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A quantitative method for hepatitis B virus covalently closed circular DNA enables distinguishing direct acting antivirals from cytotoxic agents

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ABSTRACT

Studying the biogenesis of hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) and developing anti-HBV agents require analytical methods to quantify viral DNA levels inside host cells. The well-accepted Southern blotting method is only semi-quantitative, while the other widely used methods (based on qPCR) have been questioned as to their fidelity for cccDNA quantification. In addition, Southern blotting and qPCR results barely reflect the number of host cells present in an analytical sample. We here developed new techniques that substantially improve cccDNA detection and quantification, including a sample pretreatment method that i) exploits high temperature and exonuclease V (an ATP-dependent, bidirectional exonuclease) digestion to effectively increase the amplification efficiency of HBV cccDNA by removing rcDNA and denaturing the cccDNA template, and ii) a method that splits cell samples and uses separate extraction technologies to facilitate "host normalization" based on host genomic DNA signals. Our study introduces new analytical techniques that should be useful for the basic biology and translational studies of HBV.

The covalently closed circular DNA (cccDNA) of the hepatitis B virus (HBV) represents a major obstacle for a cure of chronic hepatitis B (CHB) (Guo and Guo, 2015; Nassal, 2015; Revill et al., 2019; Seeger and Mason, 2015; Suk-Fong Lok, 2019). Specifically, current drugs for CHB only suppress viral replication; they do not disrupt viral cccDNA (Durantel and Zoulim, 2016). Southern blotting analysis is a well-accepted method for cccDNA detection, but it is time-consuming, low throughput, and only semi-quantitative. Quantitative PCR (qPCR), another widely used method, is fast, relatively high throughput, and quantitative. However, current methodologies of the qPCR-based detection of HBV cccDNA are not ideal and have been questioned for their inability to definitely differentiate cccDNA from other viral replication intermediates such as relax circular DNA (rcDNA) (Nassal, 2015). Moreover, although mitochondria DNA (mtDNA) has been used as a control of episomal DNA (Seeger and Sohn, 2014), the level of mtDNA varies depending on cell metabolic status and other conditions (Lopez-Lluch, 2017), and thus the lack of a convenient and appropriate host reference signal has complicated the intra- and inter-experiment interpretation of both qPCR- and Southern-blotting-based HBV cccDNA analytical results.

Previously reported protocols for cccDNA detection have included three steps to reduce amplification from rcDNA: 1) starting from protein-free DNA extract, which is extracted using a modified Hirt's method and typically removes most of rcDNA (Gao and Hu, 2007; Hirt, 1967; Zhou et al., 2006); 2) digesting rcDNA with Plasmid-Safe™ ATP-Dependent DNase (PSAD) (Bowden et al., 2004) or T5 exonuclease (Qu and Urban, 2019; Xia et al., 2017); 3) careful design for cccDNA-selective primers (Mason et al., 1998). However, even combining all these precautionary steps and utilizing best practices either cannot completely remove rcDNA (PSAD) or compromise cccDNA integrity for rcDNA removing (T5 exonuclease) (Ko et al., 2018). Moreover, the amplification efficiency of cccDNA can be limited as it is of supercoiled DNA structure, which is known to resist denaturation (Miller and Robinson, 1984; Newbold et al., 1995), and the low amplification efficiency results in a reduced signal-to-noise ratio in qPCR assays. We here present a simple method comprising two heat treatment steps and an enzyme digestion step that dramatically increases the amplification efficiency for HBV cccDNA in qPCR assays by both eliminating rcDNA from samples and promoting amplification from denatured cccDNA templates.

As illustrated in the schema in Fig. 1A, the samples (protein-free DNA extract) are treated with three steps prior to their use as templates for qPCR-based quantitative analysis: 1) denaturing of rcDNA via heating at 80°C for 5 min, followed by immediate cooling on ice; 2)

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digestion of the denatured rcDNA with exonuclease V (ExoV, an ATP-dependent bidirectional exonuclease for ss- and ds-DNA, developed by *New England Biolabs Inc.*) at 37°C for 30 min; and 3) denaturing of cccDNA via heating at 100°C for 20 min. We started with protein-free DNA extracted from HBV-infected HepG2-NTCP cells with aforementioned modified Hirt's method and validated our method via comparison of cccDNA quantification by qPCR method with cccDNA detection by the standard Southern blotting method. As shown in Fig. 1B and Fig. S1, heating at 80°C for 5 min was sufficient to remove canonical rcDNA band but had no or little effect on cccDNA band,

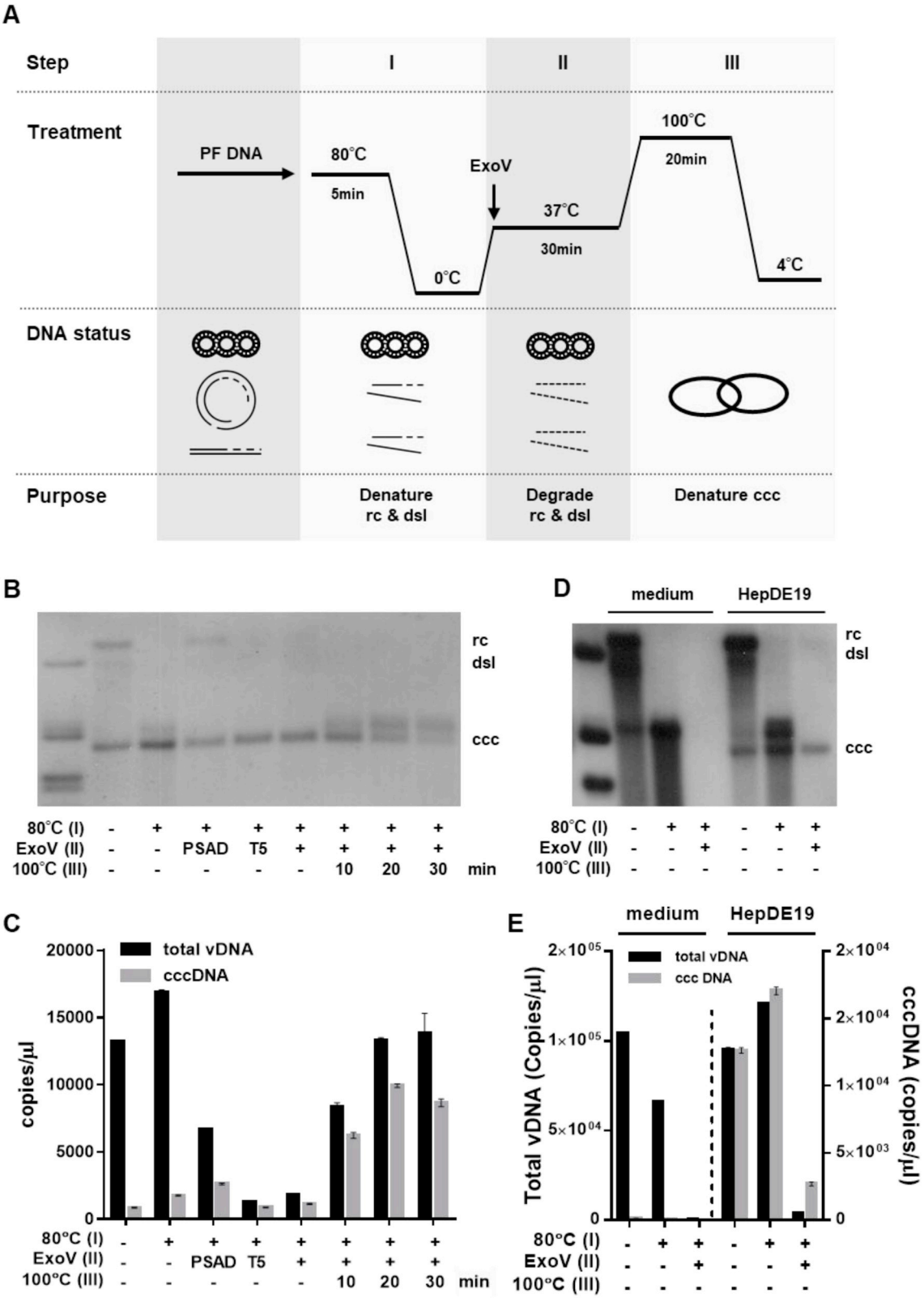

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Fig. 1. Pretreatment combining two heating steps and one ExoV digestion step completely removed rcDNA and significantly enhanced amplification efficiency of cccDNA. (A) A schema of pretreatment sequence. Viral DNA in HBV-infected HepG2-NTCP cells was extracted using a modified Hirt's method. Each 12 μg of DNA was treated following one or more steps as illustrated in (A) and digested with 20 units ExoV (*New England Biolabs Inc.*). (B) 24 in total 25 μl of treated DNA was detected via Southern blotting with DIG-labeled full-length HBV genome (DIG-high prime DNA-labeling and detection starter kit II, Roche, cat. No. 11585614910), and (C) 1 in total 25 μl of treated DNA was diluted with 24 μl ddH_2O followed by quantifying via qPCR using SYBR Green-based commercial kit (TaKaRa, RR420A) on ABI 7500 fast with a cycling condition: 95°C for 2 min for initial denaturation, 40 cycles of 95°C for 3 s and 60°C for 40 s. For comparison, two previously used nucleases, i.e., Plasmid-Safe™ ATP-Dependent DNase (PSAD, *epicentre*, 20 units at 37°C for 4 h) and T5 exonuclease (T5, *New England Biolabs Inc.*, 20 units at 37°C for 30 min) were also included (B and C). Viral DNA in the culture medium of HBV-infected HepG2-NTCP cells and viral DNA in HepDE19 cells were extracted using standard phenol extraction method and modified Hirt's method, respectively. Both extracts were incubated at 80°C for 5 min followed with or without an ExoV digestion step (5 units for DNA from culture medium, 10 units for DNA from HepDE19). The viral DNA were then (D) analyzed via Southern blotting and (E) and quantified via qPCR as (B) and (C).

indicating effective denaturation of the rcDNA but not cccDNA. Further treatment of samples with ExoV removed the denatured rcDNA with no or little effect on the quantity of cccDNA. Fig. 1C showed that ExoV treatment after heating at 80°C remarkably decreased the qPCR signal for total viral DNA (total vDNA) while only slightly decreased the signal for cccDNA.

Having verified the success of our designed rcDNA denaturation and removal steps, we next heated the sample to 100°C for 10–30 min (immediately after ExoV treatment). As shown in Fig. 1B, this heating step denatured some cccDNA molecules, resulting in a canonical intact cccDNA band and a smear new band above the cccDNA which was supposed to be denatured cccDNA with two unpaired, but physically linked strands as depicted in Fig. 1A. As 100°C heating time increased, the density of hypothetical denatured cccDNA band increased, while the band of the intact cccDNA decreased, consisting with a gradual denaturation of cccDNA during 100°C heating. A 20 min incubation at 100°C was sufficient to denature most of the cccDNA, and almost all of the cccDNA was denatured after a 30 min incubation. Quantification of cccDNA via qPCR showed that a 20 min incubation at 100°C increased the signal by 9.4-fold compared to unheated, ExoV-treated sample (6.7-fold in a repeat experiment, data not shown). Further prolonging the incubation time to 30 min increased the signal only 8.2-fold. Moreover, while a long initial denaturation step of the qPCR program (10 min) could increase the amplification efficiency of cccDNA, it failed to increase the amplification efficiency to the extent that 100°C did (Fig. S2); and the long initial denaturation step of qPCR program (10 min) led to a suboptimal linearity at amplifying a plasmid standard of HBV, compared to the initial denaturation step (2 min) we used in this study (Fig. S3). Therefore a 20 min incubation at 100°C seemed sufficient and necessary for quantification of cccDNA via qPCR (Fig. 1C).

One major challenge for quantifying cccDNA is to minimize sample treatment-related and nuclease activity-caused cccDNA loss. We compared ExoV digestion with two currently widely used nucleases, i.e., PSAD and T5 exonuclease (T5) to assess their impacts on cccDNA quantification. As shown in Fig. 1B, the upper band, corresponding to rcDNA or nicked cccDNA (comigration) (Gao and Hu, 2007) (in lane 1), was removed after heating at 80°C for 5 min (lane 2). Notably, a band at similar position was detected again in sample treated with PSAD after 80°C heating (lane 3). By comparison of lane 2–5, we speculated that the newly emerged band was nicked cccDNA converted by PSAD treatment of cccDNA. Indeed, the quantity of cccDNA was lower in PSAD-treated sample (lane 3) than that in ExoV-treated sample (lane 5) in Southern blotting assay (Fig. 1B). As for T5, no nicked cccDNA band was detected in the assay (Fig. 1B, lane4). Both Southern blotting assay and qPCR assay repeatedly showed consistent pattern for T5- and ExoV-treated samples. However, T5-treated samples repeatedly showed lower quantity of cccDNA than that of ExoV-treated samples. Fig. 1C showed that the number of cccDNA in T5-treated sample was 873 copies/μl; while in ExoV-treated sample, the number was 1130 copies/μl, indicating ~23% loss of cccDNA treated by T5 digestion comparing to that treated by ExoV (19% loss of cccDNA by T5 in a repeat experiment, data not shown). The result was consistent with a previous study reporting > 20% loss of cccDNA digested by T5 (Ko et al., 2018). It is reasonable to speculate that some cccDNA molecules were converted to nicked cccDNA and subsequently degraded by T5, hence sample with T5 digestion showed lower amount cccDNA and little if any nicked cccDNA in Southern blotting assay. Together these results supported that ExoV might be superior to PASD and T5 for cccDNA quantification. Moreover, for a given amount of viral DNA (e.g., 1.2 μg PF DNA isolated from ~1.75×10^4 HBV-infected HepG2-NTCP cells), ExoV digestion in a large range of dose (2–20 units/30 min) or reaction time (10–120 min/5 units) yielded similar quantification results of cccDNA (Fig. S4), also suggesting that ExoV is an excellent nuclease suitable for testing various samples within a relatively wide dynamic range of experimental parameters, including input amount of viral DNA, digestion time, and dose etc.

While using Southern blotting assay, we observed lower quantity of cccDNA in PSAD-treated sample (Fig. 1B, lane 3) comparing to ExoV-treated sample (Fig. 1B, lane 5), analysis by qPCR assay showed higher quantity of cccDNA in PSAD-treated sample than that in ExoV-treated sample (Fig. 1C), this is in consistent with higher amplification efficiency of cccDNA with nicked DNA in PCR without prior denaturation of the samples at 100°C.

To further validate our method, we next examined DNA samples from culture medium of HBV-infected HepG2-NTCP cells and from HBV-producing cells (HepDE19). DNA samples from the culture medium, extracted via standard phenol extraction method, contain viral ssDNA and rcDNA but no cccDNA. DNA samples from HepDE19 cells (Guo et al., 2007), extracted via the aforementioned modified Hirt's method, contain a much higher ratio of rcDNA to cccDNA than the samples from HepG2-NTCP cells (used in Fig. 1B and 1C). As shown in Fig. 1D and 1E, we found that for DNA extracted from the culture medium of HBV-infected HepG2-NTCP cells, heating at 80°C followed by ExoV treatment completely removed viral DNA as detected via Southern blotting and reduced the cccDNA signal down to an undetectable level based on qPCR. Further these experiments showed that for DNA extracted from HepDE19 cells, heating at 80°C followed by ExoV treatment completely removed rcDNA while only marginally affected cccDNA detected via Southern blotting, and reduced the ratio of total vDNA to cccDNA from 7.0 (94938:12632) down to 1.4 (3662:2694) via qPCR. We therefore concluded that the cccDNA in protein-free DNA extracts could be better quantified by qPCR assay after our new pretreatment steps are applied.

After establishment of this new method for cccDNA quantification, we next attempted to develop a method to enable a form of “host normalization” of the quantification data for viral DNA with the number of host cells present in a given sample. We previously observed that more than 80% of HepG2-NTCP cells in our culture system were arrested at G0/G1 phase after 24 h of culture (Qi et al., 2016). The amplification signal based on a particular host genome locus template should be tightly correlated with the overall cell number and should thus be suitable for host normalization of qPCR-based quantification data. Before we are able to explore this idea experimentally, we need to overcome the problem that the modified Hirt's method we originally use to extract DNA is known to precipitate large host genomic DNA fragments prior to the phenol extraction step (Hirt, 1967).

In contrast, standard phenol extraction methods recover all the viral and host DNA, but substantially decrease the signal-to-noise ratio for

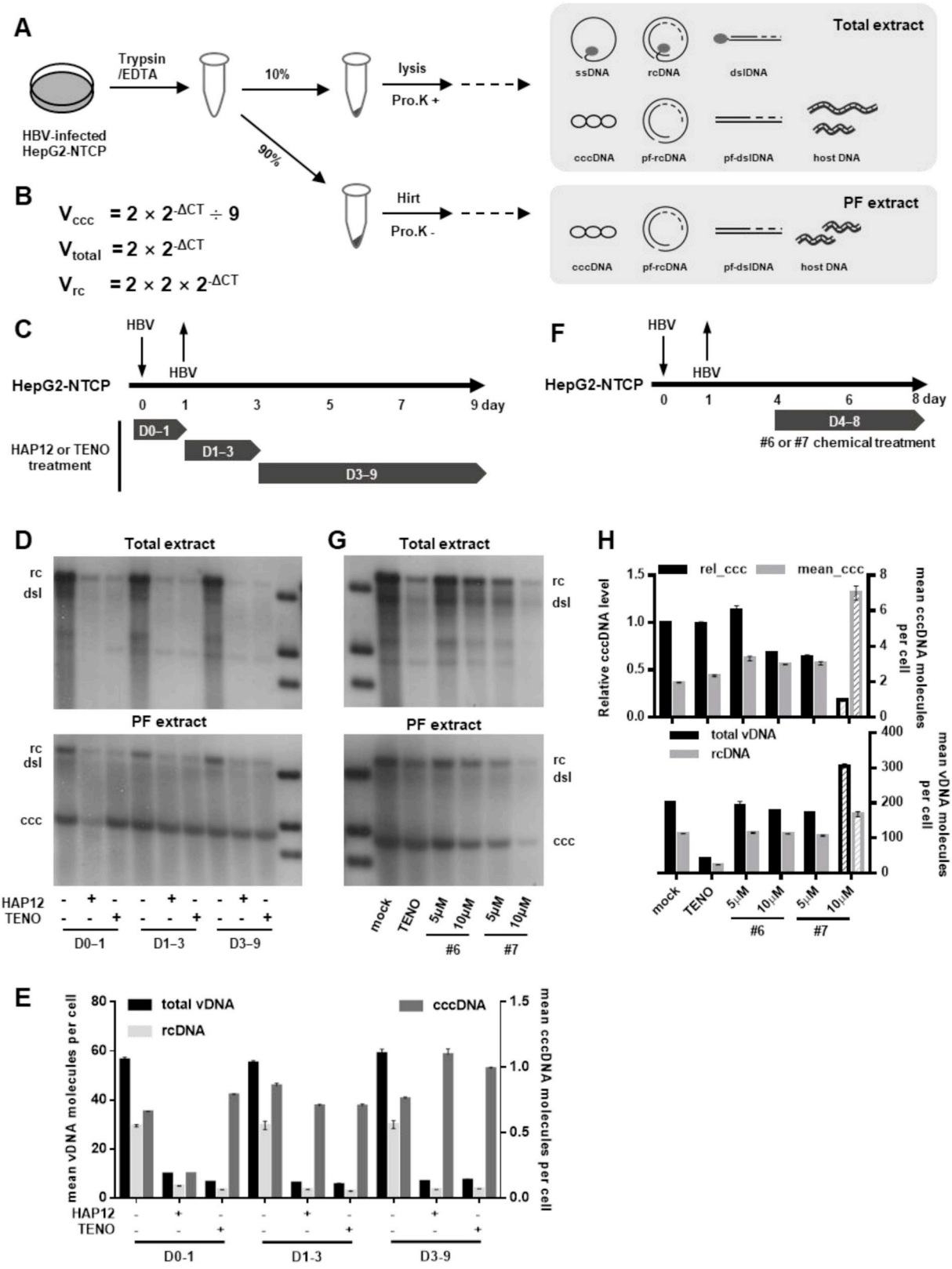

Fig. 2. Splitting cell samples for DNA extraction with separate methods enabled "host normalization". (A) Schematic illustration of cell sample splitting and DNA extraction. (B) The mathematic formulas used for results calculation following viral DNA quantification via qPCR and host normalization. Proof-of-concept experiments using (C–E) two well characterized anti-HBV drugs, HAP12 and tenofovir and (F–H) two uncharacterized compounds, #6 and #7. HepG2-NTCP cells (~0.7×10^6) were inoculated with HBV and treated with compounds as schematically illustrated in (C) and (D). Cells were then harvested, and split, and viral DNA was extracted as schematically illustrated in (A). Note that the cell samples were resuspended with small volume of PBS, e.g., 10% of final volume, before mixed with cell lysis or Hirt lysis buffer. Total extract and PF extract were dissolved with 25 μl TE buffer (10 mM Tris·HCl, pH7.5, 1 mM EDTA). (D, G) Viral DNA in 24 μl of total extract or PF extract was then analyzed via Southern blotting as Fig. 1B. (E, H) 1 μl of total extract was diluted with 24 μl ddH_2O. 1 μl of PF extract was diluted with 9 μl TE buffer and then digested with 5 units ExoV following the method described in Fig. 1. Viral DNA and host genome were then quantified via qPCR as in Fig. 1. Notice that a fixed threshold was set for the calculation of C_T values of all qPCR reactions. Notice severe cell death in sample shown in open bar with oblique line in (H).

cccDNA detection (Tuttleman et al., 1986). We therefore attempted to develop a method that combines the advantages of both extraction types to further improve the ability to precisely quantify cccDNA biogenesis, with the final aim of facilitating the evaluation of antiviral drugs. Specifically, as schematically illustrated in Fig. 2A, our method splits harvested cell sample (e.g., HBV-infected HepG2-NTCP cells) into two parts following trypsin/EDTA digestion and detachment: 10% of the precipitated cells are resuspended with cell lysis buffer (20 mM Tris·HCl, pH8.0, 5 mM EDTA, 400 mM NaCl, 1% SDS, 100 μg/ml protease K), and the remaining 90% of precipitated cells are resuspended in Hirt lysis buffer. Subsequently, standard phenol extraction is used to extract the cell lysis buffer part (termed "total extract", comprising all of the viral and host DNA), and the aforementioned modified Hirt's method is used to extract Hirt lysis buffer part (termed "PF extract", comprising the protein-free viral DNA).

These two extracts are then used for quantification of cccDNA and for host normalization. Specifically, the PF extract samples are used for the quantitation of cccDNA, and the total extract samples are used to quantify the host genomic DNA (here using the *14q24.1* locus as the amplification template for the host genomic DNA signal). Note that HepG2 cells are human hepatoma cells with hyper-diploid karyotype. The host locus chosen for normalization is *14q24.1* because previous karyotype analysis indicated that HepG2 cells stably contain two copies of chromosome 14 (Chen et al., 1993), a finding that is supported based on our unpublished deep sequencing data with our HepG2-NTCP cells. Subsequently, the cccDNA in a PF extract sample is quantified via qPCR, and the detected value is adjusted with value detected for amplification signal from the host *14q24.1* gDNA template in the corresponding total extract. In addition to quantification of cccDNA and host normalization, we also quantify total viral DNA and viral rcDNA in the total extract to enable comparison of the efficiency of viral replication. As showed in Fig. S5, the total vDNA primer was designed to target both strands of rcDNA and therefore amplifies ss-, rc- and dsl-DNA. The rcDNA primer was designed to span the nick region of minus strand of rcDNA and therefore amplifies only the plus strand of rcDNA but not ssDNA or dslDNA (See Table 1 for primer sequence). The specificity of all primers used in this study was tested for melting curve and all the amplification products showed unique melting temperature (Fig. S6).

Given two copies of the reference gene template in each cell, we use the formula in Fig. 2B to calculate the mean level of cccDNA present per cell, rather than merely showing the relative level of cccDNA. Notice that detected values for cccDNA are multiplied by 2 and divided by 9 because 1) each HepG2-NTCP cell contains two copies of the reference gene locus, and 2) the number of cells used for cccDNA quantification (i.e., PF extract) is 9-fold higher than the number of cells used for the host normalization (i.e., total extract). Total viral DNA and viral rcDNA in total extract are quantified and normalized to the host reference from the same total extract. The total vDNA is therefore multiplied simply by 2 (two copies of host reference gene locus). The rcDNA is further multiplied by 2, compared to total DNA, because that the rcDNA primer amplifies one of two strands of rcDNA, i.e., the plus strand. Notice that we design different primer pairs for host normalization of cccDNA and non-ccc viral DNA, i.e., total vDNA and rcDNA. That is because the qPCR signal, when using dye Sybr Green as we did in this study, is positively related with the length of amplification product. The amplification product of cccDNA primer (486 nt) is significantly longer than that amplified by total vDNA and rcDNA primer (< 150 nt).

Table 1
Primers for quantification of viral DNA and host genomic DNA via qPCR.

Target	Name	Sequence
Total vDNA[a]	2268F	GAGTGTGGATTCGCACTCC
	2391R	GAGGCGAGGGAGTTCTTCT
rcDNA[a]	1770F	TGTACTAGGAGGCTGTAGGCA
	1874R	GGAGGCTTGAACAGTAGGACA
cccDNA[a]	1528F	CACCTCTCTTTACGCGGACT
	2013R	ACAGAGCTGAGGCGGTATCT
14q24.1[b] (shorter)	1215F	CAAAGCAATTCTGAAAGCCA
	1360R	AGGCTTCTTGGGTAGACACC
14q24.1[c] (longer)	18501F	GGCAAGACACTTTCCTGGCA
	19096R	TTCTTGGGGCTACCTGGTTC

[a] Based on HBV genotype D (GenBank: U95551.1).
[b] Host targeting primer for host normalization of total vDNA and rcDNA.
[c] Host targeting primer for host normalization of cccDNA.

We next used our combined pre-treatment and normalization methods to test drugs that are known to exert anti-HBV effects. We first chose two well-characterized anti-HBV drugs, HAP12 (an allosteric modulator of viral nucleocapsid) (Berke et al., 2017; Guo et al., 2017) and Tenofovir (an inhibitor of viral polymerase) (Delaney et al., 2006), and designed experiments as schematically illustrated in Fig. 2C. Cells were harvested as shown in Fig. 2A at day 9 post infection (p.i.). All viral DNA in the total extract samples and the cccDNA in the PF extract samples were analyzed via Southern blotting and quantified via qPCR (with normalization). Both the Southern blotting method (Fig. 2D) and our qPCR method (Fig. 2E) repeatedly recapitulated previous findings about the antiviral effects of HAP12 and Tenofovir: 1) HAP12 inhibited cccDNA formation when added at day 0–1 p.i. rather than at other time points; 2) Tenofovir marginally affected cccDNA formation at any time point; 3) both HAP12 and Tenofovir inhibited viral replication when added after establishment of cccDNA. These observations suggest that evaluation of antiviral drugs with qPCR is as good as Southern blotting, provided that cells were processed based on the new pre-treatment method and quantified based on our new normalization scheme.

To validate whether our method does accurately reflect variations in cell numbers, we then tested drugs (#6 and #7) that were previously found in our lab to significantly decrease cccDNA levels and reduce cell viability (unpublished data). We treated HBV-infected HepG2-NTCP cells with #6 and #7 at day 4–8 p.i. as schematically illustrated in Fig. 2F. Cells were harvested at day 8 p.i. and viral DNA was extracted and quantified using our new method as introduced in Fig. 2C and 2D. Southern blotting revealed that both the vDNA and cccDNA levels decreased after treatment with #6 and #7, and both levels decreased in a dose-dependent manner for both compounds (Fig. 2G). However, when the effects of these compounds were assessed using our method with host normalization, neither the vDNA nor the cccDNA levels was found to be decreased following treatment with #6 or #7 (Fig. 2H).

These dramatically different results indicate the following point: whereas Southern blotting detects only the viral DNA present in a sample, and implies that these compounds exert antiviral effects, our method with host normalization allows us to differentiate between (i) the cytotoxicity of compounds on the host cells and (ii) the specific antiviral effects of the compounds. Thus, anti-viral drug evaluation based on Southern blotting could miss important information about cell viability and can lead to inappropriate conclusions about the apparent efficacy of an experimental treatment. These results clearly advocate for the broader use of similar host normalization steps in other assays used for the assessment of anti-viral drugs.

Previously, Long et al. developed a qPCR-based cccDNA detection method that included pretreating DNA samples (protein-free DNA extract) with heating at 85°C followed by PSAD digestion (Long et al., 2017). We here showed that ExoV is superior to PSAD for preserving the integrity of cccDNA. Recently, Luo et al. reported a non-ccc DNA-removing method by digesting protein-free extract with exonuclease I and III (Luo et al., 2017). Exonuclease III digests ss-, ds- and nick DNA and therefore removes rcDNA efficiently with unnecessity of denaturing rcDNA. However, the integrity of cccDNA was preserved at a narrow range of exonuclease III dose (two folds range) and that is unlike ExoV of which the treatment was tolerable for cccDNA quantification in a wide range of dose (2–20 units/30 min) and time (10–120 min/5 units). In addition to exonuclease digestion, we added a second high-temperature incubation to denature cccDNA prior to qPCR analysis, and we demonstrate that this additional incubation dramatically enhances the

amplification efficiency of cccDNA.

Here, we combined cccDNA quantification with a unique sample processing procedure to facilitate what we term “host normalization”. The average number of cccDNA per cell, after "host normalization", varies from 0.5 to 3 in a typical infection in our experiments. Collectively, our new pre-treatment and host normalization protocols allow us to quantify viral replication and cccDNA formation simultaneously, from a single population of cells and to normalize the detected viral DNA levels to the number of host cells present in a sample. An additional advantage of our host normalization method over quantifying vDNA with plasmid standard is that linear and relaxed circular DNA, e.g., viral ssDNA, rcDNA and host genomic DNA, are prone to be overestimated when using plasmid standard which is supercoiled and resistant to denature in qPCR assay. Together, our study demonstrates that the new analytical methods we have developed substantially advance the options for researchers studying HBV cccDNA in cells and illustrate that these new methods are attractive for new experimental designs for the evaluation of antiviral drug candidates.

Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.06.002.

References

Berke, J.M., Dehertogh, P., Vergauwen, K., Van Damme, E., Mostmans, W., Vandyck, K., Pauwels, F., 2017. Capsid assembly modulators have a dual mechanism of action in primary human hepatocytes infected with hepatitis B virus. Antimicrob. Agents Chemother. 61.

Bowden, S., Jackson, K., Littlejohn, M., Locarnini, S., 2004. Quantification of HBV covalently closed circular DNA from liver tissue by real-time PCR. Methods Mol. Med. 95, 41–50.

Chen, H.L., Chiu, T.S., Chen, P.J., Chen, D.S., 1993. Cytogenetic studies on human liver cancer cell lines. Cancer Genet. Cytogenet. 65, 161–166.

Delaney, W.E.t., Ray, A.S., Yang, H., Qi, X., Xiong, S., Zhu, Y., Miller, M.D., 2006. Intracellular metabolism and in vitro activity of tenofovir against hepatitis B virus. Antimicrob. Agents Chemother. 50, 2471–2477.

Durantel, D., Zoulim, F., 2016. New antiviral targets for innovative treatment concepts for hepatitis B virus and hepatitis delta virus. J. Hepatol. 64, S117–S131.

Gao, W., Hu, J., 2007. Formation of hepatitis B virus covalently closed circular DNA: removal of genome-linked protein. J. Virol. 81, 6164–6174.

Guo, F., Zhao, Q., Sheraz, M., Cheng, J., Qi, Y., Su, Q., Cuconati, A., Wei, L., Du, Y., Li, W., Chang, J., Guo, J.T., 2017. HBV core protein allosteric modulators differentially alter cccDNA biosynthesis from de novo infection and intracellular amplification pathways. PLoS Pathog. 13 e1006658.

Guo, H., Jiang, D., Zhou, T., Cuconati, A., Block, T.M., Guo, J.T., 2007. Characterization of the intracellular deproteinized relaxed circular DNA of hepatitis B virus: an intermediate of covalently closed circular DNA formation. J. Virol. 81, 12472–12484.

Guo, J.T., Guo, H., 2015. Metabolism and function of hepatitis B virus cccDNA: implications for the development of cccDNA-targeting antiviral therapeutics. Antivir. Res. 122, 91–100.

Hirt, B., 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. 26, 365–369.

Ko, C., Chakraborty, A., Chou, W.M., Hasreiter, J., Wettengel, J.M., Stadler, D., Bester, R., Asen, T., Zhang, K., Wisskirchen, K., McKeating, J.A., Ryu, W.S., Protzer, U., 2018. Hepatitis B virus genome recycling and de novo secondary infection events maintain stable cccDNA levels. J. Hepatol. 69, 1231–1241.

Long, Q., Yan, R., Hu, J., Cai, D., Mitra, B., Kim, E.S., Marchetti, A., Zhang, H., Wang, S., Liu, Y., Huang, A., Guo, H., 2017. The role of host DNA ligases in hepadnavirus covalently closed circular DNA formation. PLoS Pathog. 13 e1006784.

Lopez-Lluch, G., 2017. Mitochondrial activity and dynamics changes regarding metabolism in ageing and obesity. Mech. Ageing Dev. 162, 108–121.

Luo, J., Cui, X., Gao, L., 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.

Mason, A.L., Xu, L., Guo, L., Kuhns, M., Perrillo, R.P., 1998. Molecular basis for persistent hepatitis B virus infection in the liver after clearance of serum hepatitis B surface antigen. Hepatology 27, 1736–1742.

Miller, R.H., Robinson, W.S., 1984. Hepatitis B virus DNA forms in nuclear and cytoplasmic fractions of infected human liver. Virology 137, 390–399.

Nassal, M., 2015. HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B. Gut 64, 1972–1984.

Newbold, J.E., Xin, H., Tencza, M., Sherman, G., Dean, J., Bowden, S., Locarnini, S., 1995. The covalently closed duplex form of the hepadnavirus genome exists in situ as a heterogeneous population of viral minichromosomes. J. Virol. 69, 3350–3357.

Qi, Y., Gao, Z., Xu, G., Peng, B., Liu, C., Yan, H., Yao, Q., Sun, G., Liu, Y., Tang, D., Song, Z., He, W., Sun, Y., Guo, J.T., Li, W., 2016. DNA polymerase kappa is a key cellular factor for the formation of covalently closed circular DNA of hepatitis B virus. PLoS Pathog. 12 e1005893.

Qu, B., Urban, S., 2019. Quantification of hepatitis B virus covalently closed circular DNA in infected cell culture models by quantitative PCR. Bio-protocol 9 e3202.

Revill, P.A., Chisari, F.V., Block, J.M., Dandri, M., Gehring, A.J., Guo, H., Hu, J., Kramvis, A., Lampertico, P., Janssen, H.L.A., Levrero, M., Li, W., Liang, T.J., Lim, S.G., Lu, F., Penicaud, M.C., Tavis, J.E., Thimme, R., Members of the I.C.E.H.B.V.W.G.,Chairs, I.-H.S.G., Advisors, I.-H.S., Zoulim, F., July 2019. A global scientific strategy to cure hepatitis B. Lancet Gastroenterol. Hepatol. 4 (7), 545–558.

Seeger, C., Mason, W.S., 2015. Molecular biology of hepatitis B virus infection. Virology 479–480, 672–686.

Seeger, C., Sohn, J.A., 2014. Targeting hepatitis B virus with CRISPR/Cas9. Mol. Ther. Nucleic Acids 3, e216.

Suk-Fong Lok, A., 2019. Hepatitis B treatment: what we know now and what remains to Be researched. Hepatol. Commun. 3, 8–19.

Tuttleman, J.S., Pourcel, C., Summers, J., 1986. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. Cell 47, 451–460.

Xia, Y., Stadler, D., Ko, C., Protzer, U., 2017. Analyses of HBV cccDNA quantification and modification. Methods Mol. Biol. 1540, 59–72.

Zhou, T., Guo, H., Guo, J.T., Cuconati, A., Mehta, A., Block, T.M., 2006. Hepatitis B virus e antigen production is dependent upon covalently closed circular (ccc) DNA in HepAD38 cell cultures and may serve as a cccDNA surrogate in antiviral screening assays. Antivir. Res. 72, 116–124.