

A Sensitive and Specific PCR-based Assay to Quantify Hepatitis B Virus Covalently Closed Circular (ccc) DNA while Preserving Cellular DNA

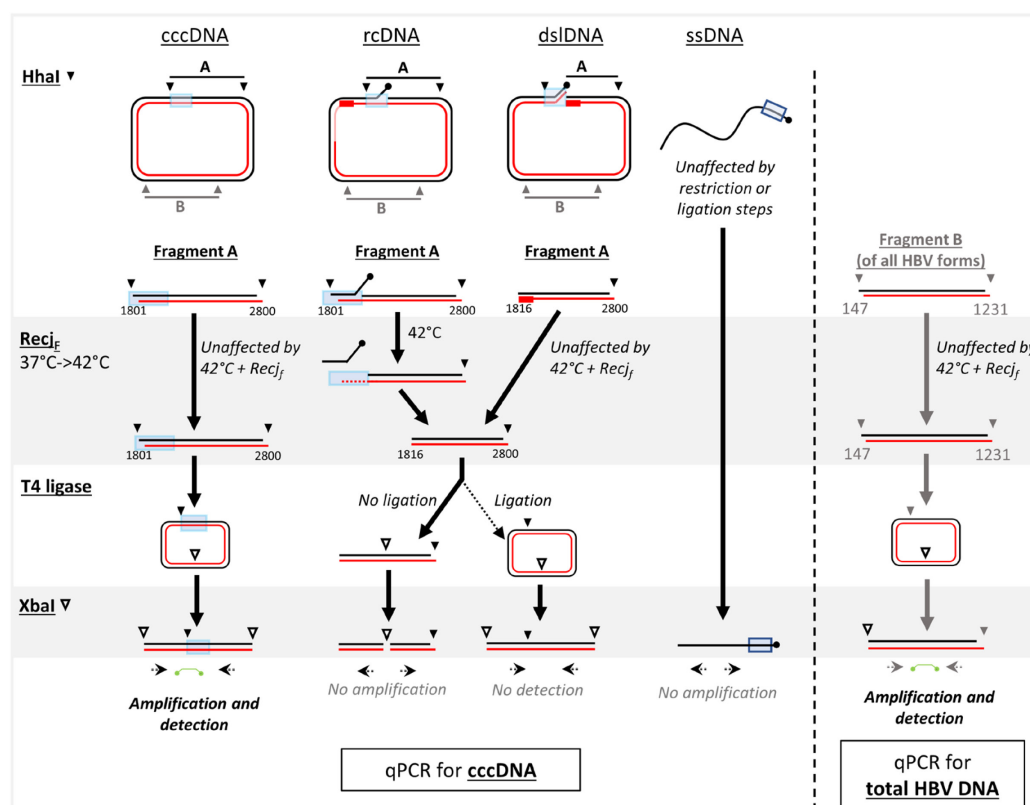
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[Abstract] Hepatitis B virus (HBV) is the major cause of liver diseases and liver cancer worldwide. After infecting hepatocytes, the virus establishes a stable episome (covalently closed circular DNA, or cccDNA) that serves as the template for all viral transcripts. Specific and accurate quantification of cccDNA is difficult because infected cells contain abundant replicative intermediates of HBV DNA that share overlapping sequences but arranged in slightly different forms. HBV cccDNA can be detected by Southern blot or qPCR methods which involve enzymatic digestion. These assays are laborious, have limited sensitivity, or require degradation of cellular DNA (which precludes simple normalization). The method described in this protocol, cccDNA inversion quantitative (cing)PCR, instead uses a series of restriction enzyme-mediated hydrolysis and ligation reactions that convert cccDNA into an inverted linear amplicon, which is not amplified or detected from other forms of HBV DNA. Importantly, cellular DNA remains quantifiable during sample preparation, allowing normalization and markedly improving precision. Further, a second linear fragment (derived from enzymatic digestion of a separate region of the HBV DNA genome and is present in all forms of HBV DNA) can be used to simultaneously quantify total HBV levels.

Graphic abstract:



Selective detection of HBV cccDNA and total HBV DNA using cinqPCR (Reproduced from Tu *et al.*, 2020a).

Keywords: Hepatitis B virus, cccDNA, Hepcludex®, Bulevirtide, Myrcludex B, cinqPCR, DNA nick, DNA repair, Viral persistence

[Background] Hepatitis B virus (HBV) is a small enveloped virus, which encapsidates a partially double-stranded circular DNA genome, the so-called relaxed circular (rc) DNA. Upon infection of human hepatocytes, the nucleocapsid is transported to the nucleus wherein the rcDNA genome is converted to covalently closed circular (ccc) DNA. This episomal form is highly stable and maintains chronic HBV infection (Tu *et al.*, 2020b). It serves as template for all viral transcripts and the pre-genomic RNA. Elimination of the cccDNA would lead to a complete cure of a chronic hepatitis B infection. Thus, the reliable quantification of cccDNA is key to developing strategies for HBV cure.

In any given infected hepatocyte, HBV DNA exists in different forms (rcDNA, double stranded linear (dsl) DNA, single stranded (ss) DNA, and cccDNA) that share overlapping sequences, but have different structures (Nassal, 2015). Thus, differentiation of cccDNA (which is present in low numbers) from other HBV DNA forms is technically challenging. Southern blot is the gold standard method used to differentiate cccDNA from other forms, though it has low throughput and poor sensitivity. PCR-based

methods are more sensitive and can be applied in a medium-throughput manner. Current protocols include an exonuclease treatment (with T5 exonuclease, plasmid-safe DNase, or a combination of Exol and ExoIII) to digest all DNA species that are not covalently closed (including cellular DNA) (Luo *et al.*, 2017; Allweiss *et al.*, 2018; Qu *et al.*, 2018). By contrast, the method described in this protocol takes advantage of particular restriction sites within the HBV DNA sequence (see graphical abstract) to selectively convert cccDNA into an amplifiable form. Therefore, our method allows the normalization to a single-copy cellular gene and total HBV DNA can be detected from the same sample, dramatically improving precision, sensitivity, and accuracy.

Materials and Reagents

Note: All stored at room temperature unless otherwise stated.

1. 24-well cell culture plates (Corning, catalog number: 3527)
2. 1.5 ml Eppendorf tubes (Sarstedt, catalog number: 72706)
3. Sealing Mats for 96-Well PCR Plates, reusable (Bio-Rad, catalog number: 2239442)
4. TwinTec PCR plates, 96 semi-skirted (Eppendorf, catalog number: 0030128.575)
5. Aluminium Foil Seals for PCR and QX100 ddPCR applications (Bio-Rad, catalog number: 181-4040)
6. P20 racked barrier LTS tips (Rainin, catalog number: RT-L20F)
7. P200 racked barrier LTS tips (Rainin, catalog number: RT-L200F)
8. HBV-susceptible cells, *e.g.*, HepG2-NTCP cells (Ni *et al.*, 2014) (maintain in 37 °C incubator at 5% CO₂ supply and 95% humidity)
9. HBV genotype D virus stock (store at -80 °C)
10. Dulbecco's Phosphate Buffered Saline (PBS, Sigma-Aldrich, catalog number: D8537)
11. Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, catalog number: 41965039, store at 4 °C)
12. Fetal bovine serum (FBS, Sigma-Aldrich, catalog number: S0615, store at -20 °C)
13. L-Glutamine (Life Technologies, catalog number: 25030024, store at -20 °C)
14. Penicillin (10,000 U/ml) Streptomycin (10 mg/ml) (Life Technologies, catalog number: 15140-122, store at -20 °C)
15. Dimethylsulfoxide (DMSO, Merck, catalog number: 102950)
16. Polyethylenglycol (PEG 8000, 40% solution in PBS, Sigma-Aldrich, catalog number: 89510)
17. Trypsin-EDTA solution (Sigma-Aldrich, catalog number: T3924, store at 4 °C)
18. NucleoSpin® Tissue kit (Macherey-Nagel, catalog number: 740952)
19. Multichannel pipette (Eppendorf, catalog number: 3125000036)
20. 10× Cutsmart buffer (NEB, catalog number: B7204, store at -20 °C)
21. *Hha*I (NEB, catalog number: R0139, store at -20 °C)
22. PCR-grade H₂O (*e.g.*, B. Braun Melsungen, for injection purposes)
23. RecJ_r (NEB, catalog number: M0264, store at -20 °C)

24. T4 DNA Ligase (NEB, catalog number: M0202, store at -20 °C)
25. 10 mM Molecular-grade ATP (NEB, catalog number: P0756, store at -20 °C)
26. *Xba*I (NEB, catalog number: R0145, store at -20 °C)
27. DNAZap PCR DNA Degradation Solutions (Thermo Scientific, catalog number: AM9890, store at 4 °C)
28. VIC-labelled TaqMan™ Copy Number Reference Assay for the human RNase P gene (Applied Biosystems, catalog number: 4403328, store at -20 °C)
29. ddPCR Supermix for Probes (Bio-Rad, catalog number: 1863010, store at -20 °C)
30. Primers/probe for cccDNA detection (ordered from Eurofins genomics, store at -20 °C)
 - a. cccDNA for: 5'-CACTCTATGGAAGGCGGGTA-3'
 - b. cccDNA rev: 5'-ATAAGGGTCGATGTCCATGC-3'
 - c. cccDNA probe: 5'-FAM- AACACATAGCGCACCAGCA-BHQ1-3'
31. Primers/probe for total HBV DNA (ordered from Eurofins genomics, store at -20 °C)
 - a. Total HBV for: 5'-GTGTCTGCGGCGTTTTATCA-3'
 - b. Total HBV rev: 5'- GACAAACGGGCAACATACCTT-3'
 - c. Total HBV probe: 5'-FAM-TGAGGCATAGCAGCAGGATG-BHQ1-3'
32. DG8 cartridges (Bio-Rad, catalog number: 186-4008)
33. DG8 gaskets (Bio-Rad, catalog number: 186-3009)
34. Droplet Reader Oil (Bio-Rad, catalog number: 186-3004)
35. Droplet generation oil for probes (Bio-Rad, catalog number: 186-3005)
36. Growth medium (see Recipes)
37. Infection medium (see Recipes)
38. Ligation mix (see Recipes)
39. Linearization mix (see Recipes)

Equipment

1. Pipette for Rainin P20 tips (Rainin, catalog number: 17014392)
2. Multichannel pipette for Rainin P200 tips (Rainin, catalog number: 17013805)
3. Thermo cycler (Analytik Jena Biometra, FlexCycler²)
4. QX200™ Droplet generator (Bio-Rad, catalog number: 1864002)
5. PX1 PCR plate sealer (Bio-Rad, catalog number: 1814000)
6. C1000 Touch™ Thermal Cycler with 96-Deep Well Reaction Module (Bio-Rad, catalog number: 1851197)
7. QX200™ Droplet Reader (Bio-Rad, catalog number: 1864003)

Software

1. QuantaSoft (Bio-Rad)

2. Microsoft Excel
3. Prism (GraphPad)

Procedure

A. Sample preparation

1. Seed 2.5×10^5 cells per well in a 24-well format in growth medium one day before infection.
2. Infect cells overnight with HBV virus stock in infection medium containing 4% PEG 8000 (Ni *et al.*, 2014).
3. Wash cells twice with PBS at room temperature (RT) and maintain in infection medium until desired harvest time.
4. Wash cells with 500 μ l PBS at RT and add 100 μ l trypsin.
5. Incubate at 37 °C until the cells detach (~5 min).
6. Resuspend the cells with 900 μ l cold medium and transfer to a 1.5 ml Eppendorf tube.
7. Centrifuge for 5 min at 500 $\times g$ at RT and remove the supernatant.
Optional: Freeze the pellet at -20 °C until further use.
8. Extract total DNA from the cell pellets using NucleoSpin® Tissue kit according to the manufacturer's instructions, but elute in 50 μ l elution buffer pre-warmed to 70 °C.

B. Inversion reaction

1. Clean a silicon sealing mat using the DNA Zap solutions, rinse in deionised water, and let dry on a paper towel.
2. Prepare the restriction digestion reaction in a 96-well plate containing 10 μ l of extracted total DNA, 2 μ l of 10 \times Cutsmart buffer, 0.5 μ l *Hha*I, 0.25 μ l *RecJ*_I, and 7.25 μ l H₂O.
3. Mix by pipetting up and down 10 times with a multichannel pipette, taking care to avoid generating bubbles.
4. Seal with the clean and dry silicon mat.
5. Run the following program in a thermo cycler: 3 cycles of 15 min at 37 °C and 15 min at 42 °C, 20 min at 80 °C, and hold at 16 °C.
6. Remove the mat carefully and add 10 μ l of the ligation mix.
7. Mix by pipetting up and down 10 times with a multichannel pipette, taking care to avoid generating bubbles.
8. Incubate in a thermo cycler at 16 °C for 2 h, 80 °C for 20 min, and hold at 16 °C.
9. Remove the mat carefully and add 5 μ l of the linearization mix.
10. Mix by pipetting up and down 10 times with a multichannel pipette, taking care to avoid generating bubbles.
11. Incubate in a thermo cycler at 37 °C for 60 min, 80 °C for 20 min, and hold at 16 °C.
Optional: Store samples at -20 °C.

C. Droplet generation and reading

1. Dilute the sample 2.5-fold by adding 52.5 μ l H₂O.
2. Mix by pipetting up and down 10 times with a multichannel pipette, taking care to avoid generating bubbles.
3. Prepare two reactions for each sample: (1) cccDNA and RNaseP cellular control and (2) total HBV DNA and RNaseP cellular control. Prepare ddPCR mix in a 96-well plate (22 μ l per reaction) (see Table 1).

Table 1. ddPCR mix composition for each reaction

Reagent	Concentration	μ l
ddPCR Supermix for probes	2×	11
TaqMan™ Copy Number Reference Assay (VIC-labelled RNaseP)	10×	1.1
Forward primer (cccDNA or total HBV DNA)	100 μ M	0.033
Reverse primer (cccDNA or total HBV DNA)	100 μ M	0.033
FAM-labelled probe (cccDNA or total HBV DNA)	100 μ M	0.033
H ₂ O		4.5
Diluted template		5.5

4. Use microplastic-free Rainin tips. Generate droplets on the ddPCR droplet generator:
 - a. Add 20 μ l of the ddPCR mix to the sample chambers of a cartridge.
 - b. Add 70 μ l of droplet generation oil using a multichannel pipette in the oil chamber.
 - c. Attach the rubber gasket, place the cartridge in the droplet generator, and run the droplet generation.
 - d. Gently transfer 42 μ l of the generated droplets using a multichannel pipette to a TwinTec 96-well plate.
 - e. Repeat Steps C4a to C4d until all samples are loaded into the TwinTec 96-well plate.
 - f. Seal the plate with an aluminum foil seal using the plate sealer.
5. **Directly after sealing**, run PCR in deep-well PCR machine with the following program: 10 min at 95 °C; 40 cycles of 10 s at 95 °C, 15 s at 54 °C, and 20 s at 68 °C; 10 min at 95 °C, and hold at 12 °C.
6. Transfer the TwinTec 96-well plate to the QX200 Droplet Reader.
7. Set up template using the Quantasoft software (as per manufacturer's user guide), reading droplets using channels for FAM (Channel 1) and VIC (Channel 2).
8. Define thresholds for positive and negative droplets based on positive and negative controls, after which the software will automatically quantify values for cccDNA per RNaseP and total HBV DNA per RNaseP.

Data analysis

In the QuantaSoft software, we use the 2D graph to draw thresholds for HBV DNA and for the RNaseP positive droplets (Figure 1). The thresholds are gated separately for all the total HBV DNA and all the cccDNA assays. For the data presentation we use the ratios total HBV DNA/RNaseP and cccDNA/RNaseP of each sample with the PoissonRatioMin and PoissonRatioMax values giving a 95% confidence interval of the technical error of the ddPCR.

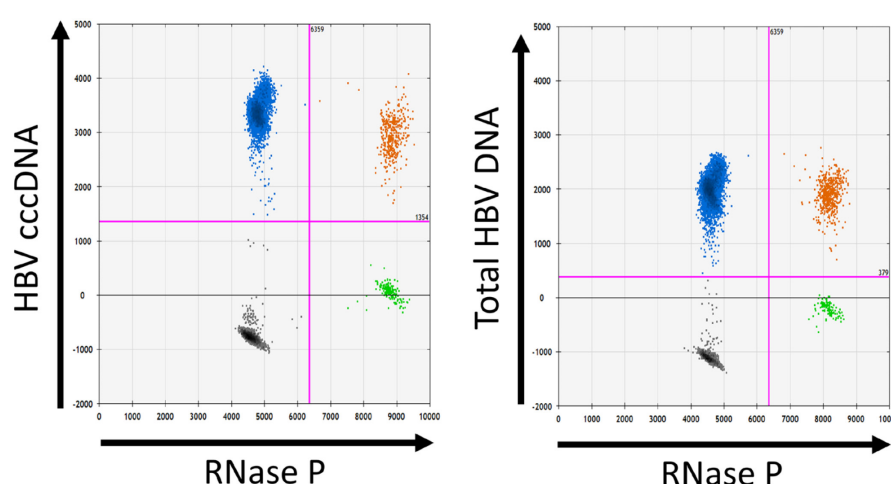


Figure 1. Data analysis using QuantaSoft. The fluorescence intensity was used to separate droplets containing amplicons detecting: HBV cccDNA (left) or total HBV DNA (right), based on FAM fluorescence (y-axis); and RNaseP, based on VIC fluorescence (x-axis). The thresholds for positive signals (pink) were gated based on positive and negative control reactions (Reproduced from Tu *et al.*, 2020a).

Notes

1. We have used this protocol with DNA extracted from cells infected in plate formats from 12-well to 96-well. In 12-well plates, we advise to elute the DNA in twice the volume.
2. For the inversion, we recommend an input of <2 µg of total DNA extract. We also recommend adding 200 ng of carrier DNA (e.g. unrelated plasmid DNA) if the total DNA input is <200 ng.
3. We usually perform HBV infections with a multiplicity of viral genome equivalents of 200. However, infection can also be performed with a smaller inoculum as the assay is sensitive enough to detect very low numbers of infected cells (Tu *et al.*, 2020a).
4. This protocol can also be used with Taqman-based qPCR instead of ddPCR.
5. This assay is only compatible with HBV sequences from genotype D which is used in most *in vitro* HBV infection assays. Unfortunately, it does not work with other genotypes due to variation

in *HhaI* restriction sites. Most *in vitro* assays with HBV are performed with the genotype D strain, this shortcoming does not allow the analysis of clinical samples.

6. Recommended controls for optimization and quality control:
 - a. **Positive control to show detection efficiency** (total HBV DNA quantification should give similar number as cccDNA quantification): HBV circDNA (Mutz *et al.*, 2018) or HBV 1.1 overlength plasmid spiked into DNA extracted from uninfected cells.
 - b. **Negative control to show selective quantification of cccDNA**: DNA extracted from heparin-purified virus (Seitz *et al.*, 2016) spiked into DNA extracted from uninfected cells.
 - c. **Negative control to determine signal from virus inoculum during cell infection**: Treatment of cells with an entry inhibitor (e.g., Myrcludex B) prior to HBV infection (Donkers *et al.*, 2017).
 - d. **No template control and uninfected cell control to determine level of non-specific amplification.**

Recipes

1. Growth medium
 - 500 ml of DMEM
 - 50 ml of FBS
 - 5 ml of L-Glutamine
 - 5 ml of Penicillin (10,000 U/ml) and Streptomycin (10 mg/ml)
2. Infection medium
 - 49 ml of Growth medium
 - 1 ml of DMSO
3. Ligation mix (10 µl per sample)
 - 3 µl of 10 mM ATP
 - 1 µl of 10× NEB Cutsmart buffer
 - 0.5 µl of T4 ligase
 - 5 µl H₂O
4. Linearization mix (5 µl per sample)
 - 0.5 µl of 10× Cutsmart buffer
 - 0.5 µl of *XbaI*
 - 4 µl H₂O

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Competing interests

Stephan Urban is co-applicant and co-inventor on patents protecting HBV preS-derived lipopeptides (bulevirtide / Hepcludex®, formerly Myrcludex B) for their use as HBV/HDV entry inhibitors. The other authors in this study declare no competing interests.

References

1. Allweiss, L., Volz, T., Giersch, K., Kah, J., Raffa, G., Petersen, J., Lohse, A. W., Beninati, C., Pollicino, T., Urban, S., Lutgehetmann, M. and Dandri, M. (2018). [Proliferation of primary human hepatocytes and prevention of hepatitis B virus reinfection efficiently deplete nuclear cccDNA *in vivo*](#). *Gut* 67(3): 542-552.
2. Donkers, J. M., Zehnder, B., van Westen, G. J. P., Kwakkenbos, M. J., AP, I. J., Oude Elferink, R. P. J., Beuers, U., Urban, S. and van de Graaf, S. F. J. (2017). [Reduced hepatitis B and D viral entry using clinically applied drugs as novel inhibitors of the bile acid transporter NTCP](#). *Sci Rep* 7(1): 15307.
3. Luo, J., Cui, X., Gao, L. and Hu, J. (2017). [Identification of an Intermediate in Hepatitis B Virus Covalently Closed Circular \(CCC\) DNA Formation and Sensitive and Selective CCC DNA Detection](#). *J Virol* 91(17): e00539-17.
4. Mutz, P., Metz, P., Lempp, F. A., Bender, S., Qu, B., Schoneweis, K., Seitz, S., Tu, T., Restuccia, A., Frankish, J., Dachert, C., Schusser, B., Koschny, R., Polychronidis, G., Schemmer, P., Hoffmann, K., Baumert, T. F., Binder, M., Urban, S. and Bartenschlager, R. (2018). [HBV Bypasses the Innate Immune Response and Does Not Protect HCV From Antiviral Activity of Interferon](#). *Gastroenterology* 154(6): 1791-1804 e1722.
5. Nassal, M. (2015). [HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B](#). *Gut* 64(12): 1972-1984.
6. Ni, Y., Lempp, F. A., Mehrle, S., Nkongolo, S., Kaufman, C., Falth, M., Stindt, J., Koniger, C., Nassal, M., Kubitz, R., Sultmann, H. and Urban, S. (2014). [Hepatitis B and D viruses exploit sodium taurocholate co-transporting polypeptide for species-specific entry into hepatocytes](#). *Gastroenterology* 146(4): 1070-1083.
7. Qu, B., Ni, Y., Lempp, F. A., Vondran, F. W. R. and Urban, S. (2018). [T5 Exonuclease Hydrolysis of Hepatitis B Virus Replicative Intermediates Allows Reliable Quantification and Fast Drug Efficacy Testing of Covalently Closed Circular DNA by PCR](#). *J Virol* 92(23): e01117-18.
8. Seitz, S., Iancu, C., Volz, T., Mier, W., Dandri, M., Urban, S. and Bartenschlager, R. (2016). [A Slow Maturation Process Renders Hepatitis B Virus Infectious](#). *Cell Host Microbe* 20(1): 25-35.

9. Tu, T., Zehnder, B., Qu, B., Ni, Y., Main, N., Allweiss, L., Dandri, M., Shackel, N., George, J. and Urban, S. (2020a). [A novel method to precisely quantify hepatitis B virus covalently closed circular \(ccc\)DNA formation and maintenance](#). *Antiviral Res* 181: 104865.
10. Tu, T., Zehnder, B., Qu, B. and Urban, S. (2020b). [De novo synthesis of Hepatitis B virus nucleocapsids is dispensable for the maintenance and transcriptional regulation of cccDNA](#). *JHEP Rep* 3(1): 100195.