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Quantification of the hepatitis B virus cccDNA: evidence-based guidelines for monitoring the key obstacle of HBV cure

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ABSTRACT

Objectives A major goal of curative hepatitis B virus (HBV) treatments is the reduction or inactivation of intrahepatic viral covalently closed circular DNA (cccDNA). Hence, precise cccDNA quantification is essential in preclinical and clinical studies. Southern blot (SB) permits cccDNA visualisation but lacks sensitivity and is very laborious. Quantitative PCR (qPCR) has no such limitations but inaccurate quantification due to codetection of viral replicative intermediates (RI) can occur. The use of different samples, preservation conditions, DNA extraction, nuclease digestion methods and qPCR strategies has hindered standardisation. Within the ICE-HBV consortium, available and novel protocols for cccDNA isolation and qPCR quantification in liver tissues and cell cultures were compared in six laboratories to develop evidence-based guidance for best practices.

Design Reference material (HBV-infected humanised mouse livers and HepG2-NTCP cells) was exchanged for cross-validation. Each group compared different DNA extraction methods (Hirt extraction, total DNA extraction with or without proteinase K treatment (+PK/−PK)) and nuclease digestion protocols (plasmid-safe ATP-dependent DNase (PSD), T5 exonuclease, exonucleases I/III). Samples were analysed by qPCR and SB.

Results Hirt and −PK extraction reduced coexisting RI forms. However, both cccDNA and the protein-free relaxed circular HBV DNA (pf-rcDNA) form were detected by qPCR. T5 and Exo I/III nucleases efficiently removed all RI forms. In contrast, PSD did not digest pf-rcDNA, but was less prone to induce cccDNA overdigestion. In stabilised tissues (eg, Allprotect), nucleases had detrimental effects on cccDNA.

Conclusions We present here a comprehensive evidence-based guidance for optimising, controlling and validating cccDNA measurements using available qPCR assays.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Persistence of the hepatitis B virus (HBV) genome, the covalently closed circular DNA (cccDNA), in the liver is a major obstacle for developing HBV cure strategies and its accurate quantification is essential in preclinical and clinical studies. However, cccDNA quantification by PCR lacks standardisation and is technically challenging due to the heterogeneous population of HBV DNA molecules present in infected cells.

WHAT THIS STUDY ADDS

⇒ Distinct cccDNA extraction and quantification protocols were cross-validated using infected tissues and cell cultures. We provide experimental evidence that coexistence of certain HBV DNA forms and potential cccDNA overdigestion due to sample preservation conditions and nuclease treatment still challenges PCR-based quantification of cccDNA. The results support recommendations for optimising, controlling and validating cccDNA measurements according to the sample type.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This work presents an evidence-based guidance for best practices to quantify cccDNA by qPCR. Information provided will assist the HBV cure research programmes aiming at assessing the impact of therapies on the HBV reservoir in preclinical studies and clinical trials.

INTRODUCTION

The hepatitis B virus (HBV) is the cause of chronic hepatitis B (CHB) and despite the availability of effective prophylactic vaccines and treatments that efficiently suppress viral replication, HBV puts 296 million carriers at risk of developing liver cirrhosis and hepatocellular carcinoma.[1] Thus, there is a strong need to develop therapies with the potential to cure CHB. The unique HBV replication strategy, however, makes viral eradication challenging.[2] Knowledge and precise monitoring of the different viral components present in infected cells and in the circulation is paramount for the development and assessment of novel therapeutic strategies.

HBV is an enveloped virus harbouring a small (3.2 kb) partially double-stranded (ds) relaxed circular DNA (rcDNA) genome which specifically

infects human hepatocytes. On infection, the rcDNA is repaired by cellular enzymes to generate a double-stranded episomal DNA genome, the covalently closed circular DNA (cccDNA)[3 4] (online supplemental figure 1). By associating with histone and non-histone proteins, the cccDNA forms a stable minichromosome within the cell nuclei that serves as transcriptional template for all viral transcripts. Nucleoside analogues (NAs) and interferon alpha are the only approved treatments for CHB but are mainly suppressive as they do not clear cccDNA from the liver. Because of the central role of the cccDNA in HBV persistence, the elimination of this molecule constitutes the ultimate goal of curative treatments.[5] Such a goal may be very difficult to achieve and current investigational approaches rather aim at achieving a functional cure, which is defined as the loss of HBsAg and undetectable HBV DNA in serum.[2] Examples of currently explored curative strategies include, among others, the use of capsid assembly modulators and entry inhibitors, which can also prevent cccDNA formation and intrahepatic amplification of the cccDNA pool,[6] degradation of HBV transcripts using siRNA technologies, immune-mediated clearance and silencing of cccDNA molecules, or its inactivation by gene editing.[7] Assessment of the impact of novel therapies on cccDNA load and activity is therefore of utmost importance. Moreover, our understanding of cccDNA biology in preclinical models and patient biopsies is still limited. Because of the paucity of biopsy material, non-invasive biomarkers (eg, HBcrAg, HBV RNA), are increasingly studied as surrogate cccDNA markers.[8] However, for the primary validation of these biomarkers, proper cccDNA quantification is needed.

A major limitation in HBV research and its translation into the clinic is the lack of standardised PCR-based methods allowing specific cccDNA quantification in HBV-infected samples. The main challenge is the reliable detection of cccDNA by quantitative PCR in the presence of high excess of rcDNA coming from virus input or coexisting replicative intermediates (RI), which are identical in sequence to the cccDNA (online supplemental figure 1). To gain specificity, PCR approaches use primers spanning the cohesive ends region of the rcDNA to preferentially detect the cccDNA (online supplemental figure 2A). Unfortunately, this approach is not completely specific since it still detects certain amounts of the highly abundant RI leading to an overestimation of cccDNA amounts.[9 10] Only when HBV replication is low (eg, during NA treatment), cccDNA quantification by PCR appears precise.[11 12] Therefore, cccDNA measurements in samples with high viral productivity or between samples with and without treatment remain challenging and reducing the levels of RI is mandatory.

To reduce the levels of HBV RI prior to PCR, various nucleases and DNA extraction methods have been proposed, although their efficacy and specificity in engaging distinct HBV DNA forms in different experimental settings vary substantially. The conventional Hirt method employs sodium dodecyl sulfate lysis and high salt precipitation of high-molecular-weight cellular chromatin and protein-bound DNA[13] thereby enriching the recovery of protein-free (pf) DNA molecules and facilitating the removal of rcDNA, which is covalently linked to the viral polymerase. Southern blot (SB) after Hirt DNA extraction allows visualisation of different HBV DNA molecules including cccDNA.[14] However, this methodology necessitates large amounts of nucleic acids and therefore lacks sensitivity; it is not suitable for liver biopsy analysis or high-throughput assays and does not allow precise quantification.

The nucleases commonly used display slightly different substrate specificities but share the common characteristic of sparing closed circular dsDNA from digestion.[11 15–18] Apart from employing different nucleases, the use of different DNA extraction procedures, qPCR conditions and normalisation increases variability and hinders reliable comparative analyses among laboratories. We, therefore, attempted to harmonise cccDNA quantification processes by comparing the most used and newer protocols through cross-validation experiments. Based on the results obtained, we propose recommendations of best practice for controlling and validating cccDNA measurements also according to the sample type.

MATERIAL AND METHODS

For generation, infection, treatment and viral characterisation of human liver chimeric mouse, as well as infection of HepG2-NTCP cell culture samples, nuclease digestion and qPCR measurements, see online supplemental material.

Preparation of liver tissue and cell samples

Liver specimens removed at the time of sacrifice were snap-frozen in 2-methylbutane and stored at −80°C until further use. Every lab received three frozen liver pieces (approximately 13 mg each) from one highly infected, untreated uPA/SCID/beige/IL2RG-/- (USG) mouse. The tissue pieces were homogenised separately in 300 µL 10 mM Tris-HCl (pH 7.5)/10 mM EDTA (pH 8.0) buffer using disposable homogenisers (Biomasher II, DWK Life Sciences, Wertheim, Germany), then pooled and distributed equally among the three DNA extractions. HBV-infected HepG2-NTCP cells were pooled before being divided and shipped to the four participating labs. Every lab received a frozen cell pellet of approximately 1×10^7 cells, which on arrival was dissolved in 900 µL 10 mM Tris-HCl/10 mM EDTA buffer and distributed equally for the three DNA extractions. Since all samples were pooled and divided equally across the DNA extractions, the results could be compared with each other directly without the need for additional normalisation to cellular DNA.

DNA extraction procedures

Total cellular DNA was isolated with the MasterPure Complete DNA and RNA Purification Kit (Epicentre, available through Lucigen, Middleton, Wisconsin, USA) as recommended by the manufacturer.[12 19] The total DNA extraction with proteinase K treatment (+PK) and the one without PK (−PK) were performed identically, except for the PK digestion step, which was omitted in the −PK extraction. Briefly, the samples were split up in several microtubes (according to the manufacturer's instructions, 7×10^5 cells or 4.4 mg tissue per 300 µL lysis buffer) and 10 mM Tris-HCl/10 mM EDTA buffer was added for a final volume of 300 µL each. One volume of double concentrated TCL buffer (Lucigen) was added to lyse the cells. Subsequently, RNA digestion was performed with 2 µL RNase A (Lucigen) for 30 min at 37°C. For the+PK extraction, 2 µL of PK (Lucigen) was added and incubated for 1 hour at 56°C. After the addition of 300 µL of MPC buffer (Lucigen), the samples were mixed and incubated on ice for 5 min, followed by 10 min high speed centrifugation to pellet the protein. DNA was recovered from the supernatant through isopropanol precipitation, dissolved in 10 mM Tris-HCl/1 mM EDTA buffer and the DNA from one extraction and sample was pooled again. The DNA content was determined by fluorometry (Qubit, Invitrogen) or spectrophotometry (NanoDrop, Thermo Fisher Scientific, Waltham, Massachusetts, USA). A detailed version of this protocol can be found on the ICE-HBV protocol database (https://ice-hbv.org/

protocol/a-modified-kit-based-hbv-protein-free-dna-extraction-from-liver-tissues-and-cell-cultures-for-hbv-cccdna-southern-blot-and-qpcr/). For a comparison of different lysis buffers, the protocol was repeated also using RLT buffer from the RNeasy Mini kit (Qiagen, Hilden, Germany). Some mouse liver tissues were immersed in Allprotect Tissue Reagent (Qiagen) as recommended by the manufacturer and stored at −20°C for several months.

One-third of the liver homogenate and cell suspension was subjected to DNA extraction according to Hirt.[13] Briefly, the samples were further diluted in 10 mM Tris-HCl/10 mM EDTA buffer to a final volume of 3 mL. Cell lysis was achieved by adding SDS (final concentration 0.6%) and 30 min of incubation at room temperature with slow agitation. After adding KCl (final concentration 0.5M), the lysates were incubated at 4°C overnight, then centrifuged for 30 min at 4°C and 14 500 g. DNA from the supernatant was extracted twice with phenol and once with phenol/chloroform/isoamyl alcohol (25:24:1), followed by an overnight ethanol precipitation. The DNA was pelleted by centrifugation, washed in 70% ethanol and dissolved in 10 mM Tris-HCl/1 mM EDTA buffer.

SB analyses were performed as previously described.[19 20] A detailed version of this protocol can be found on the ICE-HBV protocol database (https://ice-hbv.org/protocol/a-sensitive-and-rapid-southern-blot-assay-based-on-branched-dna-technology-for-the-detection-of-hbv-dna-in-cell-culture-and-liver-tissue-samples/) and as online supplemental file.

RESULTS

Comparison of cccDNA extraction protocols in HBV-infected liver tissues

Currently available and novel protocols for cccDNA extraction and quantification were collected and cross-validated according to the scheme depicted in figure 1A. Frozen liver tissues from HBV-infected humanised mice were used as reference material (online supplemental figure 3). We compared total DNA extraction methods that either included (+PK) or excluded (−PK) the PK digestion step, as well as the classical Hirt DNA extraction (Hirt) that does not include PK digestion.[13] Omission of the PK should facilitate the removal of covalently protein-bound HBV DNA and was investigated here as potential

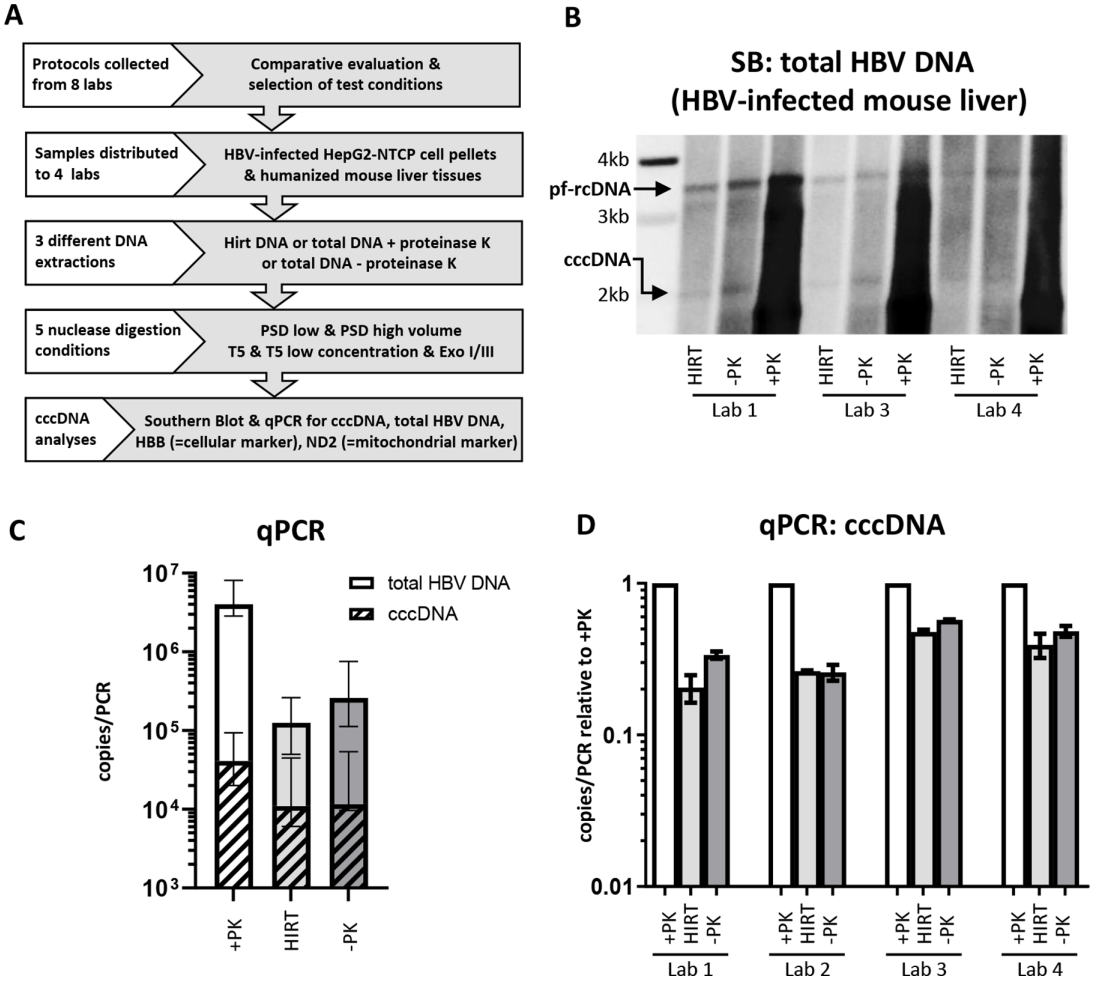

Figure 1 The effect of different DNA extraction methods on cccDNA quantification by qPCR and SB in HBV-infected USG mouse liver tissue. (A) Schematic presentation of the experimental design used for the cross-validation. (B) SB analysis on non-digested DNA extracts using HBV DNA probes in three of the labs. (C) qPCR measurements of total HBV DNA and cccDNA in the DNA extracts. Bars depict the median and range across all four labs. (D) qPCR measurements of cccDNA shown separately for every lab, relative to the amount in the +PK DNA extractions. Bars depict the mean of duplicate measurements. +PK, total DNA extraction with proteinase K digestion; −PK, total DNA extraction without proteinase K digestion; cccDNA, covalently closed circular DNA; HBV, hepatitis B virus; Hirt, Hirt DNA extraction, which does not include a proteinase K digestion; qPCR, quantitative PCR; SB, Southern blot.

alternative to Hirt extraction. A previously published cccDNA-selective PCR strategy[21] based on selective primers and a Taqman probe detecting all HBV genotypes except G (online supplemental table 1), and not cross-reacting with human or murine genomic sequences, or HBV DNA integrations,[12] was chosen for cross-validation using improved PCR conditions (for details see online supplemental figure 2A–C). The specificity for cccDNA detection was assessed on serial dilutions of serum-derived rcDNA and on mixtures of an HBV plasmid (as cccDNA surrogate) and increasing amounts of serum-derived rcDNA (online supplemental figure 2D, E). These analyses indicated that serum rcDNA can be amplified in a false-positive manner despite using cccDNA-selective PCR primers and probes when rcDNA levels exceed cccDNA levels by more than 250-fold. Under such circumstances, measured cccDNA levels increase and a precise cccDNA quantification is not possible without including strategies reducing rcDNA levels.

To evaluate the three distinct DNA extraction methods in different labs, we first analysed the liver of a highly viraemic mouse. Hirt and −PK extractions resulted in reduced HBV RI levels on the SB (figure 1B) compared with the +PK extraction. The classical pattern of a 'slow-migrating band' around 3.5 kb (known to contain pf rcDNA or deproteinated (dp)-rcDNA) and a 'fast-migrating band' at 2.1 kb (supercoiled cccDNA) was observed both after Hirt and −PK extraction procedures. The high amount of RI in the+PK extracts, however, prevented the visualisation of distinct bands. Of note, using the highly sensitive bDNA detection method, a substantial smear was still present in −PK and Hirt extracted DNA, which was absent in samples from an uninfected mouse (see example in online supplemental figure 8C), pointing out that those signals were HBV-specific. In line with the SB, qPCR measurements in all labs showed lower amounts of total HBV DNA (1.6log and 1.3log reduction) and cccDNA (0.5log and 0.4log) in Hirt and −PK extractions, respectively (figure 1C), as compared with +PK samples. The reduction of protein-bound RIs clearly improved the selectivity of cccDNA quantification by PCR (figure 1C) across all labs (figure 1D).

Normalisation of cccDNA counts to cellular levels

For all downstream cross-validation experiments, the same amount of liver tissue or cell number was used to facilitate comparison between labs and settings. In everyday practice, however, viral DNA counts have to be normalised to cell number to account for differences in the amount of input material or human hepatocyte levels when analysing chimeric livers. Accordingly, we determined the recovery of genomic DNA (gDNA) and mitochondrial DNA (mtDNA) (online supplemental figure 4A–C). Although supernatants from a classical Hirt extraction are known to harbour reduced levels of high molecular weight nucleic acids, genomic DNA was consistently extracted in our Hirt preparation, although with a trend towards a slightly reduced recovery of these DNA species in both Hirt and −PK conditions compared with the +PK extraction. However, this trend was similar across analyses, indicating that viral DNA could be normalised to gDNA or mtDNA derived from the same extraction. Moreover, a head-to-head comparison of distinct lysis buffers revealed that the TCL lysis buffer (Lucigen) led to threefold lower gDNA levels compared with RLT lysis buffer (Qiagen), although these buffers did not affect mtDNA recovery (online supplemental figure 4D–F). Such differential enrichment of gDNA will skew the normalisation of viral DNA to target cells and has to be considered in the normalisation strategy. A modified Hirt procedure using alkaline extraction,[22 23] which was tested on liver tissue and cell culture samples in two labs, showed low but still quantifiable genomic DNA recovery compared with the original Hirt (online supplemental figure 5). By enriching only supercoiled DNA species, this method also depleted all HBV DNA species except for the 'fast-migrating band'.

Comparison of different nucleases and digestion conditions in chimeric liver tissues

We next tested different nucleases and conditions to compare their efficacy. Based on existing protocols commonly used in different laboratories, T5 exonuclease was used in two settings, one being stronger ('T5': 10U, 45 min), the other one being gentler ('T5 low': 5U, 30 min) because overdigestion of cccDNA had sporadically been observed within this consortium and gentler digestion conditions were anticipated to reduce this effect. PSD was employed in a classical setting ('PSD': 10U, 6 hours in 20 µL total volume) and in a setting using more units in a higher volume ('PSD high': 30U, 2 hours in 200 µL total volume) based on experiences in tissue-derived samples. The Exo I/III combination was tested using one setting (20U ExoI, 25U ExoIII, 2 hours). All nuclease treatments reduced total HBV DNA to varying degrees (figure 2A). However, considerable variations were observed among labs. Using the classical +PK DNA extracts, 'PSD low' conditions were unable to effectively digest total HBV DNA. This was most likely caused by an inhibition of the enzyme by contaminants present in the extracts or inappropriate pH values, since 'PSD high' or the use of column-purified +PK DNA for the 'PSD low' condition remedied this issue (data not shown).

When using cccDNA selective primers, we observed a considerable reduction of cccDNA counts after nuclease treatments even in Hirt and −PK DNA extracts, indicating that residual HBV RI were still detected following these extractions in this highly infected sample (9×10^8 HBV DNA GE/mL) (figure 2B). Analysis of the human mitochondrial gene ND2 as a cellular surrogate marker for episomal DNA (figure 2C), revealed that mtDNA levels were also lower after nuclease digestion although to a lesser degree, indicating that the reduced detection of cccDNA was not only due to improved primer specificity but partially due to cccDNA loss. Although considerable variations were observed among labs, median cccDNA levels were 3.3-fold lower after T5 or Exo I/III treatments compared with PSD treatments across all DNA extracts and labs. To shed light on the reasons for these differences, we performed a head-to-head comparison of qPCR and SB analyses using the same undigested and digested samples (figure 2D). Both methods proved that all nuclease digestions reduced HBV DNA copy numbers (qPCR) and the typical smear present on the SB. However, PSD did not digest the 'slow-migrating' band where pf-rcDNA is generally found, while T5 and Exo I/III digested all HBV DNA forms except for the cccDNA-containing 'fast-migrating' band. Furthermore, cccDNA bands displayed very similar densities after exonuclease-based or PSD-based treatment within the same extraction, indicating that the higher cccDNA PCR counts observed after PSD digestion is due to a comeasurement of molecules present in the 'slow-migrating' band.

cccDNA and pf-rcDNA are detected by qPCR with the same efficiency

To assess whether the HBV DNA species present in the 'slow-migrating' band could be recognised by the cccDNA PCR with the same efficiency as the cccDNA itself, we performed

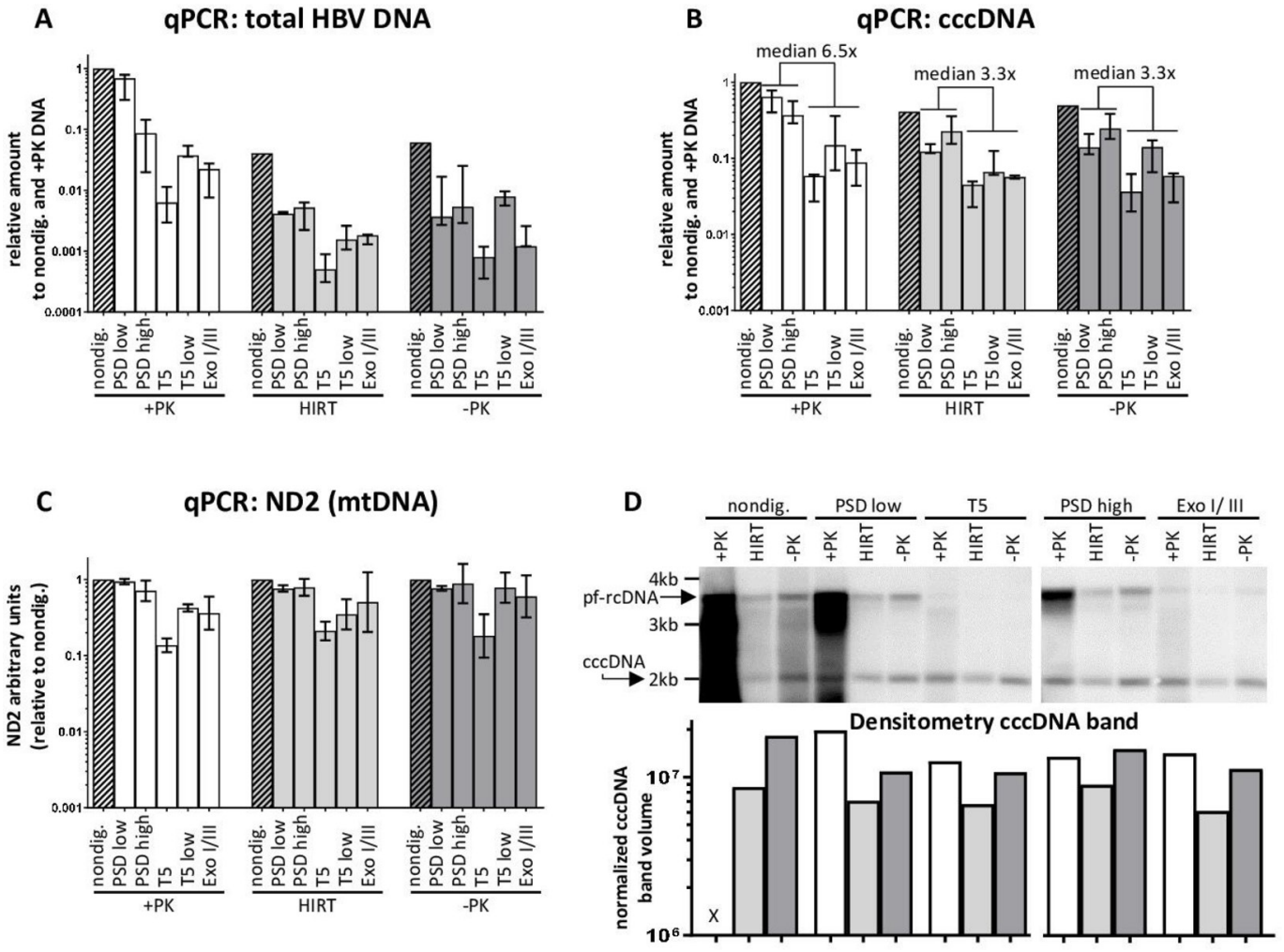

Figure 2 The effect of nuclease digestions on different DNA extracts in HBV-infected USG mouse liver tissue. The bar graphs depict qPCR measurements of total HBV DNA (A) and cccDNA (B) in all DNA extracts and labs without nuclease digestion (hatched bars) or after the indicated nuclease treatments. The bars depict the median and range of the values from three labs. Every lab performed the nuclease digestion in duplicates. The values (copies/PCR) after nuclease treatment were first normalised to the non-digested value in the respective DNA extract, then all values derived from Hirt or −PK DNA extracts were normalised to the +PK extract. (C) qPCR of mtDNA DNA via the mitochondrial gene ND2, depicted as arbitrary units normalised to the non-digested values in the respective DNA extract. (D) SB analysis in one of the labs using a second stably HBV-infected and untreated USG mouse and all three DNA extracts and four nuclease digestion conditions using HBV DNA probes (top panel) and densitometric analysis of the cccDNA band (lower panel). The 'X' denotes a sample that could not be quantified because of high background staining. The samples were run on two separate blots but identical amounts of non-digested DNA, with the help of which densitometry was normalised between blots. ND2, mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 2. +PK, total DNA extraction with proteinase K digestion; −PK, total DNA extraction without proteinase K digestion; cccDNA, covalently closed circular DNA; HBV, hepatitis B virus; qPCR, quantitative PCR; SB, Southern blot.

preparative agarose gel electrophoresis, excised and analysed by PCR the different HBV DNA bands individually. Liver samples were analysed from one untreated HBV-infected mouse and two lamivudine (Lam)-treated mice (6 and 12 weeks, respectively; online supplemental figure 3B), which were expected to have lower levels of interfering RI. DNA extracted with the −PK method was subjected to SB and qPCR after 'PSD high' and 'T5' digestion. The characteristic smear on SB composed of HBV RI in highly infected samples (figure 3A) and total HBV DNA copies by PCR (figure 3B) were reduced in Lam-treated mice, thus enabling specific cccDNA measurements by qPCR before and after PSD digestion (figure 3B). However, T5 treatment removed the 'slow-migrating' band, leading to fourfold lower cccDNA PCR measurements than PSD treatment, while the density of the faster migrating cccDNA bands remained the same regardless of the nuclease digestion (figure 3A). Interestingly, cccDNA levels appeared to decrease in a time-dependent fashion in Lam-treated mice compared with the untreated mouse.

Identical samples were used for the preparative agarose gel, where slow-migrating and fast-migrating bands were excised separately and analysed by qPCR for total HBV DNA and cccDNA (online supplemental figure 6). The cccDNA band was detected by both PCR assays in a very similar fashion and the ratio of total HBV DNA to cccDNA copies was identical across all mice and treatments except for the untreated, undigested sample (figure 3C). This observation confirmed that the 'fast-migrating' band, solely composed of cccDNA, was detected by both PCR assays. Interestingly, the same pattern was observed also when the 'slow-migrating' band was analysed, indicating that the DNA species present in this band served as similarly efficient templates for the cccDNA PCR as cccDNA itself. The slightly higher ratios in the untreated, undigested sample were

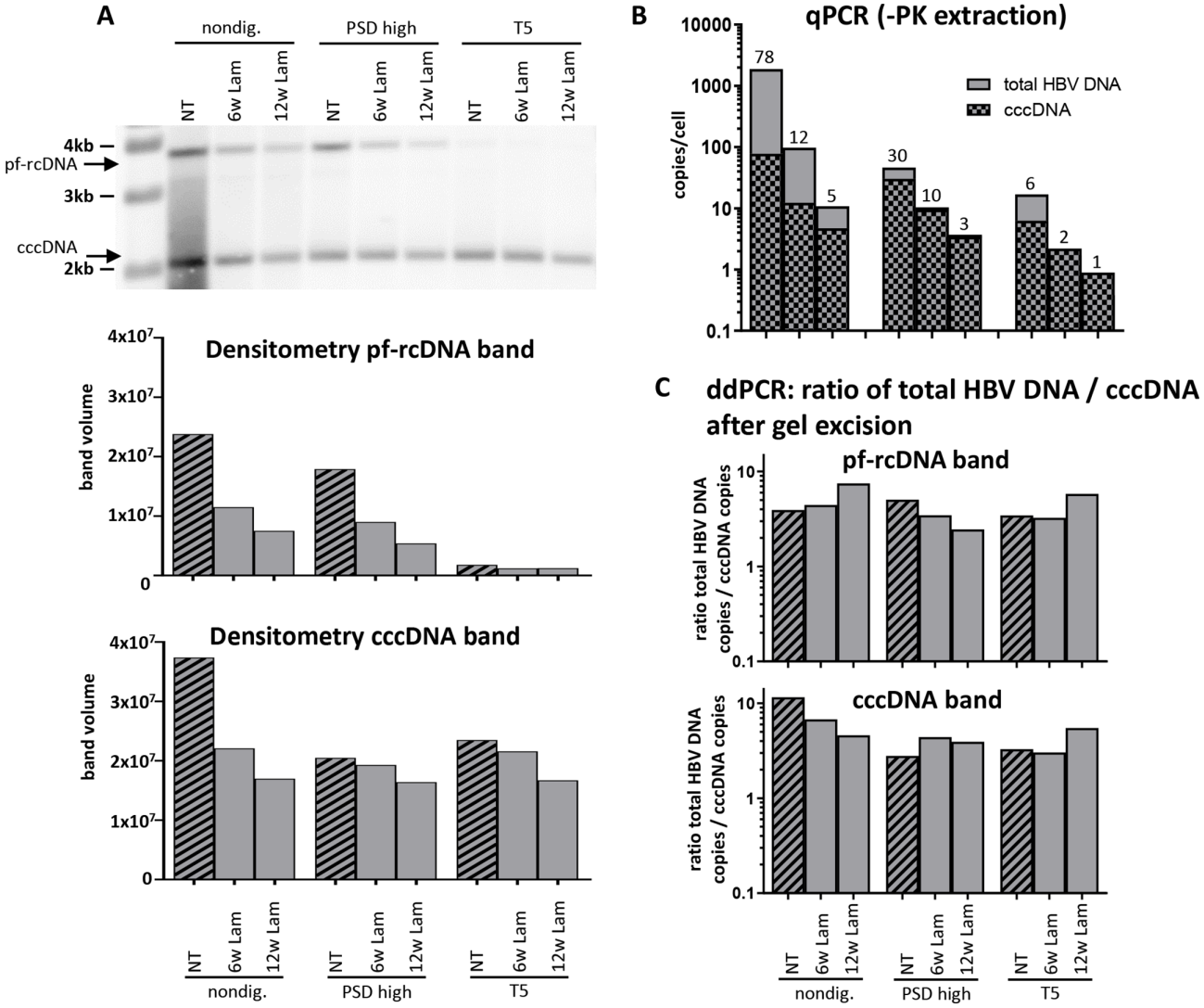

Figure 3 The pf-rcDNA is detected with the same efficiency as cccDNA by cccDNA-selective qPCR but is removed through T5 exonuclease treatment. Parallel SB (A) and qPCR (B) analysis in three stably HBV-infected USG mice (after 6 or 12 weeks of lamivudine treatment or untreated). Liver DNA was extracted with the −PK, digested with nucleases or left undigested before analysis. (A) SB using HBV DNA probes (top panel) and densitometric analysis of the pf-rcDNA (middle panel) and cccDNA band (lower panel). DNA amounts were normalised to ND2 via qPCR with human-specific primers before digestion to ensure loading of equal amounts of DNA derived from human cells. (B) qPCR measurements of total HBV DNA (grey bars) and cccDNA (checked bars) normalised to human hepatocytes via human HBB counts in the non-digested DNA extract. The cccDNA copy numbers/cell are depicted above each bar. (C) Preparative agarose gel using the same samples as in (A) and identical settings for the gel electrophoresis. The upper and lower bands were excised separately, DNA was extracted and used for qPCR analysis (total HBV DNA and cccDNA). The ratio of total HBV DNA copies to cccDNA copies was calculated for all samples derived from the upper SB band (top) and the lower band (bottom), respectively. +PK, total DNA extraction with proteinase K digestion; −PK, total DNA extraction without proteinase K digestion; cccDNA, covalently closed circular DNA; HBV, hepatitis B virus; Lam, lamivudine; NT, non-treated; qPCR, quantitative PCR; SB, Southern blot.

likely due to the codetection of HBV RI, presenting a visible smear only in this sample. Taken together, the higher cccDNA PCR counts determined in samples retaining the slow-migrating band strongly indicate that pf-rcDNA is an efficient template for the cccDNA PCR. The precise composition of this band, however, needs further investigations. Of note, a potential intermediate for cccDNA formation, the closed minus-strand rcDNA (CM-rcDNA), may also be present in that 'slow-migrating' band.[17] Nicking of the cccDNA (occurring during active transcription[7] or accidentally during the extraction process) would result in the loss of supercoiling and nicked cccDNA would migrate together with pf-rcDNA (see below and online supplemental figure 8C). Thus, quantification strategies aiming at detecting cccDNA contained in the 'fast-migrating' band should include T5 or ExoI/III nucleases, whereas PSD treatment will guarantee inclusion of potentially damaged, nicked cccDNA.

Comparison of cccDNA extraction and quantification protocols in HepG2-NTCP cells

Following the same criteria and protocols used for liver tissues, we comparatively assessed the impact of distinct DNA extraction methods on HBV DNA levels in HBV-infected HepG2-NTCP cells. Total HBV DNA qPCR measurements indicated that Hirt and −PK extractions substantially removed protein-bound HBV DNA forms by 1.7 and 1.5 logs, respectively (figure 4A). Accordingly, cccDNA counts were reduced by median 0.9 and 0.8 logs compared with+PK extractions indicating improved selectivity

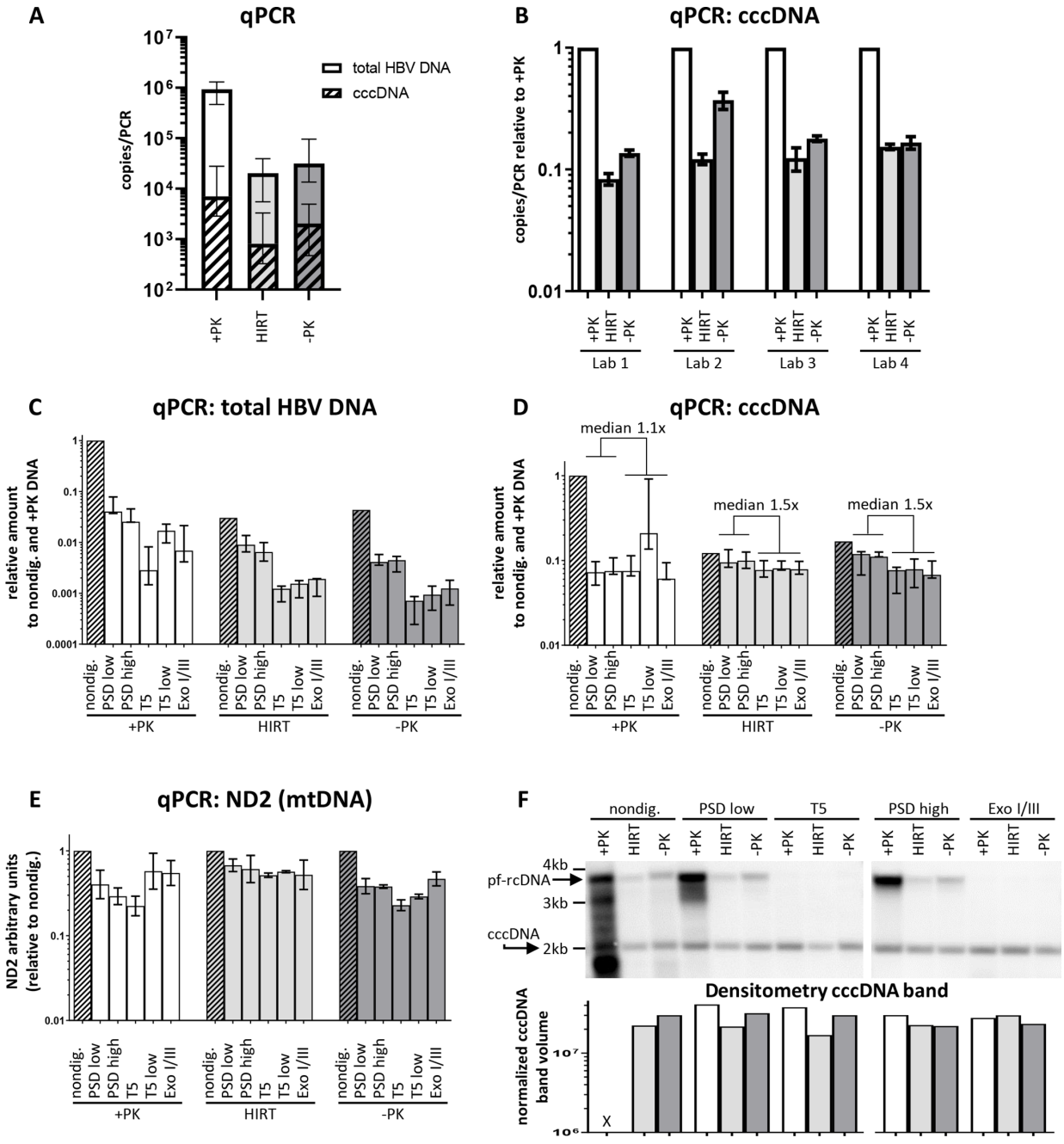

Figure 4 The effect of different DNA extraction methods and nuclease digestions on cccDNA quantification by qPCR and SB in HBV-infected HepG2-NTCP cells. (A–F) Frozen cell pellets harvested at day nine post infection were shipped to the participating labs. (A) qPCR measurements of total HBV DNA and cccDNA in the undigested DNA extracts. Bars depict the median and range across all four labs. (B) PCR measurements of cccDNA shown separately for every lab and relative to the amount in the+PK DNA extractions. Bars depict the mean of duplicate measurements. (C, D) qPCR measurements of total HBV DNA (C) and cccDNA (D) in all DNA extracts and labs either without nuclease digestion (hatched bars) or after the indicated nuclease treatments (performed in duplicates). The bars depict the median and range of the values from three labs. The values (copies/PCR) after nuclease treatment were first normalised to the non-digested value in the respective DNA extract, then all values derived from Hirt or −PK DNA extracts were normalised to the +PK extract. (E) qPCR of mtDNA DNA via the mitochondrial gene ND2, depicted as arbitrary units normalised to the non-digested values in the respective DNA extract. (F) SB using HBV DNA probes (top panel) and densitometric analysis of the cccDNA band (lower panel). The 'X' denotes a sample that could not be quantified because of high background staining. The samples were run on two separate blots with identical amounts of non-digested DNA, with the help of which densitometry was normalised between blots. +PK, total DNA extraction with proteinase K digestion; −PK, total DNA extraction without proteinase K digestion; cccDNA, covalently closed circular DNA; HBV, hepatitis B virus; qPCR, quantitative PCR; SB, Southern blot.

for cccDNA (figure 4A, B). We next applied different nuclease digestion conditions to all three DNA extraction method (figure 4C–F). In +PK extracts, the removal of RI reduced cccDNA counts by PCR. In both Hirt and −PK DNA, however, no further substantial cccDNA decline was observed after nuclease digestion, suggesting that HBV RI had been sufficiently removed through the extraction process allowing for precise cccDNA quantification. The minor reduction of cccDNA levels

after all nuclease treatments was mirrored in ND2 measurements indicating a general small loss of circular DNA molecules (figure 4E). The SB in figure 4F reproduced the characteristic HBV DNA pattern similar to liver tissues although with less RI-derived smear. Nevertheless, in Hirt and −PK DNA extracts, cccDNA levels determined by PCR were 1.5-fold lower after T5 and ExoI/III compared with PSD-based methods. Again, the 'slow-migrating' band visualised by SB was degraded only after exonuclease-based treatments.

Another factor that can influence cccDNA measurements is the presence of the inoculum, which contains high amounts of HBV particles that can persist in culture. To assess the impact of input virus on cccDNA quantification, we infected HepG2-NTCP cells with a high-titre HepAD38-derived inoculum (MOI 4000) and followed them up for 9 days in the absence or presence of the entry inhibitor bulevirtide (BLV).[24] The inoculum contained high levels of pf-rcDNA, which persisted until day nine in BLV-treated cells where the establishment of a productive infection was precluded (online supplemental figure 7A). This contaminant could be removed through 'T5' treatment, resulting in approx. twofold lower cccDNA levels detected by qPCR (online supplemental figure 7B). Of note, using this highly sensitive bDNA technology, several T5-resistant HBV DNA species were detected by SB. Interestingly, these forms are not visible under alkaline Hirt extraction conditions (online supplemental figure 5B).

Impact of DNA quality and storage conditions on cccDNA quantification

Nuclease treatment bears some risks and should be employed with caution. First, we observed high variations in terms of digestion efficiency between labs and even between replicates within one lab (data not shown). Second, excessive digestion conditions can lead to overdigestion of cccDNA. Third, the quality of the extracted DNA is of utmost importance since nicking of the cccDNA will make it a template for T5 ExoIII, but not for PSD, while all enzymes (except ExoI) will act on damaged cccDNA containing double strand breaks. Examples and explanations for risks associated with nuclease treatments can be found in online supplemental results and figure 8.

Another factor that can influence the quality of extracted DNA derives from the storage conditions. In this study, we used freshly frozen, cryopreserved samples; however, cryopreservation of liver biopsies is rarely feasible in clinical studies. Instead, tissue preservatives are used. To mimic these conditions, cryopreserved small liver pieces from two HBV-infected untreated humanised mice and two siRNA-treated mice[19] were moved to Allprotect, a common tissue preservative form Qiagen, and stored at −20°C for several months before being extracted with −PK protocols and digested with 'PSD high' or 'T5' (figure 5A). Compared with the matched fresh-frozen samples analysed in parallel, the Allprotect-stored samples showed an overall higher reduction of cccDNA levels after nuclease digestion by qPCR, with PSD reaching approximately 1log and T5 2log reductions. For comparison, the cccDNA quantified in freshly frozen liver tissue from siRNA-treated mice were not reduced by PSD treatment indicating sufficiently low HBV DNA levels for specific cccDNA quantification in this setting. Accordingly, the reduction of mtDNA was even more pronounced than the drop in cccDNA following Allprotect storage, while mtDNA levels decreased only modestly in DNA from freshly frozen tissue (figure 5B). These data hint at a general DNA damage involving both nicking and double strand breaks and warrants extreme caution when using nuclease digestion in Allprotect-stored samples.

DISCUSSION AND RECOMMENDATIONS FOR CCCDNA QUANTIFICATION

To improve our knowledge on cccDNA biology, validate serological biomarkers and assess the impact of new therapies on cccDNA levels and activity, reliable and standardised quantitative cccDNA assays are paramount. To achieve this goal in a more reliable manner then previously possible, it is critical to compare protocols and perform cross-validation experiments across laboratories. The results of this concerted effort provide novel information to guide researchers and clinical trial investigators in designing and performing protocols to accurately quantify cccDNA.

The comparative analysis of parallel qPCR and SB results was a strength of our study design and allowed us to understand how each method influences the outcome and to identify the most critical points. In figure 6, we summarised the methodologies that were validated within our consortium and the observations relevant to cccDNA quantification. Because all protocols must be adapted to the sample type, infection level and treatment, there is not one protocol that suits all needs. Instead, we provide guidance and general recommendations, which are summarised in table 1.

Through the comparison of three DNA extraction methods, we could show that not only the Hirt procedure, but also the omission of the PK digestion step from a commercial kit for fast nucleic acid purification led to efficient reduction of HBV DNA RI, thus improving the selectivity of cccDNA PCR measurements. However, −PK or Hirt-based extractions[13 22 23] might not be sufficient for specific quantification in highly replicative settings and nuclease treatment will still be needed. Therefore, we recommend performing both a DNA extraction that reduces RI and virions, a nuclease treatment to further reduce non-cccDNA species and a PCR design spanning the cohesive ends region of the rcDNA. The use of such a cccDNA-selective PCR is further supported by the fact that none of the extraction methods tested (−PK, Hirt, alkaline Hirt) was able to guarantee a complete depletion of genomic DNA, which could represent a source of integrated HBV sequences. All nucleases used here reduced RI amounts to various degrees, even when −PK or Hirt DNA extraction procedures had already lowered HBV RI levels. Nevertheless, all nucleases showed specific strengths and weaknesses. PSD did not perform well in +PK-extracted liver DNA, possibly due to impurities present in these extracts, whereas T5 and Exo I/III exonucleases performed similarly well although high concentrations and incubation times bear the risk of cccDNA overdigestion. Additionally, the efficiency of the tested nucleases varied both among labs and even within a given lab. The reasons for these variations are unclear, but could depend on small handling differences, input DNA variations and presence of sample impurities. Moreover, we showed that tissue preservatives are particularly prone to damaging the cccDNA. Consequently, nucleases induced strong reduction of cccDNA PCR levels in Allprotect-stored samples. Since patient liver biopsies are often stored in Allprotect or similar tissue preservatives, omission of nuclease digestions should be considered. Ideally, liver biopsy samples should be fresh-frozen and cryopreserved. Thus, digestion conditions have to be carefully titrated to avoid unspecific side activities potentially leading to cccDNA digestion or to insufficient digestion of other HBV DNA forms. Since it is laborious to determine whether a nuclease treatment is needed

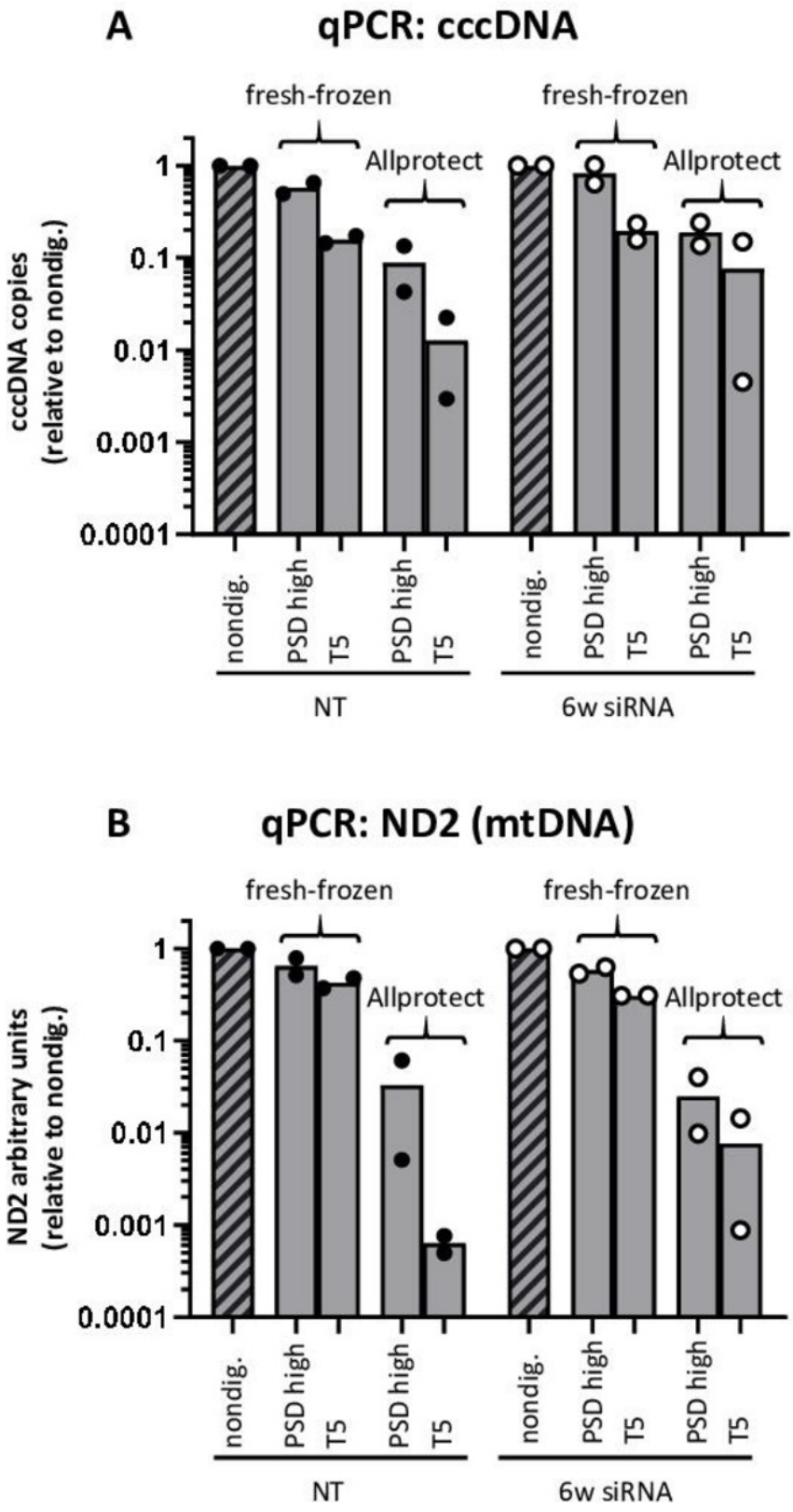

Figure 5 Nuclease treatment bears the risk of overdigesting cccDNA in Allprotect-stored samples. Comparison of nuclease digestion in fresh-frozen liver tissue or liver tissue preserved in Allprotect. Liver DNA was extracted using the −PK method from two untreated HBV-infected mice and two mice treated with siRNA targeting all HBV transcripts for 6 weeks. DNA was digested with PSD or T5 exonuclease and subjected to qPCR for cccDNA (A) and ND2 (B). Every dot depicts a single mouse, bars the median. +PK, total DNA extraction with proteinase K digestion; −PK, total DNA extraction without proteinase K digestion; cccDNA, covalently closed circular DNA; HBV, hepatitis B virus; qPCR, quantitative PCR; siRNA, small interfering RNA.

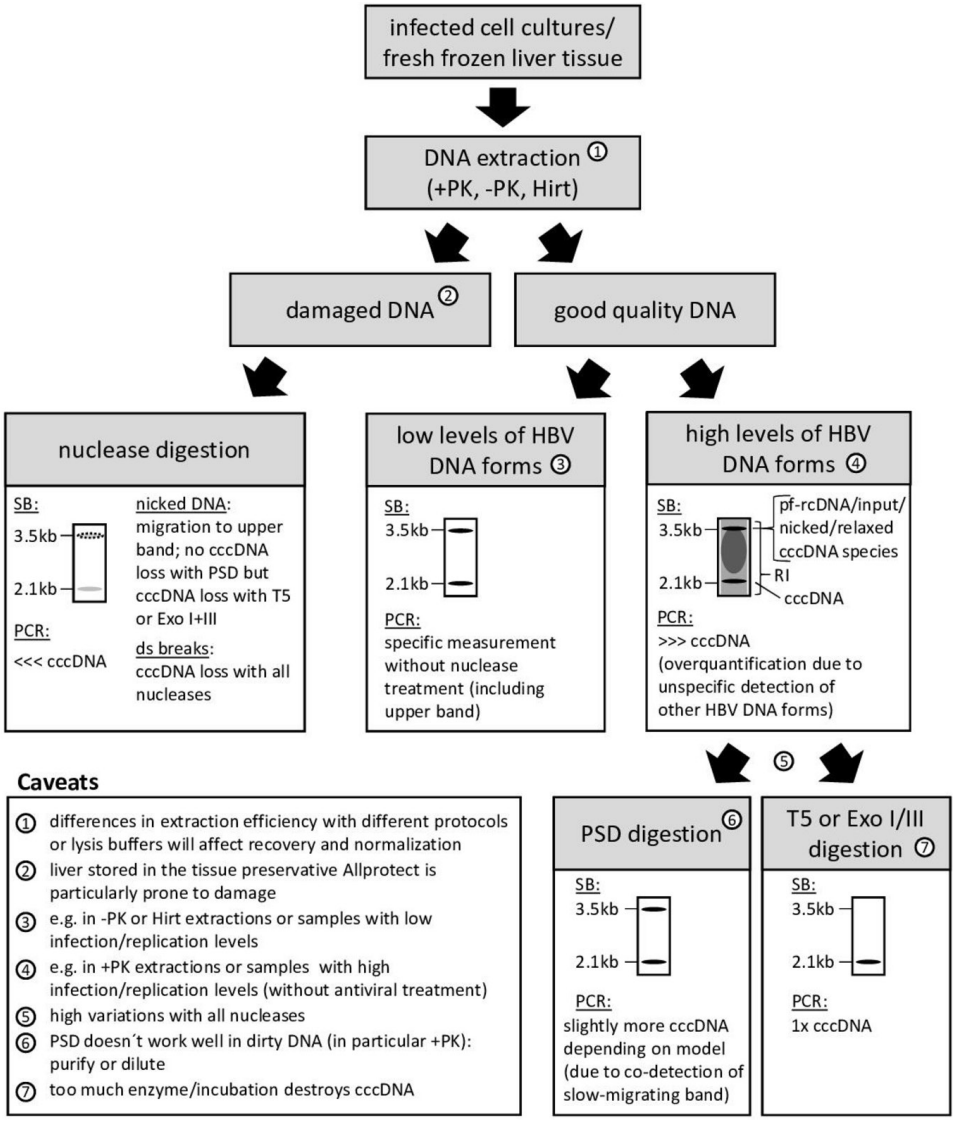

Figure 6 Schematic presentation of the main findings of the study. The flow chart summarises the experimental flow for cccDNA quantification and highlights optimal and suboptimal results for both qPCR and SB, including the major caveats. cccDNA, covalently closed circular DNA; qPCR, quantitative PCR; SB, quantitative PCR.

for every sample and condition, we recommend including it for fresh and fresh-frozen samples.

Additionally, we observed that PSD did not digest the 'slow-migrating' band, although this treatment removed most RI from −PK and Hirt samples. Furthermore, we demonstrated that without nuclease treatment, the pf-rcDNA is still present even in the setting of low replication levels and that this 'slow-migrating' band is detected by the cccDNA-selective PCR. We therefore recommend using exonuclease-based treatment when detection of this band is not wanted. The precise composition of this 'slow-migrating' band detected by SB is not entirely known but it appears to contain a mixture of molecules, which underwent removal of the covalently attached HBV polymerase but that still are unrepaired or at different phases of the repair process, such as DP-rcDNA or CM-rcDNA.[3 4] Fully repaired HBV DNA molecules that are not yet covalently closed and supercoiled (immature cccDNA) will also appear within this band.[3 4] In cell culture experiments, a prominent part of the 'slow-migrating' band might consist of input virus. Since it is unclear which factors and circumstances (infection status or treatments) may affect the conversion of rcDNA to cccDNA, the intensity of the 'slow-migrating' band may also vary between samples and experimental settings. Additionally, the choice of the extraction method will influence the detected population of HBV DNA molecules. In the side-by-side comparison of the original Hirt with a modified alkaline Hirt known to preferentially extract supercoiled DNA, the 'slow-migrating' bands—potentially resulting from circular HBV DNA with different degrees of supercoiling—appeared lost (online supplemental figure 5). Thus, further studies are needed to assess the biological meaning of such differences and cccDNA quantification strategies including the 'slow-migrating' band are justified.

For in vitro infection studies, we recommend employing exonuclease-based treatments (T5 or Exo I/III) to remove the non-cccDNA species present in the inoculum. This contaminant was present as a prominent 'slow-migrating' band on SB and persisted until day nine post infection of HepG2-NTCP cells despite the use of an entry inhibitor (bulevirtide), known to abrogate the establishment of productive HBV infection.[24] Although the example shown here used an extremely high MOI (4000),

Table 1 Recommendations for cccDNA quantification

1.	Reduce HBV RI and rcDNA through means of the DNA extraction
	► Extract DNA using Hirt or −PK method to lower RI and rcDNA contained in HBV capsids. This procedure will facilitate specific cccDNA quantification together with nuclease treatments and cccDNA-selective PCR measurements. ► PSD digestion has been shown to work more reliably after −PK or Hirt DNA extractions than after +PK extraction. Exonuclease-based methods are less frail, but low RI and rcDNA content is preferred in any case.
2.	Further reduce HBV RI and virions through nuclease digestion
	► Perform nuclease digestion to further reduce non-cccDNA species. ► To exclusively measure the cccDNA, exonuclease-based treatments should be used. To quantify both cccDNA and species contained in the 'slow-migrating' band, the use of PSD is recommended. ► The 'slow-migrating' band contains nicked cccDNA, caused mostly by technical damage. Exonuclease digestion may thus result in a loss of these cccDNA forms and lower cccDNA levels detected by PCR. ► The digestion conditions should be carefully titrated for every new sample type and extraction protocol (by PCR analysis of total HBV DNA and cccDNA in the undigested and digested sample and a direct comparison by SB) and monitored routinely (PCR analysis of total HBV DNA and cccDNA in undigested and digested samples).
3.	Assess the quality of the extracted DNA
	► Use mitochondrial DNA as a surrogate for cccDNA (circular dsDNA). A mitochondrial gene should be quantified in every sample before and after nuclease digestion. ► A reduction of <1Log10 after nuclease digestion indicates intact DNA; a reduction of >1Log10 indicates damaged DNA (DNA extract should be discarded).
4.	Determine the optimal normalisation strategy
	► Check for differential enrichment of genomic and mitochondrial DNA during the DNA extraction process to choose the optimal normalisation strategy. ► The total amount of recovered DNA can also be used for normalisation. In human chimeric mice, cccDNA numbers need to be normalised to the amounts of human hepatocytes present in every liver specimen. ► Control whether the treatments you want to test affect cell viability or mitochondria levels. Mitochondria count may vary between patients and samples.
5.	Report all methodological details for cccDNA quantification in publications
	► To allow independent assessment of the data, all methodological details should be reported including the DNA extraction method, nuclease digestion, qPCR conditions and normalisation strategy. ► Employ only one optimised method in a particular study for cccDNA extraction, nuclease digestion and qPCR quantification.
6.	Considerations for the establishment of new assays
	► For novel PCR assays, determine the degree of specificity towards cccDNA by measuring total HBV DNA and cccDNA in the untreated sample and calculate the ratio of total HBV DNA to cccDNA copy numbers. A ratio <100:1 is recommended. Copy numbers and not CT values should be used for comparison to avoid biases due to different sensitivities of PCR assays. Note that specificity ratios might also vary between types of samples and thus, falling below this ratio does not guarantee specific measurements in any case. ► Novel DNA extraction methods should assess the enrichment of cccDNA in comparison to RI and rcDNA. The optimal ratio of input sample and buffer volume has to be determined empirically. The extraction efficiency can be controlled by spiking the sample with known copy numbers of an unrelated supercoiled plasmid DNA. ► Assess the specificity to detect cccDNA in every new method including DNA extraction, nuclease digestions, PCR assays or other novel methodologies. ► Confirm the results by performing Southern blotting.

cccDNA, covalently closed circular DNA; HBV, hepatitis B virus; −PK, total DNA extraction without proteinase K digestion; +PK, total DNA extraction with proteinase K digestion; rcDNA, relaxed circular DNA; RI, replicative intermediates; SB, Southern blot.

T5 exonuclease efficiently reduced this contaminant in Hirt-extracted samples allowing specific cccDNA measurements by qPCR. Thus, it is advisable to include an entry inhibitor control in cell culture to rule out interferences due to input HBV DNA.

Because cccDNA that contains nicks or double strand breaks will become a template for nucleases and migrate together with pf-rcDNA on SB, it is of utmost importance to assess the quality of the DNA in every extract. We recommend using mitochondrial DNA—an episomal dsDNA—as a surrogate for cccDNA and to quantify a mitochondrial gene by PCR in every sample before and after nuclease digestion. Based on our experience, a reduction of less than 1log indicates sufficiently intact DNA, while more than 1log reduction indicates damaged DNA. Over-digestion of mtDNA after exonuclease-based but not PSD-based digestions, indicates that the DNA contains single-strand nicks. Consequently, cccDNA will also be nicked and reduced after exonuclease treatment. PCR assessment of mtDNA levels will indicate the quality of the DNA in the sample and after the extraction.

We acknowledge that the present methods to quantify cccDNA are not optimal yet and encourage the optimisation or development of new methods for both DNA extraction and cccDNA quantification.[25] Ideally, improved PCR-based methods should have higher specificity for cccDNA in the presence of contaminating HBV DNA species and should be robust, sensitive and suitable for all virus isolates. A milestone will be reached when novel procedures allow to avoid the use of nucleases. The implementation of digital PCR (dPCR) instead of qPCR has allowed a significant increase in sensitivity and brought the major advantage of absolute quantification without PCR standards, thus limiting the variability thereof.[12 26] However, the limited specificity for cccDNA in a complex mixture of sequence-identical HBV DNA species remains even when using dPCR. Novel methodologies to visualise cccDNA in infected cells are being developed and can complement quantitative analyses and serve to corroborate PCR and SB results.[27] Additionally, more research is needed to ascertain the nature of all HBV DNA forms, their role in the life-cycle of HBV and how they are influenced by various manipulations.

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Contributors LA, BT, JL, MLu, HG, SU, UP, SPF, MLe, FZ and MD initiated the study, designed main experiments and discussed data. CK, JL, BT and MY generated cell culture samples. LA generated liver tissue samples. LA, BT, MY, CK and BQ performed cross-validation experiments. MY, LA and JL performed Southern blot. LA analysed the data. LA and MD wrote the manuscript supported by BT, MLe and FZ. All authors read and corrected the manuscript. MD acted as guarantor of the study.

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Competing interests LA, BT, MY, CK, BQ, MLu, JL and MLe declare no conflict of interest. FZ: Consultancy for Aligos, Antios, Arbutus, Assembly, Blue Jay, Enanta, Enochian, Gilead, GSK, Zhimeng. HG: Consultancy for Aligos and Assembly; shareholder of Arbutus. UP: Consultancy for Aligos, Arbutus, Gilead, GSK, Sanofi, Vaccitech, VirBio. Research collaboration with Abbott and Roche. Share holder and board member of SCG Cell Therapy. MD: research collaboration with Gilead, MYR GmbH/Hepatera and HUMABS BioMed; consultancy for Gilead and Aligos. SPF: employment and shareholder Gilead Sciences.

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Quantification of the Hepatitis B Virus cccDNA: Evidence-based guidelines for monitoring the key obstacle of HBV cure

Short Title: cccDNA quantification harmonization

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Supplementary material and methods

Generation, infection, treatment and viral characterization of human liver chimeric. All animal experiments were performed in accordance with the European Union directive 2010/63/EU and approved by the ethical committee of the city and state of Hamburg in accordance with the ARRIVE guidelines. The generation of USG (uPA/SCID/beige/IL2RG-/-) mice reconstituted with human hepatocytes was conducted as previously reported[1]. HBV infection was established upon a single intraperitoneal injection of HBV-containing USG mouse serum (1x10^7 HBV DNA copies/mouse, genotype D, HBeAg-positive). Blood samples were taken retro-orbitally during the experiments. Viral DNA was extracted from serum samples using the QiAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany) and quantified with HBV-specific primers and probes (TaqMan® Gene Expression assay ID: Pa03453406_s1). HBV core antigen on cryostat liver sections was visualized by immunofluorescence staining as described earlier[1]. Some mice received Lamivudine (Lam) (Epivir, ViiV Healthcare, Zeist, Netherlands) for six or 12 weeks supplemented in the drinking water (20mg/100ml).

HBV infection and preparation of HepG2-NTCP cell culture samples. HepG2-NTCP cells were cultured and infected as described elsewhere[2]. Briefly, cells were infected with HepAD38-derived virus (genotype D) at an MOI of 100 genome equivalents (GE) / cell and harvested at day 9 post infection. The cells were pooled before being divided and shipped to the four participating labs. Every lab received a frozen cell pellet of approximately 1x10^7 cells, which upon arrival was dissolved in 900 µl 10 mM Tris-HCl / 10 mM EDTA buffer and distributed equally for the three DNA extractions. HepG2-NTCP cells for the SB in **Figure 4F** were infected with an MOI of 250 GE / cell and harvested at day 9 post infection as described elsewhere[3]. For **Supplementary Figure 7**, HepG2-NTCP cells were generated by transfection of expression vector pEF1/v5-His-B (Thermo Fisher Scientific) encoding the human NTCP human into HepG2 cells (ATCC). Transfected HepG2 cells were selected with G418 at 1 mg/ml for 3 weeks and the G418-resistant colonies expanded and tested for NTCP functional activity by monitoring uptake of tritium labeled taurocholate (3H(G)]-taurocholic acid 250 µCi, 9.25MBq, Perkin Elmer). Cells were infected with an MOI of 4000 GE / cell.

Alkaline Hirt DNA extraction. HBV cccDNA was extracted from HBV-infected humanized mouse liver tissue and HepG2-NTCP cells with a modified Hirt DNA extraction method as described previously[4,5]. Cell lysis in an alkaline milieu irreversibly denatures genomic DNA and all non-cccDNA HBV DNA species. After rapid neutralization, only supercoiled cccDNA renatures and remains in the supernatant after centrifugation. Briefly, 6x10^6 cells or 10mg liver tissue were diluted and homogenized in 400µl 10mM Tris-HCl (pH 7.5)/10mM EDTA (pH 8.0)/0.2% NP40. Cell lysis was achieved by the addition of 400µl 6%SDS/0.1M NaOH and 30min incubation at 37°C with agitation. The alkaline lysate was neutralized by the addition of 200µl of 3M potassium acetate (pH 4.8) and centrifuged for 15 min at 4°C and 14,500g. The supernatant was extracted with phenol (pH>7.2) followed by an additional extraction with phenol/chloroform/isoamyl alcohol (25:24:1). The DNA was precipitated with isopropanol, pelleted by centrifugation, washed twice in 70% ethanol and dissolved in 10mM Tris-HCl/1mM EDTA buffer.

Nuclease digestions. The plasmid-safe ATP-dependent DNase (PSD)[6] selectively hydrolyses linear dsDNA and with a lower efficiency single-stranded DNAs (ssDNA). Notably, it does not affect nicked circular dsDNAs (with a single discontinuous strand). PSD also digests virion-derived rcDNA[6] and intracellular capsid-associated rcDNA[7] but not rcDNA after Hirt DNA extraction[7–9]. The T5 exonuclease is a dsDNA-specific exonuclease with ssDNA-specific endonuclease activity[9] and in contrast to PSD also acts on nicked dsDNA. This bears the risk

of degrading transcriptionally active cccDNA forms that must be temporarily nicked[10]. Exonuclease I catalyzes the removal of nucleotides from linear ssDNA, while exonuclease III removes nucleotides from linear or nicked dsDNA. Together, both enzymes remove HBV RI, but leave closed single and double strands undigested[11,12].

Unless otherwise indicated, the same amount of initial starting material was used for all downstream nuclease digestion and PCR analyses to enable a direct comparison of all settings within and among the participating labs. The nuclease digestion assays were run in independent duplicates. The DNA content was determined with the Qubit™ dsDNA BR Assay Kit (Thermo Fisher Scientific) and approximately 100ng or 500ng DNA from HepG2-NTCP or mice, respectively, was used for all the following conditions: "PSD low"[6]: 10U PSD (Lucigen), 2µl 25 mM ATP, 2µl of the proprietary reaction buffer, and water for a total volume of 20 µl; incubation at 37°C for 6h, heat inactivation at 70°C for 30 min. "PSD high"[1]: 30U PSD (Lucigen), 8µl 25 mM ATP, 20 µl of the proprietary reaction buffer, and water for a total volume of 200 µl; incubation at 37°C for 2 h, heat inactivation at 70°C for 30 min. "T5"[9]: 10U T5 exonuclease (T5; NEB, Ipswich, MA, USA), 2 µl NEB reaction buffer 4, and water for a total volume of 20 µl; incubation at 37°C for 45 min, heat inactivation at 70°C for 30 min. "T5 low"[2]: 5U T5, 2µl NEB reaction buffer 4, and water for a total volume of 20µl; incubation at 37°C for 30 min, heat inactivation at 70°C for 30 min. "Exo I/III"[11,12]: 20U exonuclease I (Exo I; NEB), 25U exonuclease III (Exo III; NEB), 2µl cutsmart™ buffer (NEB), and water for a total volume of 20µl; incubation at 37°C for 2h, heat inactivation at 80°C for 20 min. Digested and nondigested samples were column-purified using the Genomic DNA Clean & Concentrator™-25 (Zymo Research, Irvine, CA, USA) before downstream analyses. In preparatory studies, the effect of heat denaturation on PSD digestion efficiency was tested in several sample types. HBV-infected HepG2-NTCP cells, rcDNA extracted from infectious mouse serum and an HBV DNA containing plasmid as a cccDNA surrogate mixed with DNA from an uninfected humanized mouse liver were used. Before digestion with PSD low and high conditions, the input DNA (500ng) was denatured by 5 min incubation at 85°C.

Quantitative PCR measurements. Quantitative PCR measurements were performed using Taqman™ chemistry and the Taqman™ Fast Advanced Master Mix (Thermo Fisher Scientific). PCR was run in triplicates. cccDNA was quantified with cccDNA-selective primers and probe as published previously[13] using improved PCR conditions (final concentration of forward primer 100 nmol/l; reverse primer 800 nmol/l; cycling conditions: 10 min initial denaturation at 95°C followed by 40 cycles of 95°C for 1 sec and 65°C for 1 min)[14] (**Supplementary Fig. 2B**). Total HBV DNA was quantified with primer and probe from the Taqman™ Gene Expression System (assay ID: Pa03453406_s1; Thermo Fisher Scientific). This assay was tested against 16 HBV isolates including genotypes A, B, C, D, E, and G as per manufacturer's specifications. The final concentration of both primers was 250 nmol/l; cycling conditions were: 20 sec initial denaturation at 95°C followed by 40 cycles of 95°C for 1 sec and 65°C for 20 sec. Known amounts of an HBV DNA plasmid were amplified to establish a standard curve for absolute quantification of cccDNA and total HBV DNA. For the absolute quantification of cccDNA in gel-extracted DNA (**Fig. 3C**), digital droplet PCR (ddPCR) was employed. Template DNA, the same primers and probe as above were diluted in ddPCR Supermix for Probes (Biorad, Feldkirchen, Germany) to the same final concentration as above. After droplet generation and PCR (cycling conditions: 10 min initial denaturation at 95°C followed by 40 cycles of 94°C for 30 sec and 65°C for 1 min), droplets were analyzed on a QX100 droplet reader (Biorad). The sensitivity of the cccDNA-selective ddPCR assay has been determined elsewhere[15]. Viral DNA was normalized to the number of human cells by measuring the human single copy gene hemoglobin beta (assay ID Hs00758889_s1) or to mitochondrial DNA by measuring the human

mitochondrial gene ND2 (assay ID #Hs02596874_g1). Human genomic DNA (Roche Applied Science, Mannheim, Germany) was used to establish a standard curve for quantification.

Preparative agarose gel electrophoresis. Total DNA extracted without proteinase K was loaded on a 1.2% TAE agarose gel. After gel electrophoresis at 25 V for 21 h, all the upper and lower HBV DNA bands were excised separately by comparison to a 1 Kb DNA ladder (Thermo Fisher Scientific). DNA was extracted from the gel with the help of the QIAquick gel extraction kit (Qiagen) following the manufacturer´s instructions. The extracted DNA was analyzed by ddPCR as described above. For comparison, a SB was prepared with equal amounts of the same samples and run in parallel. Because liver samples from three different chimeric mice were used for this assay, samples had to be normalized to the degree of human repopulation in order to compare viral loads across mice. This was done by qPCR measurements of human mitochondrial DNA (via ND2) in the undigested samples and loading of normalized amounts on the gel.

Southern blot analyses. SB analysis was performed as previously described[14,16]. Briefly, DNA was separated on an agarose gel and blotted on a Nytran membrane using downward capillary transfer. HBV DNA was detected with the help of branched DNA (bDNA) technology using the QuantiGene Singleplex assay (Thermo Fisher Scientific) and a full genome HBV probe (assay ID #VF1-12525). A detailed version of this protocol can be found on the ICE-HBV protocol database (https://ice-hbv.org/protocol/a-sensitive-and-rapid-southern-blot-assay-based-on-branched-dna-technology-for-the-detection-of-hbv-dna-in-cell-culture-and-liver-tissue-samples/).

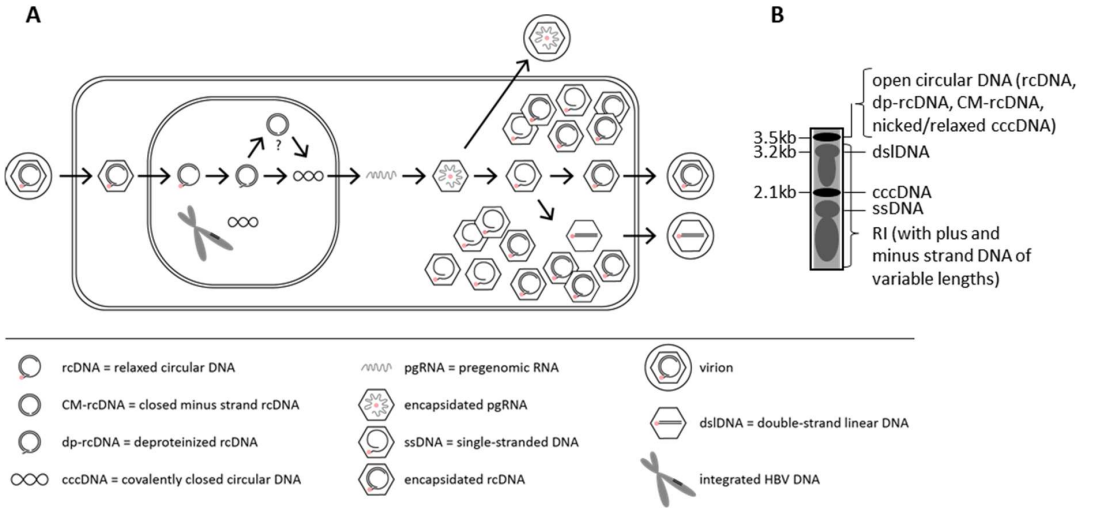
Supplementary results

Impact of DNA quality on cccDNA quantification

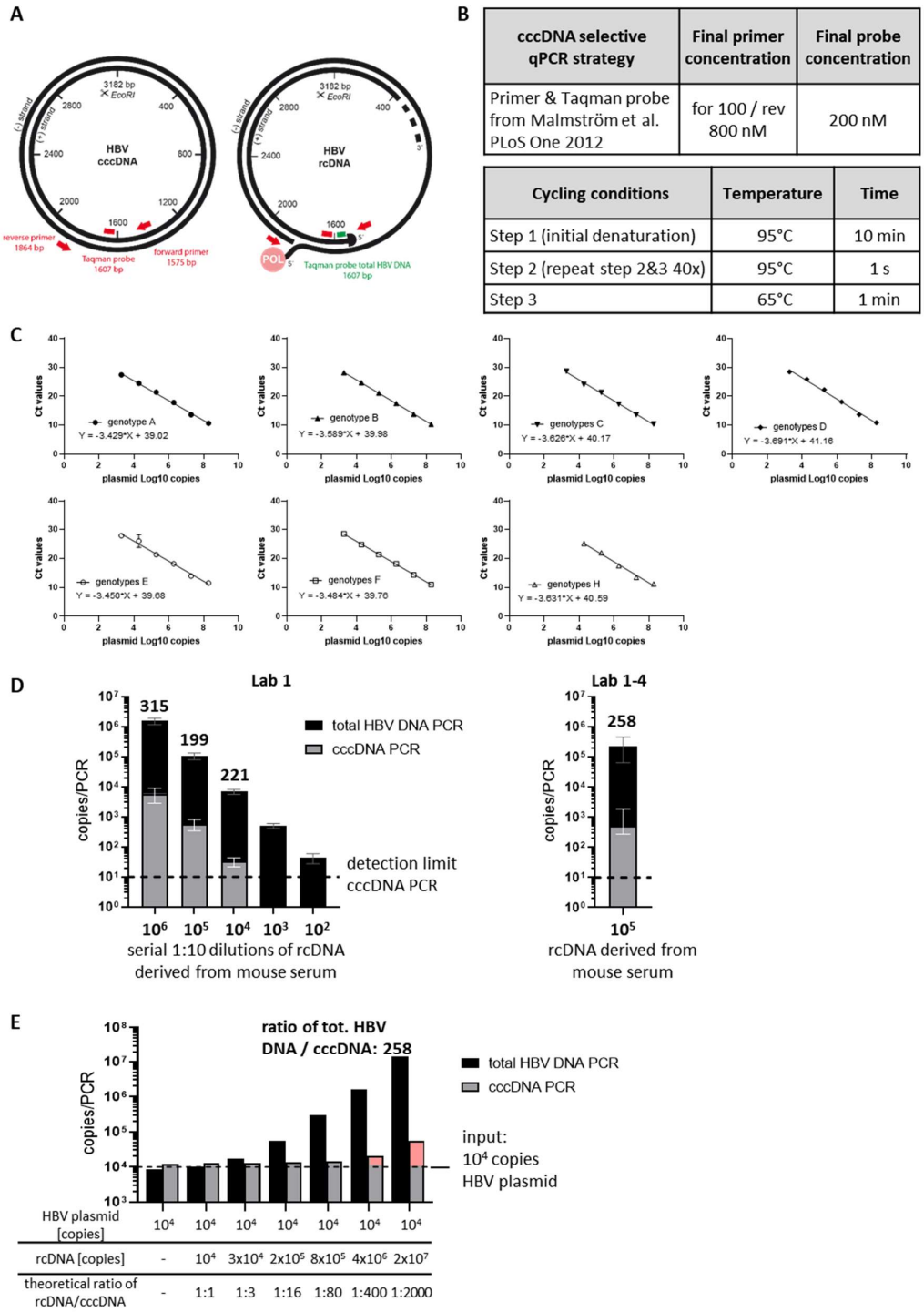
While Hirt and -PK DNA extractions were sufficient to remove replicative intermediates to a degree that makes it possible to specifically detect cccDNA (including pf-rcDNA) in low replicative samples (NA treatment), nuclease digestion was still needed in highly infected samples to remove the “upper band”. However, nuclease treatment bears some risks and should be employed with caution. First, throughout the cross-validation experiments, we observed high variations in terms of digestion efficiency between the labs and even between replicates within one lab (data not shown). Second, excessive digestion conditions could lead to over-digestion of cccDNA. We therefore compared the standard “PSD high” and “T5” conditions used throughout this study with conditions where enzymatic units were increased by 4-fold and the incubation time was extended to 6 hours (T5-augmented). The SB and densitometric analysis of the cccDNA band in -PK extracted DNA from infected mouse liver tissue shows unaltered cccDNA levels with either PSD conditions, but 2-fold lower cccDNA levels after T5-augmented conditions (**Supplementary Fig.8A**). Although the chosen conditions were extreme, these results indicate that extended digestion conditions may become detrimental when using the T5 exonuclease. In an attempt to make the pf-rcDNA accessible to digestion by PSD, we included a heat denaturation step prior to “PSD low” and “PSD high” digestion reactions[8]. Heat denaturation increased the digestion efficiency of rcDNA derived from mouse serum (**Supplementary Fig.8B**) because the enzyme could then act on the denatured single strands. However, this experiment created the highest variations and appeared to digest nicked cccDNA because of its separation into single strands. Indeed, we could observe a much stronger reduction of the cccDNA PCR counts of an HBV DNA plasmid and HBV-infected HepG2-NTCP cells using PSD and heat denaturation compared to PSD alone, indicating that the plasmid preparation contained nicks. Because of this concern and the additional need to experimentally adapt the heat denaturation conditions to the amount and viscosity of the DNA sample, this approach did not prove to be advantageous over the other conditions described here for a PCR-based cccDNA quantification.

A common characteristic of all tested nucleases is their ability to hydrolyze linear or open DNA molecules but spare covalently closed circular forms from digestion. Thus, the quality of the extracted DNA is of utmost importance. Any condition provoking nicking of the cccDNA will make it a template for T5 exonuclease and exonuclease III, but not for PSD, while all enzymes (except exonuclease I) will act on damaged cccDNA containing double strand breaks. We occasionally obtained low quality DNA with detrimental effects on cccDNA measurements after nuclease digestions. **Supplementary figure 8C** serves as an example for accidental nicking showing two independent -PK DNA extractions from the same mouse liver tissue. In one of the extracts, however, the SB revealed a prominent “slow-migrating” band and only a faint “fast-migrating” band, indicating that the cccDNA was nicked and migrated as a relaxed circular form during electrophoresis. Notably, cccDNA measurement by qPCR was still possible using PSD treatment. **Supplementary figure 8D** shows an example of -PK extracted DNA that was severely damaged, containing nicks and double-strand breaks. By comparison to the high-quality -PK extracts in the cross-validation, this sample showed 0.6log lower cccDNA levels after digestion with all nucleases. Mitochondrial DNA was reduced by 2.1log on average during all nuclease digestions demonstrating damage of this circular DNA species as well.

Supplementary figures



Supplementary figure 1: (**A**) Simplified HBV life-cycle with a focus on HBV DNA forms present in the cell. The pf-rcDNA is also known as dp-rcDNA. The removal of the viral polymerase might occur in the cytoplasm or the nucleus. The CM-rcDNA is a putative intermediate between pf-rcDNA and cccDNA. The encapsidated replicative intermediates occur with variable lengths of minus and plus strand DNA; however, only the defined intermediates i.e. pregenomic RNA, single-stranded DNA and relaxed circular DNA are depicted. The encapsidated rcDNA might be shuttling back to the nucleus to refill the cccDNA pool. (**B**) Schematic Southern blot pattern derived from highly HBV-infected samples depicting a typical picture with major bands. The blot is labelled on the right-hand side with HBV DNA forms that are present in the respective bands or smears.



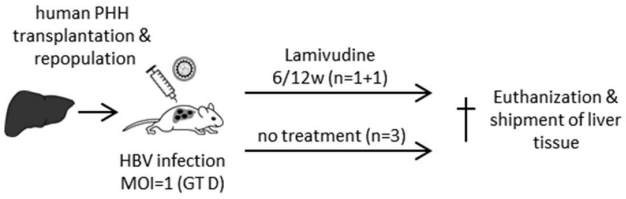
cccDNA selective qPCR strategy	Final primer concentration	Final probe concentration
Primer & Taqman probe from Malmström et al. PLoS One 2012	for 100 / rev 800 nM	200 nM

Cycling conditions	Temperature	Time
Step 1 (initial denaturation)	95°C	10 min
Step 2 (repeat step 2&3 40x)	95°C	1 s
Step 3	65°C	1 min

Supplementary figure 2: Characterization of the cccDNA-selective qPCR strategy used in this study. (**A**) A schematic presentation of the primer and probe binding sites on cccDNA and rcDNA is shown in red. The binding site of the probe for the qPCR detecting total HBV DNA is shown as a green dash. (**B**) The table summarizes the qPCR conditions for the cccDNA-selective PCR used throughout the study. (**C**) This qPCR strategy detects all HBV genotypes except genotype G with comparable efficiencies. Serial dilutions of plasmids each containing

the consensus sequence built from the HBVdb database (https://hbvdb.lyon.inserm.fr/HBVdb/) for every genotype were run in triplicates and the standard curve was computed. (**D**) rcDNA derived from two mice was analyzed by qPCR for cccDNA (grey bars) and total HBV DNA (black bars) showing the median and range. The false detection ratio (determined by the total HBV DNA copy number divided by the copy number detected by the cccDNA-selective PCR) is depicted on top of the bars and was around 250 indicating that rcDNA is amplified in the cccDNA-selective PCR in a false-positive manner with a ratio of 1:250. The PCR strategy was implemented in every lab and showed comparable cccDNA specificity in serum-derived rcDNA. (**D**) A fixed amount of an HBV containing plasmid (10^4 copies/per reaction) was mixed with increasing concentration of mouse serum derived rcDNA as indicated below the graph. Liver DNA from an uninfected USG mouse was included in every reaction to simulate conditions in actual infectious liver samples. These artificial mixtures were analyzed by qPCR for cccDNA (grey bars) and total HBV DNA (black bars). Red color indicates the fraction of cccDNA counts that is detected in a false-positive manner. The number on top of the bar depicts the false detection ratio (i.e. total HBV DNA copy numbers / cccDNA copy numbers) in this sample. This experiment illustrates the effect of this false-positive detection in mixtures of an HBV plasmid (as a surrogate for cccDNA) and increasing amounts of serum-derived rcDNA mimicking the situation in infected samples. When rcDNA levels exceed HBV DNA plasmid levels by more than 250 fold, measured cccDNA levels increase due to the false-positive detection of rcDNA and a precise quantification of cccDNA is not possible.

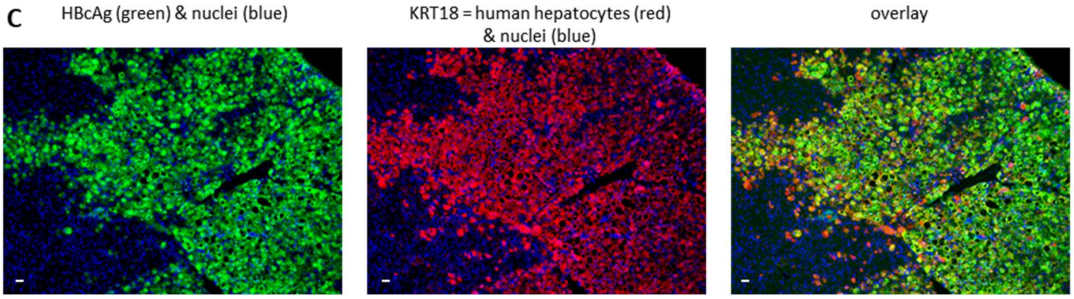
A



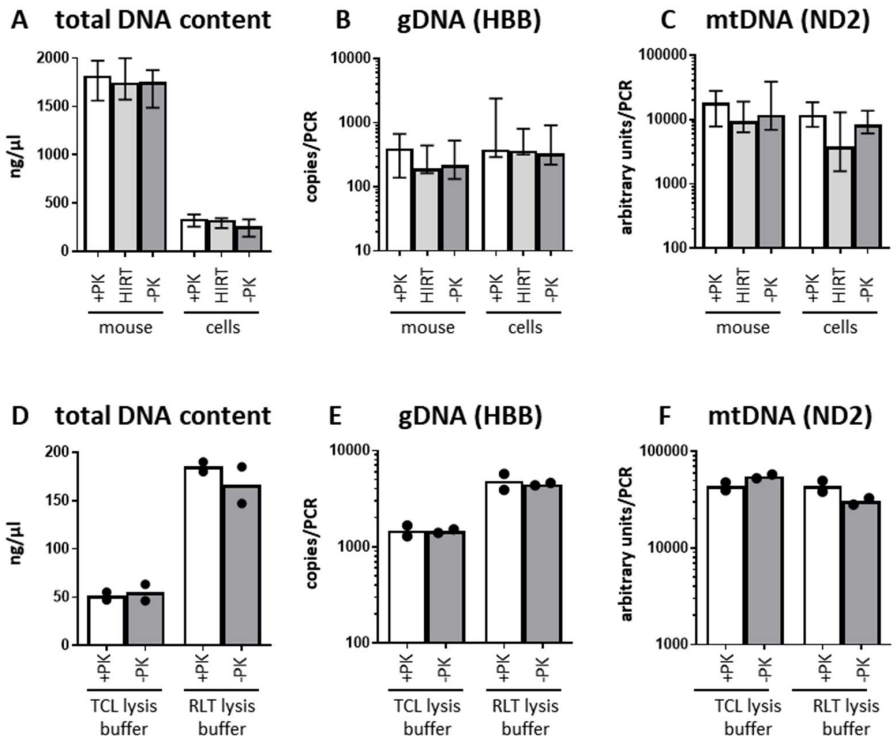
B

Treatment	Usage in study	Viral loads in serum at BL [HBV DNA copies/mL]	Viral loads in serum at EoT [HBV DNA copies/mL]	Log10 reduction to baseline
untreated control	cross-validation (Fig. 1 & 2A, B)	7.3E+08		
untreated control	parallel SB & qPCR (Fig. 2D)	9.1E+08		
untreated control	preparative gel (Fig. 3) & alkaline Hirt (Fig. S5)	6.8E+09		
6w Lamivudine	preparative gel (Fig. 3) & alkaline Hirt (Fig. S5)	1.9E+09	1.7E+07	2.1
12w Lamivudine	preparative gel (Fig. 3) & alkaline Hirt (Fig. S5)	3.1E+09	9.7E+06	2.5

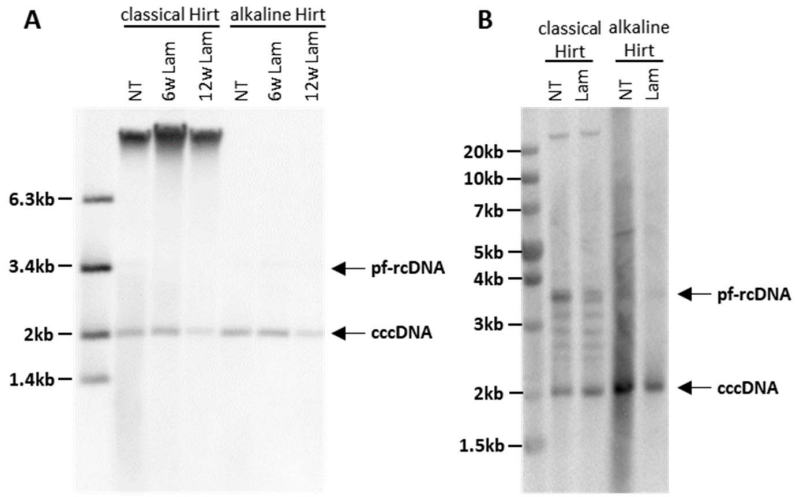
C



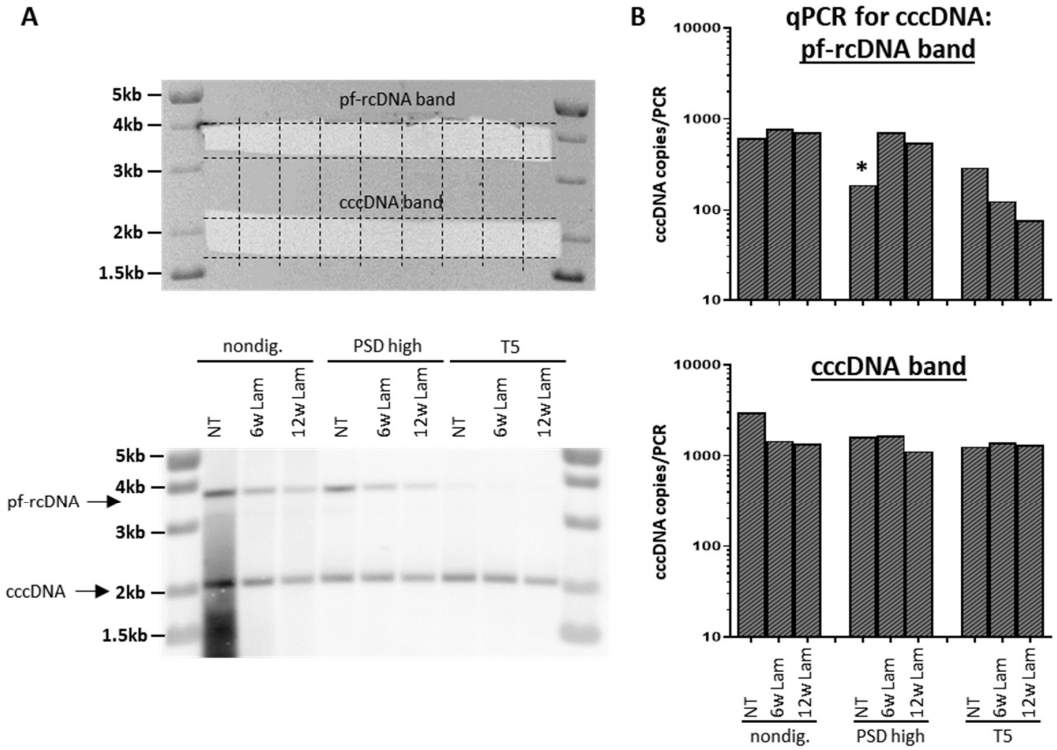
Supplementary figure 3: Sample preparation and virological characterization. (**A**) Schematic presentation of the generation and treatment of humanized liver chimeric mice used in this study. (**B**) The table summarizes the mice, their usage for the presented experiments, their final HBV DNA titers in the serum and, where applicable, their titer at the end of treatment and reductions from baseline. (**C**) Immunofluorescence co-staining for HBV core antigen (green) and the cytokeratin 18 (KRT18) in red as a marker for human hepatocytes in cryopreserved liver sections. Nuclei were stained with Hoechst 33258 (blue). Scale bar = 50 µm; BL, baseline; EoT; end of treatment.



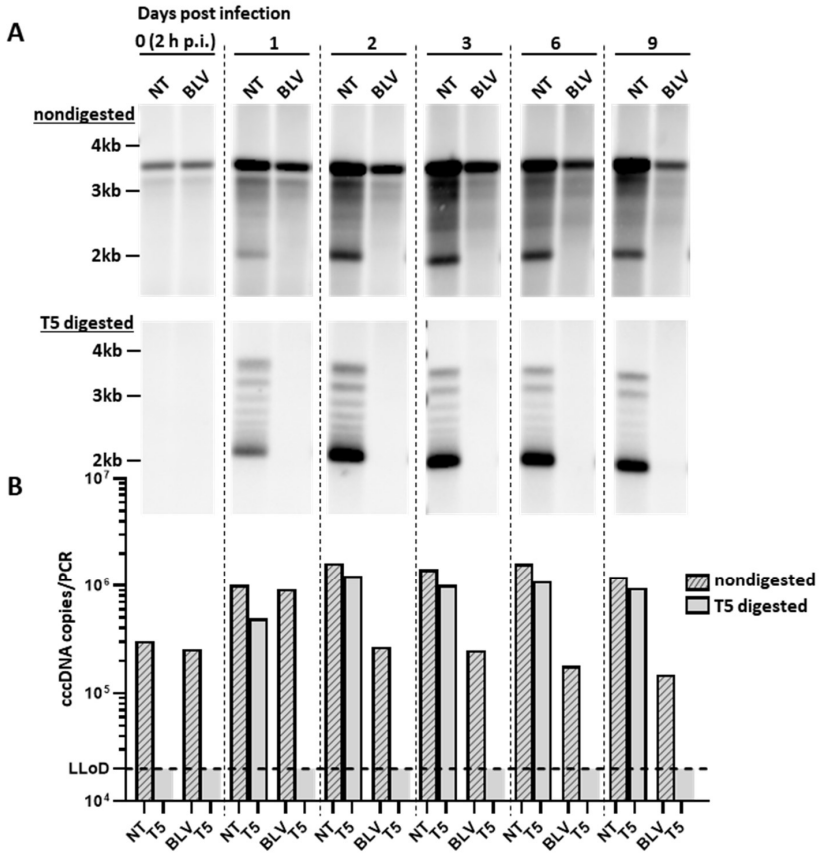
Supplementary Figure 4: The DNA extraction protocol affects the recovery of different types of DNA which in turn influences the normalization strategy. (**A-C**) The recovery of total DNA (measured by spectrophotometry) (**A**), genomic DNA (measured by qPCR for the human single-copy gene HBB) (**B**), and mitochondrial DNA (measured by qPCR for the human mitochondrial gene ND2) (**C**) are depicted as bar graphs for the three tested extractions and the two type of samples as indicated below the graphs. The bars show the median and range of the measurements in four labs. (**D-F**) The total DNA extraction with and without proteinase K treatment was tested head-to-head with the proprietary TCL buffer (Epicentre) and an alternative lysis buffer RLT (Qiagen) using two HBV-infected, untreated humanized mice. Every dot depicts one mouse, the bars the mean. The recovery of total DNA was measured by fluorometry (**D**), genomic DNA by qPCR for the human single-copy gene HBB (**E**), and mitochondrial DNA by qPCR for the human mitochondrial gene ND2 (**F**). gDNA, genomic DNA; HBB, hemoglobin subunit beta; mtDNA, mitochondrial DNA; ND2, mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 2.



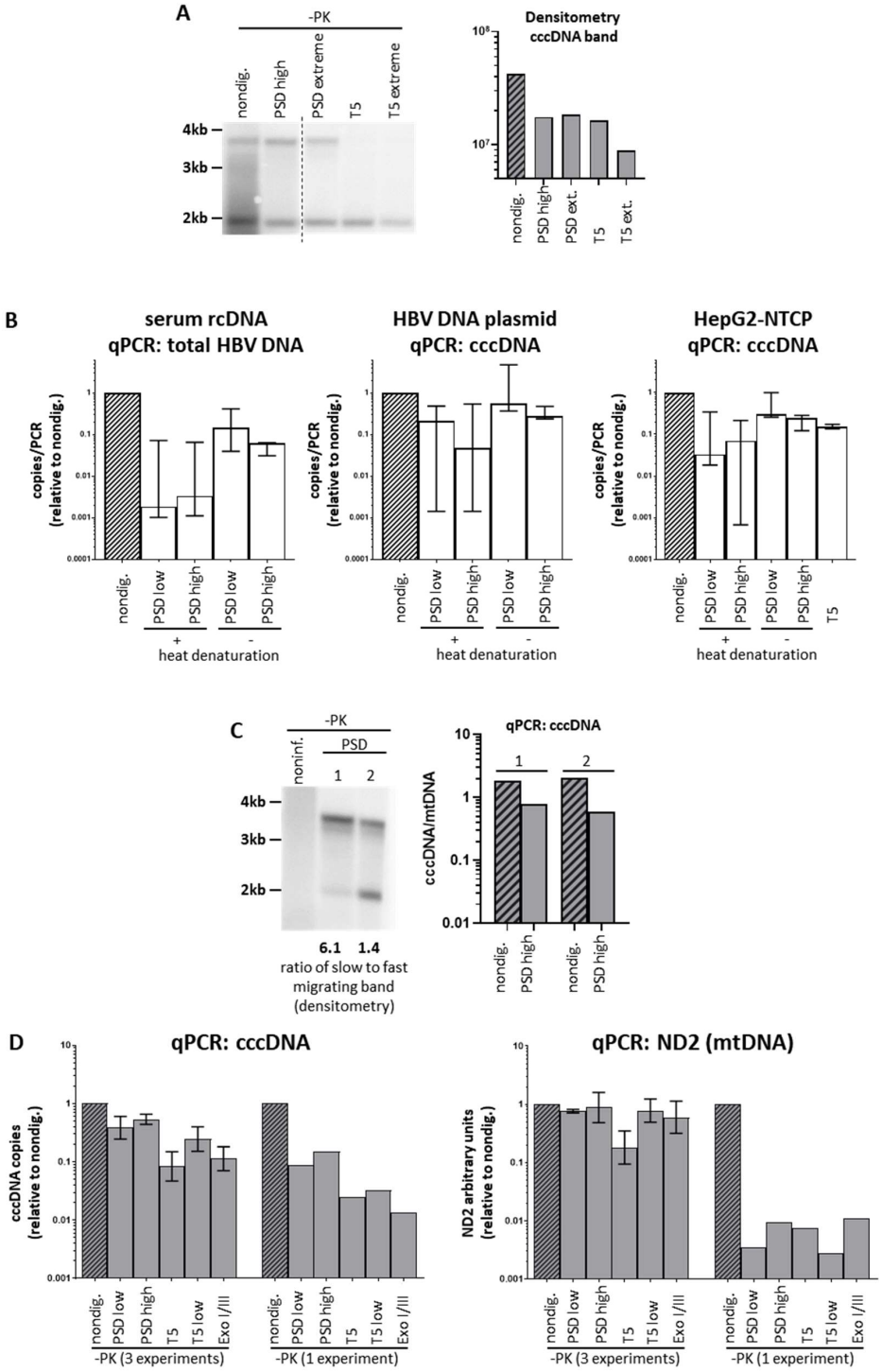
Supplementary Figure 5: Side-by-side comparison of classical and alkaline Hirt procedures. DNA was extracted with the alkaline and the classical Hirt method and subjected to SB as described in Supplementary Materials and Methods. (**A**) Liver DNA from three stably HBV-infected USG mice (after six or 12 weeks of Lamivudine treatment, or untreated, same mice as in **Figure 3**). (**B**) DNA from HepG2-NTCP cells infected with HBV (HepAD38-derived virus at MOI 250 GE / cell) and cultured for 9 days with or without Lamivudine treatment (10uM). In contrast to the classical method, cells are lysed in an alkaline milieu leading to the irreversible denaturation of genomic DNA and all non-supercoiled HBV DNA species, which are removed by centrifugation after rapid neutralization. As can be seen on the upper part of the blot, genomic DNA is visible only in the classical Hirt DNA extracts, confirming the higher capacity of alkaline extraction to deplete it. However, qPCR for HBB as an indicator of genomic DNA showed that genomic DNA was still present to varying extents (data not shown). In cell culture derived samples (**B**), most of the slow-migrating, relaxed HBV DNA forms, as well as “T5-resistant forms” (see **Supplementary Fig. 7**) are absent in the alkaline Hirt DNA extracts. These forms are not detected in either classical or alkaline Hirt extracts of mice livers (**A**), probably due to sensitivity issues or the high quality of the obtained DNA, i.e. no contribution of nicked cccDNA to the slow-migrating upper band. A smear in the alkaline Hirt-derived sample is visible in cell culture extracts (**B**, lane 4), which is absent under Lamivudine treatment (lane 5), suggesting that it is specific to HBV DNA. Lam, Lamivudine; NT, non-treated.



Supplementary Figure 6: The pf-rcDNA is detected with the same efficiency as cccDNA by cccDNA-selective qPCR. (**A**) The upper and lower bands of a parallel gel (same samples as in Figure 3) were excised separately and the DNA was extracted. The top photographs shows the agarose gel after excision; the lower photograph shows the parallel SB for comparison. (**B**) The upper graph depicts qPCR measurements for cccDNA in samples originating from the upper band in copies / PCR assay. The lower graph depicts the measurements from the lower band. The asterisk (*) marks a sample where some of the material was lost during the gel extraction process. Lam, Lamivudine; NT, non-treated.



Supplementary figure 7: Persistent pf-rcDNA from input virus interferes with cccDNA measurements in cell culture but can be removed by T5 exonuclease treatment. (**A, B**) HepG2-NTCP cells were infected with HBV (HepAD38-derived virus at MOI 4000 GE / cell) and followed up for nine days in the presence of Bulevirtide (185 nM) or without treatment. Hirt DNA was extracted at the indicated time points and subjected to SB (**A**) and cccDNA-selective qPCR (**B**) analyses either without additional nuclease digestion or after T5 exonuclease digestion. Samples treated with T5 exonuclease are shown in the bottom blot or with hatched bars, respectively. On the bottom blot, twice as much DNA as on the top blot was loaded. BLV, Bulevirtide; NT, non-treated.



Supplementary Figure 8: Nuclease treatment bears the risk of over-digesting cccDNA when digestion conditions are exaggerated or when the input DNA is damaged. (**A**) SB analysis (left) and densitometry of the cccDNA band (right) of -PK extracted liver DNA from one stably infected untreated USG mouse after various nuclease treatments. The standard conditions for PSD and T5 were compared to extreme conditions: “PSD high”: 30 U PSD in 200 µl, 2 h incubation vs. “PSD extreme”: 120 U PSD in 200 µl, 6 h incubation and “T5”: 10 U T5

exonuclease in 20 µl; 45 min incubation vs “T5 extreme”: 40 U T5 exonuclease in 20 µl; 6 h incubation. Note that the higher band volume of the nondigested sample determined by densitometry is due excessive background staining in this lane. (**B**) Comparison of PSD digestion efficiency with and without prior heat denaturation of the input DNA. DNA from HepG2-NTCP cells was extracted with the +PK extraction protocol. Serum rcDNA was extracted from humanized mouse serum using the QiAamp MinElute Virus Spin Kit. Extracted rcDNA and purified HBV DNA plasmid was diluted in +PK extracted DNA from an uninfected humanized mouse before nuclease digestion. The bar graphs show total HBV DNA or cccDNA copy numbers normalized to the nondigested levels. Bars depict the median and range of measurements from four labs. The sample type and qPCR are depicted above the respective graphs. (**C**) SB analysis (left) and qPCR for cccDNA (right) in two individual -PK DNA extracts from the same untreated HBV-infected USG mouse (denoted as 1 and 2). The ratio of upper and lower band was determined by densitometry. qPCR measurements of cccDNA normalized to mitochondrial DNA via ND2 are shown beside the blot before and after nuclease digestion. (**D**) qPCR measurements of cccDNA and ND2 in -PK extracted liver DNA from an HBV-infected USG mouse liver tissue, digested with nucleases as indicated below the graph or left undigested (hatched bars) The left-hand side of each graph shows three independent DNA extraction; the right-hand side shows the results of a failed -PK DNA extraction in one experiment, which resulted in damaged DNA. Bars depict the median of three experiments. Copy numbers per qPCR assay were normalized to the values in the nondigested sample.

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