HBV/HDV Real-TM

Handbook

for use with RotorGene™ 3000/6000 (Corbett Research), SmartCycler® (Cepheid), iQ iCycler™ and iQ5™ (Biorad), Applied Biosystems® 7300/7500 Real Time PCR Systems (Applera), MX3000P® and MX3005P® (Stratagene)

REF **V56-50FRT, V56-100FRT**

VER **01.06.2010**

Σ **50**

Σ **100**

NAME
HBV/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
HBV/HDV Real-TM is a Real-Time test for simultaneous detection of hepatitis virus B (*HBV)* DNA and hepatitis virus D (*HDV)* RNA in the clinical materials (blood plasma)

PRINCIPLE OF ASSAY
HBV/HDV Real-TM Test is based on three major processes: isolation of *virus* RNA/DNA from specimens, one-step reverse transcription of the RNA and Real Time amplification of the cDNA. *HBV* and *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. **HBV/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.
The IC amplification product is detected in the FAM/Green channel, the *HBV* DNA amplification product is detected in the JOE/Yellow/HEX/Cy3 channel and the *HDV* cDNA amplification product is detected in the ROX/Orange//TexasRed channel. The Positive Control of Extraction and Positive Control *HBV/HDV*-rec are detected in FAM/Green (IC), JOE/Yellow/HEX/Cy3 (*HBV*) and ROX/Orange/TexasRed (*HDV*) channels.

MATERIALS PROVIDED

Contents	Ref. V56-50FRT 50 reactions	Ref. V56-100FRT 100 reactions
Part N°1 – **"Controls"**[1]		
Negative Control (C-)*	2 x 1,2 ml	4 x 1,2 ml
Pos *HBV/HDV* -rec**	2 x 0,06 ml	4 x 0,06 ml
HBV/HDV IC***	2 x 0,28 ml	4 x 0,28 ml
RNA-buffer	1,2 ml	2 x 1,2 ml
HBV/HDV cDNA/IC C+	2 x 0,1 ml	4 x 0,1 ml
Part N°2–**"HBV/HDV Real-TM"**		
RT-G-mix-2	2 x 0,015 ml	4 x 0,015 ml
RT-PCR-mix-1 HDV	2 x 0,3 ml	4 x 0,3 ml
RT-PCR-mix-2	2 x 0,2 ml	4 x 0,2 ml
TaqF Polymerase	2 x 0,02 ml	4 x 0,02 ml
M-MLV Revertase	2 x 0,01 ml	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

- RNA/DNA purification kit (see RNA extraction)
- Real Time Thermalcycler
- Workstation
- Pipettors (capacity 0,5-10 µl; 5-40 µl) with aerosol barrier
- Tube racks

WARNINGS AND PRECAUTIONS

1. Wear disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterward.
2. Do not pipette by mouth.
3. Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
4. Do not use a kit after its expiration date.
5. Dispose of all specimens and unused reagents in accordance with local regulations.
6. Biosafety Level 2 should be used for materials that contain or are suspected of containing infectious agents.
7. Clean and disinfect all spills of specimens or reagents using a disinfectant such as 0,5% sodium hypochlorite, or other suitable disinfectant.
8. 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.
9. Material Safety Data Sheets (MSDS) are available on request.
10. Use of this product should be limited to personnel trained in the techniques of amplification.
11. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment and reagents in the area where you performed previous step.

STORAGE INSTRUCTIONS

Part N° 1 – "Controls" must be stored at 2-8°C.
Part N° 2 – "HBV/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 2-8°C and -20°C immediately on receipt.

STABILITY

HBV/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

HBV/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 **HBV/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-50/100** (Sacace, REF K-2-1): sample volume 100 µl
⇒ **Ribo Virus 50/100**– 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**- Automatic RNA/DNA extraction (Sacace REF SM003) sample volume 400 µl

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,
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 amples obtained at the stage of RNA/DNA 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 **HBV/HDV cDNA/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), Rox(Orange)		60	30 s	FAM, JOE/HEX/Cy3, Rox/TexasRed	
	72	15 s	–		72	15 s	–	

[1] For example Rotor-Gene™ 3000/6000 (Corbett Research, Australia)

[2] For example, SaCycler-96™ (Sacace), iQ5™/iQ iCycler™ (BioRad, USA); Mx3000P/Mx3005P™ (Stratagene, USA), Applied Biosystems® 7300/7500 Real Time PCR (Applera), SmartCycler® (Cepheid)

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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.

Internal Control is detected in the FAM/Green fluorescence channel, HBV DNA is detected in the JOE/Yellow/HEX/Cy3 fluorescence channel, HDV cDNA is detected in the ROX/Orange/TexasRed fluorescence 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	Ct channel Rox/Orange/TexasRed	Interpretation
NCS	RNA/DNA isolation	Pos (< 37)	Neg	Neg	Valid result
Pos *HBV/HDV* - rec	RNA/DNA isolation	Pos (< 37)	Pos (< 37)	Pos (< 37)	Valid result
RNA-buffer	Amplification	Neg	Neg	Neg	Valid result
HBV/HDV cDNA/IC C+	Amplification	Pos (< 37)	Pos (< 37)	Pos (< 37)	Valid result

1. The sample is considered to be positive for *HBV* DNA if its Ct value is defined in the results grid in the JOE/HEX/Cy3/Yellow channel and if it does not exceed the threshold Ct value (Ct <38).
2. The sample is considered to be positive for *HDV* RNA if its Ct value is defined in the results grid in the ROX/Orange/TexasRed channel and if it does not exceed the threshold Ct value (Ct<38).
3. The sample is considered to be negative for *HDV* and *HBV* if its Ct value is not defined in the results grid (the fluorescence curve does not cross the threshold line) in the ROX/Orange/TexasRed and JOE/HEX/Cy3/Yellow channels and the Ct value in the results grid in the IC channel (Fam/Green) does not exceed the threshold Ct value (Ct<37).
4. The sample is considered to be equivocal in case of equivocal result in any channel. The PCR analysis is recommended to be repeated.

PERFORMANCE CHARACTERISTICS

Analytical sensitivity

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

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/DNA 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 extraction procedure.
2. Weak (Ct > 37) signal on the Joe (Yellow)/Cy3/HEX and ROX/Orange/TexasRed channels: retesting of the sample is required.
3. Joe (Yellow)/Cy3/HEX or ROX/Orange/TexasRed signal with Negative Control of extraction.
    - Contamination during the 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/DNA 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-96™ is a trademark of Sacace Biotechnologies
*iCycler™ and iQ5™ are trademarks of Bio-Rad Laboratories
* Rotor-Gene™ Technology is a registered trademark of Corbett Research
*MX3000P® and MX3005P® are trademarks of Stratagene
*Applied Biosystems® is trademarks of Applera Corporation
* SmartCycler® is a registered trademark of Cepheid

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