Sacace BIOTECHNOLOGIES

HDV Real-TM

Handbook

Real Time PCR test for the qualitative detection of Hepatitis D Virus

REF V3-100FRT (Module No.1)

REF TV3-100FRT (Module No.2)

REF TV3-100FRT C (Module No.3)

Σ 100

NAME

HDV Real-TM

INTRODUCTION

Hepatitis D, also referred to as Hepatitis D virus (HDV) and classified as Hepatitis delta virus, is a disease caused by a small circular RNA virus. HDV is considered to be a subviral satellite because it can propagate only in the presence of the Hepatitis B virus (HBV). Transmission of HDV can occur either via simultaneous infection with HBV (coinfection) or via infection of an individual previously infected with HBV (superinfection).

Both superinfection and coinfection with HDV results in more severe complications compared to infection with HBV alone. These complications include a greater likelihood of experiencing liver failure in acute infections and a rapid progression to liver cirrhosis, with an increased chance of developing liver cancer in chronic infections. In combination with hepatitis B virus, hepatitis D has the highest mortality rate of all the hepatitis infections of 20%.

INTENDED USE

HDV Real-TM is a Real-Time test for the Qualitative detection of Hepatitis D Virus in human plasma and simultaneous detection of a HDV-specific Internal Control (IC), by dual color detection.

PRINCIPLE OF ASSAY

HDV Real-TM Test is based on three major processes: isolation of *virus* RNA from specimens, one-step reverse transcription of the RNA and Real Time amplification of the cDNA. *HDV* 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. **HDV 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 (V3-100FRT)

Contents	Ref. V3-100FRT 100 reactions
Part N°1 – **"Controls"**[1]	
Negative Control (C-)*	4 x 1,2 ml
Pos *HDV* RNA-rec**	4 x 0,06 ml
HDV IC RNA***	4 x 0,28 ml
RNA-buffer	2 x 1,2 ml
cDNA HDV/IC (C+)	4 x 0,1 ml
Part N°2–**"HDV Real-TM"**	
RT-G-mix-2	4 x 0,015 ml
RT-PCR-mix-1 HDV	4 x 0,3 ml
RT-PCR-mix-2	4 x 0,2 ml
TaqF Polymerase	4 x 0,02 ml
M-MLV Revertase	4 x 0,01 ml

Module No.2: Complete Real Time PCR test with RNA purification kit (TV3-100FRT)

Contents	Ref. TV3-100FRT 100 reactions
Part N° 1 – **"Ribo-Sorb":**	
Lysis Solution	2 x 22,5 ml
Washing Solution	2 x 20 ml
Sorbent	2 x 1,25 ml
RNA-eluent	10 x 0,5ml
Part N°2 – **"Controls"**[1]	
Negative Control (C-)*	4 x 1,2 ml
Pos *HDV* RNA-rec**	4 x 0,06 ml
HDV IC RNA***	4 x 0,28 ml
RNA-buffer	2 x 1,2 ml
cDNA HDV/IC (C+)	4 x 0,1 ml
Part N°3–**"HDV Real-TM"**	
RT-G-mix-2	4 x 0,015 ml
RT-PCR-mix-1 HDV	4 x 0,3 ml
RT-PCR-mix-2	4 x 0,2 ml
TaqF Polymerase	4 x 0,02 ml
M-MLV Revertase	4 x 0,01 ml

Module No.3: Complete Real Time PCR test with column RNA purification kit (TV3-100FRT C)

Contents	Ref. TV3-100FRT C 100 reactions
Part N° 1 – **"Ribo-Virus": (**see protocol cat. No. K-2/C)	
Part N°2 – **"Controls"**[1]	
Negative Control (C-)*	4 x 1,2 ml
Pos *HDV* RNA-rec**	4 x 0,06 ml
HDV IC RNA***	4 x 0,28 ml
RNA-buffer	2 x 1,2 ml
cDNA HDV/IC (C+)	4 x 0,1 ml
Part N°3–**"HDV Real-TM"**	
RT-G-mix-2	4 x 0,015 ml
RT-PCR-mix-1 HDV	4 x 0,3 ml
RT-PCR-mix-2	4 x 0,2 ml
TaqF Polymerase	4 x 0,02 ml
M-MLV Revertase	4 x 0,01 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

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

STABILITY

HDV 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

HDV Real-TM can analyze RNA extracted from plasma:

Note: Handle all specimens as if they are potentially infectious agents.

1. EDTA tubes may be used with the **HDV Real-TM**. Follow sample tube manufacturer's instructions.
2. 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.
3. Do not freeze whole blood.
4. Specimens anti-coagulated with heparin are unsuitable for this test.
5. Thaw frozen specimens at room temperature before using.
6. 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.
7. Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

RNA ISOLATION

The following isolation kits are recommended:

- ⇒ **Ribo-Sorb-100** (Sacace, REF K-2-1): sample volume 100 µl
- ⇒ **Ribo Virus 100**– spin column extraction kit (Sacace, REF K-2-C): sample volume 150 µl
- ⇒ **Magno-Virus** – Magnetic RNA/DNA extraction kit (Sacace REF K-2-16) sample volume 500 µl or 1000 µl
- ⇒ **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.

SPECIMEN AND REAGENT PREPARATION (reagents supplied with the Module No.2)

1. **Lysis Solution** and **Washing Solution** should be warmed up to 60–65^{o}C until disappearance of ice crystals.
2. Prepare required quantity of 1.5 ml polypropylene tubes including one tube for **Negative Control of Extraction** and one tube for **RNA Positive Control of Extraction**.
3. Add to each tube **10 µl** of **Internal Control** and **450 µl Lysis Solution**.
4. Add **100 µl** of **Samples** to the appropriate tube.
5. Prepare Controls as follows:
   - add **100 µl** of **Negative Control** to each of the two control tubes.
   - add **10 µl** of **Pos *HDV* RNA-rec** to the tube labeled C*pos*.
6. Vortex the tubes and centrifuge for 3-5 sec.
7. Vortex vigorously **Sorbent** and add **25 µl** to each tube.
8. Vortex for 5-7 sec and incubate all tubes for 10 min at room temperature. Vortex periodically.
9. Centrifuge all tubes for 45 sec at 10000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
10. Add **400 µl** of **Washing Solution** to each tube. Vortex vigorously and centrifuge for 45 sec at 10000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet.
11. Add **500 µl** of **Ethanol 70%** to each tube. Vortex vigorously and centrifuge for 45 sec at 10000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet.
12. Repeat step 11.
13. Add **400 µl** of **Acetone** to each tube. Vortex vigorously and centrifuge for 45 sec at 10000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet.
14. Incubate all tubes with open cap for 10 min at 60°C.
15. Resuspend the pellet in **50 µl** of **RNA-eluent.** Incubate for 5 min at 60°C and vortex periodically.
16. Centrifuge the tubes for 1 min at maximum speed (12000-16000 g). The supernatant contains RNA/DNA ready for use. The amplification can be performed on the same day of extraction. If this is not possible, the RNA preparations can be stored at -80°C for up to one month.

SPECIMEN AND REAGENT PREPARATION (reagents supplied with the Module No.3)

See protocol cat. No. K-2/C.

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,
5.0*(N+1) µl of RT-PCR-mix-2
0.5*(N+1) µl of TaqF 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 3 controls:

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

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	15 min	–	1	50	15 min	–	1
Hold	95	15 min	–	1	95	15 min	–	1
Cycling	95	5 s	–	5	95	5 s	–	5
	60	20 s	–		60	25 s	–	
	72	15 s	–		72	15 s	–	
Cycling 2	95	5 s	–	40	95	10 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)*

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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. Put the threshold line at such level where curves of fluorescence are linear.
   cDNA of HDV is detected on the JOE (Yellow)/HEX/Cy3 channel and *IC* on the FAM (Green) channel.

Results are accepted as relevant if both positive and negative controls of amplification along with negative control of 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 (< 37)	Neg	Valid result
Pos *HDV*-RNA-rec	RNA isolation	Pos (< 37)	Pos (< 37)	Valid result
RNA-buffer	Amplification	Neg	Neg	Valid result
cDNA HDV/IC (C+)	Amplification	Pos (< 37)	Pos (< 37)	Valid result

PERFORMANCE CHARACTERISTICS

Analytical sensitivity

The kit **HDV Real-TM** allows to detect *HDV* in 100% of the tests with a sensitivity of not less than 10 copies/ml. The detection was carried out on the control standard and its dilutions by negative plasma using the "Magno-Virus" extraction kit (Sacace REF K-2-16/1000) starting from a sample volume of 1 ml.

Analytical specificity

The analytical specificity of the primers and probes was validated with negative samples. They did not generate any signal with the specific *HDV* primers and probes. The specificity of the kit **HDV Real-TM** was 100%. The potential cross-reactivity of the kit **HDV Real-TM** was tested against the group control (HAV, HCV, HBV, HGV, HIV, HSV ½, EBV, CMV and other ones). It was not observed any cross-reactivity with other pathogens.

Target region: gene coding Dag

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.
        - ⇒ If the Ribo-Sorb isolation kit is used as a RNA extraction kit, 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.

EXPLANATION OF SYMBOLS

Catalogue Number

Research Use Only

Lot Number


Expiration Date

Contains reagents

Caution!

Version


Manufacturer


Temperature limitation

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