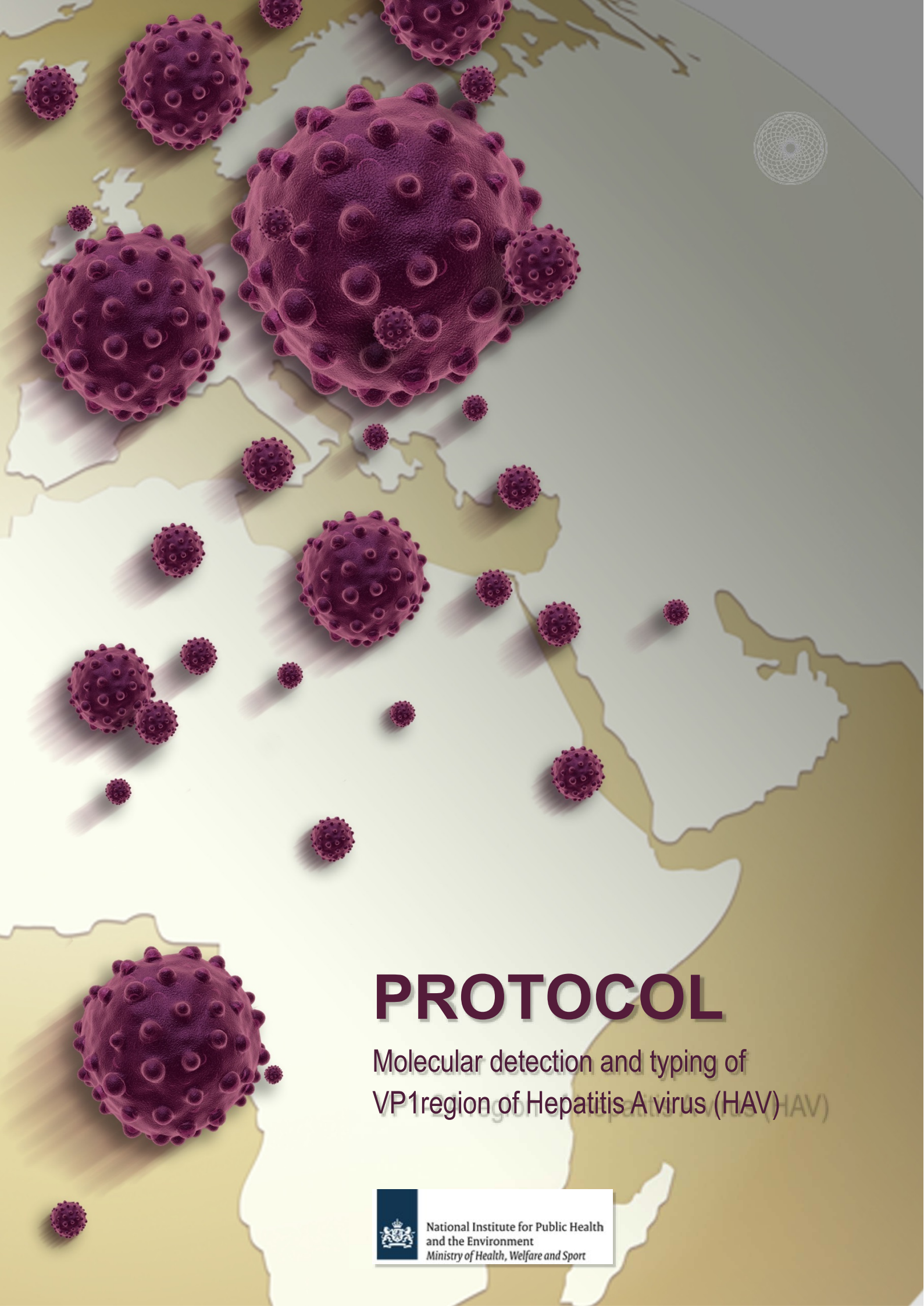

PROTOCOL

Molecular detection and typing of VP1region of Hepatitis A virus (HAV)

National Institute for Public Health and the Environment
Ministry of Health, Welfare and Sport

Contents

A. Purpose of the document

The main objective of the international HAV Network is to share aggregated epidemiological and molecular information on hepatitis A virus (HAV) strains identified in different geographic regions of the globe. This sequence-based typing database is essential for monitoring the diversity of HAV strains circulating world-wide, and is an important tool for source tracing investigations in international foodborne outbreaks.

To improve the comparability of sequences, it is necessary to share standardized protocols for detection and sequencing HAV. It is essential to have a minimum sequence length in the same selected genome region that will allow the robust comparison of different strain sequences.

This document provides information on a molecular diagnostic typing protocol for HAV, which is based on the protocols established by the reference laboratory for hepatitis A at the Dutch National Institute of Public Health and the Environment (RIVM). All laboratories wishing to implement these protocols, using similar commercial kits/reagents, should always optimize and validate the test to ensure adequate specificity and sensitivity. For any questions or additional information please contact HAV Net (havnet@rivm.nl).

B. Laboratory practical considerations:

Molecular diagnostic techniques are rapid and sensitive methods used for clinical diagnostics; however the validation of results and biosafety of laboratory personnel depend on several practical considerations:

a) Training of personnel:

- Laboratory personnel should be experienced in molecular biology techniques and familiar with protocols.

b) Biosafety:

- Diagnostic laboratory work on clinical specimens from patients, who are suspected of being infected with HAV, should be conducted in BSL 2 containment conditions with the use of appropriate personal protective equipment (PPE). Personnel should be vaccinated against hepatitis A.

c) Good laboratory practices:

- Ensuring that the recommended reagents are used and handled properly is critical, as reactions are complex and problems with a single reagent can have significant effect on the results.

d) Facilities and handling areas:

- Specimen and reagent handling facilities with appropriate room separation for various steps of RT-PCR must be in place to prevent cross-contamination. Separate rooms include a reagent preparation area, a specimen preparation area, and a amplification/detection area. The DNA-free area is the clean area and the area of amplified DNA is the contaminated area. The work flow is from clean to contaminated areas.

C. Hepatitis A virus (HAV) and genome

1. Introduction

HAV is a non-enveloped virus that belongs to the genus Hepatovirus, Picornaviridae family. HAV has a positive-polarity, single-stranded RNA that is approximately 7.5 kb in length (Nainan O et al., 2006). The genome consists of a 5′ untranslated region (UTR) of 734 to 740 nucleotides, a coding region of 2,225 to

2,227 nucleotides (regions P1, P2 and P3,) and a 3′ untranslated region of 40 to 80 nucleotides. The P1 region encodes the three major proteins of the viral capsid, VP1, VP2, and VP3. A fourth viral capsid protein (VP4), essential for virion formation, is not detected in mature viral particles. Each of the capsid proteins is cleaved from the precursor polyprotein by the viral protease 3C, encoded in the P3 region. The native con- formation of the capsid proteins VP1 and VP3 forms a single, dominant, serologic epitope on the viral capsid and elicits a neutralizing antibody response. Nonstructural proteins encoded in the P2 and P3 regions are predicted to function in RNA synthesis and virion formation. VPg (virion protein, genome linked), also encoded in the P3 region, is covalently linked to the 5′ genome terminus and involved in initiation of RNA synthesis (Nainan O. et al, 2006) (figure 1).

On table 2 are described the length (nt) of each region showed in the figure 1.

Figure 1. Hepatitis A genome structure

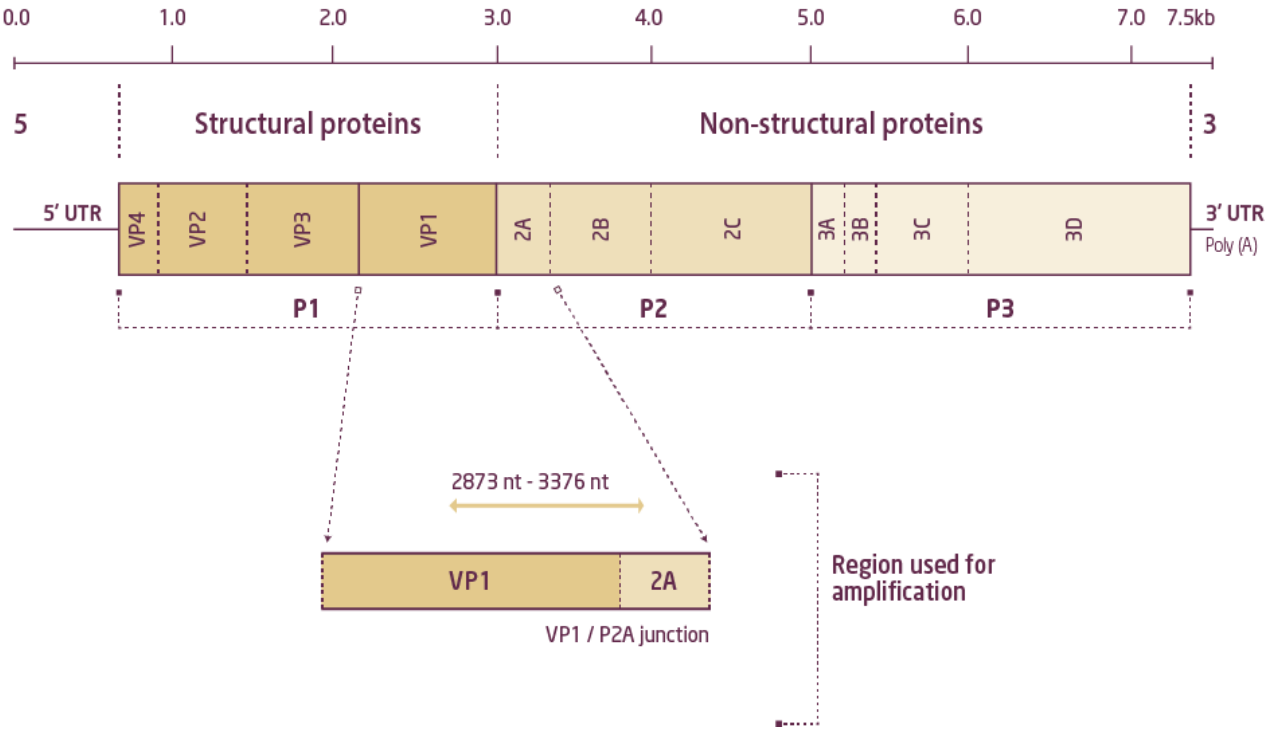

Table 1. Lengths (nt) of each HAV genomic region

Region		Length	Nucleotides
	5´UTR	734 (741)*	1-734
P1			
	VP4	62 - 68	735 - 803
	VP2	665	804 - 1469
	VP3	737	1470 - 2207
	VP1	900	2208 - 3107
P2			
	2A	567	3108 - 3674
	2B	312	3675.-.3995
	2C	1004	3996 - 5000
P3			
	3A	221	5001 - 5222
	VPg	68	5223 - 5291
	3C	656	5292 - 5948
	3D	1466	5949 - 7415
3' UTR		62	7416 - 7478

*Downstream AUG codon at nt 741 to 743 preferred for translation initiation.

2. HAV genome regions for typing

Different regions of the HAV genome have been used to characterize HAV strains and genotypes such as the C terminus of the VP3 region; the N terminus of the VP1 region; junction of the VP1/P2A regions; VP1-P2B regions and the entire VP1 region. However, no region has proved to be the best region to genotype HAV and to differentiate antigenic variants. The region selected (VP1/2A junction) to use in this protocol to type HAV strains was chosen because there is already a significant amount of information regarding sequences typed in this region which will allow us to improve our sequence comparison.

The protocol described here for typing HAV strains amplify a 503 nt fragment spanning the VP1/2A junction region that ranges from nt. 2873 to nt. 3376 (figure 1); Reference the HAV strain, genbank NC_001489. The resulting sequence is 460 nt.

D. Sequence-base typing methods

1. Type and quantity of human specimens use for molecular HAV detection

Conventionally HAV diagnosis is performed using sera/plasma specimens, but alternative stools or dried blood spots can be used (Desbois et al., 2009).

Table 2. Type and quantity of human specimens use for molecular tests

	Volume need to extract RNA (Minimum amount)	Transport and maintenance requirements
Serum or plasma samples	140 - 400 ul	Short term storage (<48 hours): room temperature or refrigerated or frozen. Storage longer than 48 hours: sera should be frozen (- 20ºC). International shipment on dry – ice.
Stools	10% stool suspension of 1 g of stool in 10ml viral transport medium	Refrigerated
Dried Blood spots	25 µl serum spotted on filter paper (1.1 cm diameter)	Room temperature

2. RNA extraction procedure

Viral nucleic acid extraction can be done using an automated system or manual extraction. RNA isolation Kit using automated MagNA Pure LC System (Roche), or manual QIAamp Viral RNA Mini Kit (Qiagen, Germany) are suitable for extractions following the manufacturer's instructions.

3. HAV genotyping by Two-step RT-PCR

The following protocols are for genotyping HAV using a conventional RT – PCR, and sequencing.

Reagents and equipment:

- 5X RT buffer Superscript III, Invitrogen
- PCR Nucleotide Mix 10mM, Roche
- Set of Primers for PCRs
- Superscript III RT 10000U (200U/µl), Invitrogen
- RNaseOUT 5000U (40 U/µl), Invitrogen·
- 0.1 M DTT, Invitrogen
- Taq polymerase buffer (1271318) Roche
- 10X Faststart Taq polymerase buffer (2161567) Roche·
- Taq Polymerase 5 U/µL
- Faststart Taq Polymerase 5 U/µL·
- Agarose
- GelRed dye
- 5x TBE
- ExoSAP-IT (USB)
- Set of Primers for sequencing
- Big Dye Terminator v3.1 (BDT), Applied Biosystems
- Sequence buffer (Big Dye Buffer), Applied Biosystems
- Thermocycler PCR/sequence PCR
- Perkin Elmer PE- 9700 Thermal Cycler

Table 3. Set of Primers for PCR (1st reaction and nested-PCR)

Name	Sequence (5' >3')	Direction	Region	PCR
HAV 6.1 codehop	TATGCYGT5TCWGG5GC5YTRGAYGG	F	VP1-2a	First-outer
HAV 10 codehop	TCYTTCATYTCWGTCCAYTTYTCATCATT	R	VP1-2a	First-outer
HAV 8.2 codehop	GGATTGGTTTCCATTCARATTGCNAAYTA	F	VP1-2a	nested-inner
HAV 11 codehop	CTGCCAGTCAGAACTCCRGCWTCCATYTC	R	VP1-2a	nested-inner

number 5 on sequence oligo´s means inosine (number depends on supplier); Y = C/T; W = A/T;
* R = A/G;N = any base

RT- PCR controls

- Positive control – The positive control used for the PCRs, is a tissue culture stock of 1* 10^7 TCID 50 /ml
- Negative control – RNAase free H_2O

Procedure

3.1 cDNA synthesis (RT- step)

a) cDNA Master mix

Table 4. Master mix

Reagent	Stock	Final concentration	Volume of reagent add per reaction (µl)
5x RT Buffer	5x	1x	2
dNTps	10 mM	1mM	1
HAV10 codehop primer inner	100 µM	5 µM	0.5
DTT	0.1M	5 mM	0.5
RNaseOUT	40 U/ ul	2 U/ ul	0.5
Superscript III RT	200 U/ ul	10 U/ ul	0.5
Final mix volume			5

b) Reaction setup:

1. Prepare cDNA master mixture
2. Add 5 µl of the RNA specimen to the 5 µl cDNA master mix (final volume = 10 µl)
3. Incubate according thermal conditions:

Table 5. Thermal conditions

Temperature (ºC)	Time (minute)
Room Temperature = 22	10
42	60
95	5

4. Prepare the PCR master mix while cDNA reactions are incubating OR cDNA can be stored at −20°C for further use.

3.2 Perform conventional PCR reactions

a) Prepare the PCR master mix (1st reaction)

Table 6. PCR master mixture

Reagent	Stock	Final concentration	Volume of reagent add per reaction (µl)
10x PCR Buffer (Taq pol 271318)	10x	1x	5
dNTPs	10 mM	0.2 mM	1
HAV 6.1 codehop primer	10 µM	1 µM	5
Taq polymerase	5 U/µl	0.05 U/ µl	0.5
H_2O (Rnase and DNAase free)			28.5
Final mix volume			40

b) Reaction set-up:

1. Prepare de master mix.
2. Add 40 µl of master mix to the cDNA tube (10 µl).
3. Mix and briefly centrifuge the tubes.
4. Run the thermal cycler program:

Table 7. Thermal cycler program

Step	Temperature (ºC)	Time (sec)
Hold	95	6 min
PCR (35 cycles)	95	30
	42	30
	60	45
Cooling	4	∞

c) Prepared the master mixture for Nested-PCR (2nd reaction)

Table 8. Nested-PCR master mix

Reagent	Stock	Final concentration	Volume of reagent add per reaction (µl)
10x PCR Buffer (FS Taq 2161567)	10x	1x	5
dNTPs	10 mM	0.2 mM	1
HAV 8.2 codehop primer	10 µM	0.8 µM	4
HAV 11 codehop primer	10 µM	0.8 µM	4
FS Taq polymerase	5 U/µl	0.05 U/ µl	0.5
H_2O (Rnase and DNAase free)			34.5
Final mix volume			49

d) Reaction set-up:

1. Prepare the nested- PCR master mix.
2. Add 1µl of the PCR product (1st reaction) to the 49 µl nested-PCR master mix.
3. Mix and briefly centrifuge the tubes.
4. Run the thermal cycler program:

Table 9. Thermal cycler program

Step	Temperature (ºC)	Time (sec)
Hold	95	6 min
PCR (40 cycles)	95	30
	60	20
	72	15
Cooling	4	∞

3.3 Product analysis

Run 10 µl each sample on 1.5% agarose gel made up with1X TBE buffer and containing GelRed dye according to manufacturer's instructions. Expected product size is 520 bp (VP1/2a), same size as the positive HAV control.

3.4 Product clean-up

It is necessary to remove RT-PCR component reagents prior to gene sequencing. Use the ExoSAP- IT Kit and follow the manufacturer's instructions.

3.5 Gene Sequencing

Table 10. Set of primers for sequencing

Name	Sequence (5' >3')	Direc-tion	Region	PCR
HAV 8.2 sequence	GGATTGGTTTCCATTCA	F	VP1-2a	sequence
HAV 11 sequence	CTGCCAGTCAGAACTCC	R	VP1-2a	sequence

a) **Prepare the master mix for sequencing**

Table 11. Sequencing master mix

Reagent	Final amount	Volume of reagent add per reaction (µl)
H_2O (RNase and DNase free)		8.75
Big Dye Buffer		7
Big Dye		1.25
Primer 1 or Primer 2	3 µM	1
Final mix volume		18

b) Reaction set-up

1. Prepare de master mix using ABI BigDye Terminator v3.1 Cycle Sequencing kits, Applied Biosystems.
2. Add 2 µl of clean PCR product to master mix.
3. Mix and briefly centrifuge the tubes.
4. Run the thermal cycler program:

Table 12. Thermal cycle program

Step	Temperature (C)	Time (sec)
Denaturation	95	10
PCR (25 cycles)	95	10
	50	5
	60	240
Cooling	4	∞

3.6 Sequence analysis with bionumerics or other software

a) Alignment

Each Forward sequence should be aligned with correspondent Reverse sequence using appropriate software.

b) Submission

Before submission to the database you should remove the codehop primers sequence in both ends of the fragment from the consensus sequence (final sequence 460 bp).

D. Additional protocol for clinical diagnosis purposes (not apply for typing)

This protocol is applied in case of uncertain IgM antibodies status, to confirm diagnosis.

1. Detection of hepatitis A virus (HAV) by two – step real-time PCR

In this protocol a fragment of 71bp at the 5' end of Non Translated Region (NTR) (nt504 – nt574) region of HAV genome (genbank NC_001489) is amplified. For the positive internal control EAV (Equine arteritis virus) is used. A fragment of 115 bp from 1ab gene is amplified. The amplified DNA is quantified by measuring the increase in fluorescence by probe hybridization and hydrolysis of a Taqman quencher/reporter probe.

Reagents and equipments:

- Random primers 20μg (500μg/ml), Promega
- PCR Nucleotide Mix 10mM, Roche·
- Water, DNase and RNase free
- 5x First Strand Buffer, Invitrogen
- 0.1 M DTT, Invitrogen
- RNaseOUT 5000U (40 U/μl), Invitrogen, cat n
- Superscript III RT 10000U (200U/μl), Invitrogen
- LightCycler TaqMan Master, Roche
- Q -solution (Qiagen)
- HAV primers and probes sets
- EAV (internal control) primers and probes set
- Plate centrifuge
- Real-Time PCR system (480 Light cycler, Roche)

Table 13. Set of Primers and probes sequences

Name (ID)	Sequence (5' >3')	Direction	Amplified Region	Expected product size
HAV 5'F	CAG TGG ATG CAT TGA GTG	F	5' NTR*	71 bp
HAV 5'R	CTA AGC ACA GAG AGG TC	R	5' NTR	
HAV 5'probe	CCT ARA GAC AGC CCT GAC	FAM/TAMRA (detection at 530 nm)	5' NTR	
EAV 2049F2	CTTGTGCTCAATTTACTGG	F	1 ab	115 bp
EAV 2147R2	CTATTCTGTACCTCTGCC	R	1 ab	
EAV 2102 probe	CTATTCTGTACCTCTGCCGCAAT	Texas Red-BHQ2 (detection at 610nm)	1ab	

Procedure

1.1 Perform cDNA synthesis (RT- step)

a) Prepare master mix A (Antisense Random Priming):

Assemble your reaction for one sample (or multiply for n samples) according the experiment volume for each reagent described below.

Table 14. Master mix A

Reagent	Stock	Final concentration	Volume of reagent add per reaction (µl)
Random primers	50 ng/µl	2.5 ng/ ul	1.5
dNTPs mix	10 mM	0.5 mM	1.5
H_2O (RNAase and DNase free)			5.8
Final mix volume			8.8

b) Prepare master mix B (RT Random Priming)

Table 15. Master mix B

Reagent	Stock	Final concen-tration	Volume of reagent add per reaction (µl)
5x RT Buffer	5x	1x	6
DTT	100 mM	5 mM	1.5
RNAseOUT	40 U/µl	2 U /ul	1.5
Superscript III RT	200 U/µl	1.33 U/µl	0.2
Final mix volume			9.2

c) Reaction setup:

1. Prepare master mixture A and B.
2. Add 12 µl of extracted RNA to the mix A and heat to 95ºC for 2 min.
3. Quickly chill the tubes on ice for at least 2 min and spin.
4. Add 9.2 µl of mix B to the 20.8 µl (8.8 µl mix A+ 12 µl RNA) and incubate according thermal conditions described below:

Table 16. Thermal conditions

Temperature (ºC)	Time (minute)
Room temperature = 22	5 -10
50	60
95	2

5. Spin and put tubes on ice
6. The cDNA is stored at −20°C for further use.

1.2 Perform Real-time PCR (LightCycler 480, Roche)

a) Prepare the PCR master mix

Table 17. PCR master mixture

Reagent	Stock	Final concentration	Volume of reagent add per reaction (µl)
Taqman Master	5X	1X	4
Q-solution	10 mM	1mM	2
HAV 5" F primer	10 µM	0.5 µM	1
HAV 5'R primer	10 µM	0.5 µM	1
HAV 5' probe	10 µM	0.25 µM	0.5
EAV 2049 F	10 µM	0.5 µM	1
EAV 2147 R	10 µM	0.5 µM	1
EAV 2102 probe	10 µM	0.25 µM	0.5
H_2O (RNase and DNAase free)			4
Final mix volume			15

b) Reaction setup

1. Prepare master mixture for real-time PCR.
2. Pipet 15 µl master mix in each of 96 wells plate.
3. Add 5 µl cDNA to each well that you are testing and seal the plate.
4. Centrifuge the plate 15 sec at 3000 rpm.
5. Put the plate in the real-time apparatus and carry out the HAV protocol with the following conditions:

Table 18. **Real-time** PCR program

STEPS	Temp Target (ºC)	Acquisition mode	Hold time (sec)	Ramp rate (ºC/sec)
Denaturation	95	None	600	4.4
Amplification (55 cycles)	95	None	20	4.4
	52	None	20	2.2
	72	Single	20	4.4
Cooling	40	none	30	2.2

6. When run is completed, click finish and select channel 530 for HAV and 610 for internal control setting to read the results.

7. Analyse real-time PCR results

   Use the LightCycler software to analyze the results.

   The Cp - value of HAV positive sample is usually between 20 and 39 cycles. The Cp of the EAV internal control is around 30 cycles.

References:

HAV Genome characterization

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