HBV Infection in Human Hepatocytes and Quantification of Encapsidated HBV DNA

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[Abstract] Human hepatic cancer cell lines such as HepG2, Huh7, and HLE cannot get infected with Hepatitis B virus (HBV) due to lack of an HBV receptor(s). Transfection with HBV genome has so far been referred as a tool to mimic HBV infection. However, since sodium taurocholate cotransporting polypeptide (NTCP) was identified as a functional receptor for HBV (Yan *et al*., 2012), hepatocyte cell lines that were stably transfected with a plasmid for NTCP expression have been used for HBV infection. This protocol is designed for infection with HBV in human hepatocyte cell line HepG2 expressing NTCP (HepG2-hNTCP-C4 cells; Iwamoto *et al*., 2014) or primary human hepatocytes (PHHs). In this section, we also describe one of the methods for the assessment of HBV infection: Quantification of the intracellular encapsidated HBV DNA.

Materials and Reagents

Materials

1. 0.1-10 µl pipet tips (Thermo Fisher Scientific, catalog number: QSP#TF104)
2. 1-200 µl and 100-1,000 µl pipet tips (Corning, catalog number: 4845 and 4846, respectively)
3. Falcon 12-well tissue culture plate (Corning, catalog number: 353043)
4. Biocoat collagen I cellware 12-well plate (Corning, catalog number: 356500)
5. 1.5 ml and 2.0 ml microcentrifuge tubes (Corning, catalog number: MCT-150-A and MCT-200-C, respectively)
6. 15 ml and 50 ml centrifuge tubes (Corning Incorporated, catalog number: 352096 and 352070, respectively)
7. 96-well fast plate (NIPPON Genetics, catalog number: 38801)

Reagents

A. Hepatocyte culture and infection with HBV
   1. Primary human hepatocytes (PHHs) (PhoenixBio Co.)
   2. HepG2-hNTCP-C4 cells (Drs. Takaji Wakita and Koichi Watashi, Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan; Iwamoto *et al*., 2014)

3. Dulbecco's Modified Eagle's Medium (DMEM) (NISSUI PHARMACEUTICAL, catalog number: 05919)
4. DMEM/F-12+GlutaMax (Life Technologies, catalog number: 31331-028)
   Note: Currently, it is "Thermo Fisher Scientific, Gibco™, *catalog number: 31331-028".*
5. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Life Technologies, catalog number: 15630-080)
   Note: Currently, it is "Thermo Fisher Scientific, Gibco™, catalog number: 15630-080".
6. Fetal bovine serum (FBS) (Life Technologies, catalog number: 10437-028)
   Note: Currently, it is "Thermo Fisher Scientific, Gibco™, catalog number: 10437-028".
7. G418 (Nacalai tesque, catalog number: 09380-86)
8. Phosphate-buffered saline (PBS) (pH 7.4)
9. HBV plasmid (pUC19-HBV, genotype C) (Dr. Yasuhito Tanaka, Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; Sugiyama *et al*., 2006)
10. FuGENE 6 transfection reagent (Promega Corporation, catalog number: E2692)
11. Opti-MEM I reduced-serum medium (Life Technologies, catalog number: 31985-070)
    Note: Currently, it is "Thermo Fisher Scientific, Gibco™, catalog number: 31985-070".
12. QIAamp DNA Blood Mini Kit (QIAGEN, catalog number: 51104)
13. Polyethyleneglycol 8000 (PEG 8000) (Sigma-Aldrich, catalog number: 81268)
14. PHHs culture media (see Recipes)
15. HepG2-hNTCP-C4 culture media (see Recipes)

B. Encapsidated HBV DNA extraction
1. Nuclease free-H_2O
2. Tris (hydroxymethyl) aminomethane (Tris) (Nacalai tesque, catalog number: 35406-91)
3. NP-40 (Nacalai tesque, catalog number: 25223-75)
4. Magnesium acetate (MgOAc) (Wako Pure Chemical Industries, Siyaku, catalog number: 130-00095)
5. Ethylenediaminetetraacetic acid (EDTA) (Wako Pure Chemical Industries, Siyaku, catalog number: 345-01865)
6. Proteases K (Life Technologies, catalog number: 25530-015)
   Note: Currently, it is "Thermo Fisher Scientific, Invitrogen™, catalog number: 25530-015".
7. Sodium dodecyl sulfate (SDS) (Wako Pure Chemical Industries, Siyaku, catalog number: 191-07145)
8. Sodium chloride (NaCl) (Nacalai tesque, catalog number: 31320-05)
9. Deoxyribonuclease I (DNase I) (Promega Corporation, catalog number: M6101A)
10. Ribonuclease A (RNase A) (Life Technologies, catalog number: 12091-021)
    Note: Currently, it is "Thermo Fisher Scientific, Invitrogen™, catalog number:

12091-021".

11. Phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich, catalog number: P2069)
12. Chloroform (KANTO CHEMICAL, catalog number: 07278-00)
13. Sodium acetate (NaOAc) (Wako Pure Chemical Industries, Siyaku, catalog number: 198-01055)
14. Glycogen (Roche Diagnostics, catalog number: 10901393001)
15. Isopropanol (Nacalai tesque, catalog number: 29113-53)
16. Ethanol (99.5%) (Nacalai tesque, catalog number: 14713-95)
17. Lysis buffer (see Recipes)

C. qPCR for quantification of HBV DNA
   1. SYBR Premix Ex Taq (2x) (Tli RNase H Plus) (Takara Bio Company, catalog number: RR420)
   2. ROX reference dye (50x) (Takara Bio Company, catalog number: A9701A)
   3. Primers for amplification of encapsidated HBV DNA by quantitative PCR
      Forward: 5'-CTTCATCCTGCTGCTATGCCT-3'
      Reverse: 5'-AAAGCCCAGGATGATGGGAT-3'
      (Product length: 222 bp, product Tm: 83.5 °C)

Equipment

1. 37 °C and 5% CO_2 cell culture incubator (WAKENBTECH CO., catalog number: 9000EX)
2. Pipettes (PIPETMAN P2, P20 and P1000) (Gilson Scientific, catalog number: F144801, F123600 and F123602, respectively)
3. High speed refrigerated micro centrifuge (KUBOTA Corporation, catalog number: 3500)
4. Vortex mixer (Labnet Internationa, catalog number: vx100)
5. Shaker (Tokyo Rikakikai Co., EYELA, catalog number: MMS-110)
6. Double aluminum block bath (SCINICS CORPORATION, catalog number: ALB-301)
7. ABI StepOnePlus™ Real-Time PCR Systems (Life Technologies, catalog number: 4379216)
   Note: Currently, it is "Thermo Fisher Scientific, Applied Biosystems™, catalog number: 4379216".
8. qPCR adhesive seal (NIPPON Genetics, catalog number: 4Ti-0560)

Procedure

A. Hepatocyte culture and infection with HBV
   1. HBV preparation and measurement of HBV virus titers:

a. Seed Huh7 cells to 10-cm dish as 1 x 10^6 cells/dish with DMEM medium supplemented with 10% FBS and cultured at 37 °C in a 5% CO_2-incubator.
b. After 24 h culture, per dish of Huh7 cells are transfected with 10 μg HBV plasmid (pUC19-HBV-C) in Opti-MEM I reduced-serum medium by FuGENE 6 transfection reagent following the manufacturer's protocol.
c. At 3 days after transfection, harvest the media in 50 ml tube and clear by centrifugation for 5 min at 5,000 rpm at 4 °C. The supernatant contains the HBV can be stocked at -80 °C until use for infection.
d. For detection the HBV DNA copies of the harvested supernatant, 100 μl supernatant mixed with 1 μl MgOAc (600 mM), 0.5 μl RNase A (20 mg/ml), 1 μl DNase I (1 unit/μl) and incubate for 3 h at 37 °C to remove the HBV plasmid. The reaction is terminated by adding 2 μl EDTA (0.5 M, pH 8.0) and incubation for 10 min at 65 °C. Then the HBV DNA is extracted using QIAamp DNA Blood Mini Kit, and HBV titers are determined by qPCR as described below (Procedure C. qPCR for quantification of HBV DNA).

2. Seed cells for HBV infection:

   PHHs are purchased from PhoenixBio Co., Ltd. (Hiroshima, Japan) and seeded on biocoat collagen I cellware 12-well plate with 1 ml of PHHs culture medium as 1 x 10^5 cells/well. HepG2-hNTCP-C4 cells are generated by Drs. Takaji Wakita and Koichi Watashi (Iwamoto *et al.*, 2014), and seeded on 12-well tissue culture plate with 1 ml of HepG2-hNTCP-C4 culture medium as 1x10^5 cells/well. The cells are cultured at 37 °C in a 5% CO_2-incubator.

3. HBV infection:

   After 24 h, PHHs or HepG2-hNTCP-C4 cells are incubated for 24 h at 37 °C with HBV stock aliquots containing the appropriate number of genome equivalents (GEq), which are diluted with 1 ml of culture medium supplemented with 4% PEG 8000. At the end of the incubation, cells are washed three times with culture medium, and harvested for analysis.

   Note: PHHs or HepG2-hNTCP-C4 cells are infected at 10 or 100 GEq/cell, respectively.

B. Encapsidated HBV DNA extraction

1. Remove culture medium, wash cells twice with cold PBS.
2. Add lysis buffer 300 μl/well.
3. Shake with 200 rpm/min for 20 min at cold room (4 °C) to disrupt cells.
4. Transfer all cell lysates to new 1.5 ml tubes.
5. Pellet nuclei and insoluble fractions by centrifugation (15,000 rpm) at 4 °C for 2 min.
6. Harvest the supernatants to new 1.5 ml tubes (on ice).
7. Make a mixture in the following order:

Mix order		Volume (μl)
1	Nuclease free-H_2O	20
2	Sample supernatant	250
3	100 mM MgOAc	18
4	RNase A (20 mg/ml)	2
5	DNase I (1 unit/μl)	10
	Total	300

8. Incubate the mixture sample at 37 °C for 6 h. (After this step, the sample can be kept at 4 °C).
9. Add 30 μl EDTA (100 mM, pH 8.0) and incubate for 15 min at 65 °C to stop the reaction.
10. Centrifuge for 10 min at 15,000 rpm at 4 °C, and then harvest all supernatants to new tubes.
11. Make a mixture in the following order:

Mix order		Volume (μl)
1	Sample supernatant	330
2	10% SDS	40
3	Nuclease free-H_2O	18
4	5M NaCl	8
5	Proteinase K (20 mg/ml)	4
	Total	400

12. Incubate the mixture sample for 2 h at 55 °C to digest the HBV core protein and release the viral genome from core particles.
13. Add 400 μl phenol:chloroform:isoamyl alcohol (25:24:1) and vortex thoroughly for approximately 1 min.
14. Centrifuge for 5 min at 15,000 rpm at room temperature.
15. Carefully transfer the upper layer to a new tube.
16. Add 400 μl chloroform and vortex thoroughly for approximately 1 min.
17. Centrifuge for 5 min at 15,000 rpm at room temperature.
18. Carefully transfer the upper layer to a new tube.
19. Add 40 μl NaOAc (3 M, pH 5.2), 1 μl glycogen (20 μg/μl) and 400 μl isopropanol.
20. Simply vortex and place on ice for 20 min.
21. Centrifuge for 20 min at 15,000 rpm at 4 °C.
22. Carefully remove the supernatant without disturbing the pellet.
23. Add 500 μl of 70% ethanol, centrifuge the sample for 10 min at 15,000 rpm at 4 °C.

24. Carefully remove the supernatant as much as possible.
25. Dry the pellet for 20 min at room temperature.
26. Resuspend the pellet with 100 µl Nuclease free-H_2O and briefly spin to collect the sample. (The purified encapsidated HBV DNA can be stock at -20 °C.)

C. qPCR for quantification of HBV DNA

1. Prepare HBV DNA standard sample:
   The HBV plasmid (pUC19-HBV) is subjected to a 10-fold serial dilutions in Nuclease free-H_2O ranging from 0.2 x 10^3 to 10^9 copies/µl (1 µg HBV plasmid (pUC19-HBV) contains 1.46 x 10^{11} DNA copies, http://www.endmemo.com/bio/dnacopynum.php). And these diluted samples are used to construct a standard curve for the quantification of HBV DNA.
2. Make a 100-fold dilution of the purified encapsidated HBV DNA samples with Nuclease free-H_2O for each qPCR template.
3. Set up the following reaction mixtures for qPCR analysis to measure the copy number of HBV DNA (for one sample):

Nuclease free-H_2O	3.8 µl
2x SYBR Premix Ex Taq	10 µl
50x ROX reference dye	0.4 µl
Forward primer (10 µM)	0.4 µl
Reverse primer (10 µM)	0.4 µl
Diluted HBV DNA sample or standard DNA sample	5 µl
Total	20 µl

   Note: Use 5 µl of nuclease free-H_2O instead of DNA sample as negative control. Each sample is prepared in triplicate.
4. The PCR plate is covered with an adhesive transparent cover, and then centrifuged shortly.
5. Set the plate in the real-time instrument and start the real-time PCR following the program as below:

Holding Stage	95 °C	10 sec		1 cycle
Cycling Stage	95 °C	5 sec		45 cycles
	60 °C	30 sec	Data collection	
Melt Curve Stage	95 °C	15 sec		1 cycle
	60 °C	60 sec	Data collection during 60 °C → 95 °C	
	95 °C	15 sec		

Recipes

1. PHHs culture media
   DMEM supplemented with 10% FBS
   100 U/ml penicillin
   100 µg/ml streptomycin
   20 mM HEPES
   44 mM $NaHCO_3$
   15 µg/ml L-proline
   0.25 µg/ml insulin
   50 nM dexamethazone
   5 ng/ml epidermal growth factor (EGF)
   0.1 mM Asc-2P
   2% dimethyl sulfoxide (DMSO)
2. HepG2-hNTCP-C4 culture media
   DMEM/F-12+GlutaMax supplemented with 10 mM HEPES
   200 U/ml penicillin
   200 µg/ml streptomycin
   10% FBS
   50 µM hydrocortisone
   5 µg/ml insulin in the presence of 400 µg/ml G418
3. Lysis buffer
   1% NP-40
   1 mM EDTA
   50 mM Tris-HCl (pH 7.4)

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