

***In vitro* Drug Susceptibility Assay for HBV Using Southern Blotting**

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[Abstract] Antiviral agents for the suppression of hepatitis B virus (HBV) have been used for treating chronic hepatitis B. However, the emergence of drug-resistant HBV is still a major problem for antiviral treatment. To identify and characterize the drug-resistant HBV, the construction of HBV replicon and *in vitro* drug susceptibility assay are essential. Here we describe the experimental methods to study drug-resistant HBV.

Materials and Reagents

A. Transfection HBV 1.2mer replicons

1. Huh7 cells (Liver Research Center, Rhode Island Hospital, Brown Medical School, Providence, USA)
2. Dulbecco modified Eagle medium (DMEM) (Life Technologies, Gibco®, catalog number: 11995)
3. Fetal bovine serum (FBS) (Life Technologies, Gibco®, catalog number: 26140-079)
4. Penicillin-streptomycin (Life Technologies, Gibco®, catalog number: 15140)
5. Lipofectamine 2000 (Life Technologies, Invitrogen™, catalog number: 11668-019)
6. Opti-MEM® I Reduced Serum Medium (Opti-MEM) (Life Technologies, Gibco®, catalog number: 31985-088)
7. HBV 1.2mer replicon [WT or Drug-resistant HBV, WT HBV1.2 replicon was kindly provided from Prof. W. S. Ryu (Yonsei University, Korea)]
8. Lamivudine (1~100 µM, provided by GlaxoSmithKline)
9. Adefovir (1~100 µM, provided by Gilead Sciences)
10. Clevudine (1~100 µM, provided by Bukwang Pharmaceutical Co.)
11. Entecavir (0.05~1 µM, Moravek)
12. Tenofovir (1~100 µM, provided by Gilead Sciences)

B. Extraction of viral DNA from HBV core particles

1. DNaseI (Takara Bio Company, catalog number: 2215B)
2. MungBean Nuclease (Takara Bio Company, catalog number: 2420B)
3. Proteinase K (Roche Diagnostics, catalog number: 03115801001)

4. Phenol-chloroform-isoamyl alcohol 25:24:1 (Sigma-Aldrich, catalog number: 77617)
5. Sodium acetate trihydrate (NaOAc) (Sigma-Aldrich, catalog number: 236500)
6. HEPES lysis buffer (see Recipes)
7. Nuclease buffer (I) (see Recipes)
8. 26% PEG solution (see Recipes)
9. Nuclease buffer (II) (see Recipes)
10. 0.5% SDS solution (see Recipes)

C. Southern blot assay and the construction of the HBV probe

1. Certified Molecular Biology Agarose (Bio-Rad Laboratories, catalog number: 161-3102)
2. Amersham Hybond-N+ membrane (GE Healthcare, catalog number: RPN203B)
3. SSC Buffer (Sigma-Aldrich, catalog number: 85639)
4. Salmon sperm DNA (Life Technologies, Invitrogen™, catalog number: AM9680)
5. Poly ethylene glycol (PEG) (Sigma-Aldrich, catalog number: P5413)
6. 50x Denhardt's solution (Sigma-Aldrich, catalog number: D2532)
7. 0.1 M sodium phosphate buffer (pH 7.0)
8. Formamide (Sigma-Aldrich, catalog number: F7503)
9. HBV 1.2mer replicon
10. Klenow fragment and 10x Klenow buffer (Takara Bio Company, catalog number: 2140B)
11. dNTP (NEB, catalog number: N0446S)
12. [α -³²P] dCTP (3,000 Ci/mmol, Perkin Elmer, catalog number: BLU513H500UC)
13. Quick Spin Columns for radiolabeled DNA purification (Sephadex G-50, Roche Diagnostics, catalog number: 11 273 973 001)
14. *Aat* II (New England BioLabs, catalog number: R0117S)
15. *Hind* III (New England BioLabs, catalog number: R0104S)
16. Random primer (Bioneer, catalog number: N-7052)
17. Na₂HPO₄ (Amresco, catalog number: 0404)
18. NaH₂PO₄ (Amresco, catalog number: 0571)
19. 0.1 M sodium phosphate buffer (see Recipes)
20. Transfer solution (I) (see Recipes)
21. Transfer solution (II) (see Recipes)
22. Transfer solution (III) (see Recipes)
23. Hybridization solution (see Recipes)
24. Washing solution (I) (see Recipes)
25. Washing solution (II) (see Recipes)

Equipment

1. 37 °C, 5% CO₂ forced-air incubator (Thermo Fisher Scientific, Forma®, model: 3131)

2. 100 mm cell culture dish (Nunc®, catalog number: 172958)
3. 6 well plate (Nunc®, catalog number: 140675)
4. Centrifuge (Eppendorf, catalog number: 5415R)
5. Pipet Aid XP (Drummond Scientific Company)
6. Electrophoresis system (Nihon Eido, catalog number: NB-1011)
7. Dry oven
8. Hybridization bottle
9. Hybridization chamber
10. Heat block

Procedure

A. Transfection of HBV 1.2mer replicons

1. One day before transfection, plate 3×10^5 Huh7 cells per well in 2 ml of growth medium (DMEM with 10% FBS, 1% Penicillin-streptomycin) on 6 well plate.
2. Two hours before transfection, change 2 ml of growth media per well at 80% cells confluence.
3. Dilute the 2 µg of HBV 1.2mer replicons in 125 µl of Opti-MEM and gently flick.
4. Dilute 4 µl of Lipofectamine 2000 in 125 µl of Opti-MEM and gently flick. Then, incubate for 5 min at room temperature.
5. After 5 min incubation, combine the diluted HBV 1.2mer replicons with the diluted Lipofectamine 2000 and gently mix by pipetting. Then, incubate for 20 min at room temperature.
6. After 4~6 h transfection, change 2 ml of growth medium per well mixed with appropriate concentration anti-HBV drugs (see Figure 2).
7. Replace the growth medium with anti-HBV drugs every day for 4 days and, then harvest the cells in 1.5 ml tube by centrifugation at 3,000 rpm for 1 min at 4 °C.

B. Extraction of core particle relative HBV DNA

1. Add 100 µl of cold HEPES lysis buffer in cell pellet and incubate on ice for 20 min.
2. Centrifuge the lysates at 13,000 rpm for 10 min at 4 °C and transfer supernatant (cell lysate, cytoplasm fraction) into new 1.5 ml tube.
3. Mix 5.67 µl of nuclease buffer (I) by pipetting and incubate for 30 min in 37 °C water bath.
4. Briefly spin down, add 40 µl of 26% PEG solution and incubate for 2 h on ice (vortexing every 30 min) and centrifuge at 13,000 rpm for 30 min at 4 °C.
5. Discard the supernatant and centrifuge at 13,000 rpm for 5 min at 4 °C.
6. Completely remove the supernatant (using pipet and KIMTECH Science Wipers).
7. Add 100 µl of nuclease buffer (II) and physically resuspend the core particles.
8. Incubate for 20 min in 37 °C water bath and briefly spin down.
9. Add 282.5 µl of 0.5% SDS solution and 12.5 µl of proteinase K (20 mg/ml) and incubate for

2~4 h in 37 °C water bath (inverting every 30 min).

10. After spin down, add 400 µl of phenol-chloroform-isoamyl alcohol and vigorously vortex for 10 sec. Then, centrifuge at 13,000 rpm for 5 min at room temperature.
11. Transfer aqueous upper layer into new 1.5 ml tube.
12. Add 40 µl of 3 M NaOAc and 800 µl of 100% ethanol. After inverting several times, precipitate the mixture overnight at -20 °C.
13. Centrifuge at 13,000 rpm for 30 min at 4 °C and throw away the supernatant.
14. Add 800 µl of 70% ethanol and gently inverting.
15. Centrifuge at 13,000 rpm for 10 min at 4 °C and throw away the supernatant.
16. Add 800 µl of 100% ethanol and gently inverting.
17. Centrifuge at 13,000 rpm for 10 min at 4 °C and throw away the supernatant.
18. Dry the HBV DNA pellet for 5~10 min (air-drying) and dissolve in 15 µl of TE buffer. Store HBV DNA at 4 °C until use.

C. Southern blot assay (gel transfer)

1. Separate HBV DNA (procedure B, step 18) on 0.8% agarose gel for 2 h at 89 V.
2. After electrophoresis, transfer the gel to tray and add transfer solution (I) to soak the gel.
3. Incubate for 10 min on shaker and rinse the gel with distilled water.
4. Add transfer solution (II) and incubate for 45 min on shaker.
5. Rinse with distilled water and add transfer solution (III).
6. Incubate over 30 min on shaker and rinse the gel with distilled water.
7. Transfer the gel to Hybond-N+ membrane for overnight using capillary method (Figure 1) and bake the membrane to fix the HBV DNA for 2 h in 80 °C dry oven.
8. Place membrane in hybridization bottle contained with 25 ml hybridization solution and incubates the membrane for 4 h in 42 °C hybridization chamber (pre-hybridization step).

D. The construction of the HBV probe

1. To construct a template for the HBV specific probe, digest HBV 1.2mer replicon with *Aat* II and *Hind* III for 2 h at 37 °C and gel purification (Ahn *et al.*, 2015).
2. To synthesis HBV specific probe, mix the 48 ng of template (step 8) and 80 ng of random primer in 50 µl of distilled water.

Note: Step 10 should be carried out during pre-hybridization step.

3. Heat the probe mixtures for 5 min at 100 °C and snap cooling on ice for 5 min.
4. After spin down, add 5µl of 10x Klenow buffer, 2.5 µl of the dNTPs mixtures (2.5 mM, except dCTP), 5 µl of ³²P-labeled dCTP (50 µCi=5 µl/labeling), and 1 µl of Klenow fragment in probe mixtures.
5. Incubate the probe mixture for 30 min at 37 °C.
6. Remove the non-labeled probe using Quick Spin Columns for radiolabeled DNA purification.

E. Southern blot assay (membrane hybridization)

1. Replace pre-warmed 25 ml of hybridization solution with ^{32}P -labeled HBV probe and incubate overnight in 42 °C hybridization chamber.
2. After hybridization step, wash the membrane with washing solution (I) for 15~20 min in 63 °C hybridization chamber.

Note: If need more washing step, repeat wash with washing solution (II). Steps 9~16 should be performed in the Radio isotope room.

3. Expose membrane to film and incubate overnight at -80 °C deep freezer. Then, develop the film.

Representative data

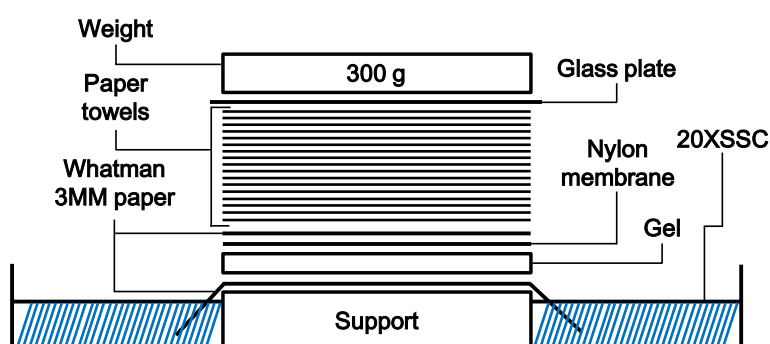


Figure 1. Scheme of capillary transfer method for Southern blotting

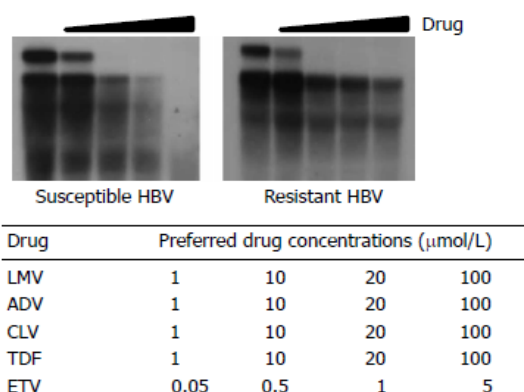


Figure 2. *In vitro* susceptibility assay of drug-resistant HBV (Kim et al., 2014). The replication of drug-resistant HBV was analyzed by Southern blot. TDF and ETV were treated with concentration as followed table (below).

Recipes

1. HEPES lysis buffer
10 mM Hepes (pH 7.5)

- 100 mM NaCl
- 1 mM EDTA
- 1% NP-40
2. Nuclease buffer (I)
 - 10 mM CaCl_2
 - 12 mM MgCl_2
 - DNaseI 0.5 unit
 - Mung Bean nuclease 7.5 unit
3. 26% PEG solution
 - 1.2 M NaCl
 - 60 mM EDTA
 - 30% sucrose
 - 26% PEG 8000
4. Nuclease buffer (II)
 - 10 mM Tris (pH 7.5)
 - 8 mM CaCl_2
 - 6 mM MgCl_2
 - DNaseI 2 unit
 - Mung Bean nuclease 3 unit
5. 0.5% SDS solution
 - 25 mM Tris (pH 7.5)
 - 10 mM EDTA
 - 100 mM NaCl
 - 0.5% SDS
6. 0.1 M sodium phosphate buffer (pH 7.0)
 - 57.7 ml of 1 M Na_2HPO_4
 - 42.3 ml of 1 M NaH_2PO_4
7. Transfer solution (I)
 - 0.25 N HCl
 - 0.6 M NaCl
8. Transfer solution (II)
 - 0.5 M NaOH
 - 1 M NaCl
9. Transfer solution (III)
 - 1 M Tris (pH 7.5~8.0)
 - 1 M NaCl
10. Hybridization solution
 - Formamide 25 ml
 - 20x SSC 12.5 ml

0.1 M sodium phosphate buufer (pH 7.0) 2.5 ml

0.5 M EDTA 0.2 ml

50x Denhardt's solution 3 ml

10% SDS 1 ml

Salmon sperm DNA (10 mg/ml) 1 ml

Distilled water 4.8 ml

11. Washing solution (I)

2x SSC

0.1% SDS

12. Washing solution (II)

0.5x SSC

0.1% SDS

Acknowledgements

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