# HCV Real-TM Genotype

## HANDBOOK

### Real Time PCR Kit for qualitative detection and differentiation of hepatitis C virus (HCV) genotypes 1a, 1b, 2, 3, 4

**REF R1-Gen**

**REF TR1-Gen**

**REF TR1-Gen-C**

**Σ 50**

## NAME

**HCV 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. Genotype 4 is principally found in the Middle East, Egypt, and central Africa.

The determination of the infecting genotype is important for the prediction of response to antiviral treatment: genotype 1 and 4 are 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 chronic condition 90 % of cases, in that time as with genotypes 2 and 3b in 33-50 %. In a number of works it is mentioned, that infection with 1b genotype have heavier current of disease with development of a cirrhosis and hepatocarcinoma.

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

kit **HCV Real-TM Genotype** is a Real-Time test for the determination of HCV-RNA genotypes 1a, 1b, 2, 3, 4 in the human plasma and simultaneous detection of HCV-specific Internal Control (IC). The **HCV Real-TM Genotype** is not meant to be used for screening of blood, plasma, serum or tissue donors for HCV, but only for determining the genotype(s) of hepatitis C virus in plasma from HCV infected individuals.

## PRINCIPLE OF ASSAY

**HCV Real-TM Genotype** is based on three major processes:

1. isolation of *HCV* RNA from specimens;
2. reverse transcription of the RNA;
3. real time PCR:
    - **PCR-mix-1-FRT HCV 1b/3** with primers and probes for subtypes 1b, 3;
    - **PCR-mix-1-FRT HCV 1a/2** with primers and probes for subtypes 1a, 2;
    - **PCR-mix-1-FRT HCV 4/IC** with primers and probes for subtypes 4 and Internal Control

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 HCV RNA.

## MATERIALS PROVIDED

### Module No.1: Real Time PCR kit (R1-Gen)

Part N° 2 – **“Controls”**

- **Negative Control***, 0,5 ml;
- **HCV Rec IC**** (Internal Control), 0,5 ml.

** must be used during the sample preparation procedure: add 100 µl of C– (Negative Control) to labeled Cneg;*

*** must be used during the sample preparation procedure (see RNA isolation)*

Part N° 3 – **“Reverta-L”:**

- **RT-G-mix-1**, 5 x 0,01 ml;
- **RT-mix**, 5 x 0,125 ml;
- **Reverse transcriptase** (M-MLV), 0,03 ml;
- **TE-buffer**, 1,2 ml.

Contains reagents for 60 tests.

Part N° 4 – **“HCV Real-TM Genotype ”:**

- **PCR-mix-1-FRT HCV 1b/3**, 0,6 mL
- **PCR-mix-1-FRT HCV 1a/2**, 0,6 mL
- **PCR-mix-1-FRT HCV 4/IC**, 0,6 mL
- **RT-PCR-mix-2-TM**, 4 x 0,3 mL.
- **TaqF Polymerase**, 4 x 0,03 mL
- Controls:
  - **HCV cDNA C+ 1b/3**, 0,2 mL;
  - **HCV cDNA C+ 1a/2**, 0,2 mL;
  - **HCV cDNA C+ 4**, 0,2 mL;
  - **TE-buffer**, 0,5 mL.

Contains reagents sufficient for 50 samples.

## Module No.2: Complete Real Time PCR test with DNA purification kit (TR1-Gen)

Part N° 1 – **"Ribo-Sorb-48":**

- **Lysis Solution**, 4 x 5,8 ml;
- **Washing Solution**, 4 x 8,0 ml;
- **Sorbent**, 4 x 0,4 ml.
- **RNA-eluent**, 4 x 0,6 ml.

Contains reagents for 48 extractions.

Part N° 2 – **"Controls"**

- **Negative Control***, 0,5 ml;
- **HCV Rec IC**** (Internal Control), 0,5 ml.

** must be used during the sample preparation procedure: add 100 µl of C– (Negative Control) to labeled Cneg;*

*** must be used during the sample preparation procedure (see RNA isolation)*

Part N° 3 – **"Reverta-L":**

- **RT-G-mix-1**, 5 x 0,01 ml;
- **RT-mix**, 5 x 0,125 ml;
- **Reverse transcriptase** (M-MLV), 0,03 ml;
- **TE-buffer**, 1,2 ml.

Contains reagents for 60 tests.

Part N° 4 – **"HCV Real-TM Genotype ":**

- **PCR-mix-1-FRT HCV 1b/3**, 0,6 mL
- **PCR-mix-1-FRT HCV 1a/2**, 0,6 mL
- **PCR-mix-1-FRT HCV 4/IC**, 0,6 mL
- **RT-PCR-mix-2-TM**, 4 x 0,3 mL.
- **TaqF Polymerase**, 4 x 0,03 mL
- Controls:
  - **HCV cDNA C+ 1b/3**, 0,2 mL;
  - **HCV cDNA C+ 1a/2**, 0,2 mL;
  - **HCV cDNA C+ 4**, 0,2 mL;
  - **TE-buffer**, 0,5 mL.

Contains reagents sufficient for 50 samples.

## Module No.3: Complete Real Time PCR test with column RNA purification kit (TR1-Gen-C)

Part N° 1 – **“Ribo-Virus”: (**see protocol cat. No. K-2/C)

Part N° 2 – **“Controls”**

- **Negative Control***, 0,5 ml;
- **HCV Rec IC**** (Internal Control), 0,5 ml.

** must be used during the sample preparation procedure: add 100 µl of C– (Negative Control) to labeled Cneg;*

*** must be used during the sample preparation procedure (see RNA isolation)*

Part N° 3 – **“Reverta-L”:**

- **RT-G-mix-1**, 5 x 0,01 ml;
- **RT-mix**, 5 x 0,125 ml;
- **Reverse transcriptase** (M-MLV), 0,03 ml;
- **TE-buffer**, 1,2 ml.

Contains reagents for 60 tests.

Part N° 4 – **“HCV Real-TM Genotype ”:**

- **PCR-mix-1-FRT HCV 1b/3**, 0,6 mL
- **PCR-mix-1-FRT HCV 1a/2**, 0,6 mL
- **PCR-mix-1-FRT HCV 4/IC**, 0,6 mL
- **RT-PCR-mix-2-TM**, 4 x 0,3 mL.
- **TaqF Polymerase**, 4 x 0,03 mL
- Controls:
  - **HCV cDNA C+ 1b/3**, 0,2 mL;
  - **HCV cDNA C+ 1a/2**, 0,2 mL;
  - **HCV cDNA C+ 4**, 0,2 mL;
  - **TE-buffer**, 0,5 mL.

Contains reagents sufficient for 50 samples.

## MATERIALS REQUIRED BUT NOT PROVIDED

- Desktop microcentrifuge for “eppendorf” type tubes
- Vortex mixer
- Disposable gloves, powderless
- Biohazard waste container
- Refrigerator, Freezer
- Real Time Thermal cycler
- Reaction tubes or plate
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Tube racks
- 70% Ethanol (freshly prepared mixture of reagent grade 96% ethanol and distilled water)
- Acetone

## STORAGE INSTRUCTIONS

Part N° 1 and 2 must be stored at 2-8°C.

Part N° 3 and 4 must be stored at -20°C.

The **HCV Real-TM Genotype** kit can be shipped at 2-8°C but should be stored at 2-8°C and -20°C immediately on receipt.

## STABILITY

**HCV Real-TM Genotype** Test is stable up to the expiration date indicated on the kit label. All components of the **HCV Real-TM Genotype** PCR kit are stable until the expiration date on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

PCR-mix-1-FRT tubes are to be kept away from light.

## QUALITY CONTROL

In accordance with Sacace’s ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

## WARNINGS AND PRECAUTIONS

The user should always pay attention to the following:

- ⚠ 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).
- 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.
- Heparin has been shown to inhibit reaction. 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.

** Only for Module No.2 and 3*

## PRODUCT USE LIMITATIONS

Use of this product should be limited to personnel trained in the techniques of DNA amplification (EN375). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

## 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 Genotype**. 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*:

⇒ **Ribo-Virus** – spin column extraction kit (Sacace, REF K-2-C/50)

⇒ **DNA/RNA Prep** – (Sacace, REF K-2-9)

⇒ **Ribo-Sorb** (Sacace, REF K-2-1)

**Please carry out the RNA extraction according to the manufacturer's instructions. Add 10 µl of Internal Control during the RNA isolation procedure directly to the sample/lysis mixture.**

** if other manufacturers' RNA isolation kits are used, please contact our customer care service at specialists@sacace.com*

## SPECIMEN AND REAGENT PREPARATION (reagents supplied with the Module No.2)

1. **Lysis Solution** and **Washing Solution** (in case of their storage at +2-8°C) should be warmed up to 60–65°C until disappearance of ice crystals.
2. Prepare 70% Ethanol.
3. Prepare required quantity of 1,5 ml polypropylene tubes including one tube for **Negative Control of Extraction** .
4. For each batch of up to 12 samples and control, add **65 µl** of **Internal Control** to one bottle of **Lysis Solution** and mix well by inverting 10-15 times. This working solution is stable for 30 min.

   *(If it is necessary to test less than 12 samples add **5 µl** of **Internal Control** and **445 µl** of **Lysis Solution** directly to each tube).*
5. Vortex thawed patient plasma specimens for 5 sec.
6. Add **450 µl** of **Lysis Solution with IC** to each of labeled tubes.
7. Add **100 µl** of **Samples** to the appropriate tube.
8. Prepare Controls as follows:
   - add **100 µl** of **C–** (**Negative Control**) to the tube labeled C*neg*.
9. Vortex the tubes and centrifuge for 7-10 sec.
10. Vortex vigorously **Sorbent** and add **25 µl** to each tube.
11. Vortex for 5-7 sec and incubate all tubes for 10 min at room temperature. Vortex periodically.
12. Centrifuge all tubes for 1 min at 10000g and using a micropipette or vacuum aspirator with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
13. Add **500 µl** of **Washing Solution** to each tube. Vortex vigorously until the sorbent is completely resuspended, centrifuge for 1 min at 10000g and using a micropipette or vacuum aspirator with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
14. Add **500 µl** of **Ethanol al 70%** to each tube. Vortex vigorously until the sorbent is completely resuspended, centrifuge for 1 min at 10000g and using a micropipette or vacuum aspirator with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between the tubes.
15. Repeat step 14.
16. Add **500 µl** of **Acetone** to each tube. Vortex vigorously until the sorbent is completely resuspended, centrifuge for 1 min at 10000g and using a micropipette or vacuum aspirator with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between the tubes.
17. Incubate all tubes with open cap for 10 min at 56°C.

18. Resuspend the pellet in **50 µl** of **RNA-eluent.** Incubate for 10 min at 56°C and vortex periodically.
19. Centrifuge the tubes for 2 min at maximum speed (12000-16000 g). The supernatant contains RNA ready for amplification. The Real Time amplification can be performed on the same day of extraction.

## SPECIMEN AND REAGENT PREPARATION (reagents supplied with the Module No.3)

See protocol cat. No. K-2/C.

## REVERSE TRANSCRIPTION PROTOCOL

1) Thaw **RT-G-mix-1** and **RT-mix**, vortex and centrifuge briefly.
2) Prepare Reaction Mix: for 12 reactions, **add 5,0 µl RT-G-mix-1** into the tube containing **RT-mix** and vortex for at least 5-10 seconds, centrifuge briefly. Add **6 µl M-MLV** into the tube with Reagent Mix, mix by pipetting, vortex for 3 sec, centrifuge for 5-7 sec (must be used immediately after the preparation).

*If it is necessary to test less than 12 samples add for each sample (N) in the new sterile tube **10*N µl** of **RT-mix, 0,4*N µl** of **RT-G-mix-1 with** and **0,5*N µl** of **M-MLV**). Mix by pipetting, vortex for 3-5 sec, centrifuge briefly.*

Table 1. Reverse transcription reaction mix preparation for less than 12 samples

| **Reagent volume for one reaction (µl)** | **10,00** | **0,4** | **0,5** |
|---|---|---|---|
| **Clinical samples** | **RT-mix** | **RT-G-mix-1** | **M-MLV** |
| *4* | *60** | *2,4* | *3,0* |
| *5* | *70* | *2,8* | *3,5* |
| *6* | *80* | *3,2* | *4,0* |
| *7* | *90* | *3,6* | *4,5* |
| *8* | *100* | *4,0* | *5,0* |

** The volumes are calcolated considering one negative control of extraction and reagents for one extra reaction*

3) Add **10 µl** of **Reaction Mix** into each sample tube.
4) Pipette **10 µl** of **RNA** samples to the appropriate tube. (*If the Ribo-Sorb isolation kit is used as a RNA 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)*.Carefully mix by pipetting.
5) Place tubes into thermalcycler and incubate at 37°C for 30 minutes.

6) Dilute 1: 2 each obtained cDNA sample with TE-buffer (add **20 µl TE-buffer** to each tube). Vortex and centrifuge briefly the tubes.
7) cDNA specimens could be stored at -20°C for a week or at -70°C up to one year.

## REAL TIME PCR

1. Thaw PCR reagents, vortex and centrifuge briefly the tubes.
2. Prepare 3 PCR tubes for each sample and mark the tubes properly (for ex. "No. sample 1b/3a", "No. sample 1a/2", "No. sample 4/IC").
3. Prepare 3 Master mixes: "1b/3", "1a/2", "4/IC". To do this, add for each sample in the new sterile tube:
   - **10 µl** of **PCR-mix-1-FRT HCV** genotype **1b/3** (or **1a/2** or **4/IC**)
   - **5 µl** of **RT-PCR-mix-2-TM**,
   - **0,5** µl of **polymerase (TaqF)**
4. Thoroughly vortex. Make sure there are no drops on the walls of the tubes; otherwise, centrifuge briefly.

Table 2. Master Mixes preparation

| **Reagent volume for one reaction (µl)** | | **10,00** | **5,00** | **0,50** |
|---|---|---|---|---|
| **Clinical samples** | **PCR reactions*** | **PCR-mix-1-FRT HCV genotype** | **RT-PCR-mix-2-TM** | **Polymerase (TaqF)** |
| *4* | *6* | *60* | *30* | *3,0* |
| *5* | *7* | *70* | *35* | *3,5* |
| *6* | *8* | *80* | *40* | *4,0* |
| *7* | *9* | *90* | *45* | *4,5* |
| *8* | *10* | *100* | *50* | *5,0* |
| *9* | *11* | *110* | *55* | *5,5* |
| *10* | *12* | *120* | *60* | *6,0* |
| *11* | *13* | *130* | *65* | *6,5* |
| *12* | *14* | *140* | *70* | *7,0* |

** minimum one Pos PCR control and one Negative extraction control must be performed for each Master Mix*

5. Pipette **15 µl** of **Master Mix** and **10 µl** of **cDNA** sample to the appropriate tube as indicated in the Diagram 1.
6. Perform for each PCR-mix-1 at least one negative control of extraction (PCR control - TE-buffer is also recommended to be used) by adding to the appropriate tube of **10 µl** of **Control** (all tubes in the position 13 of the Diagram 1).
7. Perform the Positive Control for each PCR-mix-1:
   - Add **10 µl** of Positive Control **cDNA HCV** genotypes **1b/3** to the tube with "1b/3" reaction mix labeled C+1b/3 (1st tube in the position 14 of the Diagram 1).
   - Add **10 µl** of Positive Control **cDNA HCV** genotypes **1a/2** to the tube with "1a/2" reaction mix labeled C+1a/2 (2nd tube in the position 14 of the Diagram 1).

- Add **10 µl** of Positive Control **cDNA HCV** genotype **4/IC** to the tube with "4/IC" reaction mix labeled C+4/IC (3rd tube in the position 14 of the Diagram 1).

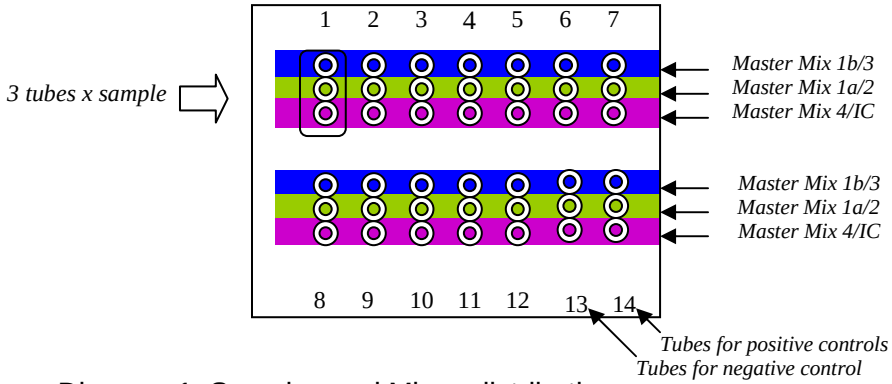


Diagram 1. Samples and Mixes distribution

8. Close tubes and transfer them into the Real Time PCR instrument.

Table 3. Real Time PCR Temperature profile.

| | 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** |
| Hold | 95 | 15 min | – | 1 | 95 | 15 min (900 s) | – | 1 |
| Cycling | 95 | 5 s | – | 5 | 95 | 5 s | – | 5 |
| | 60 | 20 s | – | | 60 | 20 s | – | |
| | 72 | 15 s | – | | 72 | 15 s | – | |
| Cycling 2 | 95 | 5 s | – | 40 | 95 | 5 s | – | 40 |
| | 60 | 20 s | FAM(Green), JOE(Yellow) | | 60 | 30 s | FAM, JOE/HEX/Cy3 | |
| | 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® 7300/7500/StepOne Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid), LineGeneK® (Bioer)*

**Rotor-type instruments**

Settings

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

**Plate-type instruments**

Settings

| Channel | Threshold |
|---|---|
| FAM | The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set the threshold at a level where fluorescence curves are linear and do not cross curves of the negative samples. |
| HEX/JOE/Cy3 | |

## DATA ANALYSIS

The fluorescence curves are analyzed with the software of Real Time PCR instruments on the 2 channels.

Table 3. HCV genotype detection

| Tube | 1b/3 | 1a/2 | 4/IC |
|---|---|---|---|
| FAM (Green) | 1b | 1a | IC |
| JOE (Yellow)/HEX/Cy3 | 3 | 2 | 4 |

The result of the experiment can be accepted only if the Positive and Negative Controls runs are valid (see table 4).

Table. 4 Results of controls

| Reaction Mix | 1b/3 | | 1a/2 | | 4/IC | |
|---|---|---|---|---|---|---|
| Channel | FAM (Green) Ct | Joe(Yellow)/ HEX/Cy3 Ct | FAM (Green) Ct | Joe(Yellow)/ HEX/Cy3 Ct | FAM (Green) Ct | Joe(Yellow)/ HEX/Cy3 Ct |
| Negative Control (Extraction) | - | - | - | - | <40 (<36 for RG) | - |
| Neg. Control (PCR) | - | - | - | - | - | - |
| $C+_{1b/3}$ | <37 | <37 | * | * | * | * |
| $C+_{1a/2}$ | * | * | <37 | <38 | * | * |
| $C+_{4}$ | * | * | * | * | - | <37 |

The results of the samples are interpreted through the presence of crossing of fluorescence curve with the threshold line (see table 5).

Table 5. Samples results.

| Tube | Channel | Ct | Result |
|---|---|---|---|
| 1b/3 | FAM | - | Genotype 3 |
| | JOE/HEX/Cy3 | 27,7 | |
| 1a/2 | FAM | - | |
| | JOE/HEX/Cy3 | - | |
| 4/IC | FAM | 35,2 | |
| | JOE/HEX/Cy3 | - | |

| Tube | Channel | Ct | Result |
|---|---|---|---|
| 1b/3 | FAM | 22,1 | Genotype 1b, 2 |
| | JOE/HEX/Cy3 | - | |
| 1a/2 | FAM | - | |
| | JOE/HEX/Cy3 | 32,1 | |
| 4/IC | FAM | 35,4 | |
| | JOE/HEX/Cy3 | - | |

| Tube | Channel | Ct | Result |
|---|---|---|---|
| 1b/3 | FAM | 30,4 | Genotype 1b, 1a |
| | JOE/HEX/Cy3 | - | |
| 1a/2 | FAM | 26,6 | |
| | JOE/HEX/Cy3 | - | |
| 4/IC | FAM | 35,0 | |
| | JOE/HEX/Cy3 | - | |

| Tube | Channel | Ct | Result |
|---|---|---|---|
| 1b/3 | FAM | 28,0 | Genotype 1b, 4 |
| | JOE/HEX/Cy3 | - | |
| 1a/2 | FAM | - | |
| | JOE/HEX/Cy3 | - | |
| 4/IC | FAM | 35,7 | |
| | JOE/HEX/Cy3 | 22,9 | |

| Tube | Channel | Ct | Result |
|---|---|---|---|
| 1b/3 | FAM | 39,4 | Genotype 4* |
| | JOE/HEX/Cy3 | - | |
| 1a/2 | FAM | - | |
| | JOE/HEX/Cy3 | - | |
| 4/IC | FAM | 35,3 | |
| | JOE/HEX/Cy3 | 22,3 | |

| Tube | Channel | Ct | Result |
|---|---|---|---|
| 1b/3 | FAM | - | Genotype 1a |
| | JOE/HEX/Cy3 | - | |
| 1a/2 | FAM | 32,0 | |
| | JOE/HEX/Cy3 | - | |
| 4/IC | FAM | 35,5 | |
| | JOE/HEX/Cy3 | - | |

| Tube | Channel | Ct | Result |
|---|---|---|---|
| 1b/3 | FAM | - | Genotype 2 |
| | JOE/HEX/Cy3 | - | |
| 1a/2 | FAM | - | |
| | JOE/HEX/Cy3 | 32,1 | |
| 4/IC | FAM | 35,0 | |
| | JOE/HEX/Cy3 | - | |

| Tube | Channel | Ct | Result |
|---|---|---|---|
| 1b/3 | FAM | - | not detected (negative) |
| | JOE/HEX/Cy3 | - | |
| 1a/2 | FAM | - | |
| | JOE/HEX/Cy3 | - | |
| 4/IC | FAM | 35,4 | |
| | JOE/HEX/Cy3 | - | |

| Tube | Channel | Ct | Result |
|---|---|---|---|
| 1b/3 | FAM | 38.9 | Genotype 2, 4* |
| | JOE/HEX/Cy3 | - | |
| 1a/2 | FAM | | |
| | JOE/HEX/Cy3 | 33,0 | |
| 4/IC | FAM | 35,8 | |
| | JOE/HEX/Cy3 | 21,1 | |

| Tube | Channel | Ct | Result |
|---|---|---|---|
| 1b/3 | FAM | - | repeat test |
| | JOE/HEX/Cy3 | - | |
| 1a/2 | FAM | - | |
| | JOE/HEX/Cy3 | - | |
| 4/IC | FAM | - | |
| | JOE/HEX/Cy3 | - | |

*Ct of the 4th genotype is lower than Ct of 1st for more than 15 cycles: the results for the genotypes 1a and 1b must not be consider. The result must be given as “positive for genotype 4”.

## SPECIFICATIONS

The sensitivity of the kit **HCV Real-TM Genotype** was evaluated using serial diluitions of recombinant positive controls. The analytical specificity of the kit was determined using the recombinant positive controls with high HCV concentrations (not less than $10^8$ copies/ml). The diagnostic specificity and sensitivity of the kit **HCV Real-TM Genotype** were analyzed also on clinical samples (blood plasma). 245 patients having diagnosis of viral hepatitis C, represented by different genotypes (1a (43 samples), 1b (66 samples), 3a (68 samples), 2 (57 samples), 4 (5 samples), 1a+1b (6 samples) were included in the trial group. As a kit of comparison the HCV Genotype Kit (Sacace) with electrophoresis detection as well as the sequencing (for samples, which are non-identified in HCV Genotype Kit) were used. The control group was presented by blood plasma taken from patients with hepatitis of other etiology and samples taken from healthy persons – donors, total 30 samples. There were used 2 control panels QCMD (Quality Control for Molecular Diagnostics) containing different genotypes of the virus – "QCMD 2006 Hepatitis C Virus Genotype Proficiency Panel" and "QCMD 2007 Hepatitis C Virus Genotype Proficiency Panel".

**Results:**

The analytical sensitivity of the kit **HCV Real-TM Genotype** was 1000 IU/ml. Using the recombinant positive controls with high HCV concentrations and blood samples taken from patients with high viral load, it was shown the complete absence of cross-reactions between the above mentioned genotypes. In the control group none of the specimens showed any reactivity with **HCV Real-TM Genotype** kit**.** The diagnostic specificity of the tested **HCV Real-TM Genotype** kit was 100% and the diagnostic sensitivity was 100%. **HCV Real-TM Genotype** kit detected the genotypes 1a, 1b, 2, 3, 4 present in the QCMD panels.

## TROUBLESHOOTING

1. Weak (Ct > 40) or no signal of the IC (Fam channel tube 4/IC) for the Negative Control of extraction.
    - The PCR was inhibited.
        - ⇒ Make sure that you use a recommended RNA extraction method and follow to the manufacturer's instructions.
        - ⇒ Re-centrifuge all the tubes before pipetting of the extracted RNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. Don't disturb the pellet, sorbent inhibit reaction.
    - 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 select for the IC detection 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 > 38) or no signal of the Positive Controls ($C+_{1b/3,}$ $C+_{1a/2,}$ $C+_{4}$).
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the amplification protocol and select the fluorescence channel reported in the manual.
    - Incorrect pipetting of reagent.
        - ⇒ Check your pipetting scheme (see table 2. Samples distribution)
3. Any signal with Negative Control of extraction (ecc. Fam channel tube 4/IC).
    - 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 between tubes.
        - ⇒ Repeat the RNA extraction with the new set of reagents.
4. Any signal with Negative Control of PCR (TE-buffer).
    - 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 last.
        - ⇒ Repeat the PCR preparation with the new set of reagents.

## KEY TO SYMBOLS USED

| | | | |
|---|---|---|---|
| REF | List Number |  | Caution! |
| LOT | Lot Number |  | Contains sufficient for <n> tests |
|  | Expiration Date | VER | Version |
|  | Store at | **NCA** | Negative Control of Amplification |
|  | Manufacturer | **NCE** | Negative control of Extraction |
|  | Consult instructions for use | **C+** | Positive Control of Amplification |
| **IC** | Internal Control | **RUO** | For Research Use Only |

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