HCV/HBV/HIV Real-TM Handbook*

Real Time PCR Kit

REF **V50-100FRT**

VER **07.10.2010**

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INTRODUCTION
Transfusion-associated transmission risk of infectious diseases has been reported worldwide. Over the past two decades, a long series of specific and non-specific measures have been introduced into the screening of blood donations in order to reduce the residual risk of transmission of bloodborne viruses. The latest specific measure has been viral nucleic acid testing (NAT), introduced by the European plasma industry in 1995, and subsequently introduced for blood donations in many countries. NAT was implemented to reinforce the safety of the blood supply; it can detect acute viral infections during the 'window period', that are not detected by the serological screening methods.
It should be noted that, contrary to the classical serologic screening methods that are always used in single donation testing, current NAT procedures usually demand pooling of blood donation samples due to the format of the employed platforms. Today, NAT implementation for HCV, HBV and HIV-1 is taken for granted in most high-income countries to ensure the maximal viral safety.

INTENDED USE
The **HCV/HBV/HIV Real-TM** kit is a qualitative in vitro test for comprehensive single-assay Real Time detection of Human Immunodeficiency Virus (HIV) RNA, hepatitis C Virus RNA and hepatitis B Virus DNA in human plasma with simultaneous detection of Internal Control (IC). This kit is intended for use as a donor screening test to detect HIV RNA, HCV RNA and HBV DNA in plasma from individual donors which may be screened as individual samples or may be tested in pools comprised of equal aliquots. The recommended quantity of the samples in one pool must be not more than 5-10 (100-200 µl of the plasma for each sample).

PRINCIPLE OF ASSAY
The **HCV/HBV/HIV Real-TM** kit is a Real-Time test for the qualitative detection of HIV RNA, HCV RNA and HBV DNA in human plasma. RNA/DNA is extracted from plasma, amplified using RT-amplification and detected using fluorescent reporter dye probes specific for HCV, HBV, HIV and IC. IC is an Internal Control and represents recombinant RNA-containing-structure which carried through all steps of analysis from nucleic acid extraction to PCR amplification-detection. Internal Control (IC) serves as an extraction and amplification control for each individually processed specimen and to identify possible reaction inhibition. HCV cDNA is detected on the Fam (Green) channel, HIV cDNA on the Joe (Yellow)/HEX/TET/Cy3 channel, HBV DNA on the Rox (Orange)/TexasRed channel and IC on the Cy5 (Red) channel.

MATERIALS PROVIDED
Part N° 1 – **"Controls"**
Part N° 2– **"HCV/HBV/HIV Real-TM":** RT Real Time;

Contents	**Ref. V50-100FRT 100 reactions**
Part N°1 – **"Controls"**	
Internal Control (IC) Rec[1]	2 x 0,5 ml
HCV/HBV/HIV Rec Pos C+[2]	2 x 0,2 ml
NCS (Neg. Control Sample) [2]	2 x 1,2 ml
Part N°2–**"HCV/HBV/HIV Real-TM"**	
RT-PCR-mix-1-TM	2 x 0,3 ml
RT-PCR-mix-2-TM	2 x 0,3 ml
Hot Start Taq Polymerase	2 x 0,03 ml
M-MLV Revertase	2 x 0,015 ml
RT-G-mix-2	2 x 0,015 ml
TE-buffer	2 x 1,2 ml
Pos HCV/HBV/HIV C+	2 x 0,1 ml

[1]*must be used during the sample preparation procedure (see RNA/DNA isolation)*
[2] *must be used during the sample preparation procedure: add 100 µl of C– (Negative Control) to labeled Cneg; add 90 µl of C– (Negative Control) and 10 µl of HCV/HBV/HIV Rec Pos C+ to the tube labeled Cpos1*

MATERIALS REQUIRED BUT NOT PROVIDED

- RNA/DNA isolation kit (see RNA/DNA isolation)
- Microcentrifuge for “eppendorf” type tubes
- Vortex mixer
- Disposable gloves, powderless
- Biohazard waste container
- Refrigerator, Freezer
- Real Time PCR System
- Microcentrifuge for Smart Cycler’s reaction tubes
- 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

Part N° 1 – **“Controls”** must be stored at 2-8°C.
Part N° 2 – **“HCV/HBV/HIV 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

HCV/HBV/HIV Real-TM 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 **HCV/HBV/HIV 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/DNA ISOLATION

The following isolation kits are recommended:

- ⇒ **Ribo-Sorb-100** (Sacace, REF K-2-1/100): sample volume 100 µl
- ⇒ **Ribo Virus 100**– spin column extraction kit (Sacace, REF K-2-C/100): sample volume 150 µl
- ⇒ **Magno-Virus** – Magnetic RNA/DNA extraction kit (Sacace REF K-2-16/200 or Sacace REF K-2-16/1000) sample volume 500 µl or 1000 µl from pool samples (up to 10 samples in a pool)
- ⇒ **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/DNA isolation procedure directly to the sample/lysis mixture.

REAGENT PREPARATION

Note: Reaction Mix volume = 25 µl

1. Thaw one set of reagents, vortex and centrifuge briefly the tubes.
2. Prepare reaction tubes.
3. Prepare **Reaction Mix** for 50 samples: add into the tube with **RT-PCR-mix-1-TM (0,3 ml) 300 µl** of **RT-PCR-mix-2-TM, 30 µl** of **TaqF Polymerase**, **15 µl** of **M-MLV Revertase** and **15 µl** of **RT-G-mix-2**. Vortex thoroughly and centrifuge briefly.

*(If it is necessary to test less than 50 samples add for each sample (N) in the new sterile tube **5*N+1 µl** of* **RT-PCR-mix-1-TM**, ***5*N+1 µl** of* **RT-PCR-mix-2-TM,** ***0,5 *N+1 µl** of **TaqF Polymerase, 0,25*N+1 µl** of **M-MML** and **0,25*N+1 µl** of* **RT-G-mix-2***)*

Example of mix preparation:

		Reagents volume for the quantity of tests (N+1)				
Reagents volume for 1 reaction, *µl*		**5.00**	**5.00**	**0.50**	**0.25**	**0.25**
Q.ty of clinical samples	**Number of PCR reactions**	**RT-PCR-mix-1-TM**	**RT-PCR-mix-2-TM**	***TaqF Polymerase***	**RT-G-mix-2**	***M-MML***
4	**8**	**45**	**45**	**4.5**	**2**	**2**
6	**10**	**55**	**55**	**5.5**	**3**	**3**
8	**12**	**65**	**65**	**6.5**	**3.5**	**3.5**
10	**14**	**75**	**75**	**7.5**	**3.5**	**3.5**
12	**16**	**85**	**85**	**8.5**	**4.5**	**4.5**
14	**18**	**95**	**95**	**9.5**	**4.5**	**4.5**
16	**20**	**105**	**105**	**10.5**	**5.5**	**5.5**
30	**34**	**175**	**175**	**18**	**9**	**9**
40	**44**	**225**	**225**	**23**	**12**	**12**
50	**54**	Whole tube	Whole tube	Whole tube	**15**	Whole tube

4. Add **10 µl** of **Reaction Mix** into each tube.
5. Add **15 µl** of **extracted RNA/DNA** sample to the appropriate tube with Reaction Mix and mix by pipetting avoiding air bubble.
   If the Ribo-Sorb isolation kit is used as a RNA/DNA 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. Prepare for each panel 2 controls:
   - add **15 µl** of **TE-buffer** to the tube labeled Negative Control;
   - add **15 µl** of **Pos HCV/HBV/HIV C+** to the tube labeled Positive Control;
7. Transfer the tubes in the thermalcycler.

TEMPERATURE PROFILE

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*
1	50	20 min	–	1	50	20 min	–	1
2	95	15 min	–	1	95	15 min	–	1
3	95	20 s	–	4	95	20 s	–	4
	46	40 s	–		46	40 s	–	
4	95	5 s	–	42	95	5 s	–	42
	60	40 s	–		60	40 s	–	
	45	30 s	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red		45	40 s	FAM, JOE/HEX, ROX/Texas Red, Cy5	

[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)

TEMPERATURE PROFILE for SmartCycler® (Cepheid)

Create a temperature profile on your SmartCycler® instrument as follows:

50°c 1200sec
95°c 900sec
(95°c 20sec; 46°c 40sec) 10 time Ramp 1°C/sec
(95°c 5sec; 60°c 40sec; 40°c 40sec) 38 time Ramp 1°C/sec

RESULTS ANALYSIS

1. The results are interpreted by the device software through the presence of crossing of fluorescence curve with the threshold line.
   HCV cDNA is detected on the Fam (Green) channel, HIV cDNA on the Joe (Yellow)/HEX/TET/Cy3 channel, HBV DNA on the Rox (Orange)/TexasRed channel and IC on the Cy5 (Red) channel

Results are accepted as relevant if positive and negative controls of amplification along with negative and positive controls of extraction are passed (see table 1).

Table 1. Results for controls

Control	Stage for control	Fam (Green)	Joe (Yellow)/ HEX/Cy3	Rox/(Orange)/ TexasRed	Cy5 (Red)	Interpretation
NCS	RNA/DNA isolation	NEG	NEG	NEG	POS	Valid result
HCV/HBV/HIV Rec Pos C+	RNA/DNA isolation	POS	POS	POS	POS	Valid result
TE-buffer	Amplification	NEG	NEG	NEG	NEG	Valid result
Pos HCV/HBV/HIV C+	Amplification	POS	POS	POS	POS	Valid result

The Ct values for the controls are specific for every lot (reported on the HCV/HBV/HIV Real-TM Data Card)

- ✓ The sample is considered to be positive for *HCV* if in the channel Fam (Green) the value of **Ct** is lower than 33.
- ✓ The sample is considered to be positive for *HIV* if in the channel Joe (Yellow)/HEX/TET/Cy3 the value of **Ct** is lower than 33.
- ✓ The sample is considered to be positive for *HBV* if in the channel *Rox (Orange)/TexasRed* the value of **Ct** is lower than 33.
- ✓ The sample is considered to be negative if in the channels Fam (Green), *Joe* (Yellow)/HEX/TET/ Cy3, Rox(Orange)/ TexasRed the Ct value is not determined (the fluorescence curve does not cross the threshold line) and in the results table on the channel Cy5(Red) the Ct value is lower than 33.

PERFORMANCE CHARACTERISTICS

Analytical specificity

Analytical specificity of the primers and probes was validated with 90 negative samples. They did not generate any signal with the **HCV/HBV/HIV Real-TM** kit primers and probes. The specificity of the kit **HCV/HBV/HIV Real-TM** was 100%
The potential cross-reactivity of the kit **HCV/HBV/HIV Real-TM** was tested also against the group control listed in the following table. It was not observed any cross-reactivity with these pathogens.

Analytical sensitivity

The sensitivity of the **HCV/HBV/HIV Real-TM** kit is:

- HCV RNA – 10 IU/ml
- HBV DNA – 5 IU/ml
- HIV RNA – 20 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.

TROUBLESHOOTING

1. Weak (Ct > 33) signal of the IC (Cy5(Red) 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. Weak (Ct > 33) signal on one of the channels: retesting of the sample is required.
3. Any signal on Fam, Joe/HEX/TET/ Cy3 or Rox channel 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

For *Research Use Only*

Lot Number


Expiration Date

Contains reagents


Caution!

Version


Manufacturer


Temperature limitation

*SaCycler-96™ is a registered 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

* only for certain countries


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