HBV Real-TM Qual

new version

Handbook

Real Time Kit for the Qualitative detection of Hepatitis B Virus in human plasma

REF V5-100 FRT

REF TV5-100 FRT

REF TV5-100 FRT C

Σ 100

NAME

HBV Real-TM Qual

INTRODUCTION

Hepatitis B virus (HBV) is a member of the Hepadnavirus family. The virus particle, (virion) consists of an outer lipid envelope and an icosahedral nucleocapsid core composed of protein. These virions are 42 nM in diameter and are sometimes referred to as "Dane particles". The nucleocapsid encloses the viral DNA and a DNA polymerase that has reverse transcriptase activity. The outer envelope contains embedded proteins that are involved in viral binding of, and entry into, susceptible cells. The virus is one of the smallest enveloped animal viruses, but pleomorphic forms exist, including filamentous and spherical bodies lacking a core. These particles are not infectious and are composed of the lipid and protein that forms part of the surface of the virion, which is called the surface antigen (HBsAg), and is produced in excess during the life cycle of the virus.

Acute infection with hepatitis B virus is associated with acute viral hepatitis – an illness that begins with general ill-health, loss of appetite, nausea, vomiting, body aches, mild fever, and dark urine, and then progresses to development of jaundice. It has been noted that itchy skin has been an indication as a possible symptom of all hepatitis virus types. The illness lasts for a few weeks and then gradually improves in most affected people. A few people may have more severe liver disease (fulminant hepatic failure), and may die as a result. The infection may be entirely asymptomatic and may go unrecognized.[16]

Chronic infection with hepatitis B virus either may be asymptomatic or may be associated with a chronic inflammation of the liver (chronic hepatitis), leading to cirrhosis over a period of several years. This type of infection dramatically increases the incidence of hepatocellular carcinoma (liver cancer). Chronic carriers are encouraged to avoid consuming alcohol as it increases their risk for cirrhosis and liver cancer. Approximately 300 million individuals are chronically infected with hepatitis B virus in the world. Enzyme-linked immunosorbent assay (ELISA)is still a main detection method for HBV infection, but ELISA result can neither efficiently reflect serum viral load or hepatitis activity nor monitor the efficacy of antiviral treatments. Currently, polymerase chain reaction (PCR) assay has been widely used for monitoring HBV load. HBV DNA monitoring has become an important tool to identify individuals with high viral replication, to monitor patients on therapy, and to predict whether antiviral therapy is successful. For example, with the introduction of new antiviral agents like lamivudine, close monitoring of patients has become increasingly important due to the occurrence of antiviral drug-resistant virus strains or the presence of flares after withdrawal of antiviral therapy.

INTENDED USE

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

PRINCIPLE OF ASSAY

HBV Real-TM Qual is a Real-Time test for the Qualitative detection of Hepatitis B Virus in human plasma. HBV DNA is extracted from plasma, amplified using Real Time Amplification and detected using fluorescent reporter dye probes specific for HBV or HBV IC. Internal Control (IC) serves as an amplification control for each individually processed specimen and to identify possible inhibition. IC is

detected in a channel other than the HBV DNA. Monitoring the fluorescence intensities during Real Time allows the detection of the accumulating product without having to reopen the reaction tube after the amplification.

MATERIALS PROVIDED

REF V5-100 FRT

HBV Real-TM Qual – PCR kit	Volume, ml	Quantity
HBV Positive Control (C+)[1]	0.06	4 tubes
Negative Control (C–)[2]	1.2	4 tubes
HBV Internal Control *IC*-rec (IC)[3]	0.28	4 tubes
RT-PCR-mix-1-TM	0.3	4 tubes
RT-PCR-mix-2-TM	0.2	4 tubes
Hot Start Taq Polymerase	0.02	4 tubes
TE-buffer[4]	1.2	2 tubes
Standard HBV		
• **QS2 HBV[5]**	0.1	4 tubes

1 must be used during the sample preparation procedure: add 90 µl of C- (Neg. Control) and 10 µl of C+ (HBV Positive Control-1) to the tube labeled Cpos;
2 must be used during the sample preparation procedure: add 100 µl of C- (Neg. Control) to the tube labeled Cneg;
3 add 10 µl of Internal Control during the DNA isolation procedure directly to the sample/lysis mixture;
4 must be used as Negative Control of Amplification;
5 must be used as Positive Control of Amplification.

REF TV5-100 FRT

HBV Real-TM Qual – PCR kit	Volume, ml	Quantity
HBV Positive Control (C+)[1]	0.06	4 tubes
Negative Control (C–)[2]	1.2	4 tubes
HBV Internal Control *IC*-rec (IC)[3]	0.28	4 tubes
RT-PCR-mix-1-TM	0.3	4 tubes
RT-PCR-mix-2-TM	0.2	4 tubes
Hot Start Taq Polymerase	0.02	4 tubes
TE-buffer[4]	1.2	2 tubes
Standard HBV		
• **QS2 HBV**[5]	0.1	4 tubes
Ribo-Sorb – Extraction kit	***Volume, ml***	***Quantity***
Lysis Solution	22.5	2 tubes
Washing Solution	20	2 tubes
Sorbent	1.25	2 tubes
RNA-eluent	0.5	10 tubes

1 must be used during the sample preparation procedure: add 90 µl of C- (Neg. Control) and 10 µl of C+ (HBV Positive Control-1) to the tube labeled Cpos;
2 must be used during the sample preparation procedure: add 100 µl of C- (Neg. Control) to the tube labeled Cneg;
3 add 10 µl of Internal Control during the DNA isolation procedure directly to the sample/lysis mixture;
4 must be used as Negative Control of Amplification;
5 must be used as Positive Control of Amplification.

REF **TV5-100 FRT C**

HBV Real-TM Qual – PCR kit	Volume, ml	Quantity
HBV Positive Control (C+)[1]	0.06	4 tubes
Negative Control (C–)[2]	1.2	4 tubes
HBV Internal Control *IC*-rec (IC)[3]	0.28	4 tubes
RT-PCR-mix-1-TM	0.3	4 tubes
RT-PCR-mix-2-TM	0.2	4 tubes
Hot Start Taq Polymerase	0.02	4 tubes
TE-buffer[4]	1.2	2 tubes
Standard HBV		
• **QS2 HBV[5]**	0.1	4 tubes
Ribo-Virus – Extraction kit	***Volume, ml***	***Quantity***
Buffer RAV1	35	2 tubes
Buffer RAW	30	2 tubes
Buffer RAV3 (concentrate)	12	2 tubes
Buffer RE	13	2 tubes
Rnase-free H_2O	13	2 tubes
Carrier RNA (lyophilized)	1 (mg)	2 tubes
Proteinase K	50 (mg)	2 tubes
Proteinase buffer	8	1 tube
Ribo Virus columns with collecting tubes (2ml)	/	100
Collecting tubes (2ml)	/	8 x 50

1 must be used during the sample preparation procedure: add 90 µl of C- (Neg. Control) and 10 µl of C+ (HBV Positive Control-1) to the tube labeled Cpos;
2 must be used during the sample preparation procedure: add 100 µl of C- (Neg. Control) to the tube labeled Cneg;
3 add 10 µl of Internal Control during the DNA isolation procedure directly to the sample/lysis mixture;
4 must be used as Negative Control of Amplification;
5 must be used as Positive Control of Amplification.

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MATERIALS REQUIRED BUT NOT PROVIDED

- DNA isolation kit (only ref. V5-100 FRT)
- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes (RCF max. 16,000 x g); Eppendorf 5415D or equivalent
- 60°C ± 2°C dry heat block
- Vortex mixer
- Pipettors (capacity 5-40 µl; 40-200 µl; 200-1000 µl) with aerosol barrier
- 1,5 ml polypropylene sterile tubes (Sarstedt, QSP, Eppendorf)
- Disposable gloves, powderless
- Tube racks
- Biohazard waste container
- 70% Ethanol (freshly prepared mixture of reagent grade 96% ethanol and distilled water)
- Acetone
- Refrigerator
- Real Time Thermal cycler
- Freezer

WARNINGS AND PRECAUTIONS

RUO For Research 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) - (only ref. TV5-100 FRT – TV5-100 FRT C)
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.

STORAGE INSTRUCTIONS

HBV Real-TM Qual must be stored at temperature ≤ -20°C. The kit can be shipped at 2-8°C for 3-4 days but should be stored at temperature ≤ -20°C immediately on receipt.

STABILITY

HBV Real-TM Qual Test 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. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

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

1. EDTA tubes may be used with the **HBV Real-TM Qual**. 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.

DNA ISOLATION

The following isolation kits are recommended:

- ⇒ **DNA/RNA-Prep** (Sacace, REF K-2-9)
- ⇒ **Ribo Virus 100**– spin column extraction kit (Sacace, REF K-2-C/100)
- ⇒ **Ribo-Sorb-100** (Sacace, REF K-2-1/100)
- ⇒ **Magno Virus (**Sacace, REF K-2-16/1000)
- ⇒ **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 during the RNA isolation procedure directly to the sample/lysis mixture.

PCR PREPARATION

1. Thaw one set of reagents, vortex and centrifuge briefly the tubes.
2. Prepare requested quantity of reaction tubes
3. To prepare the **Reaction Mix** add the following reagents in a new sterile tube:
   - **10 µl** of **RT-PCR-mix-1**,
   - **5 µl** of **RT-PCR-mix-2,**
   - **0.5 µl** of **Hot Start Taq Polymerase**,

   Vortex thoroughly and centrifuge briefly.
4. Add **15 µl** of **Reaction Mix** into each tube.
5. Add **10 µl** of **extracted DNA** sample to the appropriate tubes with Reaction Mix.
   (*If the Ribo-Sorb isolation kit is used as a RNA/DNA extraction kit, re-centrifuge all the tubes with extracted DNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. N.B. don't disturb the pellet, sorbent inhibit reaction!).*
6. Prepare for each run the following controls:
   - add **10 µl** of **QS2 HBV** into the tube labelled PCR Positive Control HBV. Mix by pipetting;
   - add **10 µl** of **TE-buffer** to the tube labelled PCR Negative Control. Mix by pipetting;

Insert the tubes in the thermalcycler and program the instrument.

Table of Reaction Mix preparation

		Reaction volume (with allowance for one extra sample)		
Reagent volume for one reaction, µl		**10.00**	**5.00**	**0.50**
Number of biological samples	Number of PCR reactions*	RT-PCR-mix-1	RT-PCR-mix-2	Hot Start Taq Polymerase
4	**7**	**80**	**40**	**4.0**
6	**9**	**100**	**50**	**5.0**
8	**11**	**120**	**60**	**6.0**
10	**13**	**140**	**70**	**7.0**
12	**15**	**160**	**80**	**8.0**
14	**17**	**180**	**90**	**9.0**
16	**19**	**200**	**100**	**10.0**
18	**21**	**220**	**110**	**11.0**
20	**23**	**240**	**120**	**12.0**
22	**25**	**260**	**130**	**13.0**
34	**37**	**380**	**190**	**19.0**
46	**49**	**500**	**250**	**25.0**

***** Number of biological samples + 2 controls of extraction + 1 control of PCR.

Amplification

Create a temperature profile on your instrument as follows:

	Rotor-type Instruments[1]			**Plate- or modular type Instruments[2]**		
Step	***Temperature, ℃***	***Time***	**Repeats**	***Temperature, ℃***	***Time***	**Repeats**
1	**95**	15 min	1	**95**	15 min	1
2	**95**	5 s	5	**95**	5 s	5
	60	20 s		**60**	20 s	
	72	15 s		**72**	15 s	
3	**95**	5 s	40	**95**	5 s	40
	60	20 s *fluorescent signal detection*		**60**	30 s *fluorescent signal detection*	
	72	15 s		**72**	15 s	

[1] For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)
[2] For example, SaCycler-96™ (Sacace), CFX-96/iQ5™ (BioRad); Mx3000/3005P™ (Agilent), ABI® 7300/7500/StepOne Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid)

Fluorescence is detected at the 4th step of Cycling 2 stage (60 °C) in FAM/Green and JOE/Yellow/Hex/Cy3 fluorescence channels.

Internal control is detected on the FAM (Green) channel, *HBV* DNA is detected on the JOE(Yellow)/HEX/Cy3 channel.

INSTRUMENT SETTINGS

Rotor-type instruments

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*from 5 Fl to 10 Fl*	*0.03*	*10 %*	*On*
JOE/Yellow	*from 5 Fl to 10 Fl*	*0.03*	*10 %*	*On*

Plate-type instruments like SaCycler-96* (Sacace), CFX-96 / iQ5 (BioRad), ABI 7300/7500/StepOne (Applied Biosystem):

Channel	Threshold
FAM	For each channel at a time set the threshold line at the level of 10-20 % of maximum fluorescence obtained for the Calibrator (QS1-HBV) in the last amplification cycle.
HEX	

** for SaCycler-96 instrument set "Criterion of the PCR positive result" to 70%, select "Multiplex Detection" as analysis type and "Threshold (Ct)" as Method.*

SmartCycler (Cepheid):

Channel	Threshold
FAM	20
Cy3	30

PROTOCOL AND DATA ANALYSIS:

Principle of interpretation is the following:

- The sample is considered **positive** for *HBV* DNA if the *Ct* value detected in the channel for the JOE/Yellow fluorophore does not exceed the boundary value specified in the table.
- The sample is considered **negative** for *HBV* DNA if the *Ct* value in the channel for the JOE/Yellow fluorophore is absent or if the *Ct* value detected in the channel for the JOE/Yellow fluorophore is greater than the specified boundary value and the *Ct* value in the channel for the FAM fluorophore does not exceed the boundary value specified in the table.
- The sample is considered **invalid** if an equivocal result (Ct value for Internal Control > boundary value or absent) is obtained in any of the channels. In this case, analysis of this sample should be repeated once again starting from the extraction.

Boundary value of the cycle threshold, Ct

Sample/Control	Channel for fluorophore	Ct boundary value	
		Rotor-type instruments	Plate-type instruments
C+	FAM/Green	25	29
	JOE/Yellow/Hex/Cy3	28	32
QS2 HBV	FAM/Green	28	32
	JOE/Yellow/Hex/Cy3	28	32
Clinical samples	FAM/Green	25	29
	JOE/Yellow/Hex/Cy3	35	35
C-	FAM/Green	25	29

The result of the analysis is considered reliable only if the results obtained for Positive and Negative Controls of amplification as well as for the Positive and Negative Control of extraction are correct (see table: Results for controls).

Results for controls

Control	Stage for control	*Ct* value in the channel for fluorophore	
		FAM	JOE
C–	DNA extraction	≤boundary value	Absent
PCE (C+)	DNA extraction	≤boundary value	≤boundary value
NCA	Amplification	Absent	Absent
QS2 HBV	Amplification	≤boundary value	≤boundary value

QUALITY CONTROL PROCEDURE

A defined quantity of Internal Control (IC) is introduced into each sample and control at the beginning of sample preparation procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition.

A negative control of extraction (NCE), negative amplification control (NCA), positive control of extraction (C+), positive amplification control (QS2 HBV) are required for every run to verify that the specimen preparation, the amplification and the detection steps are performed correctly.

If the controls are out of their expected range (see table Results for Controls), all of the specimens and controls from that run must be processed beginning from the sample preparation step.

PERFORMANCE CHARACTERISTICS

Analytical specificity

The analytical specificity of **HBV Real-TM Qual** PCR kit is ensured by selection of specific primers and probes and stringent reaction conditions. The primers and probes were tested for possible homologies to all sequences deposited in gene banks by sequence comparison analysis as well as with genomic DNA/RNA of the following organisms and viruses: hepatitis A virus; hepatitis C virus; hepatitis D virus; human immunodeficiency virus; cytomegalovirus; Epstein-Barr virus; herpes simplex virus types 1 and 2; *varicella-zoster* virus; human herpes virus types 6 and 8; parvovirus B19; tick-borne encephalitis virus; West Nile encephalitis; adenovirus types 2, 3, and 7; *Escherichia coli*; *Staphylococcus aureus; Streptococcus pyogenes, S.agalactiae;* and *Homo sapiens*. Cross-reactions for the above-mentioned organisms and viruses have not been detected.

Analytical sensitivity

The kit **HBV Real-TM Qual** allows to detect *HBV* DNA in 100% of the tests with a sensitivity specified in the table below:

Volume of sample for extraction, µl	RNA/DNA extraction kit	Analytical sensitivity, IU/ml
100	RIBO-sorb	100
200	Magno-Virus	50
1000	Magno-Virus	10

TROUBLESHOOTING

1. Weak (Ct > boundary value) 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.
    - 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. Any signal on the Joe/HEX/Cy3 channel with Negative Control of extraction (C-).
    - 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.
3. Any signal with Negative PCR Control (NCA).
    - 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

REF Catalogue Number

RUO For *Research Use Only*

LOT Lot Number

 Expiration Date

 Contains reagents

 Caution!

VER Version

 Manufacturer

 Temperature limitation

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