Hepatitis B Virus (HBV) full genome amplification and sequencing

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This protocol describes our approach at amplifying 5 overlapping PCR fragments encompassing the entire Hepatitis B virus 3020–3320 kb genome. The HBV genome is composed of nearly fully double-stranded circular DNA. One end of the full-length strand is linked to the viral DNA polymerase. The viral DNA is found in the hepatocyte nucleus soon after infection. The partially double-stranded DNA is rendered fully double-stranded by completion of the (+) sense strand and removal of a protein molecule from the (-) sense strand and a short sequence of RNA from the (+) sense strand. Non-coding bases are removed from the ends of the (-) sense strand and the ends are joined. Four known genes are encoded within this genome: C, X, P, and S. HBV is divided into four major serotypes (adr, adw, ayr, ayw) based on envelope antigenic epitopes, as well as into eight different genotypes (A-H) based on overall nucleotide sequence variation of the genome. Genotypes have a distinct geographical distribution and allow for phylogenetic epidemiology of the virus. This protocol requires the assembly of a contig formed by small (< 1 kb fragments) which are readily amplified from low quality DNA samples, avoiding tissue culture expansion methods for virus amplification.

Oligonucleotides for initial full-genomic PCR (first PCR for fragments 1 through 4)

Name	bp	Sequence	bp	%GC	Tm	Hp	Hm	Ht	Position	Ref
HBV-FO	3215	5´-TTT-TTC-ACC-TCT-gCC-TAR-TCA-´3	21	40.5	60.3	0.8	-4.3	-5.1	1821-1841	1
HBV-RO		5´-AAA-AAg-TTg-CAT-RT-gMT-gg-´3	20	40	59.4	1.6	-7.0		1806-1825	
FA1-L2	1014 948	5´-TTT-CAC-CTC-TgC-CTA-RTC-ATC-TC-´3	23	45.7	62.4	0.9	-4.3	-5.5 -5.5	1824-1845	2
FA1-L3		5´-CTT-TRg-gRC-WTR-gAC-ATY-gA-´3	20	45	59.1	2.1	-6.1		1892-1911	
FA1-R		5´-TCT-TgT-TCC-CAA-gAA-TAW-ggT-g-´3	22	40.9	60.1	-4.3	-7.0		2818-2839	
FA2-L	1074	5´-gCg-TCg-CMg-MAg-ATC-TMA-AT-´3	20	50	62	-0.1	-7.8	-6.7	2418-2438	2
FA2-R		5´-TTg-AgA-gAA-gTC-CAC-CAC-gAg-´3	21	52.4	63	0.2	-3.6		253-273	
FA3-L	1059	5´-CTg-CTg-gTM-gCT-CMA-STT-´3	18	61.1	63.7	-3.9	-6.6	-8.3	57-74	2
FA3-R		5´-gCC-TTg-TAM-gTT-ggY-gAR-AA-´3	20	45	60.4	-0.5	-6.2		1096-1115	
FA4-L	1048 1080	5´-gTA-TTg-ggg-gCS-RAR-WCT-gT-´3	20	52.5	62.8	-1.5	-9.3	-6.2 -5.6	751-770	3
FA4-L2		5´-ACT-gTC-Tgg-CTT-TYA-gYT-ATR-´3	21	40.5	59.2	0.8	-8.0		719-739	
FA4-R2		5´-AAT-TTA-TgC-CTA-CAg-CCT-CC-´3	20	45	59.4	-1.8	-5.4		1776-1795	
FA5-FO	807	5´-ggg-CgC-ACC-TCT-CTT-TAC-gC-´3	20	65	66.7	-2.0	-9.9	-6.1	1525-1541	3
FA5-RO		5´-Cgg-AAg-TgT-TgA-TAA-gAT-Agg-g-´3	22	45.5	59.7	1.5	-3.6		2310-2332	
FA5-FI	285	5´-TAA-AAg-gAC-TCT-Tgg-ACT-´3	18	38.9	54.4	-0.7	-3.5	-3.9	1655-1672	3
FA5-RI		5´-CCA-CAg-AAg-CTC-CAA-ATT-CT-´3	20	45	59.8	-0.4	-6.3		1921-1940	

Note 1: Reverse oligonucleotide primer sequences given in the above tables are the reverse-complement of sequence present in alignments and as they should be ordered for synthesis. Tm shown in ºC, Hp=hairpin structure, Hm = homodimer, Ht=heterodimer, these last three given in kcal/mole. Tm calculated using IDT's Oligo Analyzer (www.idtdna.com/calc/analyzer) at 200 nM oligo concentration, 2 mM Mg++ concentration and 0.2 mM dNTP concentration.

PCR components

Full-genomic PCR		1x (µl)
dH_2O	C*f*	5.25
Qiagent® Master Mix	1X	12.5
$MgCl_2$ @ 50 mM	2 mM	0.25
10µM Oligo mix (F+R)	400 nM	2.0
DNA	-	5
	Final volume:	25

Nested PCRs (Fragments 1 through 4))		1x (µl)
dH_2O	C*f*	2.625
Qiagent® Master Mix	1X	6.25
$MgCl_2$ @ 50 mM	2 mM	0.125
10µM Oligo mix (F+R)	400 nM	1.0
1:60 diluted 1st genomic PCR product	-	2.5
	Final volume:	12.5

Outer PCR (Fragment 5)		1x (µl)
dH_2O	C*f*	5.3
10x Buffer PCR	1X	1.25
$MgCl_2$ @ 50 mM	2 mM	0.5
dNTPs @ 10 mM	200 µM	0.25
Oligos @ 10 µM	800 nM	1
Taq (Vivantis) @ 5 IU/µL	0.04 IU/µ	0.13
DNA	-	4
	Final volume:	12.5

Nested PCR (Fragment 5)		1x (µl)
dH_2O	C*f*	7.87
10x Buffer	1X	1.25
$MgCl_2$ @ 50 mM	2 mM	0.5
dNTPs @ 10 mM	200 µM	0.25
Oligos @ 10 µM	400 nM	0.50
Taq/pfu 9:1	0.04 IU/µ	0.13
Outer PCR product (1:5 dilution)	-	2
	Final volume:	12.5

Note 2: This new protocol has been optimizied for Qiagen Hot Start Master Mix (Cat. 203443) which contains 250 units HotStarTaq DNA Polymerase, PCR Buffer with 3 mM MgCl2, and 400 µM of each dNTP).

PCR conditions

Total elapsed time 3:55 hrs.		Denaturing		Annealing	Extension		
Full genomic (fragments 1 - 4)	Temperature	95°	95°	60°	72°	72°	4º
	Time	15 min	45 sec	1.5 min	3 min	10 min	5 min
			x 30 cycles				

Total elapsed time 3 hrs.		Denaturing		Annealing	Extension		
Nested PCR's (fragments 1 – 4)	Temperature	95°	95°	50°	72°	72°	4º
	Time	15 min	45 seg	30 seg	3 min	10 min	5 min
			x 35 cycles				

Total elapsed time 1:30 hrs.		Denaturing		Annealing	Extension		
Initial and nested PCR for fragment 5	Temperature	94°	94°	52°	72°	72°	4º
	Time	3 min	15 sec	30 sec	30 sec	5 min	5 min
			x 35 cycles				

Note 3: Optimied for amplification in Applied Biosystems Simpliamp thermal cycler.

Additional notes

4. Use only pre-PCR designated area to prepare your PCR mastermix.
5. Clean pre-PCR area with 70% Ethanol before and after setting up your PCR's.
6. Prepare PCR mastermix and aliquots in ice to prevent evaporation of small volume (12 µL) PCRs.
7. Vortex all reagents prior to use except DNA polymerases, vortex mastermix after preparing.
8. First PCR product must be diluted 1:5 before adding to nested PCR (i.e., 12.5 µl PCR + 62.5 µl of dH_2O).
9. Low quality or low viral load DNA samples might work better without diluting 1st PCR product.
10. Low quality or low viral load DNA samples might amplify for partial fragments without the initial full-genomic PCR.
11. All fragments have sufficient (150 bp) overlap for contig assembly.

References

1. Modified by LgVH based on günther S, Li BC, Miska S, Krüger DH, Meisel H, Will H. A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in immunosuppressed patients. J Virol. 1995 Sep;69(9):5437-44. PMID: 7636989

2. Modified by LgVH based on Zhang Q, Wu g, Richards E, Jia S, Zeng C. Universal primers for HBV genome DNA amplification across subtypes: a case study for designing more effective viral primers.Virol J. 2007 Sep 24;4:92. Retraction (due to authorship not scientific conduct) in: Zhang Q, Wu g, Richards E, Jia S, Zeng C. Virol J. 2007;4:119. PMID: 17892576

3. SE. guerra-Palomares, CA. garcia-Sepulveda., Unpublished primers. LgVH, Facultad de Medicina, UASLP (2013).

Revision history

1.0 Original document.

1.1 Corrected FA3R primer binding site from 115 to 1115, added note on oligonucleotide sequence ordering.

1.2 Added note 5 to better explain 1:5 dilution.

2.0 Optimized protocol for use of Applied Biosystems Simpliamp cycler and Qiagen Hot Start enzyme. Primer binding positions corrected. Modified outer full genomic PCR oligo sequence to adapt to novel HBV nucleotide sequence information.

3.0 Changes to document format only.