HCV Real-TM Quant

new version

Handbook

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

REF V1-100/2 FRT

REF TV1-100/2 FRT

REF TV1-100/2 FRT C

100

NAME

HCV Real-TM Quant

INTRODUCTION

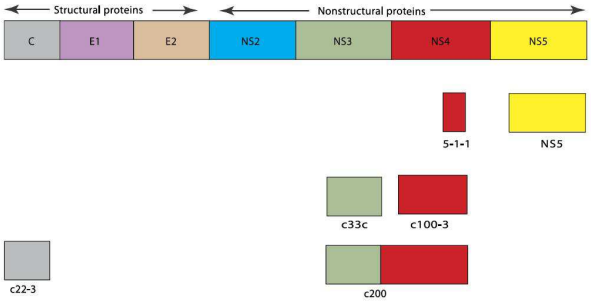
Hepatitis C virus (HCV) is a single-stranded RNA virus that belongs to the family Flaviviridae. In 1974 HCV was initially recognized as non-A, non-B hepatitis virus (NANBH) until 1989 when the etiologic agent was cloned. It is estimated by the World Health Organization that worldwide there are 170 million HCV-infected persons.

The primary mode of HCV transmission is the exposure to infected human blood via intravenous, drug use or unscreened transfusions. Nosocomial HCV transmission during dialysis, colonoscopy, and surgery has also been reported. Perinatal and sexual transmission of the virus is inefficient, but occurs more frequently if the HCV-infected mother or sexual partner is also infected with human immunodeficiency virus type 1.

Most people with acute HCV infection are asymptomatic or have mild symptoms (fatigue, nausea, jaundice) but they are unable to clear the virus and in approximately 80% of cases this leads to chronic infection. In 15 to 20% of patients chronic HCV infection progresses at a variable rate to cirrhosis, with a 1 to 4% annual risk of developing hepatocellular carcinoma.

The discovery of HCV genome in 1989 by Choo et al, paved the way for the development of serological and molecular assays for viral hepatitis C. In the first generation of an enzyme-linked immunosorbent assay (ELISA), wells of microtitre plates were coated with purified recombinant antigen c100-3 which was derived from the non-structural 4 (NS4) region of the HCV genome. However, ELISA-1 was associated with a high percentage (50% to 70%) of false positive results among low-risk blood donors and in the presence of hyperglobulinemia. Thus, second-generation anti-HCV ELISAs were developed. ELISA-2 by Ortho Diagnostics contained recombinant antigens from the core (c22-3), NS3 region (c33c), and NS4 region (c100-3) as well as a part of c100-3, named 5-1-1. Third generation anti-HCV ELISA was introduced in Europe in 1993 and in the USA in 1996. In addition to the antigens of ELISA-2, third-generation anti-HCV ELISA uses a NS5 region antigen of the viral genome. However, synthetic peptide antigens (c22 and c-100) replaced recombinant antigens of ELISA-2. Other manufactures, for example Abbott Diagnostics, used recombinant antigens derived from the same regions of HCV genome. Despite the increased sensitivity and specificity with each generation of ELISA, false-positive antibody results continue to be observed, particularly among low-risk blood donors. Thus, supplemental or confirmatory assays were developed in parallel with ELISA. The recombinant immunoblot assay (RIBA) was used extensively to confirm the presence or the absence of antibody to HCV epitopes. In RIBA, recombinant or peptide HCV antigens are blotted as separate bands onto a nitrocellulose strip flanked by a weak-positive (Level I) and a moderately positive (Level II) strip control.

Fig. 1 Genome organization of HCV and antigens licensed for diagnostic use



Since ELISA and RIBA are antibody tests, the positivity of either one or both does not necessarily indicate current HCV infection, as patients who have recovered from infection may remain anti-HCV positive for many years. Conversely, during seroconversion, antibody tests may be negative.

The direct molecular biology detection of HCV RNA by reverse-transcriptase polymerase chain reaction (RT-PCR) is considered the gold standard for the diagnosis of HCV infection.

INTENDED USE

HCV Real-TM Quant is a Real-Time test for the quantitative detection of Hepatitis C Virus in human plasma and simultaneous detection of a HCV-specific Internal Control (IC), by dual color detection.

PRINCIPLE OF ASSAY

HCV Real-TM Quant is a Real-Time test for the quantitative detection of Hepatitis C Virus in human plasma. HCV RNA is extracted from plasma, amplified using RT-amplification and detected using fluorescent reporter dye probes specific for HCV or HCV IC. HCV 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. IC is detected in a channel other than the HCV RNA.

Monitoring the fluorescence intensities during Real Time allows the detection of the accumulating product without having to re-open the reaction tube after the amplification.

MATERIALS PROVIDED

REF V1-100/2 FRT

HCV Real-TM Quant – PCR kit	*Volume, ml*	*Quantity*
DTT	–	4 tubes
RT-PCR-mix-1-TM *HCV*	0.3	4 tubes
RT-PCR-mix-2-FRT	0.2	4 tubes
Polymerase (TaqF)	0.02	4 tubes
TM-Revertase (MMlv)	0.01	4 tubes
QS1 *HCV*[1]	0.1	4 tubes
QS2 *HCV*[1]	0.1	4 tubes
Negative Control (C–)[2]	1,2 ml	4 tubes
TE-buffer[3]	1.2	4 tubes
Positive Control-1-*HCV*[4]	0.06	4 tubes
Positive Control-2-*HCV*[4]	0.06	4 tubes
Internal Control *IC*-rec (IC)[5]	0.28	4 tubes

1 *must be used as Calibration standards. Prepare two (2) tubes for each standard during the amplification procedure. Concentrations are specific for every lot (see DataCard).*
2 *must be used in the RNA/DNA extraction procedure as Negative Control of Extraction.*
3 *must be used in the amplification procedure as Negative Control of Amplification.*
4 *must be used in the RNA/DNA extraction procedure as Positive Controls of Extraction. If the extraction kit starts from 100 µl, add 90 µl of Negative Control (C-) and 10 µl of Positive Control-1-HCV and Positive Control-2-HCV to the corresponding tubes labeled Cpos1 and Cpos2. For example, if it starts from 400 µl add 390 µl of Negative Control (C-) and 10 µl of Positive Control-1-HCV or Positive Control-2-HCV respectively. Controls concentration range is specific for every lot (see DataCard);*
5 *add 10 µl of Internal Control during the RNA extraction procedure directly to the sample/lysis mixture.*

REF TV1-100/2 FRT

HCV Real-TM Quant – PCR kit	*Volume, ml*	*Quantity*
DTT	–	4 tubes
RT-PCR-mix-1-TM *HCV*	0.3	4 tubes
RT-PCR-mix-2-FRT	0.2	4 tubes
Polymerase (TaqF)	0.02	4 tubes
TM-Revertase (MMlv)	0.01	4 tubes
QS1 *HCV*[1]	0.1	4 tubes
QS2 *HCV*[1]	0.1	4 tubes
Negative Control (C–)[2]	1,2 ml	4 tubes
TE-buffer[3]	1.2	4 tubes
Positive Control-1-*HCV*[4]	0.06	4 tubes
Positive Control-2-*HCV*[4]	0.06	4 tubes
Internal Control *IC*-rec (IC)[5]	0.28	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

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4 must be used in the RNA/DNA extraction procedure as Positive Controls of Extraction. If the extraction kit starts from 100 µl, add 90 µl of Negative Control (C-) and 10 µl of Positive Control-1-HCV and Positive Control-2-HCV to the corresponding tubes labeled Cpos1 and Cpos2. For example, if it starts from 400 µl add 390 µl of Negative Control (C-) and 10 µl of Positive Control-1-HCV or Positive Control-2-HCV respectively. Controls concentration range is specific for every lot (see DataCard);

5 add 10 µl of Internal Control during the RNA extraction procedure directly to the sample/lysis mixture.

REF **TV1-100/2 FRT C**

HCV Real-TM Quant – PCR kit	*Volume, ml*	*Quantity*
DTT	–	4 tubes
RT-PCR-mix-1-TM *HCV*	0.3	4 tubes
RT-PCR-mix-2-FRT	0.2	4 tubes
Polymerase (TaqF)	0.02	4 tubes
TM-Revertase (MMlv)	0.01	4 tubes
QS1 *HCV*[1]	0.1	4 tubes
QS2 *HCV*[1]	0.1	4 tubes
Negative Control (C–)[2]	1,2 ml	4 tubes
TE-buffer[3]	1.2	4 tubes
Positive Control-1-*HCV*[4]	0.06	4 tubes
Positive Control-2-*HCV*[4]	0.06	4 tubes
Internal Control *IC*-rec (IC)[5]	0.28	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
Ribo Virus columns with collecting tubes (2ml)	/	100
Collecting tubes (2ml)	/	8 x 50

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5 add 10 µl of Internal Control during the RNA extraction procedure directly to the sample/lysis mixture.

MATERIALS REQUIRED BUT NOT PROVIDED

- RNA isolation kit
- 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. 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

HCV Real-TM Quant 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 ≤ -20°C immediately on receipt.

STABILITY

HCV Real-TM Quant 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 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.

RNA ISOLATION

The following isolation kits are recommended:

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

PCR PREPARATION

The total reaction volume is 25 µl, the volume of RNA sample is 12.5 µl.

1. Thaw one set of reagents, vortex and centrifuge briefly the tubes;
2. Prepare requested quantity of reaction tubes including 3 extraction controls, negative amplification control and 4 standards;
3. Add the entire contents of the tube with **RT-PCR-mix-2-FRT** to the tube with **DTT**. Thoroughly vortex and make sure there are no drops on the walls of the tube. Store the prepared mixture at 2–8 °C for no longer than 2 weeks.
4. Prepare in a new tube for N samples (including controls and standards) the **Reaction Mix** as follows:
   7.5 µl *N of **RT-PCR-mix-1- HCV**,
   5 µl *N of **mix RT-PCR-mix-2-FRT** and **DTT**,
   0.5 µl *N of **Polymerase (TaqF)**,
   0.25 µl *N of **TM-Revertase (MMlv),**
   Vortex thoroughly and make sure that there are no drops on the walls of the tubes.
5. Add **12.5 µl** of Reaction Mix into each tube.
6. Add **12.5 µl** of extracted RNA sample to the appropriate tubes with Reaction Mix.
7. Prepare for each run 4 standards:

- add **12.5 µl** of **QS1 HCV** into two (2) tubes each labelled QS1 HCV. Mix by pipetting;
- add **12.5 µl** of **QS2 HCV** into two (2) tubes each labelled QS2 HCV. Mix by pipetting;

8. To rule out any possible contamination, run an additional negative control of amplification reaction by adding **12.5 µl** of **TE-buffer** to the appropriate tube with Reaction Mix;

Insert the tubes in the thermalcycler and program the instrument.

Reverse transcription and Amplification Program

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	**50**	15 min	1	**50**	15 min	1
2	**95**	15 min	1	**95**	15 min	1
3	**95**	5 s	5	**95**	5 s	5
	60	20 s		**60**	20 s	
	72	15 s		**72**	15 s	
4	**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, *HCV* RNA is detected on the JOE(Yellow)/HEX/Cy3 channel.

INSTRUMENT SETTINGS

RotorGene (Corbett Research, Qiagen):

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*from 5 FI to 10 FI*	*0.03*	*10 %*	*On*
JOE/Yellow	*from 5 FI to 10 FI*	*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-HCV) 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 signal of the Internal Control cDNA amplification product is detected in the FAM channel.
- The signal of the HCV cDNA amplification product is detected in the JOE channel.

The results are interpreted by the presence (or absence) of the intercept between the fluorescence curve and the threshold line which determines the presence (or absence) of the Ct values for the sample.

Based on the Ct values and on the specified values of the calibrators, QS1 HCV and QS2 HCV, the calibration line will give the values for the number of HCV cDNA copies (JOE channel) and for the number of Internal Control cDNA copies (FAM channel) in a PCR sample.

The retrieved values are used for the HCV RNA concentration calculation, using the following formula:

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

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

Coefficient A = 1 when calculating Positive Control-1-*HCV* and Positive Control-2-*HCV* concentrations.

For example, if using MagnoVirus extraction kit which starts from 1000 µl of plasma, coefficient A will be 100/1000 = 0,1

Coefficient B is specified in the DataCard provided with the PCR kit and is specific for each lot. It cannot be used with PCR kits of different lots.

If the result is greater than 100,000,000 IU/ml, then it is interpreted as the greater than 100,000,000 IU/ml. If the obtained value is higher than the linear range, then the sample may be re-tested after 10x dilution; the produced result must be multiplied by10.

If the result is less than 60 IU/ml (extraction from 1 ml), then it is interpreted as the less than 60 IU/ml.

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

Results for controls and calibrators

Control	Stage for control	FAM/Green (IC)	JOE/Yellow/HEX (HCV)
C–	RNA extraction	+	-
PCE (C+)	RNA extraction	+	+
NCA	Amplification	-	-
QS1 HCV / QS2 HCV	Amplification	+	+

⚠ Positive Control-1-*HCV* and Positive Control-2-*HCV* results must fall in the expected range of quantitation indicated in the DataCard.

⚠ Calibration curve is valid if R^2 value is higher than 0,98.

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 HCV) 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 **HCV Real-TM Quant** 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 B 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 **HCV Real-TM Quant** allows to detect HCV RNA in 100% of the tests with a sensitivity of 60 IU/ml (value obtained using **Magno Virus -** Sacace kit, REF K-2-16/1000 starting from 1 ml of sample volume).

TROUBLESHOOTING

The results are not taken into account in the following cases:

1. If the Ct value obtained for the Positive Controls of Extraction or Amplification in the JOE channel is absent or is greater than the specified concentration range value, then it is necessary to repeat the test (from the RNA extraction stage for all samples in which HCV RNA was not found).
2. If the Ct value is obtained for the Negative Control of Extraction in the JOE channel and/or for the Negative Control of Amplification in the FAM and JOE channels, then it is necessary to repeat the test.
3. If the calculated concentrations of Positive Control-1 HCV and Positive Control-2 HCV exceed the range specified in the DataCard, then it is necessary to repeat the test (from the RNA extraction stage) for all samples.

EXPLANATION OF SYMBOLS

REF Catalogue Number

RUO For *Research Use Only*

LOT Lot Number

 Expiration Date

 Contains reagents

 Caution!

VER Version

 Manufacturer

 Temperature limitation

*SaCycler® is trademarks of Sacace Biotechnologies
*CFX-96™ 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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