

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 programed 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,

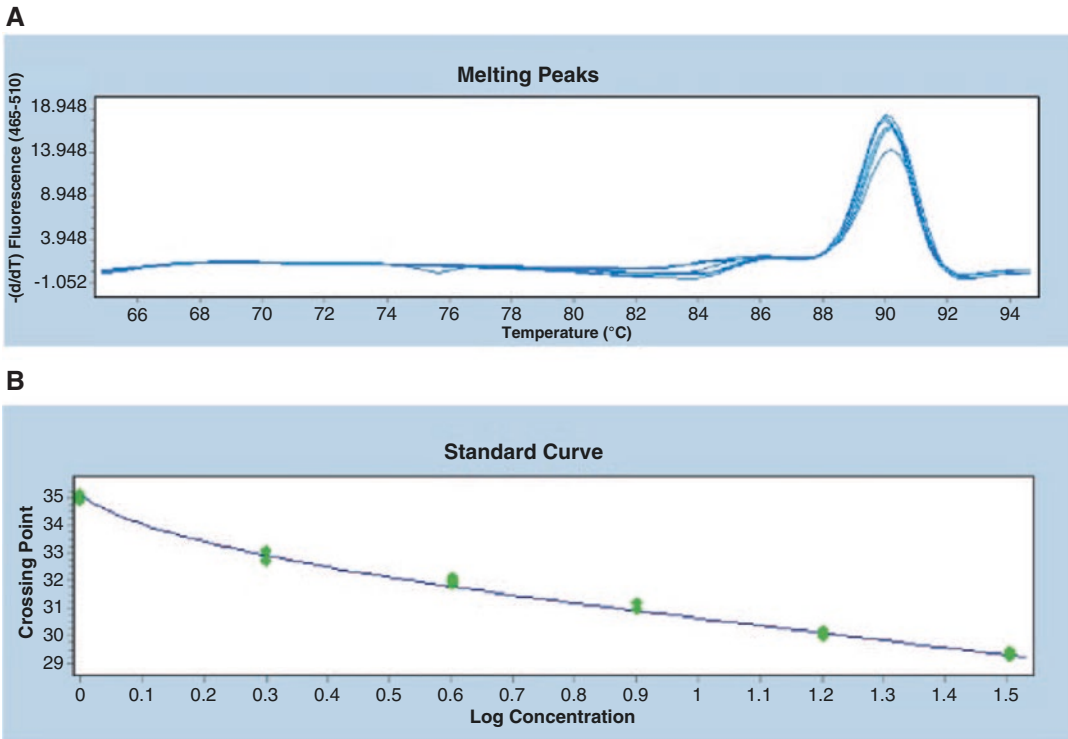


Fig. 1 Melting curve and standard curve. HBV cccDNA PCR was performed on Light Cycler™ 480 machine, melting curve (a) and standard curve (b) were analyzed

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

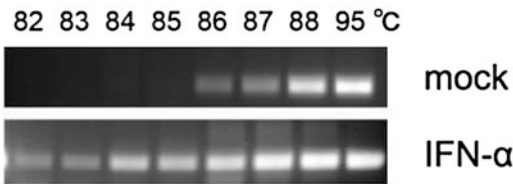


Fig. 2 3D-PCR of HBV cccDNA. HBV infected primary human hepatocyte cells were treated with 1000 IU/ml of IFN- α . 3D-PCR was performed to detect HBV cccDNA deamination 4 days after treatment

2. 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.
3. 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**).
4. 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**).
5. Amplicons were detected in a 2%-agarose gel electrophoresis (*Fig. 2*).
6. Purify DNA from gel by using “GeneJET Gel Extraction Kit” (Fermentas, St. Leon-Rot, Germany).
7. 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.
8. Extract plasmid using “GeneJET Plasmid Miniprep Kit” (Thermo Scientific, Schwerte, Germany). Dilute DNA samples

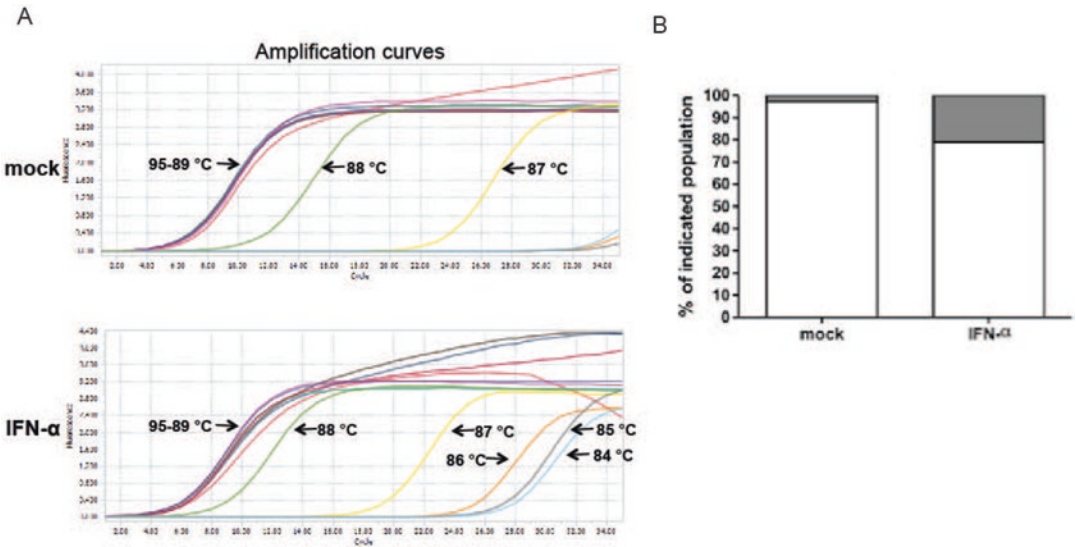


Fig. 4 Quantification of cccDNA deamination. HBV cccDNA qPCR products from mock or IFN- α treated HBV infected HepaRG were further analyzed by quantitative 3D-PCR. (a) Amplification curves were presented. (b) Proportion of deaminated cccDNA (gray) was calculated according to Cp values of different denatured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

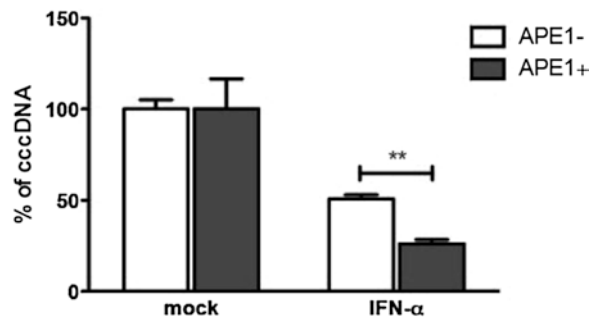


Fig. 5 Quantification of APE1 treated cccDNA. DNA extracts from HBV infected PHH cells were incubated with APE1. HBV cccDNA was quantified by qPCR

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 °C for 2 h.
3. Inactivate the reaction at 70 °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 °C for 20 min.
3. Analyze treated cccDNA by 3D-PCR as in Fig. 6 (see Note 20).

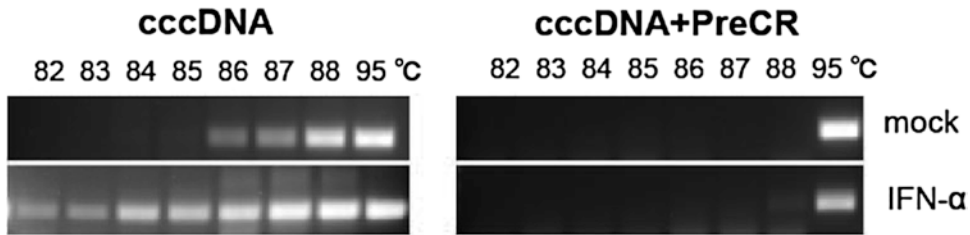


Fig. 6 3D-PCR of PreCR Repair Mix treated cccDNA. DNA extracts from HBV infected PHH cells were incubated with PreCR Repair Mix. 3D-PCR was used to detect HBV cccDNA deamination

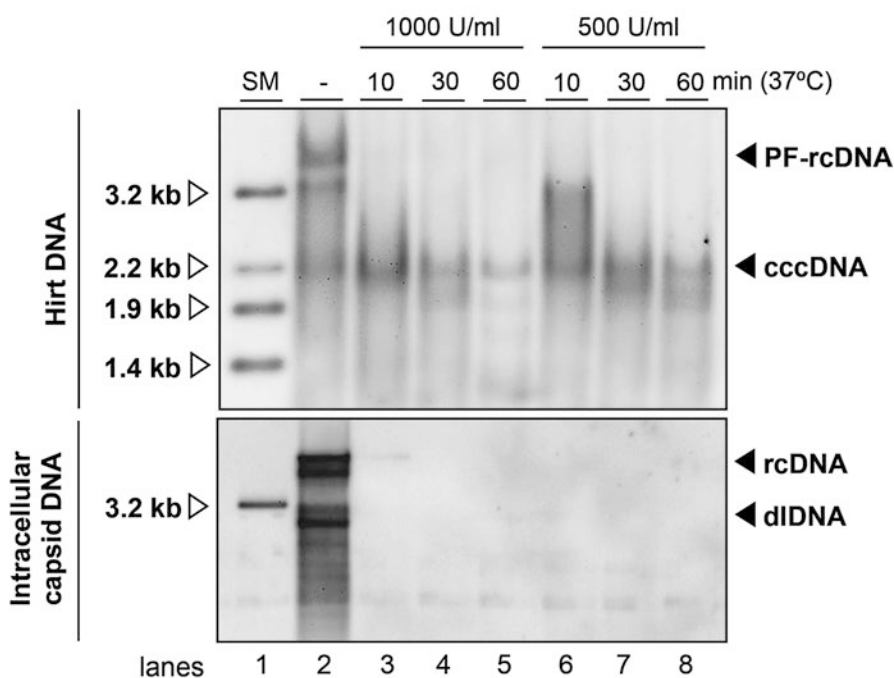
4 Notes

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: AAGTGCACACGGTTYGGCAGAT
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 > $10^5/\mu\text{l}$.

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

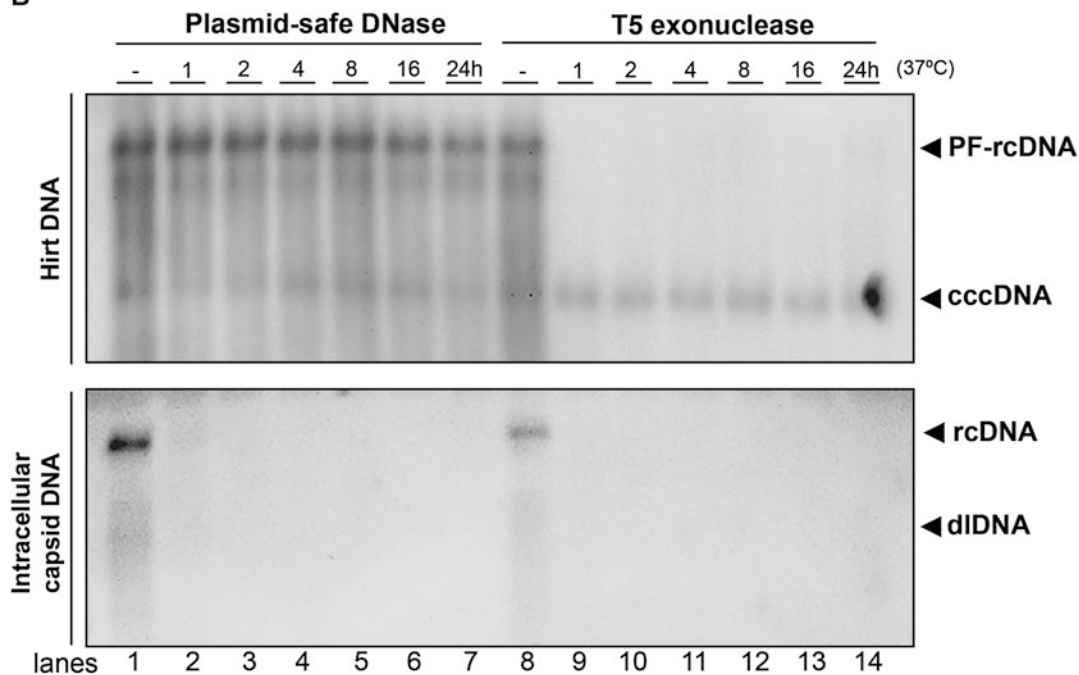


Fig. 7 Southern blot analysis of viral DNA treated with T5 exonuclease or plasmid-safe DNase. HepAD38 cells were cultured in the absence of tetracycline for 11 days to induce HBV gene expression. Protein-free DNA after Hirt lysis and DNA from intracellular capsids were extracted. (a) Samples were treated 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, 10⁷ 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).

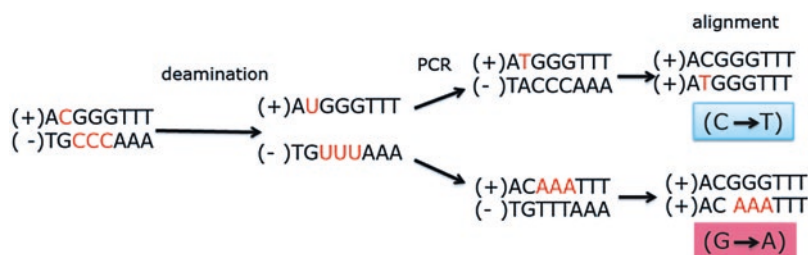


Fig. 8 Strand specific deamination. Plus strand deamination leads to C to T transition while minus strand deamination results in G to A transition

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: $2^{\Delta(Cp95^{\circ}C - Cp88^{\circ}C)}$.
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.

References

1. Nassal M (2015) HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B. *Gut* 64(12):1972–1984
2. Lucifora J, Xia Y, Reisinger F, Zhang K, Stadler D, Cheng X, Sprinzl MF, Koppensteiner H, Makowska Z, Volz T, Remouchamps C, Chou WM, Thasler WE, Huser N, Durantel D, Liang TJ, Munk C, Heim MH, Browning JL, Dejudin E, Dandri M, Schindler M, Heikenwalder M, Protzer U (2014) Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. *Science* 343(6176):1221–1228
3. Xia Y, Lucifora J, Reisinger F, Heikenwalder M, Protzer U (2014) Virology. Response to Comment on "Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA". *Science* 344(6189):1237
4. Xia Y, Stadler D, Lucifora J, Reisinger F, Webb D, Hosel M, Michler T, Wisskirchen K, Cheng X, Zhang K, Chou WM, Wettengel JM, Malo A, Bohne F, Hoffmann D, Eyer F, Thimme R, Falk CS, Thasler WE, Heikenwalder M, Protzer U (2016) Interferon-gamma and tumor necrosis factor- α produced by T cells reduce the HBV persistence form, cccDNA, without cytolysis. *Gastroenterology* 150(1):194–205
5. Liu Y, Prasad R, Beard WA, Kedar PS, Hou EW, Shock DD, Wilson SH (2007) Coordination of steps in single-nucleotide base excision repair mediated by apurinic/aprimidinic endonuclease 1 and DNA polymerase beta. *J Biol Chem* 282(18):13532–13541
6. Stenglein MD, Burns MB, Li M, Lengyel J, Harris RS (2010) APOBEC3 proteins mediate the clearance of foreign DNA from human cells. *Nat Struct Mol Biol* 17(2):222–229
7. Cai D, Nie H, Yan R, Guo JT, Block TM, Guo H (2013) A southern blot assay for detection of hepatitis B virus covalently closed circular DNA from cell cultures. *Methods Mol Biol* 1030:151–161
8. Suspene R, Guetard D, Henry M, Sommer P, Wain-Hobson S, Vartanian JP (2005) Extensive editing of both hepatitis B virus DNA strands by APOBEC3 cytidine deaminases in vitro and in vivo. *Proc Natl Acad Sci U S A* 102(23):8321–8326
9. Noguchi C, Ishino H, Tsuge M, Fujimoto Y, Imamura M, Takahashi S, Chayama K (2005) G to A hypermutation of hepatitis B virus. *Hepatology* 41(3):626–633
10. Ko C, Lee S, Windisch MP, Ryu WS (2014) DDX3 DEAD-box RNA helicase is a host factor that restricts hepatitis B virus replication at the transcriptional level. *J Virol* 88(23):13689–13698
11. Gao W, Hu J (2007) Formation of hepatitis B virus covalently closed circular DNA: removal of genome-linked protein. *J Virol* 81(12):6164–6174
12. Guo H, Jiang D, Zhou T, Cuconati A, Block TM, Guo JT (2007) Characterization of the intracellular deproteinized relaxed circular

- DNA of hepatitis B virus: an intermediate of covalently closed circular DNA formation. *J Virol* 81(22):12472–12484
13. Kock J, Rosler C, Zhang JJ, Blum HE, Nassal M, Thoma C (2010) Generation of covalently closed circular DNA of hepatitis B viruses via intracellular recycling is regulated in a virus specific manner. *PLoS Pathog* 6(9):e1001082
 14. Longo MC, Berninger MS, Hartley JL (1990) Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. *Gene* 93(1):125–128

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