Sacace BIOTECHNOLOGIES

HBV Genotype A, B, C, D Real-TM

Handbook

Real Time PCR kit for detection and differentiation of hepatitis B virus genotypes A, B, C and D in HBV-positive clinical material

REF **R5-Gen**

Σ **50**

NAME

HBV Genotype A, B, C, D Real-TM

INTRODUCTION

Hepatitis B virus (HBV) infects nearly two billion people worldwide. The hepatitis B virus (HBV) is currently categorized into eight genotypes (A to H). The HBV genotyping system was first introduced by Okamoto et al. with four genotypic groups (A to D) distinguished by 8.0 % threshold divergence between the genomes of HBV.

Global distribution of HBV genotypes and subgenotypes

Genotype	Distribution	Subgenotype	Distribution
A	Pandemic, but mostly prevalent in the USA and Northwest Europe	Aa/A1	Asia and Africa
		Ae/A2	Europe and USA
B	Northern and Southeast Asia	Bj/B1	Japan
		Ba/B2	China, Taiwan, and Vietnam
		B3	Indonesia
		B4	Vietnam
		B5	Philippines
C	Asia and Pacific region	Ce/C1	East Asia
		Cs/C2	South-east Asia
		C3	Polynesia, Solomon Islands
		C4	Northeast Australia
D	Mediterranean, the Middle East, North America, and India	D1	India, Pakistan, Iran
		D2	India, Russia, and the Baltic region
		D3	India, Pakistan
		D4	Solomon Islands, Oceania
		D5	India
E	Africa and Tunisia	NA	NA
F	Central and South America	F1	Central America, Peru, Venezuela
		F2	Venezuela
		F3	Venezuela
		F4	Bolivia
G	France, Germany, and USA	NA	NA
H	Central and South America and Mexico	NA	NA

Numerous studies have investigated the clinical implications of HBV genotypes to disease severity, response to IFN, disease chronicity and HCC

Clinical implications of HBV genotypes to disease severity, response to IFN, disease chronicity, disease transmission, and transplantation outcomes

Clinical implications	HBV genotype			
	A	B	C	D
Disease severity	• Prevalent in AS	• Less prevalent in the LC and HCC group compared to AS • HCC recurrence and metastasis: 41%	• Highly prevalent in the LC and HCC group compared to AS • HCC recurrence and metastasis: 74%	• Associated with disease severity (Child's score 2 and 3) and LC
Response to IFN	• SR: 47% • Treatment duration: 4–15 months • Follow-up period: 12 months post-treatment	• Response rates: 39–50% • Treatment duration: 16–24 weeks • Follow-up period: 48–52 weeks	• Response rate: 13–17% • Treatment duration: 16–24 weeks • Follow-up period: 48–52 weeks	• SR: 23% • Treatment duration: 4–15 months • Follow-up period: 12 months post-treatment
Disease chronicity	• Some studies report this genotype in 28.6% of acute vs. 3% of chronic patients • Others report in only 10% of acute vs. 80% of chronic patients	• Genotype B was prevalent in 26–39% of acute and only 4–11.7% of chronic patients	• Found in 84.5% of chronic vs. 59.5% of acute hepatitis patients	• Some studies report this genotype in 80% of acute vs. 11% of chronic patients • Others report 48% of chronic patients vs. 13.6% of acute patients
Sexual and vertical transmission	• 52% of genotype A patients were infected through homosexual or bisexual contact and only 16% by heterosexual	• 67% of genotype B were infected through vertical route	• 55% of genotype C were infected through vertical route	• 15% of genotype D patients were infected through homosexual or bisexual contact and 42% by heterosexual conduct • 45% of genotype D were infected through vertical route
Transplant outcomes	• Recurrence after transplantation: 20% • Recurrence after: nearly 2.7 months • ACR: 90%	• Cumulative rates of viral breakthrough after 3 years: 4% • Recurrence is less common with less severe graft damage compared to genotype C	• Recurrence after transplantation: 50–70% • Recurrence after: nearly 10 months • ACR: 60% • Cumulative rates of viral breakthrough after 3 years: 21%	• Recurrence after transplantation: 80% • Recurrence after: nearly 9.1 months • ACR: 50%

ACR, acute cellular rejection; ALF, acute liver failure; AS, asymptomatic; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; IFN, interferon; LC, liver cirrhosis; SR, sustained responder.

Genotype C is associated with rapid fibrosis development, high HCC development rate, recurrence, and metastasis compared to genotype B. It appears that genotype D may be associated with more severe disease compared to A and that genotype F is linked to high mortality rates. HBeAg-positive patients infected with genotype B have a better response compared to those with genotype C, and genotype A responds better than genotype D, especially during short term therapy. Genotype A also responds well to PEG-IFN.

INTENDED USE

HBV Genotype A, B, C, D Real-TM PCR kit is an *in vitro* nucleic acid amplification test for detection and differentiation of HBV DNA genotypes A, B, C, D in HBV-positive clinical material (blood plasma) by using real-time hybridization-fluorescence detection.

HBV Genotype A, B, C, D Real-TM kit must be used only in HBV-positive clinical material previously tested by HBV NAT method (for instance with **Sacace HBV Real-TM Quant Dx** kit).

The **HBV Genotype A, B, C, D Real-TM** assay is not for screening blood, plasma, serum or tissue donors for HBV, or to be used as a diagnostic test to confirm the presence of HBV infection.

PRINCIPLE OF ASSAY

HBV genotypes detection includes DNA isolation from biological materials and real-time PCR amplification of HBV DNA. *HBV* detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using specific *HBV* genotype primers. In real-time PCR, the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes which bind specifically to the amplified product. The real-time monitoring of fluorescence intensities during the real-time PCR allows detection of the amplified product without re-opening the reaction tubes after the PCR run. **HBV Genotype A, B, C, D Real-TM** PCR kit uses “hot-start” Taq polymerase, which greatly reduces the frequency of nonspecifically primed reactions.

The *HBV* genotype A DNA is detected in the Cy5/Red channel

The *HBV* genotype B DNA is detected in the Rox/Texas Red/Orange channel.

The *HBV* genotype C DNA is detected in the FAM/Green channel

The *HBV* genotype D DNA is detected in the JOE/HEX/Yellow channel

MATERIAL PROVIDED

Reagent	*Volume (ml)*	*Amount*
PCR-mix-1-FRT HBV-G	0.6	1 tube
PCR-mix-2-TM	0.3	1 tube
Hot Start TaqF Polymerase	0.03	1 tube
HBV DNA B/A types (C+)	0.2	1 tube
HBV DNA C/D types (C+)	0.2	1 tube
DNA-buffer	0.07	1 tube
Negative Control (C–)*	1.2	2 tubes

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

HBV Genotype A, B, C, D Real-TM PCR kit is intended for 55 tests.

MATERIALS REQUIRED BUT NOT PROVIDED

- DNA extraction kit
- Real Time Thermalcycler
- Desktop microcentrifuge for “eppendorf” type tubes
- Vortex mixer
- Disposable gloves, powderless
- Biohazard waste container
- Refrigerator, Freezer
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Disposable polypropylene PCR tubes or strips
- Tube racks

- **WARNINGS AND PRECAUTIONS**

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

SAMPLE COLLECTION, STORAGE AND TRANSPORT

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

HBV Genotype A, B, C, D Real-TM PCR kit is intended for analysis of human plasma specimens*.

- *Blood plasma:* EDTA tubes may be used with the **HBV Genotype A, B, C, D Real-TM**. 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.

**** Serum can be used also as starting material on some occasions. In this cases the analytical sensitivity of the kit HBV Genotype A, B, C, D Real-TM 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***

DNA ISOLATION

Any commercial RNA/DNA isolation kit 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 Virus** – column extraction kit (Sacace, REF K-2/C)

⇒ **Magno-Virus** (Sacace, REF K-2-16-1000)

⇒ **SaMag Viral Nucleic Acids Extraction kit** (Sacace, REF SM003)

Please carry out the DNA extraction according to the manufacturer's instructions.

PROTOCOL (total reaction vol: 25 µl)

1. Before starting work, thaw, vortex and quick spin all reagents of the kit, making sure that there are no drops on the caps of the tubes.
2. Take the required number of PCR tubes for amplification of clinical and control samples (including one negative control of extraction and two controls of amplification).
3. To prepare the reaction mixture, mix in a new sterile tube the reagents per one reaction:
   - **10 µl of PCR-mix-1-FRT HBV-G,**
   - **5 µl of RT-PCR-mix-2-TM,**
   - **0,5 µl of Hot Start TaqF Polymerase**

Thoroughly vortex and quick spin the mixture, making sure that there are no drops on the caps of the tubes.

4. Add **15 µl** of the prepared reaction mixture to each PCR tube.
5. Add **10 µl** of **DNA samples** isolated from the clinical samples to each PCR tube.
6. Run the **control reactions**:

C– - Add **10 µl** of the **DNA sample** extracted from the Negative Control to the tube labeled C– (Negative Control of Extraction)

C+ - Add **10 µl** of **Positive Control B/A types (C+)** to the tube labeled $C+_{B/A}$ (Positive Control of Amplification).

- Add **10 µl** of **Positive Control C/D types (C+)** to the tube labeled $C+_{C/D}$ (Positive Control of Amplification).

NCA - Add **10 µl** of **DNA-buffer** to the tube labeled NCA (Negative Control of Amplification).

Make sure that there are no drops on the tube walls, otherwise vortex the tubes briefly and give a quick spin.

Table. REACTION MIXTURE PREPARATION

Reagent volume for one reaction, µl		**10,00**	**5,00**	**0,50**
N. samples	N. PCR reactions	PCR-mix-1	RT-PCR-mix-2	Polymerase
4	**8**	**90**	**45**	**4,5**
5	**9**	**100**	**50**	**5,0**
6	**10**	**110**	**55**	**5,5**
7	**11**	**120**	**60**	**6,0**
8	**12**	**130**	**65**	**6,5**
9	**13**	**140**	**70**	**7,0**
10	**14**	**150**	**75**	**7,5**
11	**15**	**160**	**80**	**8,0**
12	**16**	**170**	**85**	**8,5**

AMPLIFICATION PROGRAM

1. Create a temperature profile on your instrument as follows:

	***Rotor-type instruments*[1]**			***Plate-type instruments*[2]**		
Step	***Temperature, °C***	***Time***	***Cycles***	***Temperature, °C***	***Time***	***Cycles***
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 Fluorescence acquiring		60	30 s Fluorescence acquiring*	
	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® 7500 Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid)

* Fluorescent signal is detected in the channels for the FAM, JOE, ROX and Cy5 fluorophores.

INSTRUMENT SETTINGS

Rotor-type instruments (RotorGene 6000, RotorGene Q)

Channel	**Calibrate / Gain Optimisation**	Threshold	More Settings/ Outlier Removal	Slope Correct	Eliminate Cycles before
FAM/Green	5Fl - 10Fl	0,03	20%	ON	5
JOE/Yellow	5Fl - 10Fl	0,03	10%	ON	5
ROX/Orange	5Fl - 10Fl	0,03	10%	ON	5
Cy5/Red	5Fl - 10Fl	0,03	15%	ON	5

Plate- or modular type instruments (CFX™ (BioRad); SaCycler-96™ (Sacace Biotechnologies)). For result analysis, set the threshold line at a level where curves of fluorescence are linear and do not cross curves of the negative samples.

DATA ANALYSIS

The *HBV* genotype A DNA is detected in the Cy5/Red channel

The *HBV* genotype B DNA is detected in the Rox/Texas Red/Orange channel.

The *HBV* genotype C DNA is detected in the FAM/Green channel

The *HBV* genotype D DNA is detected in the JOE/HEX/Yellow channel

The results are interpreted by the real-time PCR instrument software by the crossing or not crossing of the threshold line by the fluorescence curve (in the middle of the linear section of the fluorescence curve for the positive control (C+) in logarithmic coordinates).

The result of amplification is considered **positive** if the fluorescence curve is characteristic of real-time PCR (sigmoid-shaped) and crosses the threshold line once in the significant fluorescence increase section and if the Ct value detected in the channel is below the threshold value specified in the below table.
The result of amplification is considered **negative** if the fluorescence curve is not S-shaped and if it does not cross the threshold line (the Ct value is absent).

Boundary values of the cycle threshold, Ct

	FAM/ Green	JOE/ Yellow/Cy3	ROX/ Orange/ TexasRed	Cy5/ Red
	HBV C	HBV D	HBV B	HBV A
Sample	**Ct boundary value**			
NCA	-	-	-	-
C–	-	-	-	-
Pos C+	<28	<28	<28	<28
Clinical samples	<38	<38	<38	<38

RESULTS INTERPRETATION

The results are interpreted by the real-time PCR instrument software by the crossing or not crossing of the threshold line by the fluorescence curve.

Sample contains *HBV* type A if the Ct value detected in the Cy5 channel is less than 38.

Sample contains *HBV* type B if the Ct value detected in the ROX channel is less than 38.

Sample contains *HBV* type C if the Ct value detected in the FAM channel is less than 38.

Sample contains *HBV* type D if the Ct value detected in the JOE/HEX channel is less than 38.
Results are accepted as significant only if both positive and negative controls passed correctly (see above the table for controls).

STABILITY AND STORAGE

All components of the **HBV Genotype A, B, C, D Real-TM** PCR kit are to be stored at or less minus 20ºC. They are stable until the expiration date indicated on the label. The kit can be shipped at 2-8°C but should be stored at -20°C immediately on receipt.

SPECIFICATIONS

Sensitivity

The analytical sensitivity of **HBV Genotype A, B, C, D Real-TM** PCR kit is specified in the table below.

Biological material	Pathogen agent	Sensitivity, copies/ml
Blood plasma, blood serum	*HBV* type A, B, C, D	5×10^2

The claimed analytical features of **HBV Genotype A, B, C, D Real-TM** PCR kit are guaranteed only when additional reagents kits “Magno-Virus” or “Ribo-Virus” are used.

Specificity

– The analytical specificity of **HBV Genotype A, B, C, D Real-TM** PCR kit is ensured by selection of specific primers and probes as well as by selection of strict reaction conditions. The primers and probes were checked 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 D virus; human immunodeficiency virus; cytomegalovirus; Epstein-Barr virus; herpes simplex virus types 1 and 2; chicken pox virus; human herpes virus types 6 and 8; parvovirus B19; West Nile encephalitis; adenovirus types 2, 3, and 7. flavivirus (TBEV, Japanese B encephalitis virus, Omsk hemorrhagic fever).

Cross-reactions for the above-mentioned organisms and viruses have not been detected.

TROUBLESHOOTING

- The absence of positive signal in C+ may indicate incorrect amplification program or other errors made during PCR amplification. In this case, PCR should be carried out once again.
- Detection of any Ct value in C– suggests contamination of reagents or samples. In this case, it is necessary to repeat the analysis of all tests starting from the isolation stage and to take measures for detecting and eliminating the source of contamination.

KEY TO SYMBOLS USED

	Manufacturer		Temperature limitation
	Use by	LOT	Batch code
REF	Catalogue number	VER	Version
	Consult instructions for use		Caution
	Contains sufficient for <n> tests	C+	Positive control of amplification
		C–	Negative control of extraction

*SaCycler™ is a registered trademark of Sacace Biotechnologies
*CFX™ is a registered trademark of Bio-Rad Laboratories
* Rotor-Gene™ Technology is a registered trademark of Qiagen
* MX3005P® is a registered trademark of Agilent Technologies
*ABI® is a registered trademark of Applied Biosystems
* SmartCycler® is a registered trademark of Cepheid

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