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Amplification and sequencing of Hepatitis B virus pol gene

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ABSTRACT

The Amplification and sequencing of HBV pol gene protocol aim to present all the details from HBV DNA extraction with QIAamp DNA Blood Mini Kit (Qiagen, Werfen) and with a homemade assay for amplification/sequencing of the RT region of the HBV pol gene. This protocol is related to an ongoing publication from PLOS ONE.

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HBV DNA extraction and reagents

1 Reagent preparation

- Protease: Add 5.5 ml of protease solvent included in the kit (nuclease-free water containing 0.04% sodium azide) to the tube containing the lyophilized protease. Reconstituted protease is stable for up to two months when stored at 2-8°C. Storage at -20ºC is recommended to prolong the life of the protease. To avoid successive freezing and thawing, store protease in aliquots.
- Buffer AW1: add 125 ml of 96-100% ethanol to 95 ml of concentrated Buffer AW1 and mix by inversion 5 to10 times.
- Buffer AW2: pipette 160 ml of 96-100% ethanol into 66 ml of buffer AW2 and mix by inversion 5 to10 times.

2 HBV DNA extraction

- Heat a thermal block to 56ºC.
- Shake AL buffer before using it and if there is any precipitate, dissolve it in a bath at 56ºC.
- Prepare 350 µl of negative extraction control from a pool of negative plasmas for this virus.
- Pipette 350 µl of HBV plasma sample and positive and negative controls into 1.5ml microtubes.
- Add 35 µl of the reconstituted protease into the microtubes.
- Homogenize the AL buffer and add 350 µl to the same microtubes.
- Vortex intermittently for 15 seconds; to promote efficient lysis it is essential that the sample and the AL buffer are mixed until a homogeneous solution is obtained.
- Add 3.5 µl carrier DNA to the cap of each tube, close the tubes and mix by inversion.
  Note: carrier DNA enables viral DNA binding to the column membrane on the next step. It is not provided in the kit. Here it was used Polyadenylic Acid, PolyA, 10 mg/ml; Amersham; It should be stored at -20°C.
- Vortex intermittently for 15 seconds and spin the microtubes in the centrifuge for approximately 4 seconds.
- Incubate the tubes in the thermal block at 56ºC for 10 minutes; the amount of DNA released is maximum after lysis during this incubation step. Longer incubation periods have no effect on the extraction or quality of purified DNA.
- Spin the microtubes in the centrifuge for approximately 4 seconds
- Add 402.5µl 96-100% ethanol, vortex intermittently for 15 seconds, and then spin in the centrifuge.
- Apply the mixture from the previous step (630µl at a time), carefully, in a QIAamp Mini column incorporated into a 2ml collection tube, without letting the mixture reach the edge of the column, close the lid, and centrifuge at 6000g for 1 minute.
- Place the column in a 2ml new collection tube and discard the tube containing the filtrate.
- Repeat the two previous steps.
- Add 500µl of buffer AW1, close the lid, and centrifuge at 6000g for 1 minute.
- Place the column in a new 2ml collection tube and discard the tube containing the filtrate.
- Add 500µl of buffer AW2, close the column cover, and centrifuge at 20000g for 3 minutes.

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- Place the column in a 2ml microtube (not supplied in the kit) and discard the tube containing the filtrate.
- Centrifuge at 20000g for 1 minute to completely dry the membrane.
- Discard the 2 ml tube containing the filtrate and introduce the column into a previously identified 2 ml screw tube for elution of virus DNA.
- Apply 60µl of buffer AE in the center of the membrane, close the column lid, and incubate at room temperature for 5 minutes.
- Centrifuge at 6000g for 1 minute.
- Samples can be stored for 24 hours at 2-8°C, at -20°C for longer periods, or can be used immediately.

Homemade amplification and sequence assay

3 **Amplification of HBV Pol gene** (Biotaq DNA polimerase, 5 U/µl; Bioline/Meridian BioScience)

a) 1st round PCR

- Put the reagents from the amplification kit at room temperature, shake them slightly, spin them and place them on ice.

Note 1: the enzyme should only be removed from the freezer at the moment it is going to be used.

Note 2: Primer sequences are described in the related article of PLOS ONE.

- Mark 0.2 ml microtubes for each of the samples, for the positive control, for the negative control of extraction, and for the negative control of PCR.
- Prepare the mixture for the PCR reaction in a suitable microtube.

PCR mixture (for 1 sample)	
Water nuclease-free	- 14.45 µl
NH4 buffer (10x)	- 2.5 µl
dNTPs mix (100mM)	- 0.2 µl
FW_O (10mM)	- 1 µl
RV_O (10mM)	- 1 µl
MgCl2 (50mM)	- 0.75 µl
BioTaq enzyme	- 0.1 µl

- Distribute 20 µl of the mixture into each of the 0.2 ml microtubes (include one extra tube for a negative control of PCR technique).
- Add 5 µl of each of th e extracted samples and controls to the respective tubes. Add 5 µl of "nuclease-free" water into the PCR negative control.
- Place the tubes in a thermal cycler under the following conditions:

94ºC	4 min	
94ºC	**45 secs**	
55ºC	**30 secs**	**40 cycles**
72ºC	**1 min (+5 secs/cycle)**	
72ºC	15 min	

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4ºC ∞

- After the end of PCR reaction, save the amplified products at -20ºC or proceed with the nested PCR.

b) 2nd round PCR

- Proceed exactly as for the first PCR, for reagents and microtubes, also using the same amplification kit.
- Prepare the mixture for the PCR reaction in a 1.5 ml microtube.

PCR mixture (for 1 sample)	
Water nuclease-free	- 18.45 µl
NH4 buffer (10x)	- 2.5 µl
dNTPs mix (100mM)	- 0.2 µl
FW_I (10mM)	- 1 µ
RV_I (10mM)	- 1 µl
MgCl2 (50mM)	- 0.75 µl
BioTaq enzyme	- 0.1 µl

- Distribute 24 µl of the mixture into each of the 0.2 ml microtubes.
- Add 1µl of each of the 1st round PCR products to the respective tubes.

Note: The product obtained for the positive control in the first PCR must be diluted at 1:5 (4µl H2O + 1µl product).

- Put the 0.2ml microtubes in a thermocycler under the same cycling conditions used for the 1st round PCR.

Agarose gel electrophoresis

- Prepare a 1.5% agarose gel.
- Fill an electrophoresis cuvette with 1x TAE buffer.
- Prepare the samples to be applied to the gel, adding 10µl of each to 2µl of Orange G dye.
- To 0.5µl of the molecular weight marker (GeneRuler 100 bp DNA Ladder, 0,5 µg/µl;
- ThermoFisher Scientific), 2µl of the dye and 7.5µl of water are added to make a volume of 10µl.
- Apply the stained samples into the wells of the agarose gel and apply electric current (70 Volts) for approximately 30 minutes.
- Observe the gel in a transilluminator where a 943 bp band should be visualized for samples and positive control.

PCR products purification using a spin column system (JetQuick PCR Product Purification Spin Kit; Genomed)

- Place a 1.5 ml microtube with nuclease-free water to heat at 65ºC.
- Add 60µl of H1 kit solution to the remaining 15µl of 2nd round PCR product.
- Mix and transfer the mixture to a purification column previously inserted in a supernatant collection tube.
- Centrifuge at 15000g for 1 minute.

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- Discard the contents of the collection tube.
- Add 500µl of H2 solution to the column.
- Repeat the above centrifugation and discard steps.
- Centrifuge at 16000g for 1 minute to completely dry the column membrane.
- Insert the column into a 1.5ml microtube.
- Add 20-35µl of nuclease-free water at 65ºC to each column.
  Note: Add a higher volume, up to 50µl, if the band obtained after electrophoresis is too strong. Wait 1 minute.
- Centrifuge at 16000g for 2 minutes.
- Store purified products at -20°C or proceed with the sequencing reaction.

Sequencing Reaction (BigDye terminator v.3.1 Cycle Sequencing Kit; Applied Biosystems)

- Thaw the reagents needed for the sequencing reactions and the purified PCR products:
  BigDye terminator v.3.1 (Applied Biosystems)
  BigDye terminator 5X sequencing buffer (Applied Biosystems)
  Primers Forward and Reverse from the nested PCR (5µM)
  Nuclease free water
- Place the thawed reagents on ice and prepare a mixture for each primer as described below:

Sequencing mixture (for 1 sample)
BigDye terminator v.3.1 - 0.5 µl
Primer (5µM) - 1 µl
BigDye terminator v.3.1 Buffer (5X) - 1.75 µl

- Mix the reagents by pipetting up and down several times and make a spin to the microtube.
- Distribute 3.25 µl of each mix into the two different tubes.
- Add between 0.3 - 1 µl of the previously purified PCR product to each tube, according to the intensity of the band observed on the agarose gel.
- Complete the reaction volume to 10 µl with nuclease-free water and place the samples in the thermocycler under the following conditions:

Sequencing cycle

96ºC	1 min	
96ºC	**10 secs**	
60ºC	**4 min**	**25 cycles**
4ºC	**7 min**	
4ºC	∞	

Sequencing products purification through Ethanol/Sodium Acetate

- Prepare a sodium acetate/ethanol solution by mixing 2 µl of 3M sodium acetate (pH 4.6) with 50 µl of 100% ethanol, per reaction.
- Add 52 µl of the previous solution to each sequencing reaction and transfer to a 96 well plate.

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- Cover the plate with aluminum foil.
- Vortex the plate to mix.
- Centrifuge the plate at 2000g for 20 minutes.
- Once the centrifugation is finished, remove the aluminum foil carefully trying to not disturb the sediment.
- Immediately place several absorbent paper towels without residues on top of the plateand invert it.
- Centrifuge the inverted plate at 150g for 1 minute.
- Add 150 µl of 70% ethanol, prepared previously, and cover the plate with a new aluminum foil.
- Centrifuge at 2000g for 5 minutes.
- When the centrifugation is finished, remove again the aluminium foil carefully trying to not disturb the sediment.
- Invert the plate over clean towels and centrifuge the inverted plate at 150g for 1 minute.
- After centrifuging, allow the plate to air dry for approximately 1 minute to eliminate any remaining ethanol.
- Cover the plate with aluminum foil to store between –15ºC and –25ºC in the dark, but no longer than 2 weeks, or add 20 µl of Hi-DiTM Formamide (Applied Biosystems, USA) and move to the processing phase of the sequences in the ABI Prism 3100-Avant Genetic Analyzer according with the equipment manual.

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