HCV 1/2/3 Real-TM Genotype

Real Time PCR kit for qualitative detection of and differentiation of hepatitis C virus (*HCV*) genotypes 1, 2, and 3 RNA in HCV-positive clinical material

Handbook

REF R1-Gen-4x

Σ 50

NAME

HCV 1/2/3 Real-TM Genotype

INTRODUCTION

The hepatitis C virus is an enveloped RNA virus with a diameter of about 50 nm, classified as a separate genus (Hepacivirus) within the Flaviviridae family. The genomic organization and sequence of HCV resembles that of the pestiviruses and flaviviruses.
The genome of HCV is highly mutable. Mutations are not randomly distributed along the genome, but are most pronounced within a hypervariable region located near the N-terminus of E2. This region maps at a surface loop of the E2 protein containing a B-cell epitope that undergoes antigenic evolution over time.
HCV is classified into eleven major genotypes (designated 1-11), many subtypes (designated a, b, c, etc.), and about 100 different strains (numbered 1,2,3, etc.) based on the genomic sequence heterogeneity.
The variability is distributed throughout the genome. However, the non-coding regions at either end of the genome (5'-UTR and 3'-UTR; UTR-untranslated region) are more conserved and suitable for virus detection by PCR. The genes coding for the envelope E1 and E2 glycoproteins are the most variable. Amino acid changes may alter the antigenic properties of the proteins, thus allowing the virus to escape neutralizing antibodies.
Genotypes 1-3 have a worldwide distribution. Types 1a and 1b are the most common, accounting for about 60% of global infections. They predominate in Northern Europe and North America, and in Southern and Eastern Europe and Japan, respectively. Type 2 is less frequently represented than type 1. Type 3 is endemic in south-east Asia and is variably distributed in different countries.
The determination of the infecting genotype is important for the prediction of response to antiviral treatment: genotype 1 is generally associated with a poor response to interferon alone, whereas genotypes 2 and 3 are associated with more favourable responses. At patients with subtype 1b the disease progresses to a cronic condition 90 % of cases, in that time as with genotypes 2 and 3b in 33-50 %.
The International Consensus European Association for the Study of the Liver (EASL) recommends before beginning of antiviral therapies to carry out a liver biopsies and to determine HCV genotype. When using combination therapy with interferon and ribavirin, patients with genotypes 2 or 3 generally are treated for only 24 weeks, whereas it is recommended that patients infected with genotype 1 receive treatment for 48 weeks.

INTENDED USE

HCV 1/2/3 Real-TM Genotype PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of and discrimination between hepatitis C virus (*HCV*) genotype 1, 2, and 3 RNA in HCV-positive clinical material (blood plasma) by using real-time hybridization-fluorescence detection.

The results of PCR analysis are taken into account in complex diagnostics of disease.

PRINCIPLE OF ASSAY

Hepatitis C virus detection includes total RNA isolation from blood plasma and reverse transcription of RNA into cDNA combined with real-time PCR amplification of cDNA. *HCV* detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using specific *HCV* 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. **HCV 1/2/3 Real-TM Genotype** PCR kit uses "hot-start", which greatly reduces the frequency of nonspecifically primed reactions.

The *HCV* genotype 1 cDNA is detected in the FAM/Green channel.
The *HCV* genotype 2 cDNA is detected in the JOE/HEX/Yellow channel
The *HCV* genotype 3 cDNA is detected in the Rox/Texas Red/Orange channel.

The amplification product of an *HCV* cDNA fragment common for all HCV genotypes, which is used in this test as an internal control (IC) confirming the presence of *HCV* cDNA in tested samples, is detected in the Cy5/Red channel.

The Positive Control of Extraction, Positive Control-1-*HCV,* is detected in the FAM/Green (genotype 1) and Cy5/Red (all genotypes) channels.

The Positive Control of Amplification, Positive Control cDNA *HCV*-123, contains an *HCV* cDNA fragment common for all genotypes and is detected in FAM/Green (genotype 1), JOE/HEX/Yellow (genotype 2), ROX/Texas Red/Orange (genotype 3), and Cy5/Red (genotypes 1, 2, and 3) channels.

To optimize the laboratory analysis procedure, the same RNA isolation procedure can be used for *HCV* detection, viral load and genotyping.

CONTENT

Reagent	***Volume (ml)***	***Amount***
RT-G-mix-2	0.015	1 tube
PCR-mix-1-FRT *HCV*-1/2/3	0.6	1 tube
RT-PCR-mix-2-TM	0.3	1 tube
Hot Start Polymerase	0.03	1 tube
M-MLV Revertase	0.015	1 tube
cDNA *HCV*-1/2/3 (C+)	0.1	1 tube
Negative Control (C–)*	1.2	2 tubes
Pos *1-HCV***	0.1	1 tube

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

HCV 1/2/3 Real-TM Genotype PCR kit is intended for 55 reverse transcription and amplification reactions including controls.

MATERIALS REQUIRED BUT NOT PROVIDED

- Real Time Thermalcycler (4 channel)
- Workstation
- Pipettes (adjustable)
- Sterile tips with filters
- Tube racks
- RNA extraction kit

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.

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℃ for an additional 3 days. Alternatively, plasma may be stored at -18℃ for up to one month or 1 year when stored at -70℃.
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℃ and pr ocessed within 6 hours of collection. Plasma may be transported at 2-8℃ or frozen.
7. Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

PROTOCOL

RNA ISOLATION

The following isolation kits are recommended:

- **Ribo-Virus** (Sacace, REF K-2/C)
- **DNA/RNA-Prep** (Sacace, REF K-2-9)
- **Ribo-Sorb** (Sacace, REF K-2-1)
- **Magno-Virus** (Sacace, REF K-2-16)
- **SaMag Viral Nucleic Acid Extraction kit** (Sacace, REF SM003)

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

To prepare Positive Control of Extraction (PCE), mix **10 µl** of **Positive Control-1-*HCV*** and **90 µl of Negative Control.**

The purified RNA can be stored at 2–8 °C for at most 4 h, at temperatures not higher than minus 16 °C for 1 month, and at temperatures not higher than minus 68 °C for one year.

PREPARING TUBES FOR PCR

1. Before starting work, thaw and vortex all reagents of the kit. Make 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 two controls of extraction and one control of amplification).
3. **To prepare the reaction mixture**, mix the reagents (**10 µl of PCR-mix-FRT-*HCV*-1/2/3, 5 µl of RT-PCR-mix-2-TM, 0.25 µl of RT-G-mix-2, 0.5 µl of Polymerase and 0.25 µl of MMLV Revertase** per one reaction) in a new sterile tube. Thoroughly vortex the mixture, make 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 **RNA samples** isolated from the clinical samples to each PCR tube.
6. Run the **control reactions**:

PCE - Add **10 µl** of the **RNA sample** extracted from the Pos1-*HCV* to the tube labeled PCE (Positive Control of Extraction)

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

C+ - Add **10 µl** of **Positive Control cDNA *HCV*-123** to the tube labeled C+ (Positive Control of Amplification).

Make sure that there are no drops on the tube walls, otherwise vortex the tubes briefly.

Table. REACTION MIXTURE PREPARATION

		Reaction volume (with one extra sample)				
Reagent volume for one reaction, µl		**10.00**	**5.00**	**0.25**	**0.50**	**0.25**
N. samples	N. PCR reactions	PCR-mix-1	RT-PCR-mix-2	RT-G-mix-2	Polymerase	M-MLV Revertase
4	7	80	40	2.0	4.0	2.0
6	9	100	50	2.5	5.0	2.5
8	11	120	60	3.0	6.0	3.0
10	13	140	70	3.5	7.0	3.5
12	15	160	80	4.0	8.0	4.0
14	17	180	90	4.5	9.0	4.5
16	19	200	100	5.0	10.0	5.0
18	21	220	110	5.5	11.0	5.5
20	23	240	120	6.0	12.0	6.0
22	25	260	130	6.5	13.0	6.5
34	37	380	190	9.5	19.0	9.5
46	49	500	250	12.5	25.0	12.5

AMPLIFICATION

Rotor-Gene™ 6000/Q (Corbett Research, Qiagen)

1. Program the Rotor-Gene™ instrument according to manufacturer's manual.
2. Create a temperature profile on your Rotor-Gene™ instrument as follows:

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	**Cycle repeats**
1 (Hold)	50	30 min	–	1
2 (Hold)	95	15 min	–	1
3 (Cycling 1)	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
4 (Cycling 2)	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red	
	72	15 s	–	

SaCycler™ (Sacace Biotechnologies), iQ5™, CFX™ (BioRad); Mx3005P™ (Stratagene), Applied Biosystems® 7500 Real Time PCR (Applera), SmartCycler® (Cepheid)

1. Program the instrument according to manufacturer's manual and the Guidelines.
2. Create a temperature profile on your instrument as follows:

Step	***Temperature, °C***	**Step duration**	***Fluorescence detection***	***Cycle repeats***
1	50	30 min	–	1
2	95	15 min	–	1
3	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
4	95	5 s	–	40
	60	30 s	FAM, HEX/JOE/Cy3, ROX/Texas Red, Cy5	
	72	15 s	–	

INSTRUMENT SETTINGS

Rotor-type instruments (RotorGene 6000, RotorGene Q)

Channel	Threshold	Outlier Removal	Slope Correct
FAM/Green	*0.05*	*20 %*	*On*
JOE/Yellow	*0.05*	*20 %*	*On*
Rox/Orange	*0.05*	*20 %*	*On*
Cy5/Red	*0.05*	*10 %*	*On*

Plate- or modular type instruments SaCycler™ (Sacace Biotechnologies), iQ5™, CFX™ (BioRad); Mx3005P™ (Stratagene), Applied Biosystems® 7500 Real Time PCR (Applera), SmartCycler® (Cepheid).
For result analysis, set the threshold line at a level where curves of fluorescence are linear.

DATA ANALYSIS

- The *HCV* genotype 1 cDNA is detected in the FAM/Green channel.
- The *HCV* genotype 2 cDNA is detected in the JOE/HEX/Yellow/Cy3 channel
- The *HCV* genotype 3 cDNA is detected in the Rox/Texas Red/Orange channel.
- *HCV* cDNA fragment common for all *HCV* genotypes, which confirms the presence of the *HCV* cDNA in analyzed samples, is detected in the Cy5/Red 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 (S-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).

In all other cases, the result is considered **equivocal**.

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.

Table. Pos Controls Ct value

Maximum allowable Ct value							
Fam/Green		Joe/Yellow/HEX/Cy3		Rox/Orange/TexasRed		Cy5/Red	
PCE	C+	PCE	C+	PCE	C+	PCE	C+
Rotor-Gene 6000/Q (Corbett Research, Qiagen)							
32.0	26.0	–	28.0	–	28.0	32.0	26.0
SaCycler (Sacace Biotechnologies)							
34.0	28.0	–	30.0	–	30.0	34.0	28.0
iQ iCycler, iQ5, CFX (BioRad)							
34.0	28.0	–	30.0	–	30.0	34.0	28.0
Mx3000P (Stratagene)							
34.0	28.0	–	30.0	–	30.0	34.0	28.0
SmartCycler (Cepheid)							
34.0	28.0	–	30.0	–	30.0	34.0	30.0
Applied Biosystems® 7500 Real Time PCR (Applera)							
34.0	28.0	–	30.0	–	30.0	34.0	30.0

Table. Neg Control results

Control	Stage for control	Ct in channel				Result
		Fam/Green	Joe/Yellow/ HEX/Cy3	Rox/Orange TexasRed	Cy5/Red	
C–	RNA isolation	Neg	Neg	Neg	Neg	OK

Sample contains *HCV* genotype 1 RNA if the Ct value detected in the FAM channel is less than 36.

Sample contains *HCV* genotype 2 RNA if the Ct value detected in the HEX channel is less than 36.

Sample contains *HCV* genotype 3 RNA if the Ct value detected in the ROX channel is less than 36.

Sample contains RNA of *HCV* of another (rare) genotype if Ct values in FAM, HEX, and ROX channels are absent and the Ct value detected in the Cy5 channel is less than 36.

The **clinical sample** is regarded as **nontypeable due to a low viral load** if its analysis repeated twice starting from the extraction stage yielded a negative amplification result in all the four channels (FAM/Green, JOE/HEX/Yellow, ROX/Orange, and Cy5/Red) or a combination of equivocal and negative results was obtained.

Results are accepted as significant only if both positive and negative controls of RNA extraction and the negative controls of amplification passed correctly (see above the table for controls).

TROUBLESHOOTING

- The absence of positive signal in PCE in FAM/Green and Cy5/Red channels or the absence of positive signal in C+ in all four channels may indicate incorrect amplification program or other errors made during RNA/DNA isolation or PCR amplification. In this case, PCR should be carried out once again.
- Detection of any Ct value in C– in the results grid for FAM/Green, JOE/HEX/Yellow, ROX/Orange/TexasRed and Cy5/Red channels 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.

STABILITY AND STORAGE

All components of the **HCV 1/2/3 Real-TM Genotype** PCR kit are to be stored at or below minus 20ºC. They are stable until the expiration date indicated on the label.
Controls **cDNA *HCV*-1/2/3 (C+)** and **Pos *1-HCV*** must be stored at +2-8℃.

Positive Control cDNA *HCV*-123 and Positive Control-1-*HCV* should not be frozen/thawed more than twice. Once thawed, Positive Control cDNA *HCV*-123 and Positive Control-1-*HCV* should be stored at 2–8ºC for 6 months.

SPECIFICATIONS

Sensitivity

The analytical sensitivity of **HCV 1/2/3 Real-TM Genotype** PCR kit is specified in the table below.

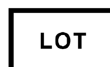
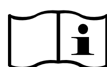
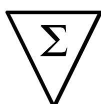
Sample volume for isolation, µl	RNA/DNA isolation method	Analytical sensitivity, IU/ml
100-150	"RIBO-sorb" "DNA/RNA-prep" "Ribo-Virus"	500
1000	"Magno-Virus"	50

The claimed analytical features of **HCV 1/2/3 Real-TM Genotype** PCR kit are guaranteed only when additional reagents kits "Magno-Virus", RIBO-sorb", "Ribo-Virus" or "DNA/RNA-prep" are used.

Specificity

The analytical specificity of **HCV 1/2/3 Real-TM Genotype** 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 B 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.
Cross-reactions for the above-mentioned organisms and viruses have not been detected.

EXPLANATION OF SYMBOLS

Symbol	Meaning	Symbol	Meaning
	Manufacturer		Temperature limitation
	Use by		Batch code
	Catalogue number		Version
	Consult instructions for use		Caution
	Contains sufficient for <n> tests	**C+**	Positive control of amplification
PCE	Positive Control of Extraction	**C–**	Negative control of extraction

* SaCycler™, is trademark of Sacace Biotechnologies
* Cycler™, iQ5™, CFX™ are trademarks of Bio-Rad Laboratories
* Rotor-Gene™ Technology is a registered trademark of Qiagen
*MX3005P® is trademarks of Stratagene
*Applied Biosystems® is trademark of Applera Corporation
* SmartCycler® is a registered trademark of Cepheid

Sacace Biotechnologies Srl
via Scalabrini, 44 – 22100 – Como – Italy Tel +390314892927 Fax +390314892926
*mail: **info@sacace.com** web: **www.sacace.com***