IVD For *in Vitro* Diagnostic Use

CE

HAV Real-TM

Handbook

Real Time Kit

REF V4-50FRT

50

NAME

HAV Real-TM

INTRODUCTION

Hepatitis A is a liver infection caused by the hepatitis A virus (HAV). HAV is usually spread from person to person when an uninfected person ingests food or beverages that have been contaminated with the stool of a person with the virus: faecal-oral transmission. Unlike hepatitis B and C, hepatitis A infection does not cause chronic liver disease and is rarely fatal, but it can cause debilitating symptoms. The symptoms of hepatitis A range from mild to severe, and can include fever, malaise, loss of appetite, diarrhea, nausea, abdominal discomfort, dark-colored urine and jaundice (a yellowing of the skin and whites of the eyes).

Improved sanitation and Hepatitis A immunization are the most effective ways to combat the disease. Adequate supplies of safe-drinking water and proper disposal of sewage within communities, combined with personal hygiene practices, such as regular hand-washing, reduce the spread of HAV.

INTENDED USE

HAV Real-TM is Real-Time amplification test for the qualitative detection of Hepatitis A (HAV) RNA in clinical specimens (plasma, feces, etc) and water.

PRINCIPLE OF ASSAY

HAV Real-TM Test is based on three major processes: isolation of HAV RNA from specimens, one-step reverse transcription of the RNA and Real Time amplification of the cDNA. *HAV* detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using specific primers and detection via fluorescent dyes. These dyes are linked with probes of oligonucleotides which bind specifically to the amplified product. The real-time PCR monitoring of fluorescence intensities allows the accumulating product detection without reopening of reaction tubes after the PCR run. **HAV Real-TM** PCR kit is a qualitative test which contain the Internal Control (IC). It must be used in the isolation procedure in order to control the process of each individual sample extraction and serves also to identify possible reaction inhibition.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (V4-50FRT)

Contents	Ref. V4-50FRT 55 reactions
Part N°2 – **"Controls"**[1]	
Negative Control (C-)*	2 x 0,5 ml
Pos *HAV*-RNA-rec**	0,1 ml
Internal Control (IC) RNA***	0,5 ml
RNA-buffer	0,6 ml
Pos Control cDNA HAV/IC (C+)	0,1 ml
Part N°3–**"HAV Real-TM"**	
RT-G-mix-2	0,015 ml
RT-PCR-mix-1-TM	0,6 ml
RT-PCR-mix-2-TM	0,3 ml
Hot Start Taq Polymerase	0,03 ml
M-MLV Revertase	0,015 ml

* *must be used in the isolation procedure as Negative Control of Extraction.*
** *must be used in the isolation procedure as Positive Control of Extraction.*
*** *add 10 µl of Internal Control RNA during the RNA purification procedure directly to the sample/lysis mixture*

MATERIALS REQUIRED BUT NOT PROVIDED

- Desktop microcentrifuge for "eppendorf" type tubes
- Vortex mixer
- Disposable gloves, powderless
- Biohazard waste container
- Refrigerator, Freezer
- Real Time Thermal cycler
- Reaction tubes or plate
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Tube racks
- 70% Ethanol (freshly prepared mixture of reagent grade 96% ethanol and distilled water)
- Acetone

WARNINGS AND PRECAUTIONS

IVD ***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

1. ⚠ Lysis Solution contains guanidine thiocyanate*. Guanidine thiocyanate is harmful if inhaled, or comes in contact with skin or if swallowed. Contact with acid releases toxic gas. (Xn; R: 20/21/22-36/37/38; S: 36/37/39).
2. Wear disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterward.
3. Use routine laboratory precautions. Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas. Do not pipette by mouth.
4. Do not use a kit after its expiration date.
5. Do not mix reagents from different kits.
6. Dispose all specimens and unused reagents in accordance with local regulations.
7. Heparin has been shown to inhibit reaction. The use of heparinized specimens is not recommended.
8. Avoid repeated thawing and freezing of the reagents, this may reduce the sensitivity of the test.
9. Once the reagents have been thawed, vortex and centrifuge briefly the tubes.
10. Prepare quickly the Reaction mix.
11. Specimens may be infectious. Use Universal Precautions when performing the assay.
12. Specimens and controls should be prepared in a laminar flow hood.
13. Handle all materials containing specimens or controls according to Good Laboratory Practices in order to prevent cross-contamination of specimens or controls.
14. Clean and disinfect all spills of specimens or reagents using a disinfectant such as 0,5% sodium hypochlorite, or other suitable disinfectant. Follow by wiping down the surface with 70% ethanol.
15. Avoid contact of specimens and reagents with the skin, eyes and mucous membranes. If these solutions come into contact, rinse immediately with water and seek medical advice immediately.
16. Material Safety Data Sheets (MSDS) are available on request.
17. Use of this product should be limited to personnel trained in the techniques of amplification.
18. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Extraction Area and moving to the Amplification Area. Do not return samples, equipment and reagents in the area where you performed previous step. Personnel should be using proper anti-contamination safeguards when moving between areas.

**** Only for Module No.2 and 3***

STORAGE INSTRUCTIONS

Part N° 1 and 2 must be stored at 2-8°C. Part 3 **"HAV Real-TM " must be stored at -20°C.**
The kit can be shipped at 2-8°C for 3-4 days but should be immediately stored at -20°C on receipt.

STABILITY

HAV Real-TM is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

HAV Real-TM can analyze RNA extracted from:

- ***Plasma:***
  - EDTA tubes may be used with the **HAV Real-TM Quant**. Follow sample tube manufacturer's instructions.
  - Whole blood collected in EDTA should be separated into plasma and cellular components by centrifugation at 800-1600 x g for 20 min within six hours. The isolated plasma has to be transferred into a sterile polypropylene tube. Plasma may be stored at 2-8°C for an additional 3 days. Alternatively, plasma may be stored at -18°C for up to one month or 1 year when stored at -70°C.
  - Do not freeze whole blood.
  - Specimens anti-coagulated with heparin are unsuitable for this test.
  - Thaw frozen specimens at room temperature before using.
  - Whole blood must be transported at 2-25°C and processed within 6 hours of collection. Plasma may be transported at 2-8°C or frozen.
  - Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.
- ***Feces:***
  - Prepare 20% feces suspension by adding in 5 ml tube of 4ml of Saline Solution and 1,0 gr (approx. 1,0 ml) of feces. Vortex to get the homogeneous suspension and centrifuge for 5 min to 7000-12000g and using a micropipette with a plugged aerosol barrier tip transfer in a new sterile 1,5 ml tube 50 µl of the supernatant.
- ***Water:***
  - Centrifuge 10-20 ml for 10 min at maximum speed. Discard the supernatant and leave about 100 µl of solution for DNA extraction

Specimens can be stored at +2-8°C for no longer than 12 hours, or frozen at -20°C to -80°C.
Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

RNA ISOLATION

Any commercial RNA/DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the "SAMPLE COLLECTION, STORAGE AND TRANSPORT" paragraph, could be used.
Sacace Biotechnologies recommends to use the following kits:

⇒ **Ribo-Sorb** (Sacace, REF K-2-1)
⇒ **Ribo-Virus** (Sacace, REF K-2/C)
⇒ **SaMag Viral Nucleic Acids Extraction kit** (Sacace, REF SM003)

Please carry out the RNA extraction according to the manufacturer's instructions. Add 10 µl of Internal Control during the RNA isolation procedure directly to the sample/lysis mixture.

RT AND AMPLIFICATION

Total reaction volume is **25 µl**, the volume of RNA sample is **10 µl**.

1 Prepare the reaction mix for required number of samples.

2 For N reactions mix in a new tube:

10*(N+1) µl of RT-PCR-mix-1-TM,
5.0*(N+1) µl of RT-PCR-mix-2
0.5*(N+1) µl of Taq Polymerase
0.25*(N+1) µl of RT-G-mix-2
0.25*(N+1) µl of MMlv

3 Vortex the tube, then centrifuge shortly. Add **15 µl** of prepared reaction mix into each tube.

4 Using tips with aerosol filter add **10 µl** of RNA samples obtained at the stage of RNA isolation and mix carefully by pipetting**.**
N.B. If the Ribo-Sorb isolation kit is used as a RNA extraction kit, re-centrifuge all the tubes with extracted RNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. N.B. don't disturb the pellet, sorbent inhibit reaction

5 Prepare for each panel 2 controls:

- add **10 µl** of **RNA-buffer** to the tube labeled PCR Negative Control;
- add **10 µl** of **Pos Control cDNA HAV(C+)** to the tube labeled C_{pos};

Create a temperature profile on your Real-time instrument as follows:

	Rotor type instruments[1]				*Plate type or modular instruments*[2]			
Stage	*Temp, °C*	*Time*	*Fluorescence detection*	*Cycle repeats*	*Temp,°C*	*Time*	*Fluorescence detection*	*Cycle repeats*
Hold	50	30 min	–	1	50	30 min	–	1
Hold	95	15 min	–	1	95	15 min	–	1
Cycling	95	5 s	–	5	95	5 s	–	10
	60	20 s	–		60	20 s	–	
	72	15 s	–		72	15 s	–	
Cycling 2	95	5 s	–	40	95	5 s	–	40
	60	20 s	FAM(Green), JOE(Yellow)		60	30 s	FAM, JOE/HEX/Cy3	
	72	15 s	–		72	15 s	–	

[1] *For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)*
[2] *For example, SaCycler-96™ (Sacace), CFX/iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7300/7500/StepOne Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid), LineGeneK® (Bioer)*

Rotor-type instruments

Settings

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct	Dynamic tube
FAM/Green	*from 3 Fl to 8 Fl*	0.03	10 %	On	On
JOE/Yellow	*from 3 Fl to 8 Fl*	0.03	10 %	On	On

Plate-type instruments

Settings

Channel	Threshold
FAM	The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set the threshold at a level where fluorescence curves are linear and do not cross curves of the negative samples.
HEX/Joe/Cy3	

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RESULTS ANALYSIS

1. The results are interpreted by the device software through the presence of crossing of fluorescence curve with the threshold line.
   cDNA of HAV is detected on the JOE(Yellow)/HEX/Cy3 channel and *IC* on the FAM (Green) channel.

Results are accepted as relevant if positive and negative controls of amplification and extraction are passed (see table 1).

Table 1. Results for controls

Control	Stage for control	Ct channel Fam (Green)	Ct channel Joe (Yellow)/ HEX/Cy3	Interpretation
NCS	RNA isolation	Pos (< 33)	Neg	Valid result
Pos HAV-RNA-rec	RNA isolation	Pos (< 33)	Pos (< 30)	Valid result
RNA-buffer	Amplification	Neg	Neg	Valid result
cDNA HAV C+	Amplification	Pos (< 33)	Pos (< 30)	Valid result

PERFORMANCE CHARACTERISTICS

The kit **HAV Real-TM** allows to detect HAV in 100% of the tests with a sensitivity of not less than 500 copies/ml.

TROUBLESHOOTING

1. Weak or absent signal of the IC (Fam (Green) channel): retesting of the sample is required.
   - The PCR was inhibited.
     ⇒ Make sure that you use a recommended RNA extraction method and follow the manufacturer's instructions.
     ⇒ Re-centrifuge all the tubes before pipetting the extracted RNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. Don't disturb the pellet, sorbent inhibit reaction.
   - The reagents storage conditions didn't comply with the instructions.
     ⇒ Check the storage conditions
   - The PCR conditions didn't comply with the instructions.
     ⇒ Check the PCR conditions and for the IC detection select the fluorescence channel reported in the protocol.
   - The IC was not added to the sample during the pipetting of reagents.
     ⇒ Make attention during the RNA extraction procedure.
2. Weak (Ct > 37) signal on the Joe (Yellow)/Cy3/HEX channel: retesting of the sample is required.
3. Joe (Yellow)/Cy3/HEX signal with Negative Control of extraction.
   - Contamination during RNA extraction procedure. All samples results are invalid.
     ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol.
     ⇒ Use only filter tips during the extraction procedure. Change tips among tubes.
     ⇒ Repeat the RNA extraction with the new set of reagents.
4. Any signal with Negative PCR Control.
   - Contamination during PCR preparation procedure. All samples results are invalid.
     ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
     ⇒ Pipette the Positive controls at the end.
     ⇒ Repeat the PCR preparation with the new set of reagents.

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
IVD	For *in Vitro* Diagnostic Use	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**C–**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
	Expiration Date	**IC**	Internal Control

* SaCycler™ is a registered trademark of Sacace Biotechnologies
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* ABI® is a registered trademark of Applied Biosystems
* LineGeneK® is a registered trademark of Bioer
* SmartCycler® is a registered trademark of Cepheid

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