HDV Real-TM Quant

Handbook

Real Time PCR Kit for the Quantitative detection of Hepatitis D Virus in human plasma

REF **V3-100/2FRT**

Σ **100**

NAME

HDV Real-TM Quant

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

HDV Real-TM Quant assay is proposed for management of chronically infected patients to specify their evolutionary profiles before, during, and after treatment, and to study the natural history of HDV infection. It could also be used in large-scale prospective studies to define treatment guidelines and to evaluate the efficacy of new drugs.

PRINCIPLE OF ASSAY

HDV Real-TM Quant 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 Quant** PCR kit is a quantitative 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

Contents	Ref. V3-100/2FRT 120 reactions
Part N°1 – **"Controls"**[1]	
Negative Control (C-)*	4 x 1,2 ml
Pos1 *HDV* RNA-rec**	4 x 0,06 ml
Pos2 *HDV* RNA-rec**	4 x 0,06 ml
HDV IC-rec***	4 x 0,28 ml
TE-buffer	4 x 1,2 ml
Part N°2–**"HDV Real-TM Quant"**	
DTT	4 tubes
RT-PCR-mix-1-TM	4 x 0,3 ml
RT-PCR-mix-2-TM	4 x 0,2 ml
Hot Start Taq Polymerase	4 x 0,02 ml
M-MLV Revertase	4 x 0,01 ml
Standard HDV[1]	
• QS1 HDV/IC	4 x 0,1 ml
• QS2 HDV/IC	4 x 0,1 ml

* must be used in the isolation procedure as Negative Control of Extraction: add 100 µl of C– (Negative Control) to tube labeled Cneg;

** must be used in the isolation procedure as Positive Controls of Extraction. add 90 µl of C– (Negative Control) and 10 µl of HDV Rec Pos controls to the tubes labeled Cpos1 and Cpos2;

*** add 10 µl of Internal Control RNA during the RNA purification procedure directly to the sample/lysis mixture

[1] *Standards' and controls' concentrations are specific for every lot.*

MATERIALS REQUIRED BUT NOT PROVIDED

- RNA isolation kit (see 11. RNA isolation)
- Desktop microcentrifuge for "eppendorf" type tubes
- Vortex mixer
- Disposable gloves, powderless
- Biohazard waste container
- Refrigerator, Freezer
- Real Time Thermal cycler
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Tube racks

WARNINGS AND PRECAUTIONS

1. Wear disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterward.
2. Use routine laboratory precautions. Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas. Do not pipette by mouth.
3. Do not use a kit after its expiration date.
4. Do not mix reagents from different kits.
5. Dispose all specimens and unused reagents in accordance with local regulations.
6. Heparin has been shown to inhibit reaction. The use of heparinized specimens is not recommended.
7. Avoid repeated thawing and freezing of the reagents, this may reduce the sensitivity of the test.
8. Once the reagents have been thawed, vortex and centrifuge briefly the tubes.
9. Prepare quickly the Reaction mix.
10. Specimens may be infectious. Use Universal Precautions when performing the assay.
11. Specimens and controls should be prepared in a laminar flow hood.
12. Handle all materials containing specimens or controls according to Good Laboratory Practices in order to prevent cross-contamination of specimens or controls.
13. 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.
14. 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.
15. Material Safety Data Sheets (MSDS) are available on request.
16. Use of this product should be limited to personnel trained in the techniques of amplification.
17. 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.

STORAGE INSTRUCTIONS

The kit HDV Real-TM Quant 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 Quant 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 Quant 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 Quant**. 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.

* ***Serum can be used also as starting material on some occasions. In this cases the analytical sensitivity of the kit HDV Real-TM Quant is the same, but the clinical sensitivity may be significantly decreased because of the precipitation of viral particles during the clot retraction phase of serum preparation.***

RNA ISOLATION

The following isolation kits are recommended:*

- ⇒ **Ribo-Sorb** – sorbent extraction kit (Sacace, REF K-2-1): sample volume 100 µl
- ⇒ **Ribo Virus** – spin column extraction kit (Sacace, REF K-2-C): sample volume 150 µl
- ⇒ **DNA/RNA Prep** – extraction kit (Sacace, REF K-2-9): sample volume 100 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.

** if other manufacturers' RNA isolation kits are used, please contact our customer care service at specialists@sacace.com*

RT AND AMPLIFICATION

Note: Reaction Mix volume = 25 µl

1. Thaw one set of reagents, vortex and centrifuge briefly the tubes.
2. Prepare reaction tubes or PCR plate.
3. Prepare **Reaction Mix** for 30 samples: add into the tube with **DTT, 300 µl** of **RT-PCR-mix-1**, **200 µl** of **RT-PCR-mix-2, 20 µl** of **Hot Start Taq Polymerase** and **10 µl** of **M-MLV Revertase**. Vortex thoroughly and centrifuge briefly.

*(If it is necessary to test less than 25 samples add the entire content of the tube with **RT-PCR-mix-2** to the tube with **DTT** and vortex for at least 5-10 seconds. This mix is stable for 1 month at -20°C. Add for each sample (N) in the new sterile tube **7,5*N µl** of **RT-PCR-mix-1, 5,0*N µl** of the mixture of **RT-PCR-mix-2** and **DTT**, **0,5 *N µl** of **TaqF Polymerase** and **0,25*N µl** of **M-MLV**)*

4. Add **12,5 µl** of **Reaction Mix** into each tube.
5. Add **12,5 µl** of **extracted RNA** sample to the appropriate tube with Reaction Mix and mix by pipetting.

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!

6. Carry out the control amplification reactions:

PCE 1 -Add **12,5 µl** of **of RNA sample** isolated from **Pos1** ***HDV*** **RNA-rec** to the tube for positive control of extraction 1;

PCE 2 -Add **12,5 µl** of **of RNA sample** isolated from **Pos2** ***HDV*** **RNA-rec** to the tube for positive control of extraction 2;

C– -Add **12,5 µl of RNA sample** isolated from Negative Control to the tube for negative control of extraction;

QS_1 -Add **QS1 HDV/IC** to the two tubes for positive control of amplification 1 (**12,5 µl µl per each tube**);

QS_2 -Add **QS2 HDV/IC** to the two tubes for positive control of amplification 2 (**12,5 µl µl per each tube**).

Thoroughly mix by pipetting. Avoid air bubbling.

To rule out possible contamination, run an additional control reaction:

NCA -Add **12,5 µl** of **TE-Buffer** to the tube for negative control of amplification.

7. Close the tubes and transfer them into the Real Time PCR instrument.

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)*

*Deg/Sec = 2,0

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	

RESULTS ANALYSIS

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.

The concentrations of Positive samples (in the JOE/Yellow/HEX channel) and Internal Control (in the FAM/Green channel) are determined automatically on the basis of Ct values (by the crossing of the fluorescence curve with the threshold line set at a certain level) and of the specified values of DNA calibrators (QS1, QS2). HDV RNA concentration is calculated with the following formula:

$$\frac{\textit{HDV}\text{ cDNA copies per PCR-sample}}{\text{IC cDNA copies per PCR-sample}} \times \text{coefficient A} \times \text{coefficient B} = \text{copies } \textit{HDV}\text{ RNA/ml of plasma}$$

$$\text{Coefficient A} = \frac{100}{\text{extraction volume, µl}}$$

Coefficient A is useful to optimize the obtained value according to the sample starting volume recommended in the RNA extraction kit. For instance, the starting volume using the Ribo-Sorb and DNA/RNA Prep is 100 µl and therefore the coefficient A is 1 (in this case the coefficient may not be used), while the volume using Ribo-Virus kit is 150 µl and the coefficient A is 0,66.

Coefficient B (IC copies/ml of plasma) is specified in the Data card enclosed in the PCR kit and it's specific for each lot.

If the result is more than 100,000,000 copies/ml, it will be displayed as more than 100,000,000 copies HDV/ml. If the result is more than the linear measurement range, the sample can be analyzed after 10x dilution and the obtained result should be multiplied by 10.

If the result is less than 300 copies/ml in case of extraction from 100 µl, less than 150 copies/ml in case of extraction from 200 µl, or less than 30 copies/ml in case of extraction from 1 ml, then it will be displayed as less than 300, less than 150, or less than 30 copies HDV/ml, respectively.

Results are accepted as relevant if all positive and negative controls 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 (< 32)	Neg	Valid result
Pos1 *HDV*-RNA-rec	RNA isolation	Pos (< 32)	Pos (should be in the range specified in the Lot Data Card)	Valid result
Pos1 *HDV*-RNA-rec	RNA isolation	Pos (< 32)	Pos (should be in the range specified in the Lot Data Card)	Valid result
RNA-buffer	Amplification	Neg	Neg	Valid result
QS1 HDV/IC	Amplification	Pos	Pos	Valid result
QS2 HDV/IC	Amplification	Pos	Pos	Valid result

PERFORMANCE CHARACTERISTICS

Analytical sensitivity

The kit **HDV Real-TM Quant** allows to detect *HDV* in 100% of the tests with a sensitivity of not less than 30 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.

Linear range

The kit **HDV Real-TM Quant** is linear from 30 to 100 000 000 copies/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 Quant** was 100%. The potential cross-reactivity of the kit **HDV Real-TM Quant** 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

* SaCycler™ is a registered trademark of Sacace Biotechnologies
* CFX™ and iQ5™ are registered trademarks of Bio-Rad Laboratories
* Rotor-Gene™ is a registered trademark of Qiagen
* MX3005P® is a registered trademark of Agilent Technologies
* 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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