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Sleeping Beauty Transposon-based System for Rapid Generation of HBV-replicating Stable Cell Lines

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[Abstract] The stable HBV-transfected cell lines, which based on stable integration of replication-competent HBV genome into hepatic cells, are widely used in basic research and antiviral drug evaluation against HBV. However, previous reported strategies to generate HBV-replicating cell lines, which primarily rely on random integration of exogenous DNA by plasmid transfection, are inefficient and time-consuming. We newly developed an all-in-one Sleeping Beauty transposon system (denoted pTSMP-HBV vector) for robust generation of stable HBV-replicating cell lines of different genotype. The pTSMP-HBV vector contains HBV 1.3-copy genome and dual selection markers (mCherry and puromycin resistance gene), allowing rapid enrichment of stably-transfected cells via red fluorescence-activated cell sorting and puromycin antibiotic selection. In this protocol, we described the detailed procedure for constructing the HBV-replicating stable cells and systematically evaluating HBV replication and viral protein expression profiles of these cells.
Keywords: HBV, HBV-replicating cell lines, Sleeping Beauty transposon system, HBV 1.3-copy genome, HepG2

[Background] Chronic hepatitis B virus (HBV) infection is currently a major public health burden, affecting over 240 million individuals globally (Witt-Kehati *et al.*, 2016). Patients with chronic HBV have an elevated risk of chronic active hepatitis, cirrhosis, or primary hepatocellular carcinoma (HCC) (Schweitzer *et al.*, 2015). Current treatments with interferon-α or nucleoside analogs do not eradicate the virus, and their effects on clearing hepatitis B surface antigen (HBsAg) are limited (Lucifora and Protzer, 2016; Soriano *et al.*, 2017). Therefore, there is an urgent need for the development of novel antiviral inhibitors (Nassal, 2015).

A cell culture model for evaluating the activity of new agents against HBV is an important tool for new drug development. The stable HBV-replicating cell lines, which carry replication-competent HBV genome stably integrated into the genome of human hepatoma cell lines (Huh7 and/or HepG2), are widely used to evaluate the effects of antiviral agents (Witt-Kehati *et al.*, 2016). The stable HBV-producing human hepatoma cell lines (HepG2.2.15 and HepaAD38) integrated the D-genotype HBV genome, which are widely used in antiviral research (Chang *et al.*, 1987; Ladner *et al.*, 1997). However, stable HBV-producing cell lines of genotypes A, B, and C are not commonly used in the research field. Therefore, there is a need to develop cell lines of HBV genotypes A-C for drug development.

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The Sleeping Beauty (SB) transposon system, derived from teleost fish sequences, is extremely effective at delivering DNA to vertebrate genomes, including those of humans (Structure of SB can be seen in Figure 1A). Sleeping Beauty transposition is a cut-and-paste process, during which the element 'jumps' from one DNA molecule to another (Figure 1B) (Ivics and Izsvak, 2011). Since its reconstruction in 1997 from the salmonid fish genome (Ivics *et al.*, 1997), the SB system has been undergoing several modifications to improve its efficacy (Geurts *et al.*, 2003; Baus *et al.*, 2005; Score *et al.*, 2006). The development of the hyperactive transposase SB100X has increased approximately 100-fold of efficiency compared with the first-generation transposase (Mátés *et al.*, 2009), which is expected to facilitate widespread applications in functional genomics and gene therapy (Izsvak and Ivics, 2004).

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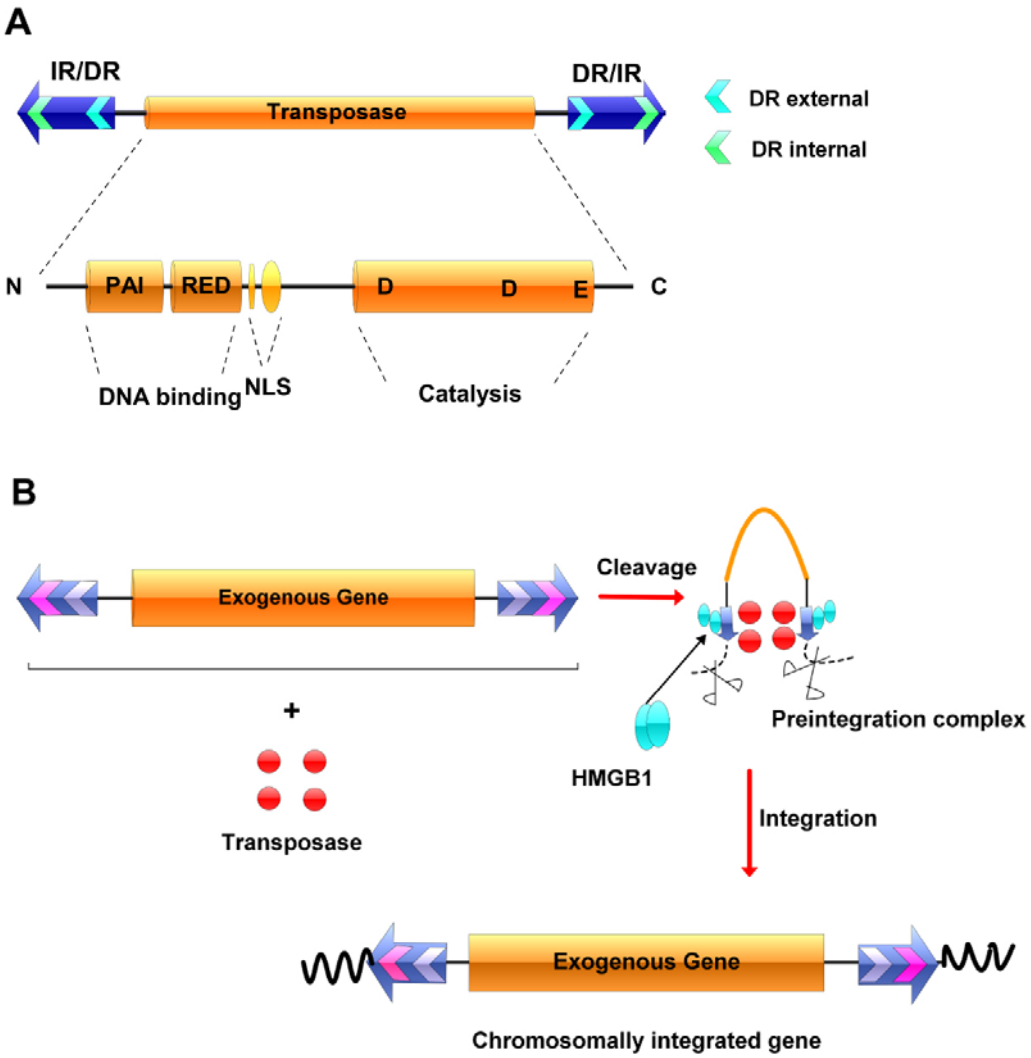

Figure 1. The *Sleeping Beauty* transposable element and its transposition. A. The *Sleeping Beauty* (SB) system. The transposase gene (yellow rectangle) is flanked by terminal inverted repeats (IR/DRs, blue arrows), each containing two binding sites for the transposase (small green arrows). The transposase consists of an N-terminal, DNA-binding domain (PAI and RED), a nuclear localization signal (NLS), a C-terminal and catalytic domain (DDE). B. Transposition. The transposase gene within the element can be replaced by a therapeutic gene, and the resultant transposon can be maintained in a simple plasmid vector. The transposase is supplied *in trans*. The transposase binds to its binding sites within the IR/DR repeats and, together with host factors such as HMGB1, forms a synaptic complex, in which the ends of the transposon are paired. The transposon is excised from the donor molecule and integrates into a new location.

Materials and Reagents

1. Pipette tips, 10 µl (Haimen plastic, 20111088)
2. Pipette tips, 200 µl (Corning, Axygen®, catalog number: T-200-Y-R)
3. Pipette tips, 1 ml (Haimen plastic, 20111011)

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4. Cell culture plate (100 mm) (Corning, catalog number: 430167)
5. Cell culture plate (60 mm) (Thermo Fisher Scientific, catalog number: 150288)
6. 15 ml tube (Thermo Fisher Scientific, catalog number: 339651)
7. 70 μm cell strainer (Fisher Scientific, Fisherbrand, catalog number: 22-363-548)
8. Cell culture plate (6 well) (Corning, catalog number: 3516)
9. Cell culture plate (24 well) (Corning, catalog number: 3524)
10. Cell Imaging Plate, 24 well, glass bottom (Eppendorf, catalog number: 0030741021)
11. Nylon membranes (Roche Diagnostics, catalog number: 11417240001)
12. Human hepatoma HepG2 cells (Originally from the China Centre for Type Culture Collection, Wuhan, China)
13. Minimum essential medium (MEM, powder) (Thermo Fisher Scientific, Gibco™, catalog number: 41500-083)
14. Fetal bovine serum, Qualified, Australia Origin (Thermo Fisher Scientific, catalog number: 10099141)
15. X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics, catalog number: 06366236001)
16. Opti-MEM (Thermo Fisher Scientific, Gibco™, catalog number: 31985070)
17. 0.25% Trypsin-EDTA (1x), Phenol Red (Thermo Fisher Scientific, Gibco™, catalog number: 25200-114)
18. Puromycin (Thermo Fisher Scientific, Invitrogen™, catalog number: A1113803)
19. Mouse anti-HBcAg (Innodx Biotechnology, catalog number: 2A7-21 [it's new anti-HBc mAb, available on request])
20. Alexa Fluor® 488 Donkey Anti-Mouse IgG (H+L) (Thermo Fisher Scientific, catalog number: A-21202)
21. DAPI (Thermo Fisher Scientific, Invitrogen™, catalog number: D1306)
22. Micrococcal nuclease (Takara Bio, catalog number: D2910)
23. Proteinase K (Takara Bio, catalog number: D9033)
24. Ethanol, C_2H_6O, AR (Xilong Scientific, catalog number: 1030029AR)
25. Oligonucleotides

Name	Sequence (5'-3')	Size (bp)
KHF2	TTTCACCTCTGCCTAATCAT	20
KHR1	TCAGAAGGCAAAAAAGAGAGTAACTC	26
KHP4 (Probe)	CCTTGGGTGGCTTTGGGGCATGGA (marked in the 5 'end with the fluorophore HEX and the 3 'end with the quencher BHQ1)	24
HBX-probe F	AAACTGCAGGATGGCTGCTAGGCTGTACTG	30
HBX-probe R	CCCTCGAGGGCAGAGGTGAAAAAGTTGC	28

26. dNTP Mixture 2.5 μM (Takara Bio, catalog number: D4030A)

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27. DIG Easy Hyb Granules (Roche Diagnostics, catalog number: 11796895001)
28. PrimeSTAR GXL DNA Polymerase (Takara Bio, catalog number: DR050A)
29. Premix Ex Taq™ (Probe qPCR) (Takara Bio, catalog number: RR390A)
30. Casein 10x blocking buffer (Sigma-Aldrich, catalog number: B6429-500ML)
31. Anti-DIG (AP) antibody (Roche Diagnostics, catalog number: 11093274910)
32. CDP-Star AP substrate (Roche Diagnostics, catalog number: 12041677001)
33. Universal DNA Purification Kit (TIANGEN Biotech, catalog number: DP214-03)
34. Diagnostic kit for Hepatitis B surface antigen (CLEIA) (Wantai Biological Pharmacy, catalog number: HBV-1396)
35. Diagnostic kit for Hepatitis B e-antigen (ELISA) (Wantai Biological Pharmacy, catalog number: HBV-0396)
36. ED-11 (Innovax Biotechnology)
37. Virus DNA/RNA Extraction kit (GenMag Biotechnology, catalog number: NA007)
38. Mycoplasma-free neonatal bovine serum (Tianhang Biotechnology, catalog number: 11011-8615)
39. Sodium chloride (NaCl, AR) (Xilong Scientific, catalog number: 1001012AR)
40. Potassium chloride (KCl, cell culture) (Sigma-Aldrich, catalog number: P5405)
41. Hydrochloric acid (HCl, AR) (Xilong Scientific, catalog number: 1029013AR)
42. Sodium hydroxide (NaOH, AR) (Xilong Scientific, catalog number: 1001037AR)
43. Disodium hydrogen phosphate ($Na_2HPO_4·12H_2O$, AR) (Xilong Scientific, catalog number: 1001067AR)
44. Potassium dihydrogen phosphate (KH_2PO_4, AR) (Xilong Scientific, catalog number: 1002048AR500)
45. Sodium bicarbonate ($NaHCO_3$, AR) (Xilong Scientific, catalog number: 44558)
46. Paraformaldehyde (Sigma-Aldrich, catalog number: 16005-1KG-R)
47. Triton X-100 (AMRESCO, catalog number: 0694)
48. Bovine albumin (Low endotoxin) (ICPbio International, catalog number: ABRE-1KG)
49. Tris base (SEEBIO BIOTECH, catalog number: 183995)
50. Tris-saturated phenol (Solarbio, catalog number: T0250)
51. Ethylenediaminetetraacetic acid (EDTA, AR) (Xilong Scientific)
52. NONIDET® P-40 Substitute (AMRESCO, catalog number: M158-500ML)
53. Calcium chloride ($CaCl_2$, AR) (Xilong Scientific, catalog number: U1000566-500g)
54. Sodium dodecyl sulfate (SDS) (Merck, catalog number: 428015)
55. Chloroform (AR) (Xilong Scientific, catalog number: 1039013AR500)
56. Isoamyl alcohol (AR) (Xilong Scientific, catalog number: U1001975-500ml)
57. Maleic acid (Sigma-Aldrich, catalog number: M0375)
58. Tween-20 (BBI Solutions, catalog number: TB0560-500ml)
59. Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-$Na_2·2H_2O$, AR) (Xilong Scientific, catalog number: 100186)

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60. Acetate (CH_3COOH, AR) (Xilong Scientific, catalog number: 1029047AR)
61. Sodium citrate ($C_6H_5Na_3O_7$, AR) (Xilong Scientific, catalog number: 1001059AR)
62. Verson buffer (see Recipes)
63. PBS buffer (see Recipes)
64. 4% paraformaldehyde (see Recipes)
65. 0.2% Triton X-100 (see Recipes)
66. 3% BSA (see Recipes)
67. NET buffer (see Recipes)
68. 1.2 M $CaCl_2$ (see Recipes)
69. 0.5 M EDTA (see Recipes)
70. 10% SDS (see Recipes)
71. Phenol/chloroform/isoamyl alcohol (25:24:1) (see Recipes)
72. Maleic acid buffer (see Recipes)
73. Washing buffer (see Recipes)
74. Detection buffer (see Recipes)
75. 50x TAE buffer (see Recipes)
76. Depurination buffer (see Recipes)
77. Denaturation buffer (see Recipes)
78. Neutralization buffer (see Recipes)
79. 10x SSC (see Recipes)
80. 2x SSC/0.1% SDS (see Recipes)
81. 0.5x SSC/0.1% SDS (see Recipes)

Equipment

1. Pipettes (Mettler-Toledo International, RAININ, model: Pipet-Lite)
2. CO_2 Incubator (Thermo Fisher Scientific, catalog number: 3111)
3. Centrifuge (Thermo Fisher Scientific, model: Heraeus™ Pico™ 17)
4. Sorvall refrigeration Centrifuge (Thermo Fisher Scientific, model: Sorvall™ ST 16R)
5. BD FACS Aria III (BD, model: FACSAria™ III)
6. High Content Screening System (PerkinElmer, model: Opera Phenix™)
7. Water bath (Grant Instruments, model: GD100)
8. UV cross-linking instrument (Shanghai SIGMA High-tech, model: SH4)
9. Bio-Rad vacuum blotter (Bio-Rad Laboratories, model: Model 785)
10. Multifunctional molecular hybridization oven (UVP, model: HM-4000)
11. ImageQuant LAS4000 mini (GE Healthcare, model: ImageQuant LAS 4000 mini)
12. Plastic film sealing machine (Shanghai Mingwei, model: F-400)
13. Biomek NX^P (Beckman Coulter, model: Biomek NX^P)
14. Microplate reader (Autobio, model: PHOmo)

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15. Orion II Microplate Lumimometer (Titertek-Berthold, model: Orion II)
16. Electrophoresis apparatus (Bio-Rad Laboratories, catalog number: 1645050)
17. LightCycler® 96 (Roche Molecular Systems, model: LightCycler® 96)

Procedure

A. Generation of HBV-replicating stable cell lines
   1. Transfection of HepG2 cells
      a. Grow HepG2 cells in MEM/10% FBS at a 37 °C, CO_2 incubator. Split the cells to a 10-cm plate 24 h before transfection; the cell density should reach 70-90% at the time of DNA transfection.
      b. Dilute 20 μg each of pTSMP-HBV1.3 plasmids of genotype Ae (AY707087), Ba (GU357842), Ce (GU357845) and D1 (GU357846) with 2 ml opti-MEM serum-free medium, respectively. Mix gently.
         Note: The map of pTSMP-HBV1.3 plasmid and the genome organization of HBV1.3 used in this study are shown in Figures 2A and 2B, respectively. The construction of pTSMP-HBV1.3 plasmids can be referred to (Wu et al., 2016).

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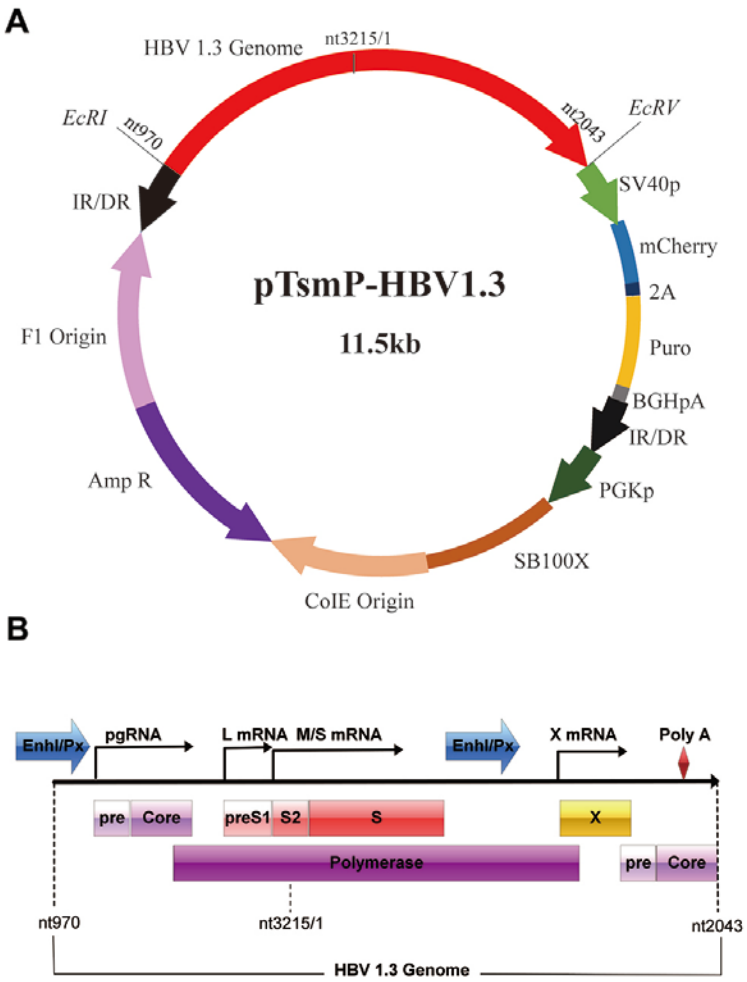

Figure 2. Schematic representation of the pTsmP vector (A) and HBV 1.3 genome construction (B)

c. Pipet the X-tremeGENE HP DNA Transfection Reagent (60 µl) directly into the medium containing the diluted DNA without contact with the walls of the plastic tubes. Mix gently.
d. Incubate the transfection reagent: DNA complex for 15 min at room temperature.
e. Add the transfection complex to the cells in a dropwise manner.
f. Following transfection, incubate cells for 48 h at 37 °C in a 5% CO_2 incubator; successful transfected cells with activated red fluorescence (mCherry positive) can be visualized by fluorescence microscopy and sorted by flow cytometry.

2. Flow cytometric sorting of successful transfected cells
   a. Wash the cells with 5 ml Verson buffer (see Recipes) and discard the supernatant.
   b. Add 500 µl 0.25% trypsin-EDTA to the cells and incubate at 37 °C for 3 min.
   c. Add 5 ml MEM/10% FBS to stop digestion.
   d. Transfer 2 x 10^7 cells into a 15 ml tube.
   e. Centrifuge cell 1,000 *x g* for 3 min, and then discard supernatant.
   f. Wash with 5 ml PBS buffer (see Recipes).

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g. Centrifuge cells at 1,000 *x g* for 3 min, and then discard supernatant.
h. Resuspend cells in 7 ml PBS buffer, and then filter through a 70 μm cell strainer.
i. Run samples on BD FACS Aria III machine, the excitation light required is 561 nm (Figure 3).

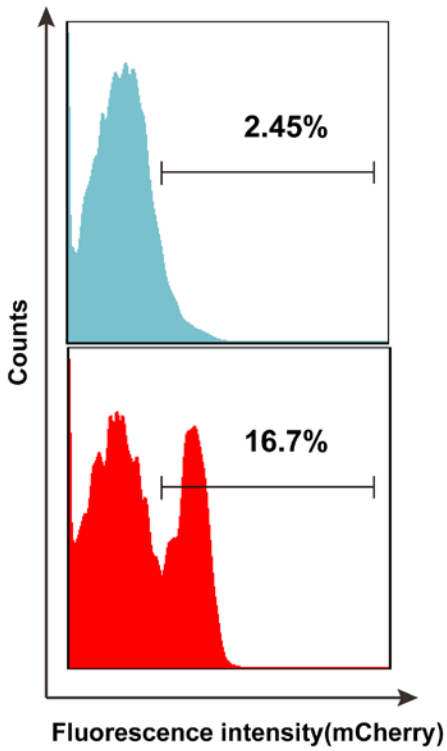

Figure 3. The first round of mCherry-activated FACS sorting. Negative cell with unexpressed mCherry is HepG2 (blue peak), positive cell with expressed mCherry is HepG2-pTsmp-1.3HBV-S11 (red peak).

j. The sorted mCherry-expressing cells are cultured in a 6-well culture plate for expansion in the presence of puromycin selection (2 μg/ml). Refresh Culture medium containing puromycin every 2 days.
k. On the following day, when cell confluence is 70-80%, the cells are subjected to the second round of mCherry fluorescence-activated FACS sorting and further puromycin selection.
l. After about a 4-round of mCherry fluorescence-activated FACS sorting and puromycin-resistant cell selection, over 90% cells are puromycin resistant and stably expressed mCherry.
m. These cells are further propagated. After 10 passages, nearly 100% of cells of the 4 HBV cell lines persistently express mCherry and grow well in the presence of puromycin.

B. Characterization of HBV-replicating stable cell lines
1. Immunofluorescence of hepatitis B virus core antibody

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a. Cell preparation

Seed 4 x 10^5 pTSMP-HBV1.3 cells and 3.5 x 10^5 parental HepG2 cells (serve as a negative control) on the cell imaging plate with a density of 70%. Incubate the cells at 37 °C and 5% CO_2 in an incubator for 24 h.

b. Cell washing

Discard the medium and wash once with 500 µl PBS.

c. Fixation

Add 250 µl per well of 4% formaldehyde (see Recipes) along the side of cell plate. Allow cells to fix in the dark for 15 min at room temperature (RT). Rinse three times in 1 ml 1x PBS for 3 min each.

d. Permeabilization

Incubate the samples for 5 min in 250 µl 0.2% Triton X-100 (see Recipes) per well. Rinse three times in 1 ml 1x PBS for 3 min each.

e. Blocking

Incubate cells with 250 µl 3% BSA (see Recipes) in PBS at RT for 1 h to block potential non-specific binding of the antibody, then discard the supernatants.

f. Primary antibody

Incubate the cells with 250 µl anti-HBcAg antibody (1:1,000 dilute in 3% BSA) for 1 h at RT (or overnight at 4 °C) in the dark. Next, remove the solution and wash cells 3 times with 1 ml/well PBS for 3 min each.

g. Secondary antibody

Incubate the cells with 250 µl secondary antibody (Alexa Fluor® 488 Donkey Anti-Mouse IgG (H+L), 1:1,000 dilute in 3% BSA) for 30 min at RT in the dark. Remove the solution and wash 3 times with 1 ml/well PBS for 3 min each in the dark.

h. Counterstaining

Incubate the cells with 250 µl DAPI (1:2,000 dilute in 3% BSA) for 5 min at RT in the dark. Discard the solution and wash 3 times with 1 ml/well PBS for 3 min each.

i. Photographs

Photograph in High Content Screening System (Figure 4). The setting used in the imager for imaging (Table 1).

Table 1. The setting used in the High Content Screening System for imaging

Channel	Excitation wavelength (nm)	Excitation time (msec)	Exciting power (%)
DAPI	488	100	50
mCherry	561	200	50
HBcAg	488	200	50

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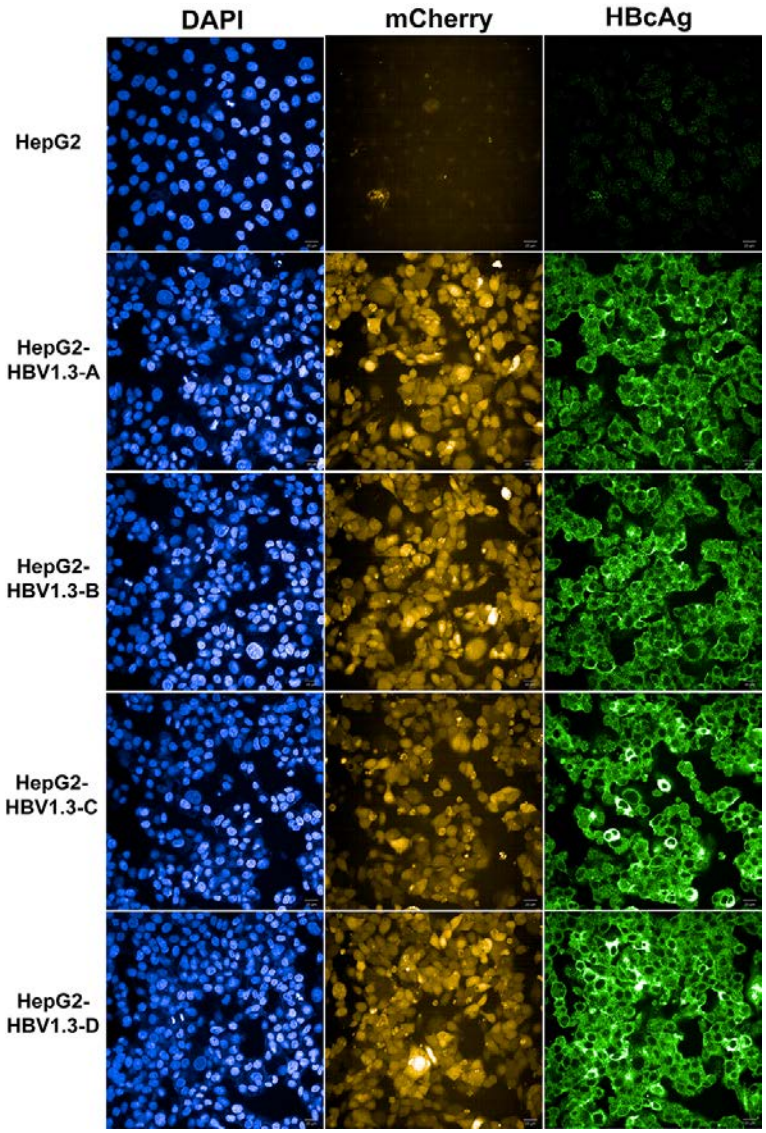

Figure 4. Immunofluorescence for HBcAg of HBV cell lines (Confocal mode, 40x water lens)

2. Southern blot
   a. Cell: Plate 4 x 10^6 cells in the 6 cm dish with 60-70% confluent. Culture cells for 2 days and proceed to the next experiment.
   b. Extraction of HBV total DNA
      i. Discard the supernatant of cells and wash 2 times with cold PBS.
      ii. Add 600 µl NET buffer (see Recipes) in cell pellet and incubate on ice (or 4 °C) for 1 h. *Note: We usually add 200-300 µl in one well of a 6-well plate; or 400-600 µl a 6 cm dish; or 1.2-1.8 ml in a 10 cm dish.*
      iii. Centrifuge the lysates at 13,400 *x g* for 20 min at 4 °C and transfer the supernatant (600 µl) into a new 1.5 ml EP tube.
      iv. Add 2 µl Micrococcal nuclease (10 mg/ml) and 3 µl 1.2 M $CaCl_2$ (final concentration of 6 mM) (see Recipes) and incubate for 30 min in a 37 °C water bath.

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v. Add 30 µl 0.5 M EDTA (final concentration of 25 mM) (see Recipes) and incubate for 15 min in a 65 °C water bath.
vi. Add 6.68 µl Proteinase K (19 mg/ml) and 32.1µl 10% SDS (see Recipes) and incubate overnight (At least 12 h but no more than 18 h) in a 50 °C water bath (final concentration of 200 µg/µl proteinase K and 0.5% SDS).
vii. Add an equal volume of phenol/chloroform/isoamylalcohol (approx. 600 µl), mix thoroughly by inverting the tube several times and incubate at room temperature (15-25 °C) for 5 min. Centrifuge at 14,000 *x g* for 10 min and transfer the aqueous phase into a new 1.5 ml EP tube.
viii. Repeat the above step (Step B2b) vii twice.
ix. Precipitate DNA by adding twice the volume of absolute ethanol and 1/10 volume of sodium acetate and then placing at -80 °C for 2 h. Centrifuge at 14,000 *x g* for 10 min at 4 °C. Carefully decant supernatant.
x. Wash the DNA pellet with 500 µl 75% ethanol and centrifuge at 14,000 *x g* for 5 min. Carefully decant supernatant.
xi. Air-dry pellet for 5-10 min and re-dissolve DNA in 35 µl distilled deionized water. Store HBV DNA at -80 °C until use.

c. The construction of the HBV probe
i. Prepare PCR reaction solution according to the following components:

5x PrimeSTAR GXL buffer	5 µl
HBx-probe-F (10 µM)	1 µl
HBx-probe-R (10 µM)	1 µl
dNTP Mix (2.5 µM)	2 µl
DIG-dUTP	2 µl
pGEM-1.3 HBV	1 µl
PrimeSTAR GXL DNA polymerase	1 µl
ddH_2O	37 µl

ii. PCR reaction

95 °C	5 min	
95 °C	30 sec	40 cycles
58 °C	30 sec	
58 °C	40 sec	
58 °C	10 min	

iii. Purify the HBV probe by using Universal DNA Purification Kit.

d. Evaluate the effect of the probe
i. The probe is diluted to 2 ng/µl as the first gradient and 10-fold down-diluted to a total of 5 gradients with a minimum concentration of 0.2 pg/µl.

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ii. Drop the diluted probe on a piece of nylon membrane via pipette, 5 µl each (final loading: 10,000 pg, 1,000 pg, 100 pg, 10 pg, 1 pg). Keep a certain distance between each point to avoid contamination.
iii. UV irradiation (1.5 J/cm^2) for 3 min.
iv. Block for 30 min with 1x blocking buffer (Casein blocking buffer [10x] diluted in Maleic acid buffer [see Recipes]).
v. Incubate for 40 min with anti-DIG (AP) antibody (1:3,000 diluted in 1x blocking buffer).
vi. Wash nylon membrane 3 times with washing buffer (see Recipes) for 10 min each.
vii. Rinse with detection buffer (see Recipes) for 2 min.
viii. Add the AP substrates to the membrane to completely infiltrate it.
ix. Exposure by using ImageQuant LAS4000 mini. The intensity of your experimental probe is required to make the point of 2 pg/µl or below visible to yield satisfactory hybridization results. If the probe signal is weaker than this, it will be difficult to detect signals from low abundance species (Figure 5).

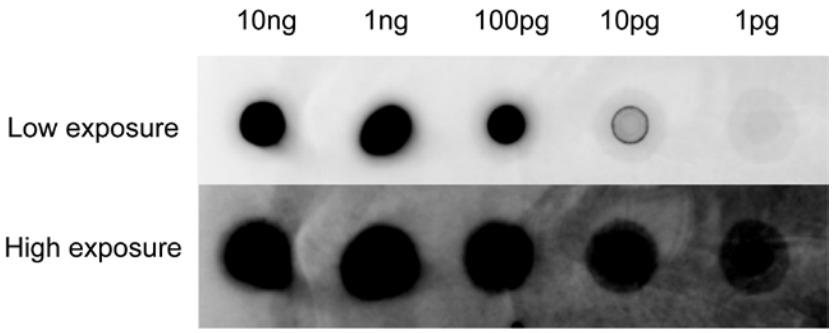

Figure 5. Southern blotting analysis for the effect of probe

e. Southern blot analysis
i. Separate the DNA samples by electrophoresis through 1.2% agarose gel at 80 V for 2 h in 1x TAE buffer (see Recipes).
ii. After electrophoresis is completed, gel is treated as followed (The following steps are operated on a shaker):
1) Transfer the gel to a tray and add depurination buffer (see Recipes) to soak the gel, incubate for 15 min on the shaker at RT and shake gently (Breaking the DNA into smaller pieces, thus allowing more efficient transferring from the gel to membrane).
2) Remove the depurination buffer. Rinse the gel twice with ddH_2O for 1 min each.
3) Rinse twice with denaturation buffer (see Recipes) for 15 min each.
4) Rinse the gel twice with ddH_2O for 1 min each.
5) Rinse with neutralization buffer (see Recipes) for 10 min.
iii. Transfer DNA from the gel to nylon membrane with 10x SSC for 1.5 h by vacuum blotter (Figure 6).

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Note: Wet the precut nylon membrane in double distilled water by slowly lowering the membrane at a 45-degree angle to the water. Then, wet the membrane and the filter paper in 10x SSC; Place the gel and nylon membrane in the order shown in Figure 6; Remove bubbles between the gel and membrane; Start the vacuum source and slowly turn the bleeder valve clockwise until the gauge reads at 5 inches of Hg.

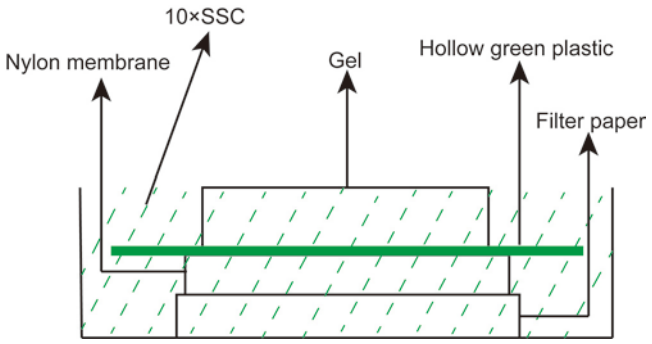

Figure 6. Scheme of capillary transfer method for Southern blotting

iv. After gel transfer, fix the DNA to the nylon membranes by UV crosslinking at 1.5 J/cm^2 for 3 min.

Note: Irradiation time = irradiation dose (mJ/cm^2)/irradiation intensity (mW/cm^2), the irradiation intensity of this instrument is 9.0 mW/cm^2.

v. Pre-hybridization step

Place membrane in hybridization plastic film contained with 10 ml DIG Easy Hyb buffer and incubate the membrane for 2 h in 42 °C hybridization chamber with gentle agitation.

vi. Hybridization

1) Denature the DNA probe at 95 °C for 5 min and immediately place on ice for 2 min.
2) Replace pre-warmed 10 ml of DIG Easy Hyb buffer with 300 ng DIG-dUTP- labeled HBV probe and incubate overnight in a 42 °C hybridization chamber with gentle agitation.

vii. Wash

1) Wash 2 times with 2x SSC/0.1% SDS buffer for 5 min at room temperature.
2) Wash 2 times with pre-warmed 0.5x SSC/0.1% SDS buffer for 5 min at 65 °C.

Note: Shake the buffer before use.

viii. Detection

1) Rinse with Maleic acid buffer (see Recipes) for 2 min.
2) Block for 30 min with 1x blocking buffer (10x blocking buffer diluted with Maleic acid buffer).
3) Incubate for 40 min with anti-DIG(AP) antibody (1:3,000 diluted in 1x blocking buffer)
4) Wash 3 times with 100 ml washing buffer for 15 min each.

Note: Washing buffer shake evenly before use.

5) Equilibrate for 3 min in 20 ml detection buffer.

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6) Add the membrane with AP substrate in dropwise so that to completely infiltrate.
7) Expose the membrane continuously and take images using ImageQuant LAS4000 mini.

3. Detection of the levels of viral markers in supernatants
   a. Cell
      i. Seed 3.5 x 10^5 pTSMP-HBV1.3 cells in the 24-well plates with a density of 60-70%.
      ii. Replenish and collect the culture medium for measurement of viral antigens and HBV DNA every 2 days.
      iii. Monitor the levels of these viral markers in supernatants for each cell line during 1 month.
   b. Viral antigens
      i. Measure viral antigens (HBsAg and HBeAg) in culture medium by chemiluminescence method using commercial assay kits (Wantai, Beijing, China).
      ii. Antigen (HBsAg and HBeAg) standards are standardized patient serum, with the original concentration are 90,000 IU/ml and 10,000 Ncu/ml respectively.
      iii. The standard of HBeAg is diluted with 20% NBS into six gradient concentrations, 10, 5, 2.5, 1.25, 0.25, 0.05 Ncu/ml respectively.
      iv. The standard of HBsAg is diluted with ED-11 into six gradient concentrations, 45, 9, 1.8, 0.36, 0.072, 0.0144 IU/ml respectively.
   c. HBV total DNA
      i. HBV total DNA extraction
         1) Extract the total DNA in culture medium by using virus DNA/RNA extraction kit on Biomek NX^P (Beckman, California, USA).
         2) The standard of HBV total DNA is standardized patient serum with the original concentration is 1.5 x 10^8 IU/ml, diluting with 10-fold dilutions in PBS into six gradients.
      ii. Prepare PCR reaction solution according to the following components.

2x Premix Ex Taq (Probe qPCR)	10 μl
KHF2 (100 μM)	0.1 μl
KHR1 (100 μM)	0.1 μl
KHP4 (100 μM)	0.05 μl
DEPC water	4.75 μl
Template	5 μl

      iii. Real-time PCR reaction is performed with LightCycler 96.

95 °C 30 sec
95 °C 5 sec
60 °C 30 sec } 45 cycles
Hex channel

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Notes

1. The state of the cells is critical to the transfection efficiency and cell state after sorting.
2. During the sorting process, cells should be sorted at 4 °C, and the flow rate should be controlled below 9 µl/min. This is crucial for the cell state after sorting.
3. The number of cells after sorting should at least be enough to plate in 48-well plates so that the cells are more likely to survive.

Recipes

1. Verson buffer(1 L)
   0.4 g KCl
   0.06 g KH_2PO_4
   8.0 g NaCl
   0.35 g $NaHCO_3$
   0.08 g $Na_2HPO_4·12H_2O$
   0.2 g EDTA
   Filter sterilize (0.22 µm) or sterilize by autoclavation
2. PBS buffer
   145 mmol/L NaCl
   2.68 mmol/L KCl
   10 mmol/L $Na_2HPO_4·12H_2O$
   1.76 mmol/L KH_2PO_4
   Adjust to pH 7.4
   Filter sterilize (0.22 µm) or sterilize by autoclavation
3. 4% Paraformaldehyde
   4 g Paraformaldehyde
   100 ml PBS
   1-2 drops 10 M NaOH
   Store at 4 °C and protect from light
4. 0.2% Triton X-100
   200 µl Triton X-100
   100 ml PBS
   Store at 4 °C
5. 3% BSA
   3 g BSA
   100 ml PBS
   Sub-package and store at -20 °C

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6. NET buffer
   50 mM Tris-base (pH = 8.0)
   1 mM EDTA
   100 mM NaCl
   0.5% NP-40
7. 1.2 M $CaCl_2$
   13.32 g $CaCl_2$
   100 ml ultra-pure water
   Store at 4 °C
8. 0.5 M EDTA
   Store at 4 °C
9. 10% SDS
   100 g electrophoresis grade SDS
   Add ddH_2O to 1 L
   Heat to 68 °C
   Adjust to pH 7.2
10. Phenol/chloroform/isoamyl alcohol (25:24:1)
    Equilibrate phenol, chloroform and isoamyl alcohol in a ratio of 25:24:1, then store in a brown glass bottle and cover with a layer of Tris-Cl, store at 4 °C until use
11. Maleic acid buffer
    0.1 M Maleic acid
    0.15 M NaCl
    Adjust to pH 7.5
    Sterilize by autoclavation
12. Washing buffer
    0.1 M Maleic acid
    0.15 M NaCl
    3 ml/L Tween 20
    Adjust to pH 7.5
    Sterilize by autoclavation
13. Detection buffer
    0.1 M Tris-base
    0.1 M NaCl
    Adjust to pH 9.5
    Sterilize by autoclavation
14. 50x TAE buffer
    242 g Tris-base
    100 ml EDTA (0.5 M, pH 8.0)
    57.1 ml acetate

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Add ddH_2O to 1 L
Adjust to pH 8.5

15. Depurination buffer
    0.2 N HCl
    Sterilize by autoclavation
16. Denaturation buffer
    0.5 M NaOH
    1.5 M NaCl
    Sterilize by autoclavation
    Neutralization buffer
    1 M Tris-base
    1.5 M NaCl
    Sterilize by autoclavation
17. 10x SSC
    1.5 M NaCl
    0.15 M Sodium citrate
    Adjust to pH 7.0
    Sterilize by autoclavation
18. 2x SSC/0.1% SDS
    200 ml 10x SSC
    10 ml 10% SDS
    Add ultra-pure water to 1 L
    Sterilize by autoclavation
19. 0.5x SSC/0.1% SDS
    50 ml 10x SSC
    10 ml 10% SDS
    Add ultra-pure water to 1 L
    Sterilize by autoclavation

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