

A modified kit-based HBV protein-free DNA extraction from liver tissues and cell cultures for HBV cccDNA Southern blot and qPCR

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Introduction

- cccDNA quantification from total DNA preparation by qPCR with cccDNA-selective primers is challenging because of the coexisting HBV DNA forms (replicative intermediates, protein-free rcDNA (aka deproteinated rcDNA), virion-derived rcDNA), which are detected despite selective PCR conditions if in excess over cccDNA. Detection by Southern blot after Hirt DNA extraction is regarded as the “gold standard” but lacks sensitivity and is very laborious.
- This protocol was developed to reduce HBV non-cccDNA forms during the total DNA extraction process and improve cccDNA quantification by qPCR and Southern blot. It serves as a rapid and reliable alternative to classical Hirt DNA extraction. It has been evaluated in different laboratories for its use in cell culture samples (HepG2-NTCP, primary human hepatocytes) and humanized mouse liver tissue(1).
- The protocol follows the instructions from the MasterPure Complete DNA and RNA Purification Kit (Epicentre/Lucigen) but omits the proteinase K digestion step. Theoretically, the viral polymerase remains covalently attached to the rcDNA or other HBV replicative intermediates when proteinase K digestion is omitted and such protein-bound DNA forms are subsequently removed during the protein precipitation step, thus mainly the protein-free rcDNA and cccDNA are extracted in the end.
- Caution must be taken when handling samples with high HBV replication levels since the reduction of HBV RI might not be sufficient for selective qPCR measurements and an additional nuclease digestion prior to qPCR is warranted in any case.

Materials and Reagents

- MasterPure Complete DNA and RNA Purification Kit (#MC85200 Epicentre, available through LucigenEpicentre): From this kit you will use the 1x and 2x Tissue and Cell Lysis solution (TCL), the MPC protein precipitation reagent, the RNase A, and the proteinase K (PK). All components but the 2xTCL can also be purchased separately.
- Tissue and Cell Lysis solution (#MTC096H Lucigen)
- MPC protein precipitation reagent (#MMP095H Lucigen)
- RNase A (5µg/µl #MRNA092 Lucigen)
- Proteinase K (50µg/µl #MPRK092 Lucigen)
- TE buffer (10:10): 10 mM Tris-HCl (pH 7.5) and 10 mM EDTA (pH 8.0)
- TE buffer (10:1): 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA (pH8.0)
- Isopropanol
- 70% ethanol
- reaction tubes (1.5 ml and 2 ml)

- heating block or bath
- NanoDrop or Qubit (Qubit™ dsDNA BR Assay Kit, catalog number Q32853)
- For liver tissue: dry ice, 10 cm petri dish, razor blades or scalpel, forceps, and pestles for homogenization of the tissue (plastic or metal pestles for the use in 1.5ml microcentrifuge tube, or glass Dounce homogenizer)

Experimental Procedures

Initial steps for liver tissues

1. Prepare all necessary reagents for the extraction, set a heating block to 37° C, label the tubes, have the liver pieces ready on dry ice.
2. Cut off a <10 mg piece of every liver sample to be processed: Place petri dish on dry ice, cut the tissue with a razor blade or scalpel on the dish, transfer the piece in a labelled pre-chilled tube using pre-chilled forceps. Don't let the tissue thaw at that time, leave the samples on dry ice until the next step.

Note 1: *The kit must not be overloaded with sample material. The manual recommends to use 1 - 5 mg tissue per extraction; if you have more, the samples needs to be split and processed separately. In our hands 10 mg per extraction is the upper limit to be used. 10 mg roughly corresponds to a cube with 2 mm edges. Liver pieces can be weighed using precision scales.*

3. Let the tissue drop into a 1.5 ml tube filled with 300 µl TE (10:10) at room temperature. Homogenize the tissue with a pestle. (Try to smash the tissue between the side of the tube and the pestle while moving the pestle up and down and twisting it simultaneously). When the liver is homogenized (no clumps, homogeneous yellow-brown color), place it on ice.
4. Add 300 µl 2xTCL (= 600 µl), mix briefly by pipetting and leave on ice while you proceed with the next liver piece.

Initial steps for cell cultures

1. Before harvest, wash the cells once with PBS, then add TCL buffer to every plate/well and mix well by pipetting. This step needs to be adapted to the number of cells per well. Typically, on a 12-well plate, 0.5 million HepaRG, 1 million PHH, or 1 million HepG2-NTCP cells are present per well and can be lysed with 600 µl TCL. On a 6-well plate, 2 million PHH or HepG2-NTCP cells per well can be lysed with 900 µl TCL buffer. In the following we will describe the procedure using 600µl TCL buffer.

Note 2: *The kit must not be overloaded with sample material. According to the manufacturer, 0.5 - 1 million mammalian cells can be processed in 300 µl of TCL buffer. If you have more, the amount of TCL buffer needs to be scaled up or the sample must be split and processed separately.*

2. Scrape cells off the plates and transfer to a 1.5 ml tube. Place on ice.

Continued DNA extraction for tissues and cells

Note 3: *At this point, the proteinase K digestion, which is skipped here, would take place. However, we strongly recommend testing this protocol with and without PK in comparison - at least during the set-up phase - in order to assess the actual reduction of HBV DNA intermediates with this protocol. The tissue homogenate or cell lysate can be split up in halves at this point and one half can be used for the PK digestion (add 2µl of PK and incubate for 1 h at 56°C), while the other half without PK will stand on ice in the meantime. The amount of total HBV DNA (measured by qPCR) in the samples without PK can be expected to be around 1-2 log lower than in the same samples processed with PK.*

1. Add 2 μ l RNase A to every sample and incubate at 37° C for 30 min.
2. Place samples on ice and add 300 μ l of MPC reagent to every tube (total volume= 900 μ l) and mix thoroughly by inverting the tubes several times. Incubate on ice for 5 min to ensure proper protein precipitation.
3. Centrifuge in a table-top microcentrifuge at full speed (12,000 rpm) and 4° C for 10 min.
4. Transfer the supernatant to a fresh 2 ml microcentrifuge tube. In case the supernatant still contains parts of the white protein pellet, which is sometimes difficult to avoid, centrifuge again (full speed (12,000 rpm) and 4° C for 5 min) and transfer supernatant to a fresh tube.
5. Add 900 μ l isopropanol (total volume = 1800 μ l) and invert several times for DNA precipitation (approx. 40x).
6. Centrifuge in a table-top microcentrifuge at full speed and 4° C for 10 min.
7. Discard the supernatant and wash the pellet with 1 ml 70% ethanol.
8. Repeat the centrifugation and the washing step.
9. After the last centrifugation, remove as much liquid as possible and let the pellet dry completely.
10. Once the pellet has dried, immediately dissolve it in an appropriate volume of TE (10:1). For a 10 mg liver piece 70 μ l have proven adequate, and for 1 million cells, a volume of 50 μ l.
11. Let the samples incubate overnight at 4 °C or heat briefly to 37° C (15 min). Flick the tubes or pipet up and down to mix. Make sure the DNA is well dissolved before you continue.
12. Determine the DNA content of the samples using a spectrophotometer (Nanodrop) or a fluorometer (Qubit). The Qubit provides specific DNA measurements also in the presence of contaminants and therefore is the method of choice for the quantification of liver-derived DNA. With this protocol, liver-derived DNA is typically not entirely pure and will thus be over-quantified with the Nanodrop.
13. The DNA is now ready for downstream applications. For qPCR, total HBV DNA and cccDNA can be quantified and results can be normalized to genomic DNA using, for instance, beta globin as a reference gene, or mitochondrial DNA (for instance the mitochondrial gene ND2) or initial sample cell counts. Samples can be used for Southern blot either directly or after an additional nuclease digestion. The latter step might be necessary to reduce background staining when the replication level in the sample was high.

Note 4: Normalization is a critical point for PCR quantification of viral DNA. We have made the observation that DNA recovery of genomic DNA as well as mitochondrial and HBV DNA is similar for DNA extraction with or without the proteinase K digestion and thus, both DNA species can be used for normalization.

Note 5: This protocol ideally reduces total HBV DNA by 10 – 100-fold compared to the extraction with proteinase K digestion. However, in highly infected samples, this reduction might not be sufficient to facilitate specific cccDNA PCR measurements. Therefore, we recommend including a nuclease digestion before PCR for all the extracted samples. Details for nuclease digestion protocols can be found here elsewhere. Because cccDNA that contains nicks or double strand breaks will become a substrate for nucleases, it is of utmost importance to obtain high-quality DNA and to assess its quality in every extract. We recommend to use mitochondrial DNA - a covalently closed circular DNA - 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 1 Log₁₀ indicates sufficiently intact DNA, while a reduction of more than 1 Log₁₀ indicates damaged DNA and should not be used.

References

1. Allweiss L, Giersch K, Piroso A, Volz T, Muench RC, Beran RK, et al. Therapeutic shutdown of HBV transcripts promotes reappearance of the SMC5/6 complex and silencing of the viral genome in vivo. Gut. 2021 Jan 28;