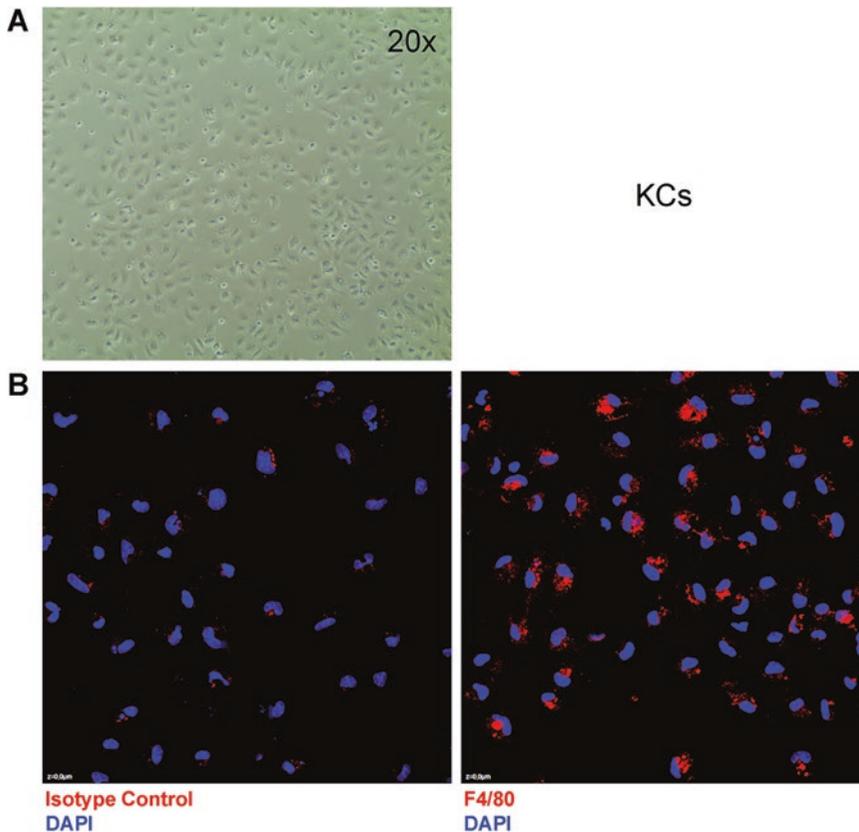


Fig. 3 Culturing and staining of KCs. KCs were isolated and cultured overnight. Cell morphology was examined by phase-contrast microscopy using EVOSTM XL Core Imaging System from AMG. The identity of isolated KCs was assessed by immunofluorescence staining of cell-specific marker—F4/80. Nuclei were counterstained with DAPI (*blue*). KCs displayed an irregular morphology (**a**) and F4/80 expression (**b**, *red*). The purity of KC culture exceeds 90% as judged by fluorescence microscopy

4 Notes

1. The concentration of rat tail collagen I for coating is between 5–8 $\mu\text{g}/\text{cm}^2$; cm^2 refers to the surface area of the bottom of the well. Collagen I is dissolved in 0.02 N acetic acid. Add the desired volume of collagen I to plate, and shake or tap the plate to distribute the collagen evenly. Then let the plate dry under UV for 1.5–2 h, or overnight. Rinse with PBS before use. Collagen-coated plates may be sealed and stored at 4 °C for months.
2. This is a non-recirculating perfusion, and all liquids will run out the inferior vena cava. Therefore, it is necessary to lay some absorbent bench pads down on the bench. The hard board which is used for fixing mice should also be covered by a pad. If possible, it is recommended to place the fixation board inside a shallow-wall container.
3. Carefully examine and remove any visible bubbles in the pumping system before starting the perfusion. Start the pump right before punching the needle into the portal vein to remove any unseen air in the tip region of the needle which may block microcapillaries and cause insufficient perfusion of some liver lobes.
4. If the liver does not blanch instantly after starting the perfusion, particularly after the inferior vena cava has been cut, there are two common reasons: (1) the operator missed the portal vein and (2) microcapillaries were blocked by air bubbles.
5. Alternatively, it is also possible to perform a retrograde perfusion by needling the inferior vena cava and cutting the portal vein for draining. However, this may result in decrease of cell yields.
6. One technique which may increase the cell yields and reduce total digestion time is to press the inferior vena cava for 5–10 times during digestion. This may cause the liver to swell and increase the dissociation of liver tissue and therefore increase the final yields.
7. Avoid breaking the gall bladder during the whole procedure, as this will reduce yields and viability of the cells.
8. All steps must be performed gently with care. The cells are very fragile at this point and are susceptible to shearing damage.

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Partial Hepatectomy and Castration of HBV Transgenic Mice

Yongjun Tian and Jing-hsiung James Ou

Abstract

Hepatitis B virus (HBV) is a hepatotropic virus. Its infection can cause liver injury and regeneration, and its replication is affected by the gender. Transgenic mice that carry a 1.3-mer overlength HBV DNA genome productively replicate HBV in hepatocytes and have been very useful for studying the replication and pathogenesis of HBV in vivo. By using this mouse model, the relationship between HBV and liver injury and regeneration as well as the effect of the gender on HBV gene expression and replication has been studied. In this chapter, we describe the surgical procedures of partial hepatectomy and castration and provide examples to demonstrate how these surgical procedures may be used to study the effect of HBV on liver regeneration and the effect of androgen on HBV replication.

Key words HBV transgenic mice, Partial hepatectomy, Liver injury and regeneration, Castration, Gender effect

1 Introduction

Hepatitis B virus (HBV) is an important human pathogen that chronically infects ~350 million people in the world. There are three times more male HBV carriers than female HBV carriers, indicating a gender effect on HBV replication [1]. HBV is hepatotropic, and its infection can cause liver injury. Hepatocytes are normally quiescent. However, they can enter the cell cycle and proliferate upon liver injury to fully restore the liver mass within a short period of time [2]. HBV transgenic mice that carry a 1.3-mer overlength HBV genome can productively replicate HBV to high levels in the liver and have been used extensively to study HBV replication and pathogenesis in vivo [3, 4]. The research using these mice had led to many important findings. For example, by using these mice, we found that the male sex hormone androgen could enhance HBV gene expression and replication [5], which provided an explanation to the gender disparity of chronic HBV carriers. We had also used 70% partial hepatectomy (PHx) in the

past to induce liver injury and regeneration to study their effects on HBV replication and the effect of HBV on liver regeneration [5]. The procedures for 70% PHx by en bloc ligation of liver lobes were first described for rats [6]. These procedures were subsequently modified and used for mice [7–12]. Here we provide a protocol describing this surgical technique, which takes 15–20 min to complete, for the removal of two-thirds of the liver in mice for studying liver regeneration and the effect of HBV on it. We also describe a simple surgical procedure to remove the testicles of male mice for studying the effect of androgen on HBV replication.

2 Materials

2.1 Mouse

Male C57BL/6 mice 10–14 weeks of age (approximately 25 g body weight) were used. All mice were maintained under pathogen-free conditions. Animal handling and care were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the University of Southern California.

2.2 Anesthesia

1. Inhaled anesthetic agent: isoflurane, 1–4%, continuous via nose cone.
2. Injectable anesthetic agent: ketamine, 80–100 mg/kg, and xylazine, 5–10 mg/kg.

2.3 Analgesia

1. Buprenorphine, 0.02–0.05 mg/kg, subcutaneous injection once every 6–12 h for at least 48 h after surgery.
2. Meloxicam, 1–2 mg/kg. Orally administered, once a day.

2.4 Reagents

1. 0.9% wt/vol NaCl.
2. 70% vol/vol ethanol.
3. Betadine.

2.5 Equipment

1. 1 and 5 mL syringes.
2. Isoflurane vaporizer.
3. Gaymar Stryker Lightweight Mul T Pad Temperature Therapy Pads.
4. Half-curved, blunt microsurgery scissors.
5. Curved microsurgery needle holder.
6. Extra fine Graefe forceps.
7. Curved forceps.
8. Operating scissors.

9. Needle holder.
10. Cotton swab.
11. Suture for peritoneum closure.
12. Styrofoam pad.
13. 4-0 black braided silk, surgical suture for knots on liver lobes.
14. 5-0 black biodegradable sutures.

3 Methods

3.1 Anesthetization

1. The inhaled anesthetic is administered using a commercial anesthesia system (Harvard Apparatus, MA, USA, Cat: 726421). The mouse is placed into an induction chamber (Harvard Apparatus, MA, USA, Cat: 727110) supplied with 2% isoflurane and oxygen. Isoflurane and oxygen with a flow rate of 5 L/min are used in the induction phase and 0.5–2.0 L/min in the maintenance phase (*see* **Notes 1–3**).
2. The injectable anesthetics used are ketamine (80–100 mg/kg) and xylazine (5–10 mg/kg) (*see* **Note 1**).

3.2 Partial Hepatectomy

1. After anesthesia, the mouse abdominal wall is shaved, and the animal is transferred to a warming pad. All feet of the animal are fixed to the operation table (*see* **Notes 4 and 5**).
2. Disinfect the skin with a surgical scrub containing 2% povidone-iodine solution.
3. A midline abdominal skin and muscle incision (about 2 cm long) is performed to expose the xiphoid process.
4. To achieve an adequate surgical field, use retractors for optimal retention of the bilateral costal bows and the fixation forceps to pull the lower abdominal wall.
5. Gently pull down the median lobe with a saline-moistened cotton swab, and cut the falciform and triangular ligament with a curved microsurgery scissor to the surface level of the suprahepatic inferior vena cava.
6. Lift the median and left lateral lobes with a saline-moistened cotton swab. Gently pull down the stomach and cut the membrane that links the caudate and the left lateral lobes.
7. Gently lift the left lateral lobe with a cotton swab, and place the 4-0 silk on the base of it using the microdissecting forceps. Pull the left lateral lobe down over the suture to its original position, and check the right end and left end of the suture with the microdissecting forceps, to make the suture go around the lobe.
8. Use the microsurgery needle holder and the microdissecting forceps to tie the two ends of the suture over the top of the left

lateral lobe, and make the knot as close to the base of the lobe as possible.

9. Cut the tied lobe just above the suture with the microsurgery curved scissors.
10. Gently lift the right portion of the median lobe, place the thread for the second knot at the right portion of the median lobe and avoid the gall bladder. Pull the right portion of median lobe down over the suture.
11. Use the microsurgery needle holder and the microdissecting forceps to tie the two ends of the suture over the top of the right portion of median lobe, placing the knot as close to the base of the lobe as possible.
12. Cut the tied lobe just above the suture with the microsurgery curved scissors.
13. Place the thread for the third knot at the base of the left portion of the median lobe. Pull the left portion of median lobe down over the suture.
14. Use the microsurgery needle holder and the microdissecting forceps to tie the two ends of the suture over the top of the left lateral lobe, placing the knot as close to the base of the lobe as possible.
15. Cut the tied lobe just above the suture with the microsurgery curved scissors.
16. Close the peritoneum and the skin with 5-0 biodegradable sutures.
17. After closing the abdomen, wipe the skin surrounding the suture with Betadine, and place the mouse on a warming pad for recovery.

3.3 Castration

1. The anesthetized animal is placed in dorsal recumbency with tail toward the surgeon.
2. Remove the hair over the scrotum of the mouse using a clipper or depilatory cream. Remove the loose hair with gauze.
3. Disinfect the skin of surgical area and the scrotum with 70% alcohol followed by 2% povidone-iodine solution.
4. Midline skin and muscle incisions are made in the scrotum.
5. Soak a sterile gauze pad with sterile isotonic saline.
6. Push out the first testicle out of the tunica. Gently raise it to expose the underlying blood vessels and tubules and rest it on the saline-soaked gauze.
7. The fat surrounding the vas deferens and spermatic blood vessels may be gently removed using dry sterile gauze to facilitate cauterization.

8. Cauterize the vas deferens and spermatic blood vessels. Place removed testicle aside. Check for bleeding.
9. Use sterile cotton-tipped swabs to gently return any remaining tissues into the scrotum.
10. Procedure is repeated with the other testicle.
11. Hold the edges of the incision together with forceps, and close the skin incision with 5-0 biodegradable sutures.
12. After closing the abdomen, wipe the skin surrounding the suture with Betadine.

3.4 Postoperation Care and Estimation of Animal Condition

1. As anesthesia can reduce animal body temperature, a warmer needs to be used to maintain the body temperature immediately after surgery until the animal has completely recovered from anesthesia.
2. For partial hepatectomy, the postoperation observation was performed once every 2 h for 12 h after the surgery and thereafter once every 4 h. An analgesic agent was given intramuscularly every 12 h for 5 days after surgery.
3. Castrated male mice must live alone in a cage.
4. If aseptic techniques are followed, antibiotics are not necessary. If an infection develops, gentamicin can be given intramuscularly at a dosage of 5 mg/kg for 5 days.

3.5 Anticipated Results

1. Mice should recover within minutes after surgery. No mortality of the animal will be observed if the PHx is performed correctly. Perioperative mortality is frequently due to technical errors, such as the accidental penetration into the chest cavity during the cutting of the falciform ligament or by the tips of the forceps when placing silk suture underneath the liver lobe, or a bowel injury during the opening of the peritoneum. Minimal morbidity within the first 12 h after surgery includes shivering, decreased mobility, and hunched posture.
2. The hepatocellular proliferation can be evaluated either by the bromodeoxyuridine (BrdU) incorporation assay or by the analysis of PCNA or Ki67-positive cells in the liver section (Fig. 1a) [13–15]. It is detected at 24 h after surgery and peaks at about 36 h. In young adult mice, the liver regeneration, which can be evaluated by volumetric measurement of liver mass (liver weight/body weight ratio), is completed within 7–14 days (Fig. 1b). This liver regeneration rate is not affected by HBV (Fig. 1b).
3. The level of circulating androgen will decrease within 1 week after castration. This will lead to the reduction of HBV titers in mice (Fig. 2).

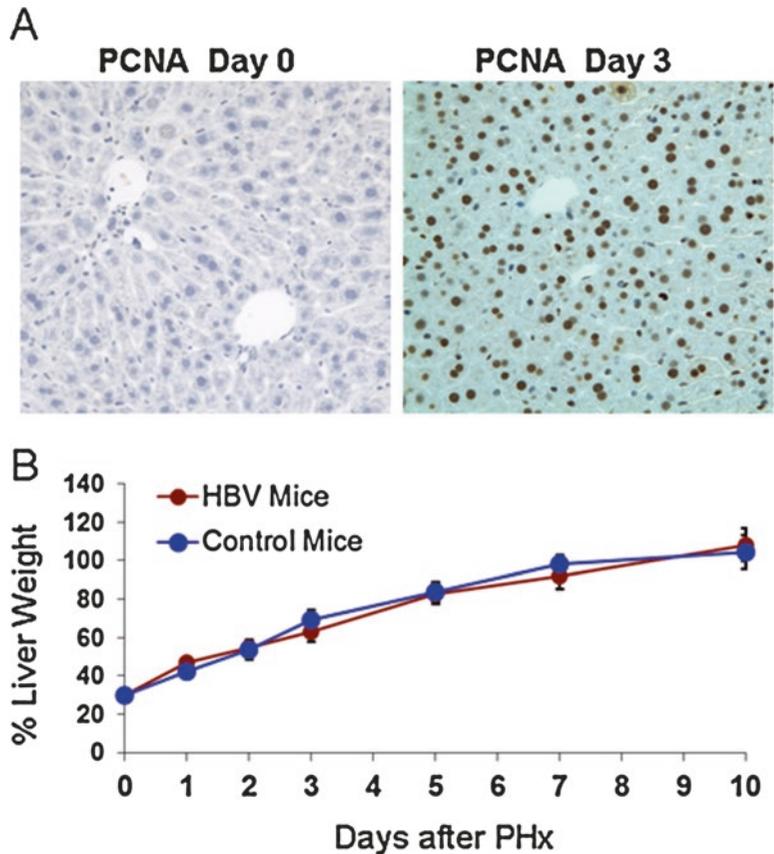


Fig. 1 Studies of liver regeneration after PHx. (a) PCNA staining of liver tissue sections prior to and 3 days after PHx. (b) Lack of effect of HBV on liver regeneration. Ten-week-old male mice were subjected to 70% PHx and sacrificed at different time points. Liver/body weight ratios were analyzed at the time points indicated

4 Notes

1. Isoflurane is an inhalant anesthetic and the first choice for these surgical procedures, as it is safe and the animal recovers fast after surgery. As the use of isoflurane requires a vaporizer, which may not always be available, ketamine/xylazine may be used as the alternative anesthetics.
2. Since mice cannot throw up, there is no need to deprive food and water for 12 h before surgery.
3. Mice need to be kept well hydrated prior to surgery, as dehydration can lead to death during surgery. Mice can be treated with 1 mL 0.9% NaCl via hypodermic injection prior to surgery to prevent dehydration.
4. Age can affect the liver regeneration rate. Young mice restore their liver mass more rapidly than old mice. If age is not a

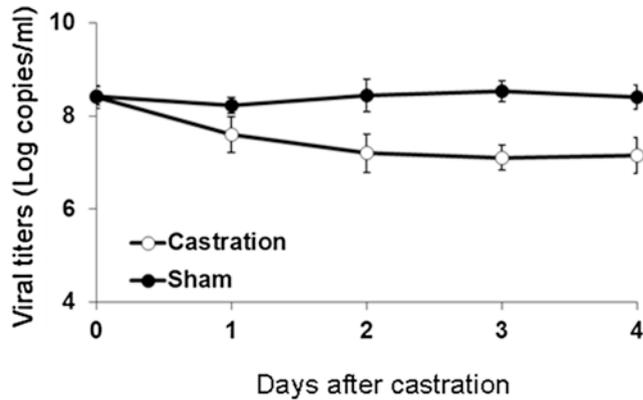


Fig. 2 Effect of castration on HBV replication in transgenic mice. HBV transgenic mice were castrated, and the mouse sera were collected at weeks 0, 1, 2, 3, and 4 for titration of HBV DNA using the real-time PCR [5]. The sham-operated mice were used as the control. The results shown at individual time points represent the means plus standard deviations of at least three mice

consideration for the study, 8–14-week-old mice are preferred for PHx.

- If the BrdU incorporation assay is used to monitor hepatocellular DNA synthesis, mice should be injected with 50 mg/kg BrdU 2 h before sacrifice. The liver is then isolated and sectioned for immunohistochemical staining using the anti-BrdU antibody.

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Studying HBV Infection and Therapy in Immune-Deficient *NOD-Rag1^{-/-}IL2RgammaC-null* (NRG) Fumarylacetoacetate Hydrolase (Fah) Knockout Mice Transplanted with Human Hepatocytes

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Abstract

Chimeric mouse models with a humanized liver provide a unique tool to study hepatic virus diseases, including viral infection, viral pathogenesis, and antiviral therapy. Here we describe a detailed protocol for studying hepatitis B infection in NRG-derived fumarylacetoacetate hydrolase (Fah) knockout mice repopulated with human hepatocytes. The procedures include (1) maintenance and genotyping of the homozygous NRG-fah/fah mutant mice (NRG/F), (2) intrasplenic injection of human hepatocytes, (3) NTBC drug reduction cycling to improve human hepatocyte repopulation, (4) human albumin detection, and (5) HBV infection and detection. The method is simple and allows for highly reproducible generation of NRG/F-hu Hep mice for studying HBV infection and therapy.

Key words Humanized mouse model, Chimeric liver, NRG/F mouse, HBV, NTBC

1 Introduction

Human hepatitis B virus (HBV) is a strict hepatotropic- and species-specific virus. Currently, only a very few species are reported to be permissive for its productive infection, including tree shrew [1, 2] and primates (such as chimpanzee [3]). With the ban of chimpanzees in scientific research [4], small animal models have become attractive alternatives for studying HBV infection and evaluating HBV antivirals. Since the development of the first HBV transgenic mouse model in the 1990s [5], attempts have been made to recapitulate the HBV life cycle and pathology in mice, including the direct delivery of the HBV genome either via tail vein hydrodynamic injection of plasmids [5, 6] or infection with adeno-associated virus (AAV) vectors harboring the HBV genome [7] (*see* detailed review [8]). Unfortunately, the HBV covalently

closed circular DNA (cccDNA), which is the essential template for progeny HBV production and for the viral persistence in hepatocytes, is poorly reproduced in the above models, suggesting that mouse hepatocytes cannot support the complete HBV life cycle or at least do so too inefficiently to make the cccDNA reach a detectable level. Thus, these models are of limited use for the study of the kinetics of HBV infection.

Sodium-taurocholate cotransporting polypeptide (NTCP, also known as SLC10A1), a cotransporter participating in the enterohepatic circulation of bile acids, was recently identified as the HBV infection receptor [9], and overexpression of NTCP in HepG2 and Huh7 cells renders them permissive for HBV infection *in vitro*. NTCP transgenic mice, however, still fail to support HBV infection, indicating that mouse hepatocytes somehow restrict the HBV infection in the early steps of infection in an unidentified manner [10].

These limitations led to the development of chimeric mouse models with humanized liver. Currently, three main strains of humanized mice with primary human hepatocytes are available for modeling HBV replication and testing HBV antivirals, including uPA mice [11, 12], Fah knockout mice [13–15], and TK-NOG (herpes simplex virus (HSV) thymidine kinase (TK)) transgenic mice [16]. These three strains allow for specific killing of mouse hepatocytes and support very high levels of human hepatocyte repopulation. uPA mice with human adult hepatocytes were the first useful model for supporting high-level HBV replication. However, overexpression of uPA in an uncontrolled manner always leads to high lethality of newborn pups, resulting in expensive and time-consuming colony maintenance [17]. To overcome this drawback, the latter two models, the Fah knockout and TK transgene overexpression, eliminate mouse hepatocytes in a more controlled, inducible manner. The protocols for generating humanized mouse liver using uPA mice [18] and TK-NOG mice [19] have been reported recently. In this chapter, we focus on the immune-deficient Fah knockout mice.

The mouse Fah gene encodes the last enzyme in the tyrosine catabolism pathway and is preferentially active in mouse hepatocytes. Knockout of Fah results in the hepatic accumulation of toxic tyrosine metabolic intermediates and the subsequent death of mouse hepatocytes. However, the liver injury can be controlled by administration or withdrawal of 2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), which blocks the accumulation of toxic intermediates caused by Fah mutations [13, 20]. Maintenance of the Fah knockout mouse colony is easy and only requires supplementation of the water with NTBC. Increasing degrees of liver damage can easily be achieved by supplying water with reduced level of NTBC, such as 25%, 12%, 6%, and 0%, as described in details in the following protocol.

Immune-deficient Fah knockout mice with different backgrounds, including B57 and NOD, are generated for repopulation with human hepatocytes for different purposes [14, 15, 20]. Mice with NOD background, for example, can support a higher level of human hematopoietic stem cell repopulation [21]. For the purposes of adult human hepatocyte transplantation, however, there is no significant difference between Fah knockout mice with different backgrounds. In this chapter, we describe in detail the procedure for repopulation of NRG/F knockout mice, as an example, with human hepatocytes for HBV infection studies. This protocol should be applicable to Fah knockout mice of other backgrounds as well. In particular, the optimized protocol for NTBC cycling will help to prevent the loss of transplanted mice.

2 Materials

2.1 Solution

1. 0.5% sodium bicarbonate.
2. NTBC, 2-(2-Nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (Sigma-Aldrich).
3. Povidone-iodine.
4. KAPA Express Extract Kits (Kapa Biosystems, Inc.).
5. Quantitative PCR reagent. Thermo scientific ABsolute qPCR SYBR Green ROX Mix.
6. PCR primers (*see* Subheading 3).
7. Bethyl Laboratories Human Albumin ELISA Quantitation Set.
8. ELISA Coating Buffer, 0.05 M Carbonate-Bicarbonate, pH 9.6.
9. ELISA Wash Solution, 50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0.
10. ELISA Blocking Solution, 50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0.
11. Sample/Conjugate Diluent, 50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20.
12. Enzyme Substrate, TMB Microwell Peroxidase Substrate System (2-C) (KPL, Kirkegaard & Perry Laboratories, Inc.).
13. ELISA Stop Solution, 1 M H₂SO₄.
14. HBV patient serum (SeraCare Life Science).
15. Freshly made Avertin. Mix 0.25 g of 2,2,2-Tribromoethanol, 99% (Alfa Aesar) with 0.5 ml of tert-amyl alcohol (Sigma-Aldrich) and dissolve it in 20 ml of water at 50 °C. Filter-sterilize through 0.2- μ m filter.
16. Bleach (10%).

17. ddH₂O molecular biology grade (Sigma-Aldrich).
18. 70% ethanol.
19. 1× DPBS (Gibco).

2.2 Equipment

1. Surgery tools: scissors, curved blunt-ended forceps, wound clips and wound clip hold, and suture.
2. Scalpel.
3. Water bath.
4. 0.22 μm filter Millex® Syringe Filters (Millipore).
5. Realtime PCR machine.
6. 0.5 and 1 ml insulin syringe.
7. Hair shaver.

3 Methods

3.1 Prepare NTBC Drug Solution (1000×)

1. Dissolve 8 g of NTBC in 1 L of 0.5% sodium bicarbonate at 65 °C for 30 min. Shake every 3–5 min until all the compound is dissolved (*see Note 1*).
2. Filter the solution using a 0.22 μm filter. Allocate and store at –20 °C.

3.2 NRG/F Mouse Genotyping and Colony Maintenance

3.2.1 Mouse Genomic DNA Extraction

1. Genomic DNA was extracted from mouse tail tips using the KAPA Mouse Genotyping Kit according to the instructions (*see Note 2*).
2. For one sample, mix the 10× KAPA Express Extract Buffer (5 μl/sample), 1 U/μl KAPA Express Extract Enzyme (1 μl/sample), and ddH₂O (44 μl/sample). The volume can be scaled up accordingly for more samples.
3. Heat at 75 °C for 15 min and inactivate the enzyme at 95 °C 5 min (*see Note 2*).
4. The sample can be stored at 4 °C for 1 week or frozen at –20 °C for long term.

3.2.2 Q-PCR Detection of Fah Deletion Homozygous Mice

1. Genomic DNA extract was diluted at a ratio of 1:20 (5–95 μl ddH₂O) and use 5 μl as template.
2. For the Fah gene knockout detection, using the following primers, FAH-QF (TTGAATCTTGAAGAATGGTTTGAGC) and FAH-QR (AATGCCAACATGATCATCCTAGG), amplicon size 148 bp. Mix the two primers together to get a 10 μM stock.
3. For the mouse genome DNA control, using the following primers, mGAPDH (450 + 26) (AACCACGAGAAATATGACA ACTCACT) and mGAPDH (584 – 20) (GGCATGGACTGT GGTCTAGA), amplicon size 134 bp. Mix the two primers together to get a 10 μM stock.

4. Q-PCR reaction setup. 2× SYBR buffer 7.5 μl, mixed primer (10 μM) 0.105 μl, ddH₂O 2.4 μl, and diluted sample 5 μl.
5. Program for Q-PCR. Step 1: 50 °C 2 min. Step 2: 95 °C 15 min. Step 3: 95 °C 15 s. Step 4: 60 °C 1 min; go to **step 3** for 35 cycles.
6. Data analysis. Normalize the FAH-KO to mGAPDH. NoFAH-KO signal will be detected in negative controls. Set heterozygous mice to 1, and the homozygous mice should be around 2.

3.2.3 NRG/F Homozygous Mice Maintenance

NRG/F homozygous mice must be maintained with 100% NTBC (8 μg/ml). The NTBC drug is pretty stable up to 3 weeks in the cage. Residual water can be filtered and reused when the water bottle is changed.

3.3 Transplantation with Human Adult Hepatocytes

3.3.1 Hepatocyte Preparation

1. Suspensions of fresh isolated hepatocytes are commercially obtained from Triangle Research Lab. Disinfect the tube surface with 70% ethanol after opening the package.
2. Centrifuge cells at 100×g for 10 min.
3. Take 5 ml of the supernatant medium to another tube for resuspending the cell pellet in **step 5**.
4. Carefully discard most of supernatant, keeping less than 0.5 ml residual medium.
5. Resuspend the cells and transfer to a 1.5 ml Eppendorf tube. Adjust the volume with supernatant medium to 50–70 μl per million cells.
6. Count the cells and check cell viability.

3.3.2 Intrasplenic Injection

Throughout the procedure, standard sterile surgical techniques are used, including bead sterilization of instruments before and in between animals and the use of sterile (prepackaged) gloves. The entire surgery procedure should be performed in a laminar flow hood. It will take 15 min if performed by one person, and it can be completed in 5–6 min by two people. The mice will wake up in 2–3 h. If bleeding occurs and cannot be stopped, mouse must be euthanized via CO₂.

1. Mouse anesthesia. Intraperitoneally inject 500–800 μl of Avetin, depending on the weight of the mouse. It will take 1–2 min to completely anesthetize the mouse. Check whether the mouse is fully asleep by squeezing the mouse foot pad.
2. Shave the left side abdomen around the spleen.
3. Lay the mouse down on autoclaved paper towel and sterilize shaved region by sequentially using povidone-iodine and 70% ethanol on the body surface.
4. Open the skin of abdomen with a scissor. The incision size is about 1.5–2 cm.

5. Open the abdomen muscle with a scissor. The incision size is about 0.5–1 cm.
6. Gently pull out the fat pad under the spleen using a curved blunt-ended forceps.
7. Slowly inject one million hepatocytes in 50–70 μl into the lower pole of the spleen at a speed about 50 μl in 5 s.
8. After injection, pull out the needle very slowly to prevent bleeding (*see Note 3*).
9. Close abdominal muscle layer by muscle suture.
10. Close skin layer by skin staples.
11. Wound clips will be removed within 10 days after surgery.

3.4 NTBC Cycling

The timing of NTBC cycling is very important, since NTBC controls the level of liver damage (*see Note 4*). The cycle is listed below and summarized in Table 1. The remaining NTBC water can be reused at each step. For instance, in each cycle, just to add 50 ml of 100% NTBC water to 150 ml autoclaved water to get 25% NTBC water, 96 ml of 25% NTBC water to 104 ml autoclaved water to get 12% NTBC water, and 100 ml of 12% NTBC water to 100 ml autoclaved water to get 6% NTBC water. A lot of NTBC drug will be saved in this manner.

Cycle 1

- Day 0, right after surgery, change water to 25% of NTBC (2 $\mu\text{g}/\text{ml}$).
 Day 2, change water bottle to 12% NTBC (0.96 $\mu\text{g}/\text{ml}$).
 Day 4, change water bottle to 6% NTBC (0.48 $\mu\text{g}/\text{ml}$).
 Day 7, change water to 0% NTBC.
 Day 21, change the water to 100% NTBC.

Cycle 2

- Day 28, change water to 25% of NTBC (2 $\mu\text{g}/\text{ml}$).
 Day 30, change water bottle to 12% NTBC (0.96 $\mu\text{g}/\text{ml}$).
 Day 32, change water bottle to 6% NTBC (0.48 $\mu\text{g}/\text{ml}$).

Table 1
Schedule of NTBC drug recycling (*see Note 4*)

Cycle	NTBC concentration				
	25%	12%	6%	0%	100%
1	Day 0	Day 2	Day 4	Day 7–21	Day 21–28
2	Day 28	Day 30	Day 32	Day 35–49	Day 21–28
3	Day 56	Day 58	Day 60	Day 63–77	Day 77–84
4	Day 84	Day 86	Day 88	Day 91–	

Day 35, change water to 0% NTBC.

Day 49, change the water to 100% NTBC.

Cycle 3

Day 56, change water to 25% of NTBC (2 µg/ml).

Day 58, change water bottle to 12% NTBC (0.96 µg/ml).

Day 60, change water bottle to 6% NTBC (0.48 µg/ml).

Day 63, change water to 0% NTBC.

Day 77, change water to 100% of NTBC (2 µg/ml).

Cycle 4

Day 84, change water to 25% of NTBC (2 µg/ml).

Day 86, change water bottle to 12% NTBC (0.96 µg/ml).

Day 88, change water bottle to 6% NTBC (0.48 µg/ml).

Day 91, change water to 0% NTBC.

3.5 Human Albumin Detection

A modified protocol from Bethyl Laboratories Human Albumin ELISA Quantitation Set (*see Note 5*).

1. Plate coating. Coating antibody dilution 1:1000 (*see Note 5*). Add 100 µl of diluted coating antibody to each well. Note: Run each standard or sample in duplicate. Incubate at room temperature (20–25 °C) for 1 h. Wash plate five times.
2. Add 200 µl of Blocking Solution to each well. Incubate at room temperature for 30 min. Wash plate five times.
3. Dilute the mouse serum sample during **step 1** and **step 2**. 100× during week 3–7 and 1000× thereafter. Standard dilution to 400, 200, 100, 50, 25, 12.5, 6.25, and 3.125 ng/ml.
4. Add 100 µl of standard or sample to well. Incubate at room temperature for 1 h. Wash plate five times.
5. Prepare 1:10,000 dilution of detection antibody dilution (*see Note 5*). Add 100 µl of diluted HRP detection antibody to each well. Incubate at room temperature for 1 h. Wash plate five times.
6. Add 100 µl of TMB Substrate Solution to each well. Develop the plate in the dark at room temperature for 10–15 min. Stop reaction by adding 50 µl of Stop Solution to each well. Measure absorbance on a plate reader at 450 nm.

3.6 HBV Infection and Detection

3.6.1 Inoculate NRG/F-hu Hep Mice with HBV Serum

For infection studies, human serum containing high titer HBV DNA (>10e9 genome copies/ml) is used. NRG/F-hu Hep mice with serum hALB >0.5 mg/ml are infected with HBV virus through the retro-orbit vein, <50 µl/injection.

1. Anesthetize with Avertin, intraperitoneally inject 500–800 µl, depending on the weight of the mouse. It will take 1–2 min to completely put down the mice. Check the anesthesia effect by squeezing the mouse foot pad.

2. Lay down the mouse to autoclaved paper towel. Immobilize the mouse head with thumb and point fingers.
3. Inject the diluted HBV virus (<50 μl) into the retro-orbit. Decontaminate the needle with 10% bleach.

3.6.2 Detection of HBV DNA in Mouse Serum (See Note 6 and Fig. 1)

1. Serum was collected from tail vein, and HBV viral DNA was extracted with QIAamp[®] MinElute[®] Virus Spin Kit according to the instruction. Elute HBV DNA using 50 μl elution buffer.
2. The following primers are used to quantify the HBV virus load. Primer 1, HBV2270F GAGTGTGGATTTCGCACTCC, and Primer 2, HBV2392R GAGGCGAGGGAGTTCTTCT.
3. Q-PCR reaction setup. 2 \times SYBR buffer 7.5 μl , mixed primer (10 μM) 0.105 μl , ddH₂O 2.4 μl , and diluted sample 5 μl . Prepare duplicate wells for each sample.
4. HBV standard. Use human patient sample with known titer HBV.
5. Program for Q-PCR. Step 1: 50 $^{\circ}\text{C}$ 2 min. Step 2: 95 $^{\circ}\text{C}$ 15 min. Step 3: 95 $^{\circ}\text{C}$ 15 s. Step 4: 60 $^{\circ}\text{C}$ 1 min; go to **step 3** for 40 cycles.

4 Notes

1. The NTBC can be dissolved in 100% DMSO, in which case it does not need to be heated. If dissolved with DMSO, special filters resistant to DMSO should be used.
2. Foot, finger, and ear tissues can also be used. The KAPA express extract takes much less time. Other methods to extract genomic DNA using Protease K digestion can be also be used to extract genomic DNA. Unlike digestions with Proteinase K, KAPA Express Extract does not completely degrade the tissue. There will be intact tissue visible in the tube after lysis. This does not have a negative impact on the downstream PCR.
3. Ligation is required sometimes, but it is not necessary if a small volume of cells is injected and the needle is pulled out very slow. We only use one million cells in the NRG/F mice. Up to five million cells have been used in published reports [20]. Our experience is that large-volume (100 μl) injection with a high concentration of cells usually causes bleeding.
4. If the surgery is not successful, mice will soon die (days 2–10). At around day 21 in the first cycle and day 49 in the second cycle, the NTBC must be put back to 100%. Otherwise, the majority of the transplanted mice will be lost. We have observed a big loss of mice at week 3 and week 7 after surgery if NTBC is not adjusted back to 100%. The time of cycling also relies on the amount and proliferation capacity of human hepatocytes

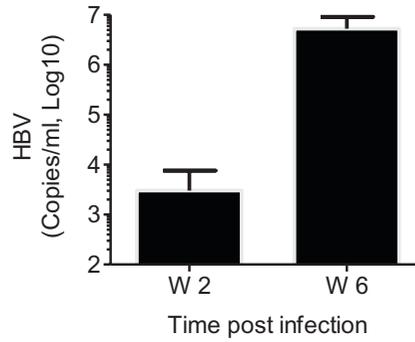


Fig. 1 HBV titers in the NRG/F-hu Hep mice. HBV serum diluted with $1 \times$ DPBS (1.0×10^7 genomic DNA in $50 \mu\text{l}$) was inoculated through retro-orbital injection. Serum HBV titers were measured using quantitative PCR. An increased HBV viral load in the serum was detected from week 2 and 6 postinfection

injected. If more cells (>1 million cells) are use, the interval of NTBC 0% can be extended. If the transfected adult hepatocytes are highly proliferative, the interval of NTBC 0% can be extended. However, this requires consistent checking of the mouse health status.

- Coating antibody is used at 1:100 dilution in the original manufacturer's protocol. However, 1:100 dilution usually gives a high background. Use of the coating antibody at 1:1000 dilution will not only reduce the background but also greatly reduce the cost. Similarly, the detection antibody is used at 1:10,000 dilution instead of 1:7500, as described in manufacture guide.
- We usually use quantitative PCR to detect the HBV viral genomic DNA (Fig. 1). HBsAg ELISA can be used to detect HBsAg.

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Measurement of Antiviral Effect and Innate Immune Response During Treatment of Primary Woodchuck Hepatocytes

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Abstract

An estimated 350 million people are chronically infected with hepatitis B virus (HBV), and over one million people die each year due to HBV-associated liver diseases, such as cirrhosis and liver cancer. Current therapeutics for chronic HBV infection are limited to nucleos(t)ide analogs and interferon. These anti-HBV drugs in general reduce viral load and improve the long-term outcome of infection but very rarely lead to a cure. Thus, new therapies for chronic HBV infection need to be developed by utilizing liver cell lines and primary cultures and small laboratory animals capable of replicating HBV or surrogate hepadnaviruses for antiviral testing. Natural infection with woodchuck hepatitis virus (WHV), a hepadnavirus closely related to HBV, occurs in woodchucks. Chronic WHV infection has been established over decades as a suitable model for evaluating direct-acting antivirals as well as vaccines, vaccine adjuvants, and immunotherapeutics because animals are fully immunocompetent. Before HBV-specific compounds are applied to woodchucks, they are usually tested in primary woodchuck hepatocytes (PWHs) replicating WHV at high levels for confirming drug specificity against viral or host targets. Here we describe a protocol for the isolation of PWHs from liver of WHV-infected woodchucks, maintenance in culture, and use in assays for determining antiviral efficacy, safety, and associated host innate immune response of new experimental drugs. Exemplary assays were performed with the nucleoside analog, lamivudine, and the immunomodulator, interferon-alpha.

Key words Primary woodchuck hepatocytes, Antiviral treatment, Innate immune response, Woodchuck hepatitis virus, Lamivudine, Interferon-alpha

1 Introduction

Chronic infection with hepatitis B virus (HBV) is responsible for approximately 1.2 million deaths per year worldwide due to HBV-associated liver diseases, including hepatic cirrhosis and hepatocellular carcinoma (HCC) [1]. It is estimated that more than two billion people have serological evidence of previous or current HBV infection and that over 350 million individuals are chronic carriers of HBV [1]. The immunologic mechanisms that

predispose to the development of chronic HBV infection are not completely understood. However, innate immune response to HBV is believed to play a crucial role in protection against viral infection, and development of an efficient innate immune response is probably needed to avoid viral persistence and subsequent progression to chronic hepatitis B. Furthermore, the contribution of innate immune response to antiviral efficacy observed following therapy with nucleos(t)ide analogs and (pegylated) interferon-alpha (IFN- α) is not well studied. These limitations are mainly due to the lack of immunocompetent, small laboratory animals susceptible to HBV infection and of cell lines suitable for virus propagation.

The Eastern woodchuck (*Marmota monax*) is naturally infected with the woodchuck hepatitis virus (WHV), a hepadnavirus which is closely related to human HBV in regard to genome organization and replication cycle [2]. Neonatal infection of woodchucks with WHV parallels the main route of human (vertical) transmission for chronic HBV infection and displays a disease course similar to that in HBV-infected patients. Thus, chronic WHV infection in woodchucks is a fully immunocompetent model for studying chronic hepatitis B- and HBV-induced HCC, and the recent comparison of hepatic transcriptional profiles in woodchucks and humans with acute self-limiting and chronic hepadnaviral infections identified important parallels in the antiviral immune responses [3, 4]. Chronic WHV carriers have extensively been used to evaluate efficacy and safety of experimental drugs, therapeutic vaccines, and immunomodulators [5]. Such studies have demonstrated the need for more detailed investigation of the innate immune response in woodchucks and to develop new molecular and cellular assays for measuring this response in liver cells. Primary woodchuck hepatocytes (PWHs) can be generated from the liver of chronic WHV carrier woodchucks. The derived cells replicate WHV at rather high levels in culture and retain their morphological characteristics for more than a month [6, 7]. Thus, WHV-replicating PWHs represent a cell culture model for various research and antiviral therapeutic applications and allow the measurement of innate immune response during treatment with anti-HBV drugs such as viral polymerase inhibitors and host immunity modulators.

Lamivudine (3TC) is a nucleoside analog licensed for the treatment of chronic HBV infection [8]. The prodrug is converted into its triphosphate form and then competes with the natural cytosine triphosphate for binding at the active site of the HBV polymerase. The incorporation of 3TC at the end of the growing viral DNA strands terminates chain elongation and thereby blocks viral replication. 3TC has been demonstrated to have antiviral efficacy against WHV in vitro and in vivo [6, 9, 10].

Interferon-alpha (IFN- α) is a pleiotropic cytokine and used for the treatment of chronic HBV infection as a recombinant protein

or in pegylated form [8]. The direct antiviral property of IFN- α results in the expression of various IFN-stimulated genes (ISGs) with antiviral effect functions within the HBV-infected hepatocyte [11]. The immunomodulatory property of IFN- α includes upregulation of immune cells such as natural killer cells and activation of CD8+ T cell function [12]. IFN- α has been demonstrated to have antiviral efficacy against WHV in vitro and in vivo [10, 13–15]. For the treatment of PWHs with IFN- α , a recombinant woodchuck interferon- α 5 protein (rwIFN- α 5) was used [16].

This chapter describes a method for the isolation of woodchuck PWHs from the liver of chronic WHV carrier woodchucks, their culture, and use in assays for determining antiviral efficacy based on the changes of WHV pregenomic (pg) RNA, safety based on cytotoxicity, and associated innate immune response based on the expression changes of selected host genes during treatment with 3TC and rwIFN- α 5. The availability of these in vitro assays facilitates the testing of antiviral effects and the characterization of host immune response of larger numbers of new experimental drugs, with applications to the continued modeling and therapy of chronic HBV infection in the woodchuck.

2 Materials

Unless indicated otherwise, prepare all solutions and buffers with DNase- and RNase-free water at room temperature. Use good sterile handling practice by working under a laminar flow, tissue culture hood, or a class II biological safety cabinet. Store all solutions and buffers at 4 °C unless indicated otherwise. Follow all appropriate waste disposal regulations when discarding materials in contact with virus and/or drugs.

2.1 Cell Culture Media

1. Hanks' Balanced Salts Solution (HBSS): HBSS and 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.0–7.4.
2. William's Media E (WME): WME, 5% (v/v) heat-inactivated fetal bovine serum (FBS), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 100 μ g/mL of the aminoglycoside gentamicin.
3. Alpha minimum essential medium (α MEM): α MEM, 5% (v/v) FBS, 10 mM HEPES, and 100 μ g/mL gentamicin. α MEM is used for diluting out the collagenase enzyme to be applied during liver perfusion (*see below*).
4. α MEM-collagenase: α MEM, 10 mM HEPES, and 0.03% (w/v) collagenase (Roche Diagnostics, IN, USA) (*see Note 1*).
5. Complete medium: WME, 5% (v/v) FBS, 10 mM HEPES, 100 μ g/mL gentamicin, 2 μ g/mL glucagon (Sigma Aldrich,

MO, USA), 5 mL ITS+1 solution (Discovery Labware, MA, USA), and 2 mM L-glutamine (Gibco, Life Technologies, NY, USA) (*see Note 2*).

2.2 Antiviral Drug and Immunomodulator

1. 3TC (Tokyo Chemical Industry America, OR, USA): Weigh out the powder using an analytical scale and dissolve in water for preparing a 100 mM stock solution. Store the stock solution at -20°C until use. Dilute the stock solution further at 1:400 or 1:1000 using complete medium for obtaining working solutions of 250 and 100 μM . Use the working solutions immediately.
2. rwINF- $\alpha 5$ (Hoffmann-La Roche, Basel, Switzerland): Dilute the stock solution (1 mg/mL) at 1:100 using complete medium for obtaining an intermediate solution (10 $\mu\text{g}/\text{mL}$). Store the intermediate solution at -20°C until use. Dilute the intermediate solution further at 1:100, 1:200, or 1:1000 using complete medium for obtaining working solutions of 0.01, 0.005, and 0.001 $\mu\text{g}/\text{mL}$. Use the working solutions immediately.

2.3 Cell Culture Plates

1. Reconstitute 10 mg of lyophilized rat tail collagen (Roche Diagnostics) under a sterile hood. Mix 10 mL of water with 20 μL of glacial acetic acid (Fisher Scientific, PA, USA) within a 50 mL conical plastic tube. Use a syringe and a filter containing a 0.22 μm cellulose acetate membrane for sterile filtration of the mixture into a fresh tube.
2. Add the entire 10 mL of water/acid solution to the 10 mg of collagen by using a new syringe with a needle for sticking through the rubber stopper of the glass bottle for obtaining a final concentration of 1 mg/mL.
3. Mix the collagen with the water/acid solution overnight at 4°C in the cold room using a rotating device.
4. The next morning, add 10 μL of collagen solution to each well of a 48-well (flat bottom) cell culture plastic plate (Cellstar) (Greiner Bio-One, Germany) under a sterile hood. Use a sterile pipette plastic tip (Gilson, WI, USA) for spreading homogeneously the collagen solution on the surface of the well bottom.
5. Allow the plate to dry under the airflow of the hood for at least 1 h before plating PWHs (*see Note 3*).

2.4 Cytotoxicity Assay

1. CellTiter-Glo One Solution (Promega, WI, USA).
2. μClear opaque 96-well plates (Greiner Bio-One).

2.5 RNA Isolation

1. TRI Reagent RT (Molecular Research Center, OH, USA) is a pre-prepared solution that combines phenol and guanidine thiocyanate in a monophasic solution and facilitates effective

inhibition of RNase activity. A biological sample such as liver or hepatocytes is homogenized or lysed in TRI Reagent RT and the homogenate/lysate then separated into aqueous and organic phases by bromoanisole within the BAN Phase Separation Reagent (Molecular Research Center) following centrifugation. After the phase separation, RNA remains in the aqueous phase, while DNA and proteins are sequestered into the interphase and organic phases.

2. 1.5 mL plastic microcentrifuge tubes (VWR International, PA, USA).
3. Isopropanol (BDH Chemicals, PA, USA).
4. 75 % ethanol (BDH Chemicals).
5. Kimberly-Clark Professional Wypall L40 wipes (VWR).
6. Ultra Pure water (Hardy Diagnostics, CA, USA).

2.6 Complementary (c) DNA Synthesis

1. High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA).
2. PCR tube strips and caps (USA Scientific, FL, USA).
3. Reverse WHV primer (position 2579 to 2556 in WHV7 genome) as described [17]: 5'-TGG CAG ATG GAG ATT GAG AGC-3' (Integrated DNA Technologies, IA, USA).

2.7 Real-Time Polymerase Chain Reaction (PCR)

1. TaqMan Gene Expression Master Mix kit (Applied Biosystems).
2. MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems).
3. MicroAmp Adhesive Film (Applied Biosystems).
4. WHV primers and probe (these and other primers and probes below were obtained from Integrated DNA Technologies):
 - (a) Forward primer (position 2504–2524 in WHV7 genome) as described [17]: 5'-AGA AGA CGC ACT CCC TCT CCT-3'.
 - (b) Reverse primer (*see* above).
 - (c) Probe (position 2531–2556 in WHV7 genome) as described [17]: 5'-/56-FAM/AGA AGA TCT CAA TCA CCG CGT CGC AG/3BHQ_1/-3'.
5. Interferon regulatory factor 9 (IRF9) primers and probe:
 - (a) Forward primer: 5'-CCC AGA CTG ACC GTG TAT AAA G-3'.
 - (b) Reverse primer: 5'-AGC CAC TCT CCA AAC AGA AC-3'.
 - (c) Probe: 5'-/56-FAM/AAC AAT GGC TGT AGT TCC TCA GAG GG/3IABkFQ/-3'.

6. Interferon-induced guanosine-5'-triphosphate (GTP)-binding protein MX1:
 - (a) Forward primer: 5'-GCT GGA CGA AGG AAA GGC-3'.
 - (b) Reverse primer: 5'-AGT GGC AGG GAT TTA CAG ATG-3'.
 - (c) Probe: 5'-/56-FAM/AGC TCA GTG GTC AGT CTC TCC GC/3IABkFQ/-3'.
7. 18S rRNA:
 - (a) Forward primer: 5'-GTA ACC CGT TGA ACC CCA TT-3'.
 - (b) Reverse primer: 5'-GGG ACT TAA TCA ACG CAA GC-3'.
 - (c) Probe: 5'-/56-FAM/GCA ATT ATT CCC CAT GAA CG/31ABkFQ/-3'.

2.8 Equipment

1. Analytical scale such as the Mettler-Toledo ML54 Analytical Balance (Mettler-Toledo, OH, USA).
2. Rotating device such as the BioMixer (Benchmark, NJ, USA).
3. Culture pan, temperature-adjustable such as the VWR Tissue Flotation Water Bath (VWR).
4. Flow rate-adjustable, peristaltic mini-pump, tubing, and canula (Fisher Scientific).
5. Speed- and time-adjustable rocking instrument such as the Timed Rock N Roller Mixer (Laboratory Instruments, MD, USA).
6. Refrigerated centrifuge such as the Eppendorf Centrifuge 5810 R (Brinkman Instruments, NY, USA).
7. Cell incubator such as the Binder CO₂ Incubator (Binder, NY, USA).
8. Microscope such as the Biostar Inverted Biological Microscope (Reichert-Jung, NY, USA).
9. Orbital shaker such as the BioMixer (Benchmark Scientific, NY, USA).
10. Luminescence reader such as the Centro LB960 microplate luminometer (Berthold Technologies, Germany).
11. Microcentrifuge such as the Microfuge 22R Centrifuge (Beckman Coulter, CA, USA).
12. Spectrophotometer such as the NanoDropTM 8000 spectrophotometer (Thermo Scientific, MA, USA).
13. PCR instrument such as the 2720 Thermal Cycler (Applied Biosystems).
14. Real-time PCR instrument such as the 7500 Real-Time PCR System (Applied Biosystems).

2.9 Others

1. Sterile scalpels.
2. Sterile gauze.
3. Cell strainer (Falcon).
4. Hemocytometer such as the Bright-Line Hemocytometer (Hausser Scientific, PA, USA).
5. Trypan blue solution.

3 Methods

3.1 Isolation and Maintenance of Primary Woodchuck Hepatocytes (PWHs)

Primary cultures of nonproliferating woodchuck hepatocytes are derived from the liver by using the collagenase perfusion technique as described previously [7]. This method allows converting the entire liver into a suspension of intact hepatocytes and provides a high initial cell yield, with up to 98% cell viability under optimal conditions. Perfusion results into the swelling of liver lobes due to the expansion of extracellular space and into the digestion of intracellular collagenous tissue by collagenase. The expansion/digestion then leads to cell dissociation [18].

1. Following euthanasia of a woodchuck chronically infected with WHV, remove aseptically the entire liver and place into a sterile glass beaker containing HBSS/0.5 mM EDTA buffer on wet ice (Fig. 1).
2. Place the liver into a temperature-adjustable culture pan under a sterile hood.
3. Perfuse each lobe for approximately 10 min with ice-cold HBSS/0.5 mM EDTA using a flow rate-adjustable, peristaltic pump and appropriate tubing and cannula for the removal of blood (Fig. 2) (*see Note 4*). A flow rate of approximately 3.5 mL/min is recommended for perfusion.



Fig. 1 Liver of a chronic WHV carrier woodchuck

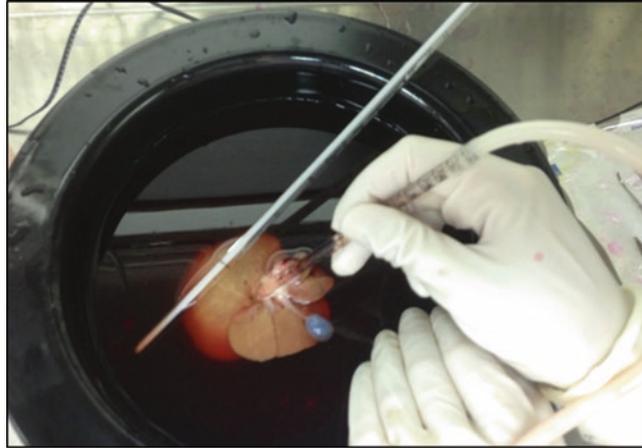


Fig. 2 Perfusion of a liver lobe with ice-cold HBSS/0.5 mM EDTA within a temperature-adjustable culture pan

4. When the liver starts blanching, perfuse the lobes further with a total of 100 mL WME/HEPES/gentamicin/FBS medium for restoring Ca^+/Mg^+ ions using the peristaltic pump.
5. Discard the medium from the culture pan and continue to perfuse the liver with a total of 300 mL of 37 °C warm α MEM-collagenase medium. Administer the medium through each lobe for approximately 10 min using the peristaltic pump. Once the 300 mL of medium are used, α MEM-collagenase medium is recycled from the culture pan which is adjusted to keep the temperature at 37 °C.
6. After the collagenase perfusion, cut the liver in smaller pieces with a sterile scalpel and mince the tissue pieces with sterilized tweezers.
7. Incubate the liver pieces in α MEM-collagenase medium for 1 h at 37 °C by placing the culture pan on a speed- and time-adjustable rocking instrument to support thorough dissociation of hepatocytes.
8. Filter the resulting hepatocyte solution into 50 mL conical plastic tubes by using sterile gauze placed over the tube opening. Place plastic tubes with filtered hepatocytes on wet ice. Dependent on the organ size, use approximately 6–8 plastic tubes per liver.
9. Pellet hepatocytes at $50 \times g$ for 4 min at 4 °C by using a refrigerated centrifuge and discard the supernatant.
10. Suspend cell pellets in fresh, ice-cold α MEM/HEPES/gentamicin/FBS medium and concentrate hepatocytes into four plastic tubes.
11. Pellet again hepatocytes at $50 \times g$ for 4 min at 4 °C, discard the supernatant, and suspend cell pellets in fresh, ice-cold

α MEM/HEPES/gentamicin/FBS medium. Repeat this washing step at least three more times to remove cellular debris.

12. After the final wash, suspend cell pellets in WME/FBS/HEPES/gentamicin medium.
13. Remove remaining cellular debris by filtering the hepatocyte suspension through a 70 μ m cell strainer into new tubes (Fig. 3).
14. Count a hepatocyte aliquot with a hemocytometer using trypan blue solution for determination of viability. Adjust the cell concentration to 2×10^5 viable cells/mL with WME/FBS/HEPES/gentamicin medium.
15. Plate 500 μ L of hepatocyte solution into each well of a 48-well plate pre-coated with rat tail collagen (Fig. 4).

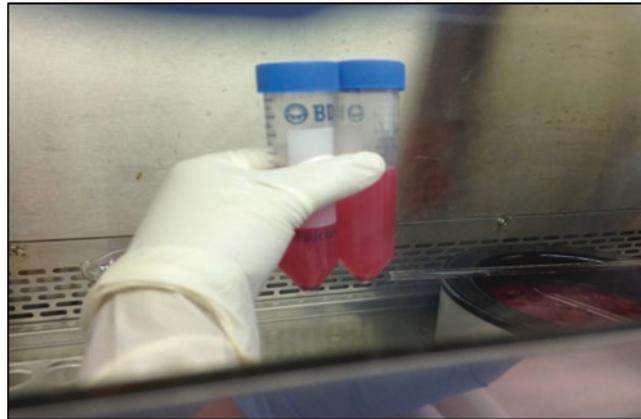


Fig. 3 Concentrated woodchuck hepatocytes in complete medium following removal of cellular debris

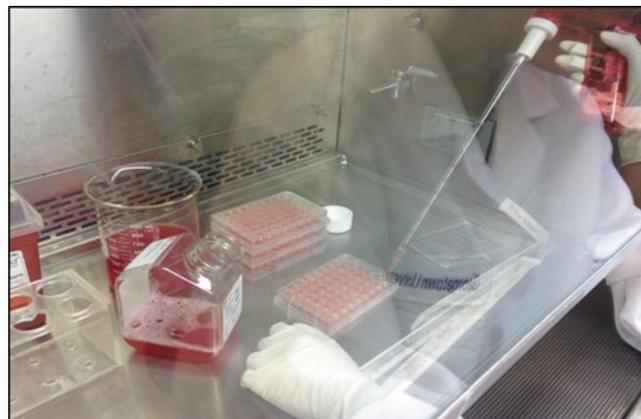


Fig. 4 Plating of 1×10^5 viable cells into each well of a 48-well plate pre-coated with rat tail collagen

16. Place the plates into an incubator and allow hepatocytes to attach to the pre-coated well bottom for at least 4 h at 37 °C and 5% CO₂.
17. Confirm cell attachment with a microscope, discard the old medium, and replace with 500 µL of complete medium per well.
18. Following incubation of cells for 2 days at 37 °C and 5% CO₂, use hepatocytes for the desired experimental application, including antiviral treatment and host immune response determination.

3.2 Treatment of PWHs with Antiviral Drugs and Immunomodulators

1. Remove medium from hepatocytes and add 500 µL of complete medium per well containing 3TC (e.g., at concentrations of 100 µM and 250 µM) and rwIFN-α5 (e.g., at concentrations of 0.001, 0.005, and 0.01 µg/mL) to selected wells. Add 500 µL of complete medium to control wells.
2. Incubate plates at 37 °C and 5% CO₂ for the desired duration of treatment (e.g., for 1–14 days/6–336 h).
3. Remove medium every second day (every 48 h) and replace with 500 µL of fresh complete medium per well containing 3TC, rwIFN-α5, or placebo (i.e., only complete medium).

3.3 Test of Drug-Associated Cytotoxicity in PWHs

For determining the percentage of viable hepatocytes during treatment with 3TC, rwIFN-α5, and placebo, a cytotoxicity assay such as the CellTiter-Glo One Solution Assay is applied. Cytotoxicity is usually tested at pretreatment (0 h) and thereafter at days 1 (24 h), 3 (72 h), 5 (120 h), 7 (168 h), 10 (240 h), and 14 (336 h) of treatment. The manufacturer's instructions are followed.

1. Thaw the CellTiter-Glo One Solution overnight at 4 °C.
2. The next morning, equilibrate the solution to room temperature and then place the bottle in a water bath at 22 °C. Prior to use, mix the solution gently by inverting the bottle several times.
3. In the meantime, equilibrate PWHs to room temperature by placing the plate under a sterile hood for 30 min.
4. Remove the cell supernatant and add 100 µL of CellTiter-Glo One Solution to each well (*see Note 5*).
5. Place the plate for 2 min on an orbital shaker for inducing cell lysis.
6. For stabilizing the luminescence signal, incubate the plate at room temperature by placing under a hood for 1 h.
7. Remove 100 µL of supernatant from the lysed cells and transfer into the wells of an opaque 96-well plate.
8. Measure the luminescent signal by placing the plate into a luminescence reader instrument.

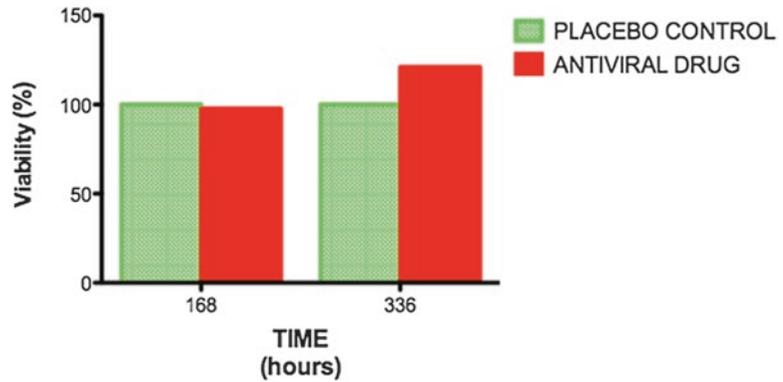


Fig. 5 Changes in cell viability following treatment of PWHs from a chronic WHV carrier woodchuck with an antiviral drug (*red bars*) for 7 days (168 h) and 14 days (336 h). Cell viability for placebo-treated control PWHs (*green bars*) was set at 100% and is shown for comparison at both time points. Compared to the placebo control, changes in viability of antiviral treated PWHs were minimal indicating no drug-associated cytotoxicity during prolonged treatment

9. Obtain luminescence data and calculate the change in viability of treated PWHs from untreated and/or placebo-treated control PWHs set at 100%.
10. Plot cell viability percentages for each drug, concentration, and treatment duration for determining cytotoxicity during treatment with antiviral drugs and immunomodulators (Fig. 5).

3.4 Isolation of RNA from PWHs

For determining changes in WHV replication activity such as the concentration of intracellular WHV pgRNA, cellular RNA is isolated during treatment of PWHs with 3TC, rwIFN- α 5, or placebo. Cellular RNA is also utilized for assaying changes in the expression of selected host innate immune response genes. Isolation of cellular RNA is usually performed at pretreatment (0 h) and thereafter at days 1 (6, 12 and 24 h), 3 (72 h), 5 (120 h), and 7 (168 h) of treatment.

1. Remove supernatant from each well (and store at -80°C if needed for subsequent applications).
2. Add 500 μL of TRI Reagent RT per well and leave the solution in contact with hepatocytes at room temperature for at least 10 min to allow optimal cell lysis.
3. Transfer the entire lysis solution into a labeled 1.5 mL plastic microcentrifuge tube (*see Note 6*).
4. Add 25 μL of BAN Phase Separation Reagent to the lysate and mix by vortexing for 15 s.
5. Spin the sample for 15 min at 4°C at full speed using a refrigerated centrifuge (do not exceed $20,000\times g/14,000$ rpm).

6. Without disturbing the interphase, transfer up to 250 μL of liquid from the aqueous phase into a fresh microcentrifuge tube.
7. Add 250 μL of ice-cold isopropanol. Vortex and incubate at room temperature for 10 min.
8. Spin the sample for 15 min at 4 $^{\circ}\text{C}$ at full speed. As RNA is insoluble in isopropanol, a pellet will be visible after the centrifugation. Discard carefully the supernatant without disturbing the pellet.
9. Add 1 mL of ice-cold 75% ethanol to the microcentrifuge tube.
10. Wash the pellet by spinning for 5 min at 4 $^{\circ}\text{C}$ at full speed. Carefully discard the supernatant without disturbing the pellet. Repeat the ethanol wash one more time.
11. Following the last ethanol wash, remove all ethanol from the microcentrifuge tube with a pipette or by using a small paper tissue such as a folded Kimberly-Clark wipe.
12. Let the pellet air dry at room temperature for 5 min.
13. Add 12 μL of RNase-free water to the sample and mix well to solubilize the RNA pellet.
14. For determination of RNA concentration within the sample, use a spectrophotometer for measuring the 260, 280, and 230 optical density values for calculation of concentration and judgment of purity of isolated cellular RNA.
15. Store RNA sample at -80°C for later processing or use immediately for the synthesis of cDNA (*see below*) (*see Note 7*).

3.5 cDNA Synthesis for Assay of WHV pgRNA

The synthesis of the first strand of cDNA from cellular total RNA is performed by using the reverse WHV primer. This primer binds to a region within the WHV core and polymerase open reading frames (ORFs) which is only present in the WHV pgRNA/precure mRNA [17].

1. Add 50 ng of cellular RNA in a total volume of 8 μL per tube of a PCR tube strip.
2. Add 0.2 μL of reverse primer (100 μM).
3. Incubate the RNA/primer solution at 65 $^{\circ}\text{C}$ for 5 min in a PCR instrument or thermal cycler.
4. Following incubation, immediately place the PCR tube strip on wet ice for 5 min.
5. Utilizing the High-Capacity cDNA Reverse Transcription kit, add 2 μL of RT buffer (10 \times), 0.8 μL of deoxynucleotide (dNTP) mix (100 mM; 25 \times), 1 μL of multiscribe reverse transcriptase enzyme (50 U/ μL) and 8 μL of RNase-free water to the RNA/primer solution.

6. Perform the cDNA reaction in a PCR instrument or thermal cycler using the following conditions: 10 min at 25 °C, 120 min at 37 °C and 5 min at 85 °C; thereafter cool to 4 °C.
7. Store cDNA sample at –80 °C for later processing or use immediately for PCR amplification (*see* below).

3.6 cDNA Synthesis for Assay of Host Innate Immune Response Gene Expression

The synthesis of the first strand of cDNA from cellular total RNA is performed by using random primers from the High-Capacity cDNA Reverse Transcription kit.

1. Add 1 µg of cellular RNA in a total volume of 14.2 µL per tube of a PCR tube strip.
2. Incubate the RNA solution at 65 °C for 5 min in a PCR instrument or thermal cycler.
3. Following incubation, immediately place the PCR tube strip on wet ice for 5 min.
4. Utilizing the High-Capacity cDNA Reverse Transcription kit, add 2 µL of RT buffer (10×), 0.8 µL of dNTP mix (100 mM; 25×), 2 µL of random primer mix (10×), 1 µL of multiscribe reverse transcriptase enzyme (50 U/µL), and 6 µL of RNase-free water to the RNA solution.
5. Perform the cDNA reaction in a PCR instrument or thermal cycler using the following conditions: 10 min at 25 °C, 120 min at 37 °C, and 5 min at 85 °C; thereafter cool to 4 °C.
6. Dilute the cDNA sample 1:5 with RNase-free water.
7. Store cDNA sample at –80 °C for later processing or use immediately for PCR amplification (*see* below).

3.7 Amplification of WHV pgRNA

Real-time polymerase chain reaction (PCR) is a technology to amplify very small amounts of DNA/cDNA by applying steps involving denaturation, annealing, and extension, thereby generating millions of copies of a particular target DNA sequence. For determining changes in WHV replication activity during drug treatment, a primer and probe set is used that specifically amplifies cDNA representing WHV pgRNA [17].

1. Add 5 µL of cDNA sample per well of a 96-well PCR plate. Use triplicate wells for each sample to account for any variability.
2. Add 5 µL of a standard dilution series of a (linearized) plasmid containing one copy of the WHV7 DNA genome [9, 17] per well of the same PCR plate. Use triplicate wells for each plasmid concentration ranging from 2×10^1 to 2×10^7 WHV copies/mL.
3. Utilizing the TaqMan Gene Expression Master Mix kit, add 10 µL of master mix and 4.6 µL of DNase-free water to each well.

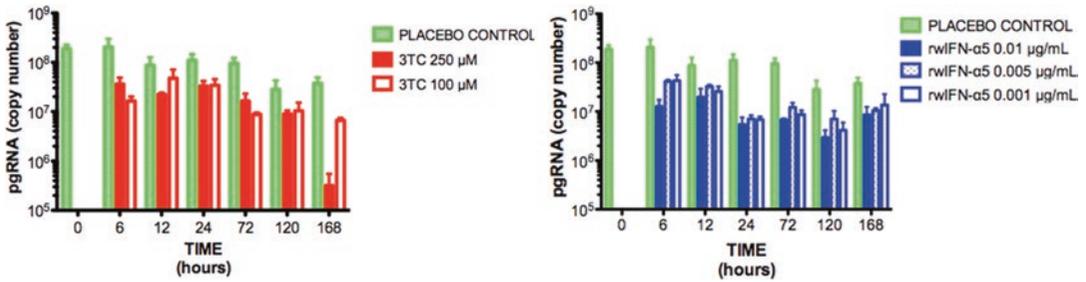


Fig. 6 Changes in WHV pgRNA copy number following treatment of PWHs from a chronic WHV carrier woodchuck with 3TC at concentrations of 100 and 250 μM (red bars) or rwIFN- α 5 at concentrations of 0.001, 0.005, or 0.01 $\mu\text{g}/\text{mL}$ (blue bars) for 1 day (6, 12 and 24 h), 3 days (72 h), 5 days (120 h), and 7 days (168 h). WHV pgRNA copy numbers for untreated/placebo-treated control PWHs (green bars) are shown for comparison at pretreatment (0 h) and at each other time point. Compared to the placebo control, the highest concentration of 3TC reduced the copy number of intracellular WHV pgRNA by approximately 2 \log_{10} after 7 days (168 h) of treatment. Compared to the placebo control, all concentrations of rwIFN- α 5 reduced the copy number of WHV pgRNA by more than 1 \log_{10} between 1 and 5 days (24–120 h) and the antiviral effect appeared more pronounced than that of 3TC during this time

4. Add 0.18 μL of WHV forward and reverse primers (each 100 μM) and 0.05 μL of WHV probe (100 μM) to each well.
5. Perform the amplification on a real-time PCR instrument using the following conditions: 2 min at 50 $^{\circ}\text{C}$, 10 min at 95 $^{\circ}\text{C}$, 40 cycles of 15 s at 95 $^{\circ}\text{C}$, and 1 min at 65 $^{\circ}\text{C}$.
6. Following completion of the amplification, obtain Ct values for WHV pgRNA samples and plasmid dilutions. Average Ct values from triplicate wells (see **Note 8**).
7. Calculate the copy numbers of WHV pgRNA in all samples by using the slope and intercept parameters derived from the standard curve for the plasmid dilutions.
8. Plot copy numbers of WHV pgRNA for each drug, concentration, and treatment duration for determining antiviral efficacy during treatment with 3TC or rwIFN- α 5 (Fig. 6).

3.8 Amplification of Host Immune Gene Expression and Normalization with 18S rRNA

For determining changes in host innate immune response during drug treatment, the expression of selected immune genes is analyzed, including IRF9 and MX1. Upregulated MX1 expression is associated with the resolved outcome of acute WHV infection and with the antiviral efficacy mediated by immunomodulators in chronic WHV carrier woodchucks [3, 4, 19]. IRF9 is involved in the Jak/STAT pathway that is activated when (pegylated) IFN- α binds to its receptor resulting in the induction of antiviral ISGs such as MX1 and 2',5'-oligoadenylate synthetase (2'5'-OAS) [20]. Primer and probe sets that bind to a region within the cDNA representing woodchuck IRF9 and MX1 are used for amplification. For normalization of cellular RNA concentration in different samples, a primer and probe set is utilized that specifically amplifies cDNA

representing 18S rRNA. This primer and probe set binds to a region within the human 18S rRNA cDNA sequence which is highly conserved in the 18S rRNA molecule of different species, including the woodchuck.

1. Add 5 μL of diluted cDNA sample per well of a 96-well PCR plate. Use triplicate wells for each sample.
2. Utilizing the TaqMan Gene Expression Master Mix kit, add 10 μL of master mix and 4.6 μL of DNase-free water to each well.
3. For amplification of IRF9 or MX1, add 0.18 μL of the respective forward and reverse primers (each 100 μM) and 0.05 μL of the respective probe (100 μM) to selected wells.
4. For amplification of 18S rRNA, add 0.18 μL of 18S rRNA forward and reverse primers (each 100 μM) and 0.05 μL of 18S rRNA probe (100 μM) to selected wells.
5. Perform the amplification on a real-time PCR instrument using the following conditions: 2 min at 50 $^{\circ}\text{C}$, 10 min at 95 $^{\circ}\text{C}$, 40 cycles of 15 s at 95 $^{\circ}\text{C}$, and 1 min at 65 $^{\circ}\text{C}$.
6. Following completion of the amplification, obtain Ct values for IRF9 and MX1 and for 18S rRNA. Average Ct values from triplicate wells (*see Note 8*).
7. Normalize IRF9 and MX1 expression to 18S rRNA expression by using the formula $2^{\Delta\text{Ct}}$, where ΔCt indicates the difference in the threshold cycle between 18S rRNA and target gene (i.e., IRF9 or MX1).
8. Plot the normalized expression of IRF9 or MX1 for each drug, concentration, and treatment duration for determining induction/changes of host innate immune response during treatment with 3TC and rwIFN- $\alpha 5$ (Fig. 7).

4 Notes

1. For a total of 500 mL of medium, add 0.3 g of collagenase powder into a 50 mL conical plastic tube (Falcon, Corning Life Science, Mexico). Add 50 mL of αMEM /HEPES medium to the tube, mix well, and sterile filter the solution back into the bottle with the remaining medium using a syringe and a filter containing a 0.22 μm cellulose acetate membrane. Equilibrate αMEM -collagenase medium in a 37 $^{\circ}\text{C}$ water bath for at least 1 h prior to the collagenase step.
2. It is recommended to prepare two 500 mL bottles of complete medium for obtaining sufficient amounts of medium during isolation, maintenance, and treatment of PWHs. Dissolve glucagon in 5 mL of acidic water. Acidic water is prepared by

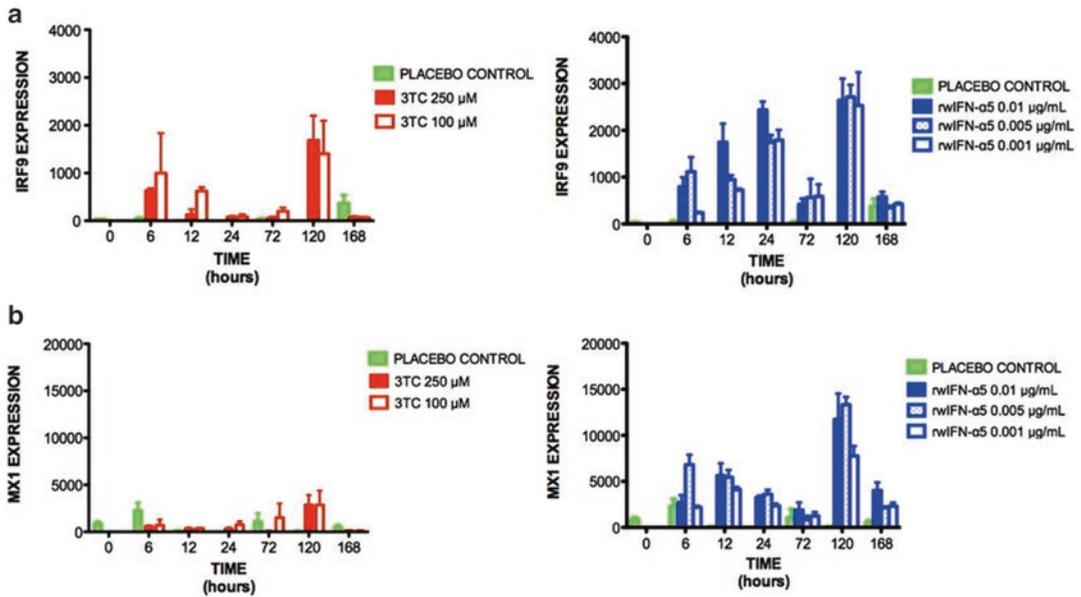


Fig. 7 Induction of (a) IRF9 and (b) MX1 gene expression following treatment of PWHs from a chronic WHV carrier woodchuck with 3TC at concentrations of 100 and 250 μ M (red bars) or rwIFN- α 5 at concentrations of 0.001, 0.005, or 0.01 μ g/mL (blue bars) for 1 day (6, 12, and 24 h), 3 days (72 h), 5 days (120 h), and 7 days (168 h). Gene expression in untreated/placebo-treated control PWHs (green bars) is shown for comparison at pretreatment (0 h) and at each other time point. Compared to the placebo control, 3TC at both concentrations appeared to induce IRF9 expression after 1 day (6 h) and more so after 5 days (120 h) of treatment. Induction of MX1 expression by 3TC was sporadic but appeared somewhat upregulated after 5 days (120 h) of treatment. Compared to the placebo control, all concentrations of rwIFN- α 5-induced IRF9 and MX1 expression after 1 day (6–24 h) and especially after 5 days (120 h) of treatment and the expression levels were more pronounced than those mediated by 3TC. Significant expression of IRF9 and MX1 correlated well with the reductions in WHV pgRNA copy numbers (Fig. 6) suggesting an important role of both genes in the *in vitro* antiviral effects mediated by rwIFN- α 5

adding 1 μ L of 1 N HCL to 5 mL of water. Sterile filter the glucagon solution into a 50 mL conical plastic tube using a syringe and a filter containing a 0.22 μ m cellulose acetate membrane. Add 5 mL of WME medium to the glucagon glass bottle, dissolve any remaining powder, and sterile filter into the plastic tube. Divide the 10 mL glucagon/WME solution between the two bottles of complete medium. Add 5 mL of ITS + 1 solution and 5 mL of L-glutamine (100 \times) to each bottle.

- Collagen-prepared plates can be stored at 4 $^{\circ}$ C for approximately 1 week by wrapping into a sealed plastic bag. If using such stored plates, allow the collagen to re-equilibrate to room temperature by placing under a hood for at least 1 h.
- If not already performed immediately after euthanasia, remove the gallbladder and any surrounding connective or fat tissue with a sterile scalpel and forceps.

5. Wear gloves when removing the seal of the CellTiter-Glo One Solution bottle for avoiding any contamination with adenosine triphosphate (ATP).
6. If cellular RNA does not need to be isolated immediately, store the cell lysate within a microcentrifuge tube at -80°C . Storage will allow processing the sample up to several months later.
7. Although not required when using TRI Reagent RT and BAN Phase Separation Reagent per the manufacturer's instructions, digestion of the isolated RNA with DNase I before use in the subsequent cDNA synthesis for removal of residual DNA contamination is recommended.
8. For comparison of Ct values obtained by different PCR amplifications, it is recommended to define parameters such as threshold (e.g., 0.05) and baseline (e.g., cycles 3–9 or 3–15) which are then applied to each PCR run.

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