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A Southern Blot Assay for Detection of Hepatitis B Virus Covalently Closed Circular DNA from Cell Cultures

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Abstract

Chronic hepatitis B remains a substantial public health burden affecting approximately 350 million people worldwide, causing cirrhosis and liver cancer, and about 1 million people die each year from hepatitis B and its complications. Hepatitis B is caused by hepatitis B virus (HBV) infection. As an essential component of the viral life cycle, HBV covalently closed circular DNA (cccDNA) is synthesized and maintained at low copy numbers in the nucleus of infected hepatocytes, and serves as the transcription template for all viral RNAs. Therefore, cccDNA is responsible for the establishment of viral infection and persistence. The presence and longevity of cccDNA may also explain the limitations of current antiviral therapy for hepatitis B. Thus, understanding the mechanisms underlying cccDNA formation and regulation is critical in understanding the HBV pathogenesis and finding a cure for hepatitis B. Here we describe a protocol for HBV cccDNA extraction and detection in detail. The procedure includes two major steps: (1) HBV cccDNA extraction by Hirt protein-free DNA extraction method and (2) HBV cccDNA detection by Southern blot analysis. The method is straightforward and reliable for cccDNA assay with cell culture samples, and it is useful for both HBV molecular biology and antiviral research.

Keywords

HBV; cccDNA; Hirt extraction; Southern blot

1 Introduction

Hepatitis B virus (HBV) is the causative agent for a chronic viral infection of the liver called “chronic hepatitis B,” and its most severe consequence, hepatocellular carcinoma (HCC) [1]. HBV is a partially double-stranded DNA virus that belongs to the *Hepadnaviridae* family. The virion has an outer lipid envelope, inside of which there is an icosahedral nucleocapsid composed of 180 or 240 copies of viral core proteins. The nucleocapsid encloses the genomic relaxed circular DNA (rcDNA) and a viral DNA polymerase which is covalently attached to 5′ end of one of the DNA strands [2, 3].

HBV is a naturally noncytopathic virus that mainly infects hepatocytes, although it has been detected in extrahepatic sites [4, 5]. Although tissue culture models of HBV infection are limited [6, 7], studies of viral entry indicate that infection of the hepatocyte is thought to be through endocytosis upon the engagement to a yet-to-be identified plasma membrane receptor [8]. The viral genome is then transported into the nucleus, where it converts to

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covalently closed circular DNA (cccDNA), which is the transcription template for all viral RNAs [9, 10]. A longer than full genome-length viral RNA transcript, called pregenomic RNA (pgRNA), is exported into the cytoplasm and packaged into nucleocapsid, inside of which it undergoes reverse transcription to generate viral minus-strand DNA, and then plus-strand DNA synthesis to yield rcDNA [2, 3]. The mature nucleocapsids are either packaged by viral envelope proteins and egress as virion particles to further spread the infection or shuttled back into the nucleus to amplify the cccDNA reservoir through the so-called intracellular amplification pathway, resulting in a steady-state level of cccDNA ranging from 1 to 50 copies per infected cell [2]. Therefore, the sustained level of cccDNA in the infected cells is critical for establishment and persistence of chronic HBV infection.

Currently available HBV therapeutics, specifically interferon-α and nucleos(t)ide analogues, fail to eradicate the cccDNA reservoir from infected hepatocytes although the viral DNA replication can be substantially suppressed [11, 12]. Consequently, failure to repress or eliminate the cccDNA results in viral rebound after the cessation of therapy [12, 13]. Moreover, the cccDNA could “inherit” and amplify drug-resistant mutations and evade nucleos(t) ide treatment [11].

Therefore, there is an urgent need for the development of novel therapeutic agents that directly target the cccDNA formation and maintenance. To serve such a purpose, a reliable cccDNA measurement method is required. Here we describe a protocol being used for HBV cccDNA extraction and detection from cell cultures [14 – 16], which is modified from previously reported methods for avihepadnavirus cccDNA assay, i.e., duck hepatitis B virus (DHBV) [17, 18].

For cccDNA extraction from HBV-replicating cells, a Hirt protein-free DNA extraction procedure is employed [19]. In principle, sodium dodecyl sulfate (SDS) is first applied to break down all the lipid membranes and viral capsids to release all the viral nucleic acids. A high concentration of salt (NaCl) is then added to precipitate down high-molecular-weight cellular chromatin and protein covalently bonded DNA in SDS–protein complexes, including HBV DNA replicative intermediates with viral polymerase covalently attached. Protein-free DNA stays in the supernatant and can be further purified by organic phenol. HBV cccDNA, a protein-free DNA, is one of the major viral products of this process. Another protein-free viral DNA, specifically deproteinized rcDNA (DP-rcDNA), which is a putative precursor of cccDNA, can be extracted simultaneously as well [14, 20].

Currently, there are two major means of detecting HBV cccDNA. One of those is a polymerase chain reaction (PCR)-based method, such as real-time PCR and rolling circle PCR [21, 22]. Both PCR methods amplify cccDNA by specifically designed primers. Although the sensitivity of PCR detection is satisfactory, the major drawback is that the PCR amplification signals not only come from the cccDNA but also can come from other co-purified viral DNA, such as replicative intermediates in total DNA preparation, or DP-rcDNA from Hirt extraction, especially during the later PCR cycles.

Alternatively, the cccDNA can be directly detected by Southern blot analysis through DNA hybridization, which not only detects the cccDNA but also distinguishes the cccDNA from

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other viral DNA species by differential migration during agarose gel electrophoresis. In this chapter, we describe in detail the Southern blot procedure for HBV cccDNA detection and quantification. Although this protocol is not amenable to high-throughput screening for antiviral compounds, it could serve as a suitable and reliable method for hit validation in secondary antiviral assays [16].

2 Materials

2.1 Extraction of cccDNA from Cell Culture

1. 1 M Tris–HCl, pH 7.5.
2. 0.5 M ethylenediamine tetraacetic acid (EDTA), pH 8.0.
3. TE buffer (10:10): 10 mM Tris–HCl, pH 7.5 and 10 mM EDTA.
4. TE buffer (10:1): 10 mM Tris–HCl, pH 7.5 and 1 mM EDTA.
5. Phenol: Equilibrated with 10 mM Tris–HCl (pH 8.0) and 1 mM EDTA.
6. Phenol/chloroform: Phenol:chloroform:isoamyl alcohol (25:24:1), saturated with 10 mM Tris–HCl (pH 8.0) and 1 mM EDTA.
7. 10 % SDS.
8. 5 M NaCl.
9. 100 % ethanol, and 70 % ethanol.

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, and store at room temperature.
3. Agarose (molecular biology grade).

2.3 Probe Preparation for HBV DNA Detection

1. pGEM-3Z vector (Promega) with an insertion of genome-length HBV fragment (i.e., EcoRI linearized), in which the transcription of sense RNA is under the control of the prokaryotic SP6 promoter.
2. 800 Ci/mmol [α- ^{32}P] UTP (PerkinElmer).
3. Riboprobe® in vitro transcription system-SP6 (Promega).
4. 5 M NH_4 OAc.
5. 10 mg/mL yeast RNA (Ambion).
6. Isopropanol.
7. Formamide.

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2.4 Southern Blotting and DNA Hybridization

1. Depurination buffer: 0.2 M HCl (*see* Note 1).
2. Denaturing buffer: 0.5 M NaOH, 1.5 M NaCl.
3. Neutralization buffer: 1.5 M NaCl, 1 M Tris–HCl, pH 7.4.
4. 20× SSC: 3 M NaCl, 0.3 M sodium citrate.
5. Amersham Hybond—XL membrane (GE Healthcare).
6. Whatman 3 MM chromatography paper.
7. UV crosslinker.
8. EKONO™ hybridization buffer (G-Biosciences).
9. Wash buffer: 0.1 % SDS, 0.1× SSC.
10. Phosphorimager exposure and scan system.

3 Methods

3.1 Cell Culture and Compound Treatment

1. Seed HBV stable cell lines, such as HepG2.2.15 [23], or tetracycline-inducible (tet-off) HepAD38 [24], or tetracycline-inducible (tet-off) HepDE19/DES19 cells [14], in collagen-coated 6-well plates at approximately 100 % confluence with appropriate culture medium (*see* Notes 2 and 3).
2. When the cell monolayer reaches complete confluence (normally 24–48 h post seeding), change the culture medium with fresh medium (*see* Note 4). For tetracycline-inducible cell lines, remove the tetracycline from culture medium to induce HBV replication.
3. For antiviral assays, add testing compounds at serial non-cytotoxic doses in the culture medium, and set solvent (i.e., dimethyl sulfoxide) as treatment control (*see* Note 5).
4. Repeat compound treatment with medium change every other day; harvest the cells at day 10 post treatment.

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[1]0.2 M HCl needs to be freshly prepared, as the solution will gradually lose the acid concentration over time.
[2]Collagen-coated plate is ideal for cells to form confluent monolayers, especially for HepG2-based cells.
[3]HepG2.2.15 cell line is cultured with DMEM/F12 medium supplemented with 10 % fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, plus 400 μg/mL G418. Tetracycline-inducible (tet-off) cell lines are maintained in the same way as the HepG2.2.15, but with the addition of 1 μg/mL tetracycline.
[4]To obtain a high level of HBV replication in tetracycline-inducible cell lines, it is critical to withdraw the tetracycline when the cells reach confluence, as HBV replication will arrest cell growth [25, 26].
[5]Addition of the compounds at the start of induction of HBV replication, before the cccDNA has formed, will expose the establishment phase of the cccDNA metabolism to possible inhibition, but will still allow detecting agents that are able to remove the cccDNA itself after it becomes established.

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3.2 cccDNA Extraction from Cell Culture

1. Lyse cells by adding 1.5 mL of TE buffer (10:10) and 0.1 mL of 10 % SDS into each well of a 6-well cell culture plate. Gently mix and incubate the plate for 30 min at room temperature.
2. Transfer the viscous cell lysate to a 15 mL centrifuge tube (*see* Note 6). Add 0.4 mL of 5 M NaCl and gently invert the tube ten times. Let the tube sit at 4 °C for at least 16 h to efficiently precipitate proteins and protein-associated DNAs.
3. Centrifuge at 14,500 × *g* for 30 min at 4 °C. Transfer the supernatant to a fresh 15 mL centrifuge tube. To remove the residual protein from the supernatant, add an equal volume of phenol (approx. 2 mL) to the supernatant and mix thoroughly by hand shaking for 10 s (*see* Note 7). Centrifuge at 3,500 × *g* for 10 min at 4 °C, and transfer the aqueous phase to a fresh 15 mL tube. Repeat phenol extraction step. Add equal volume of phenol/chloroform (approx. 2 mL), mix thoroughly by hand shaking, centrifuge at 3,500 × *g* for 10 min at 4 °C, and transfer the aqueous phase to a fresh 15 mL tube.
4. Add two volumes of 100 % ethanol (approx. 4 mL) and mix thoroughly by inverting the tube several times. Incubate at room temperature overnight to precipitate DNA (*see* Note 8).
5. On the second day, centrifuge the tube at 3,500 × *g* for 30 min at 4 °C, and discard the supernatant. Add an equal volume of 75 % ethanol (approx. 2 mL) and gently flick the tube to wash the DNA pellet. Centrifuge at 3,500 × *g* for 15 min at 4 °C.
6. Discard the supernatant. Allow the pellet to air-dry for about 10 min at room temperature. Dissolve the DNA pellet in 25 μL TE buffer (10:1).

3.3 Agarose Gel Electrophoresis

1. Prepare 150 mL of 1.2 % gel by mixing 1.8 g agarose, 5 mL of 30× TAE buffer, and 145 mL of distilled water in a microwavable flask. Microwave the flask until the agarose is thoroughly dissolved. Let it cool down to about 50 °C at room temperature. Pour the liquid gel into the gel tray (12 cm × 10 cm) with an appropriate comb inserted. Let the gel solidify at room temperature before removal of the comb.
2. Prepare 600 mL of gel running buffer by diluting 20 mL of 30× TAE buffer with deionized water.

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[6]Use wide-mouth plastic transfer pipette to transfer the lysate into centrifuge tube.
[7]Use leakproof tube with screw cap. Do not shake the tube too vigorously or too long, which may physically nick the supercoiled cccDNA.
[8]Because high concentration of salt is used in Hirt extraction, incubation of DNA solution at low temperature will result in a lot of salt co-precipitating with DNA.

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3. Prepare samples by adding approximately 3 μL of 10× loading buffer to 25 μL of each sample. Pipette up and down to mix well.

4. Assemble submarine gel-running unit by correctly positioning the gel box, adding running buffer into the chamber, and loading 28 μL of each sample into a separate well. Connect the gel-running unit to a power supply and run gel at 25 V overnight.

3.4 Southern Blot

1. Disconnect the gel-running unit and take out the gel carefully.

2. Submerge the gel in a tray containing freshly prepared 0.2 M HCl. Agitate gently for 10 min at room temperature to depurinate the DNA samples from the gel. The bromophenol blue dye in the gel should gradually turn grey during this process.

3. Rinse the gel three times with distilled water.

4. Submerge the gel in a tray of denaturing buffer, and agitate gently for 1 h at room temperature. The loading dye in the gel should turn back to blue during this process.

5. Rinse the gel three times with distilled water.

6. Submerge the gel in a tray of neutralization buffer, and agitate gently for at least 1 h at room temperature.

7. Soak the Hybond-XL membrane (12 × 10 cm) in a tray with deionized water for 5 min at room temperature. Replace the water with 20× SSC and continue to agitate for 15 min.

8. To transfer DNA from the gel to the Hybond-XL membrane, assemble the transfer apparatus as follows: Layer two sheets of Whatman 3 MM filter paper in the gel transfer tray and pour 20× SSC transfer buffer on top to completely wet the Whatman papers. Smooth out the bubbles in between the Whatman papers and the tray gently by a plastic roller, and get rid of the excess amount of the transfer buffer. Place the gel on top of the Whatman papers with the side containing the loading wells facing down; make sure that there is no air bubble between the Whatman papers and the gel. Place the presoaked Hybond-XL membrane on top of the gel and use a plastic roller to get rid of the air bubbles between the gel and the membrane. Layer two sheets of 20× SSC pre-wet Whatman papers (12 × 10 cm) on top of the membrane; use plastic roller to remove air bubbles in between. Place a stack of dry paper towels (about 4 in.) precut to the gel size on top of the Whatman papers. Seal the transfer tray with 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 24–48 h (*see* Note 9).

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9. After gel transfer, disassemble the blot apparatus and flip over the membrane with the gel still attached, and mark the position of each loading well on the membrane by a pencil (*see* Note 10). Wrap up the gel and paper towels by plastic wrap and discard.

10. Cross-link the Hybond-XL membrane in a UV crosslinker chamber with UV energy dosage at 120 mJ/cm^2. The membrane can be directly subject to hybridization, or sandwiched between two sheets of Whatman filters and stored at −20 °C.

3.5 Probe Preparation for HBV DNA Detection (See Note 11)

1. Prepare rNTP (supplied with Riboprobe® system) solution by mixing 50 μL of each 10 mM rATP, 10 mM rGTP, 10 mM rCTP, and 0.2 mM UTP.

2. Add 4 μL of 5× reaction buffer, 4 μL of rNTP solution prepared from the previous step, 2 μL of 100 mM DTT, 0.25 μg of SalI-linearized pGEM-HBV DNA template (*see* Note 12), 1 μL of RNasin, and 1 μL of SP6 polymerase into a nucleasefree 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 °C for 1 h.

3. To remove the DNA template, add 1 μL of RNase-free RQ1 DNase to the reaction and incubate at 37 °C for 15 min.

4. Add 15 μL of 5 M NH_4 OAc 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 × *g* for 30 min at 4 °C, and discard the supernatant carefully without disturbing the pellet. Dissolve the probes 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 (*see* Note 13).

3.6 Hybridization (See Note 11)

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 14).

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[9]When assembling the Southern blot transfer apparatus, avoid possible short circuits of capillary liquid flow. The DNA will not be efficiently transferred if the blot short-circuits. Use parafilm strips to seal the edges of the gel.

[10]After transfer, the gel becomes thinner. Use the pencil tip to penetrate the loading wells and mark their positions on the membrane. This step is for labeling the DNA-binding side of the membrane and tracking the running lanes with orientation.

[11]All radioactive materials and procedures should be handled strictly by following the institutional laboratory safety regulation rules.

[12]Linearization of the plasmid pGEM-HBV by SalI is an optimal step to produce “run-off” transcripts derived from the HBV sequence only. Other restriction enzyme, which singly cuts the vector sequences downstream of SP6-HBV transcription cassette but does not leave a 3′ overhang, can also be used.

[13]^{32}P-radiolabeled HBV DNA probes prepared by random priming or end labeling can also be used in the hybridization.

[14]If using other commercial hybridization buffers, follow the pre-hybridization and hybridization conditions recommended by the manufacturers.

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2. Replace the pre-hybridization buffer with 5 mL of fresh EKONO™ 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 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, making sure that the membrane is not cracked or wrinkled during this process. Seal the membrane with plastic wrap. Place the membrane in a phosphorimager cassette with the DNA-binding side facing up. Layer the phosphor imaging screen on top facing the DNA-binding side of the membrane. Close the cassette tightly and expose for 1–2 h in the dark.

6. Scan the phosphor imaging screen with phosphorimager system. Store the membrane properly and erase the signal in the phosphor imaging screen with intense light for reuse.

7. A typical phosphor image of HBV DNA Southern blot is shown in Fig. 2 (also *see* refs. 14, 15). Quantify the signal intensity of the cccDNA bands with software provided by the phosphorimager system. When testing antiviral compounds, compare the signals from treatment wells with solvent control well and determine the compound's effective concentration inhibiting cccDNA production by 50 % (EC_{50} values) (*see* Note 15).

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[15]In HBV stable cell lines, the cccDNA is exclusively synthesized from the rcDNA through the "intracellular amplification pathway". If cccDNA reduction is observed under compound treatment, the effect of test compound on viral core DNA replication and cccDNA stability should be analyzed as previously described [16].

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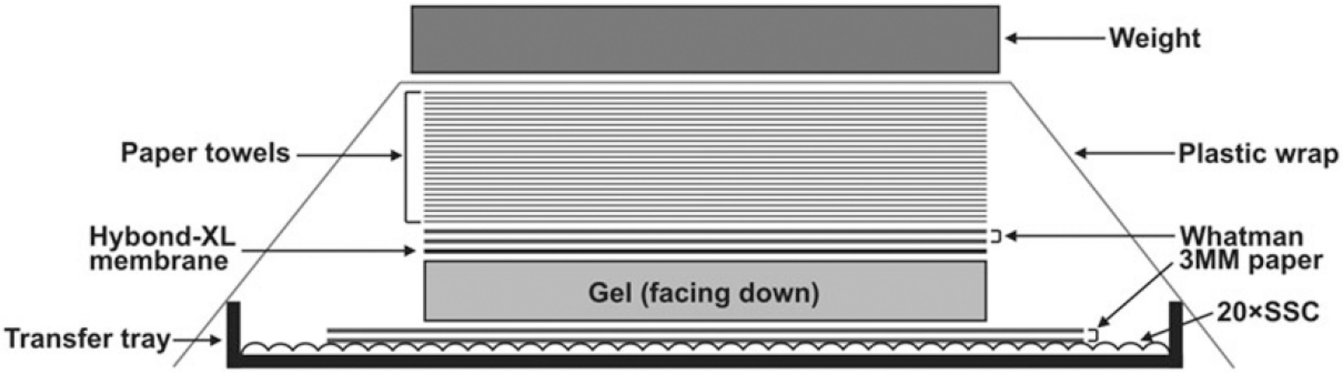

Fig. 1.
Assembly of HBV DNA Southern blot transfer apparatus. See text for details

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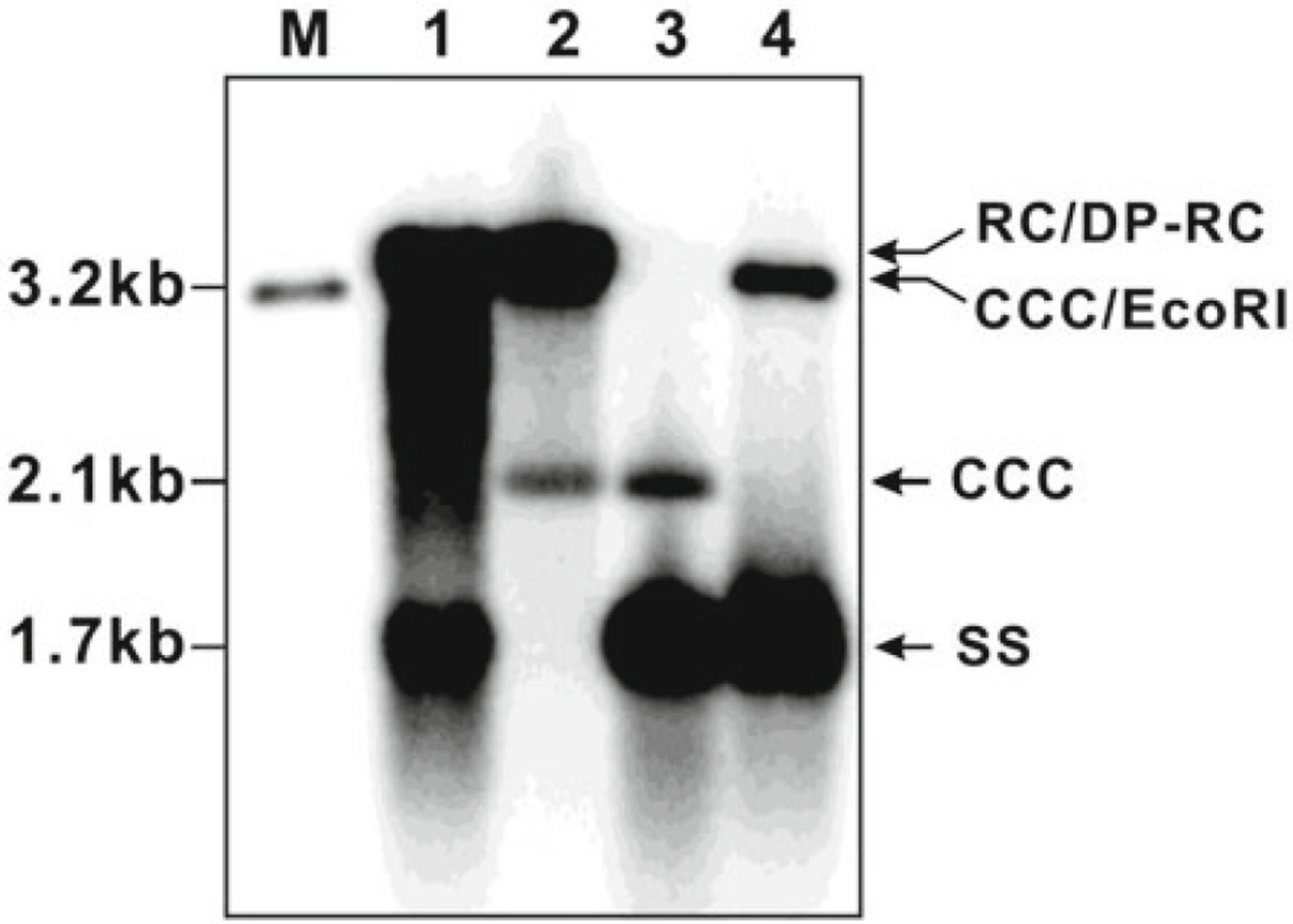

Fig. 2.

Detection of HBV DNA by a Southern blot assay. A typical pattern of HBV cytoplasmic core DNA (*lane 1*) and Hirt DNA (*lane 2*) upon Southern blot hybridization is shown. *RC* relaxed circular DNA, *DP-RC* deproteinized RC DNA, *CCC* covalently closed circular DNA, *SS* single-stranded (−) DNA, *M* HBV genomic length DNA marker. The approximate size of each HBV DNA species is labeled on the *left*. To further validate the authenticity of HBV cccDNA, the Hirt DNA sample in *lane 2* has been heated to 85 °C for 5 min before gel loading, a condition that denatures DP-rcDNA into SS DNA, while the cccDNA stays undenatured and its electrophoretic mobility remains unchanged (*lane 3*). The DNA sample from *lane 3* is further digested with EcoRI, in which condition the cccDNA is linearized to a genome-length double-stranded DNA (*lane 4*). Reproduced from [15] with permission from Elsevier