HCV Real-TM Quant Dx Handbook

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

CE 1023

IVD **For Quantitative *inVitro* Diagnostic Use**

REF **V1-96/3FRT**

Σ **96**

For use with Rotor-Gene® 6000/Q (Qiagen)

For use with SaCycler-96™ (Sacace)

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KEY TO SYMBOLS USED

REF	List Number	Σ	Contains sufficient for <n> tests
LOT	Lot Number	VER	Version
IVD	For *InVitro* Diagnostic Use	CONTROL INT	Internal control
	Temperature limitations	CONTROL 1	High Positive Control
	Manufacturer	CONTROL 2	Low Positive Control
	Consult instructions for use	CONTROL -	Negative Control
	Expiration Date	CAL 1	Standard 1
	Caution!	CAL 2	Standard 2
	Keep away from sunlight		

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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.

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.

HCV RNA testing done with the quantitative kit provides prognostic information regarding likelihood of treatment response to interferon monotherapy, interferon plus ribavirin combination therapy and peginterferon plus ribavirin combination therapy. Sustained virological response is defined as testing negative for HCV RNA, 6 months after cessation of therapy. Recent studies suggest that the rate of

response to therapy is also important. For instance, conversion to an HCV RNA negative test result after 4 weeks of therapy constitutes a rapid virological response and it is a strong predictor of treatment success. Patients who have not had an early virological response, defined as at least a 2-log decline in HCV RNA, after 12 weeks of therapy are unlikely to respond with an additional 36 weeks of therapy and should stop therapy.

INTENDED USE

HCV Real-TM Quant Dx kit is a Real-Time Amplification test for the Quantitative detection of Hepatitis C virus RNA in human plasma. **HCV Real-TM Quant Dx** Real Time assay is not for screening blood, plasma, serum or tissue donors for HCV, or to be used as a diagnostic test to confirm the presence of HCV infection.

PRINCIPLE OF ASSAY

HCV Real-TM Quant Dx Kit is a Real-Time PCR test for the Quantitative detection of Hepatitis C Virus. HCV RNA is extracted from plasma, amplified using real time amplification and detected using fluorescent reporter dye probes specific for HCV and the simultaneous detection of a HCV-specific Internal Control (IC), by dual color detection.

During each round of thermal cycling, amplification products dissociate to single strands at high temperature allowing primer annealing and extension as the temperature is lowered. Exponential amplification of the product is achieved through repeated cycling between high and low temperatures, resulting in a billion-fold or greater amplification of target sequences. Amplification of both targets (HCV and IC) takes place simultaneously in the same reaction. Monitoring the fluorescence intensities during Real Time allows the detection and quantification of the accumulating product without having to re-open the reaction tube after the real time amplification.

Internal Control (IC) serves as an extraction and an amplification control for each individually processed specimen and to identify possible inhibition. IC is detected in a channel other than the HCV RNA. HCV-IC-L is a lyophilized Internal Control and represents recombinant RNA-containing-structure which carried through all steps of analysis from nucleic acid extraction to PCR amplification-detection. The presence of HCV Rec IC allows not only to monitor the extraction procedure and to check possible PCR inhibition but also to verify possible losses of the RNA during extraction procedure thus enabling to calculate precisely the HCV viral load.

The assay is standardized against the 4th WHO International Standard for Hepatitis C Virus for Nucleic Acid Amplification Techniques (NIBSC code: 06/102 15) and results are reported in International Units/mL (IU/mL).

The target sequence for the **HCV Real-TM Quant Dx** assay is in the 5´utr region of the HCV genome. This region is specific for HCV and is highly conserved.

MATERIALS PROVIDED

Sacace HCV Real-TM Quant Dx Amplification Reagent Kit

RT-PCR REAGENT PACK

- 96 vials (0,2 ml) with lyophilized amplification reagents

Sacace HCV Real-TM Quant Dx Control Kit[1, 2]

CONTROL INT

- 4 vials with lyophilized reagent HCV-IC-L

CONTROL 1

- 4 vials with lyophilized reagent HCV-Pos1-L C+ - Sacace HCV Real-TM Quant Dx High Positive Control*

CONTROL 2

- 4 vials with lyophilized reagent HCV-Pos2-L C+ - Sacace HCV Real-TM Quant Dx Low Positive Control*

CONTROL –

- 4,0 vials, 4,0 ml per vial with Negative Control

Sacace HCV Real-TM Quant Dx Calibrator Kit[1,2]

CAL 1

- 4 vials with lyophilized reagent HCV Quantitative Standard 1

CAL 2

- 4 vials with lyophilized reagent HCV Quantitative Standard 2

[1]Standards' and controls' concentrations are specific for every lot.

[2]must be used during the sample preparation procedure (see RNA isolation)

MATERIALS REQUIRED BUT NOT PROVIDED

- RNA isolation kit (see RNA isolation)
- Desktop microcentrifuge for "eppendorf" type tubes
- Vortex mixer
- Disposable gloves, powderless
- Biohazard waste container
- Refrigerator, Freezer
- Real Time Thermal cycler
- Biological safety cabinet approved for working with infectious materials
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Tube racks

WARNINGS AND PRECAUTIONS

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

1. Read the instructions in this package insert carefully before processing samples. Strict compliance with the user manual is required for optimal PCR results.
2. Wear disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterward.
3. Use routine laboratory precautions. Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas. Do not pipette by mouth.
4. Do not use a kit after its expiration date.
5. Do not mix reagents from different kits.
6. To reduce the risk of nucleic acid contamination due to aerosols formed during pipetting, pipettes with aerosol barrier tips must be used. Change aerosol barrier pipette tips between all liquid transfers.
7. During preparation of samples, compliance with good laboratory practices is essential to minimize the risk of cross-contamination between samples as well as the inadvertent introduction of nucleases into samples during and after the extraction procedure. Proper aseptic technique should always be used when working with RNA or DNA.
8. Use only powder-free gloves.
9. Dispose all specimens and unused reagents in accordance with local regulations.
10. Heparin has been shown to inhibit reaction. The use of heparinized specimens is not recommended.
11. Specimens may be infectious. Use Universal Precautions when performing the assay.
12. Specimens and controls should be prepared in a laminar flow hood.
13. Handle all materials containing specimens or controls according to Good Laboratory Practices in order to prevent cross-contamination of specimens or controls.
14. 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.
15. 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.
16. This product does not contain potentially infectious components. Material Safety Data Sheets (MSDS) are available on request.
17. Use of this product should be limited to personnel trained in the techniques of amplification.
18. 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.
19. It is possible that not all known HCV genotypes are detected.

STORAGE AND SHIPPING INSTRUCTIONS

All components of the **HCV Real-TM Quant Dx** PCR kit must be stored at +2-8ºC when not in use. All components of the **HCV Real-TM Quant Dx** 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.

HCV Real-TM Quant Dx PCR kit can be shipped at a temperature between 4°C and 37°C for up to 2 weeks.

STABILITY

HCV Real-TM Quant Dx 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. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results. When a positive or negative control value is out of the expected range, it may indicate deterioration of the reagents. Associated test results are invalid and samples must be retested. Assay recalibration may be necessary.

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.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

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

Current studies refer to EDTA or Citrate Phosphate Dextrose (CDP) plasma as the most suitable sample materials for HCV detection. Therefore, we recommend the use of these materials with the **HCV Real-TM Quant Dx**. The internal validation of the **HCV Real-TM Quant Dx** assay has been performed using human CDP plasma samples. Sample materials other than plasma are not validated.

HCV Real-TM Quant Dx assay is only for use with human plasma specimens

1. EDTA or CDP tubes may be used with the **HCV Real-TM Quant Dx**. Follow sample tube manufacturer's instructions.
2. Whole blood collected in EDTA or CDP 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.

INTERFERING SUBSTANCES

Elevated levels of bilirubin (≥15 mg/dl) and lipids (≥800 mg/dl) do not influence the system. Heparin (≥10 IU/ml) affects the PCR. Samples that have been collected in tubes containing heparin as anticoagulant should not to be used. Also, samples of heparinized patients must not be used.

REAGENTS PREPARATION

Before starting any **HCV Real-TM Quant Dx** protocol prepare the following reagents:

1. Choose the requested quantity of lyophilized controls and calibrators and centrifuge briefly.
2. Add Negative Control(CONTROL -) as for table below:

Lyophilized reagent	CONTROL - , µl
CAL 1	1200
CAL 2	1200
CONTROL 1	1200
CONTROL 2	1200
CONTROL INT	300

3. Close the tubes and incubate all tubes for 2 min at room temperature. Vortex periodically.
4. Centrifuge the tubes for 5 sec.
5. Dissolved reagents must be stored at 2-8 °C and always protected from light up to 30 days (do not freeze!)

RNA ISOLATION

Any commercial RNA/DNA isolation kit, if IVD-CE 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:

- ⇒ **Magno-Virus** (Sacace, REF K-2-16): sample volume 1000 µl, recommended elution volume 50 µl;
- ⇒ **SaMag Viral Nucleic Acids Extraction kit** (Sacace, REF SM003): automated nucleic acid purification system: sample volume 400 µl, recommended elution volume 50 µl.

Please carry out the RNA extraction according to the manufacturer's instructions. Add 10 µl of CONTROL INT during the RNA isolation procedure directly to the sample/lysis mixture in all samples, controls, calibrators (see Internal Control).

High and Low Positive Controls (CONTROL 1 and CONTROL 2) as well as Neg. Control (CONTROL -) must be always used with patient specimens during RNA isolation procedure. These controls must be treated in the same way as patient specimens following the Handbook of used RNA purification kit.

INTERNAL CONTROL

HCV-IC-L represents recombinant RNA-containing-structure which carried through all steps of analysis from nucleic acid extraction to PCR amplification-detection. The presence of HCV-IC-L allows not only to monitor the extraction procedure and to check possible PCR inhibition but also to verify possible losses of the RNA during extraction procedure thus enabling to calculate precisely the HCV viral load.

An IC threshold cycle [Ct] assay validity parameter is established during a calibration run. A defined, consistent quantity of IC(10 µl) is introduced into each specimen, calibrator, and control at the beginning of sample preparation and measured on the real time pcr instrument to demonstrate proper specimen processing and assay validity.

The median amplification cycle at which the IC target sequence fluorescent signal is detected during the calibration procedure (two calibrators are run in replicates of three) establishes an IC Ct validity range to be met by all subsequent processed specimens.

The established range of samples IC Ct = *Medium IC Ct of 6 calibrators ± 2,5 cycles*

Specimens whose IC Ct value exceeds the established range must be retested starting from sample preparation.

ASSAY CALIBRATION

A calibration curve is required to quantitate the HCV RNA concentration of specimens and controls. Two assay calibrators are run in replicates of three to generate a calibration curve (HCV concentration versus the threshold cycle [Ct] at which a reactive level of fluorescent signal is detected). The calibration curve slope and intercept are calculated and stored on the instrument. The concentration of HCV RNA in a sample is calculated from the stored calibration curve.

The quantitative standards CAL1 and CAL2 must be treated in the same way as patient specimens.

Before the first use of a new lot of **HCV Real-TM Quant Dx,**6 calibrators run must be performed beginning from RNA extraction procedure to generate a calibration curve (two calibrators are run in replicates of three):

- Prepare 3 sample preparation tubes for CAL1, 3 tubes for CAL2 for each calibration run.
- Add 10 µl of CONTROL INT to each tube;
- Add CAL1 and CAL2 to the appropriate tubes in the quantity indicated in the manual of RNA purification kit (for example, 1000 µl using Magno-Virus kit (Sacace, REF K-2-16)

Follow the sample preparation procedure described in the Handbook of used RNA purification kit.

ASSAY PROTOCOL

Note: Reaction Mix volume = 50 µl

⚠ *No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution*

REAL TIME PCR CALIBRATION PROCEDURE:

1. Prepare 6 reaction tubes with lyophilized reagents to perform PCR of extracted calibrators.
2. Add **50 µl** of eluted samples obtained from RNA purification of 6 calibrators.

Follow the procedure for amplification and detection as described in this manual.

Once an **HCV Real-TM Quant Dx** calibration run is accepted and stored, it may be used until the lot in use expires. During this time, all subsequent samples may be tested without further calibration by importing the experiment with Calibration Curve in the experiment with clinical samples using the same **HCV Real-TM Quant Dx** lot number.

REAL TIME PCR SAMPLE PROCEDURE:

1. Prepare requested quantity of reaction tubes with lyophilized reagents to perform PCR of extracted samples and controls.
2. Add **50 µl** of eluted samples obtained from RNA purification step.

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

Create a temperature profile on your Real-time instrument as follows:

Stage	*Temp,°C*	*Time*	*Fluorescence detection*	*Cycle repeats*
Hold	50	15 min	–	1
Hold	95	15 min	–	1
Cycling	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling 2	95	5 s	–	40
	60	30 s*	FAM, JOE/HEX/Cy3	
	72	15 s	–	

** 20 s using Rotor-Gene™ 6000/Q instrument*

QUALITY CONTROL PROCEDURE

A defined quantity of Internal Control (IC) is introduced into each sample at the beginning of sample preparation procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition.

One replicate each of the Negative Control (CONTROL -), the HCV High (+) Control (CONTROL 1) and the HCV Low (+) Control (CONTROL 2) must be included in each test run.

If the controls are out of their expected range (see Data Sheet), all of the specimens and controls from that run must be processed beginning from the sample preparation step.

RESULTS INTERPRETATION

The Internal Control (IC) is detected on FAM/Green channel and HCV RNA on Joe/Yellow/HEX channel.

Each DNA amplification is associated with generation of a fluorescence signal measurable in FAM/Green channel (for IC) or in Joe/Yellow/HEX channel (for HCV RNA) resulting in a sigmoid growth curve (log scale).

The data analysis is performed according to manufacturer's instructions (Operation manual Rotor-Gene™ 6000/Q, Qiagen and Instrument Manual SaCycler-96™, Sacace) using the respective software and considering the recommendations of the handbook.

Check the obtained results to ensure that the run is valid and to interpret results.

HCV RNA is determined basing on CT values and as standard curve resulting from analysis of quantitation standards.

HCV RNA concentration is expressed in IU/ml.

If the result is more than 100,000,000 IU/ml, then it will be displayed as the result more than 100,000,000 IU HCV/ml. If the result is more than the linear measurement range, the sample can be analyzed after 10x dilution and the obtained result should be multiplied by 10.

If the result is less than 13 IU/ml in case of extraction from 1 ml, then it will be displayed as the result less than 13 IU HCV/ml.

PERFORMANCE CHARACTERISTICS

The **HCV Real-TM Quant Dx** Kit was evaluated according to the common technical specifications (CTS) for in vitro diagnostic medical devices (2009/108/EC) and European Pharmacopoeia 7.0, p. 2.6.21. Nucleic acid amplification techniques (07/2010:20621).

DIAGNOSTIC CHARACTERISTICS EVALUATION

As a result of clinical tests, the diagnostic characteristics of the kit were established: diagnostic sensitivity and diagnostic specificity total for all types of biomaterials.

Number of studies conducted to establish diagnostic characteristics:

RESULT	TOTAL
True positive	100
False positive	0
True negative	100
False negative	0
Total	200

Diagnostic sensitivity

Diagnostic sensitivity= TP/(TP+FN) x 100% with 95% confidence interval, where TP - true positive results obtained by tested kit and reference methods, FN - false negative results in case of negative result obtained by tested kit and positive result obtained by reference methods.

Diagnostic specificity

Diagnostic specificity= TN/(TN+FP) x 100% with 95% confidence interval, where TN - true negative results obtained by tested kit and reference methods, FP - false positive results in case of positive result obtained by tested kit and negative result obtained by reference methods.

Diagnostic characteristics:

Number of samples (n) - 200;

Diagnostic sensitivity - 100,0 % (95 % CI: 96,38-100 %);

Diagnostic specificity - 100,0 % (95 % CI: 96,38-100 %).

Thus, according to the data analysis (n=200 samples) it was found that the diagnostic sensitivity of the kit is 100,0 % (95 % CI: 96,38-100 %), diagnostic specificity is 100,0 % (95 % CI: 96,38-100 %).

ANALYTICAL SENSITIVITY

The analytical sensitivity or Limit of Detection (LOD) is defined as the smallest amount of HCV RNA that can be precisely detected with a probability of 95% or greater. The LOD in consideration of RNA purification from the sample is determined using HCV-positive clinical specimens in combination with a particular extraction method. HCV RNA was extracted with Magno-Virus purification kit (Sacace, REF K-2-16) according to the manufacturer's instructions.

The LOD of the **HCV Real-TM Quant Dx** assay is 13 IU/mL with the 1.0 mL sample preparation procedure.

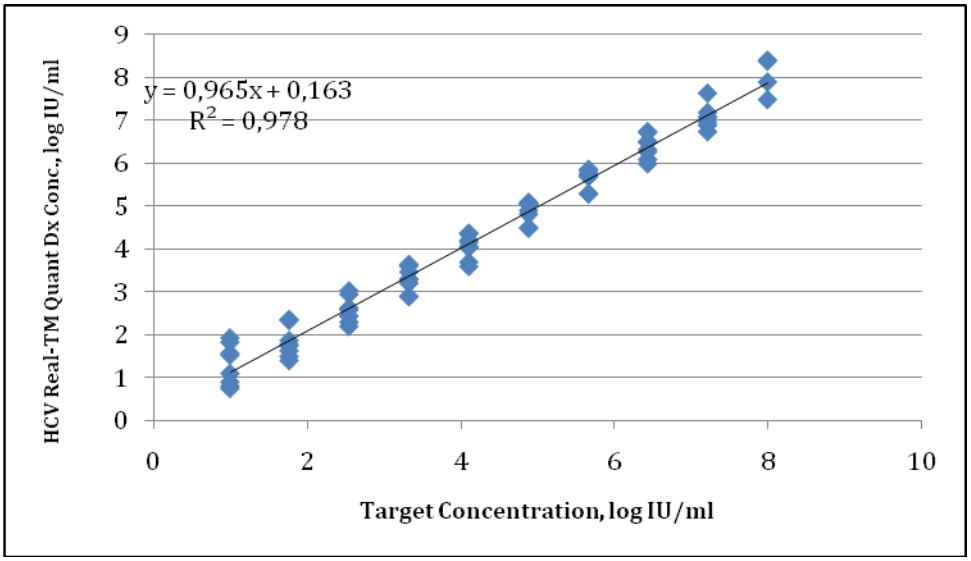
The LOD was determined by testing dilutions of the 4th WHO International Standard for Hepatitis C Virus for Nucleic Acid Amplification Techniques (NIBSC code: 06/102 15) prepared in HCV negative human plasma. The results were determined by Probit analysis.

LINEAR RANGE

Linear range or Linearity of an assay is its ability to obtain test results that are directly proportional to the concentration of the nucleic acid. In other words, it is the interval between the upper and the lower concentration where test results are directly proportional to the concentrations.

The upper limit of quantitation for the **HCV Real-TM Quant Dx** assay is 100 million IU/mL and the lower limit of quantitation is equal to the Limit of Detection (13 IU/mL with the 1.0 mL sample preparation procedure).

The linear range of the **HCV Real-TM Quant Dx** kit was determined by analyzing a dilution series (from 8,00 log IU/ml to 1,00 log IU/ml) of an HCV synthetic quantitative standard calibrated against the 4th WHO International HCV RNA Standard. The results, representative of the **HCV Real-TM Quant Dx** assay linearity, are shown in Figure 1.



SPECIFICITY

The analytical specificity of the primers and the probes was validated with 100 negative plasma samples obtained from the European Directorate for the Quality of Medicine & HealthCare (EDQM). They did not generate any signal with the specific HCV primers and probes. The specificity of the kit **HCV Real-TM Quant Dx** was 100%

GENOTYPE DETECTION

Genotypes 1a, 3a, 5a were tested from QCMD HCV genotype 2010 panel; genotypes 1b, 4h were tested from QCMD HCV genotype 2011. The detectability of all relevant subtypes and genotypes has thus been ensured. All tested HCV RNA genotypes give positive results with **HCV Real-TM Quant Dx** kit.

CROSS-REACTIVITY

The potential cross-reactivity of the kit **HCV Real-TM Quant Dx** was tested against the group control listed in the following table. It was not observed any cross-reactivity with these pathogens.

Table 1: Testing the specificity of the kit with other pathogens:

Control group	Results HCV (Joe/Yellow channel)	Results IC (Fam/Green channel)
Cytomegalovirus (CMV)	-	+
Epstein Barr virus (EBV)	-	+
Hepatitis B virus (HBV)	-	+
Hepatitis D virus (HDV)	-	+
Hepatitis A virus (HAV)	-	+
Parvovirus B19	-	+
Herpes Zoster Virus (VZV)	-	+
Human immunodeficiency virus 1	-	+
Herpes simplex virus 1	-	+
Herpes simplex virus 2	-	+
Human herpes virus 6	-	+
Human herpes virus 8	-	+
West Nile Virus (WNV)	-	+
Tick-borne encephalitis virus (TBEV)	-	+
Adenovirus type 2	-	+
Adenovirus type 4	-	+
Adenovirus type 7	-	+
Rotavirus A	-	+
Escherichia coli (E.coli)	-	+
Staphylococcus aureus	-	+
Streptococcus pyogenes	-	+
Streptococcus agalactiae	-	+

ROBUSTNESS

The robustness of the assay is a measure of its capacity to remain unaffected by small and accidental variations in method parameters and provides an indication of its reliability during normal usage.

As **HCV Real-TM Quant Dx** kit contains RT-PCR REAGENTS PACK 96 vials with ready-to-use lyophilized reagents, the only probable variation during normal usage of the kit could be the different volume of added eluted samples obtained from RNA purification step.

The robustness of **HCV Real-TM Quant Dx** kit was evaluated adding different volume of eluted sample (from 46 to 52 µl). 20 HCV RNA negative plasma pools spiked with HCV RNA to a final concentration of 3 times 95 per cent cut-off value were added with small variations (from 46 to 52 µl) to the vials containing lyophilized reagents. All tested samples were found positive for HCV RNA.

PRECISION

The precision of the HCV Real-TM Quant Dx kit was evaluated for the manual sample preparation using the SaCycler-96 instrument.

The precision data of the HCV Real-TM Quant Dx kit allow the determination of the total variance of the assay. The total variance consists of the intra-assay variability (variability of multiple results of samples of the same concentration within-run), the inter-assay variability (variability of multiple results of the assay generated on different days) and the inter-lot variability (variability of multiple results of the assay using different lots). The data obtained was used to determine the standard deviation and the coefficient of variation for the specific pathogen and the internal control.

The total variability calculated was:

mean SD Log Ct HCV	mean SD Log Ct IC
0,003	0,003

mean CV % Ct HCV	mean CV % Ct IC
0,79	0,79

Intra-assay variability

SD log Ct HCV	SD log Ct IC
0,002	0,003

CV % Ct HCV	CV % Ct IC
0,53	0,65

Inter-assay variability

SD log Ct HCV	SD log Ct IC
0,003	0,003

CV % Ct HCV	CV % Ct IC
0,70	0,63

Inter-lot variability

SD log Ct HCV	SD log Ct IC
0,005	0,005

CV % Ct HCV	CV % Ct IC
1,15	1,10

CROSS CONTAMINATION

The cross contamination of the HCV Real-TM Quant Dx kit was evaluated for the manual sample preparation using RotorGene Q instrument. To demonstrate absence of cross contamination a panel of 20 samples consisting of alternate samples of negative plasma spiked with high concentration of HCV and negative plasma samples was tested.

All the positive samples were correctly detected and gave a fluorescence positive reaction with good and clear sigmoid-shaped fluorescence curves and a Cycle threshold (Ct) less than 35 both in HCV channel and in Internal Control channel.

All the negative samples gave no fluorescence signal in the HCV channel and the Internal Control channel showing no cross contamination at all.

PROTOCOL FOR ROTORGENE™ 6000/Q

1. Switch on instrument by pressing the main switch, start Rotor-Gene software
2. Start a New Run

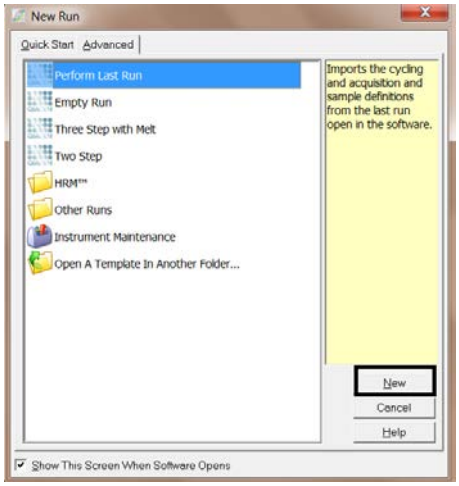


Figure 1. Start "New Run"

3. Select the appropriate rotor, check the "Locking Ring Attached" box and click "Next".

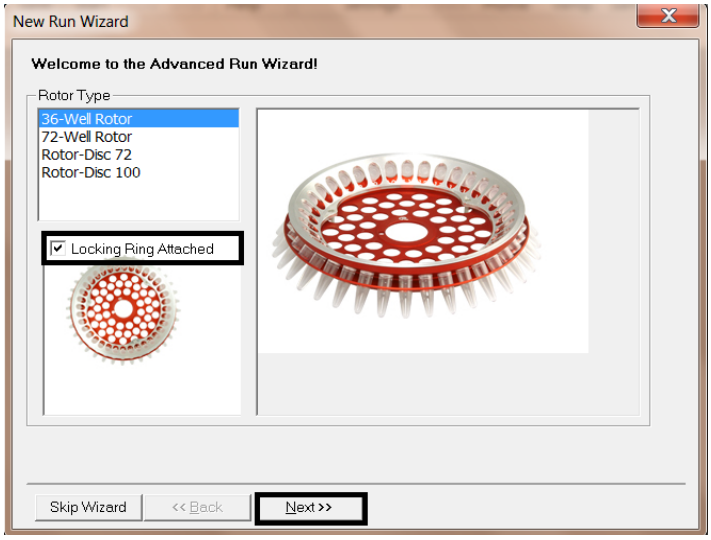


Figure 2. Rotor and Locking Ring

4. Input operator's name, notes, and reaction volume (50). Subsequently press "Next".

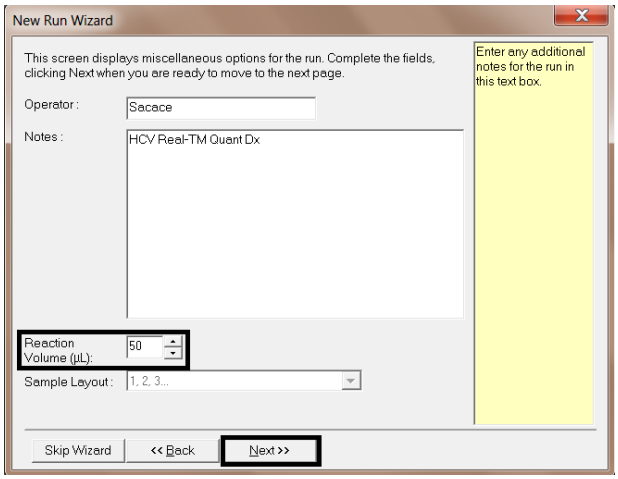


Figure 3. Reaction Volume

5. Click the “Edit Profile” and program the temperature profile.

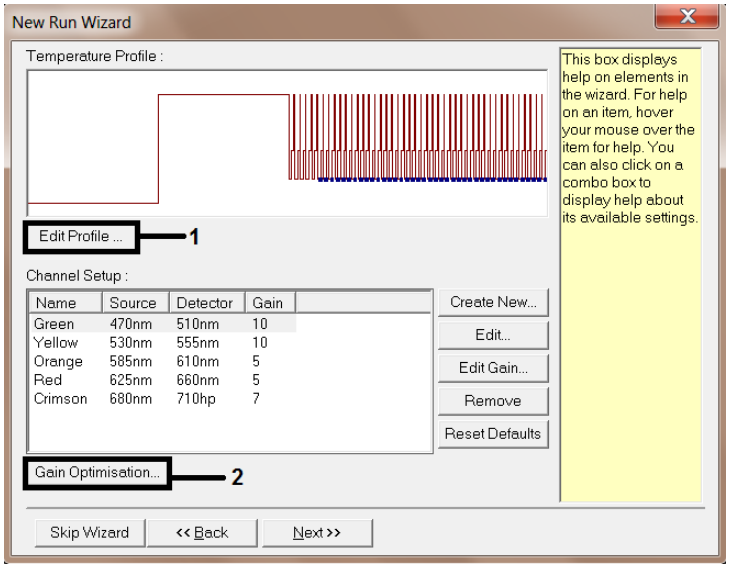


Figure 4. Edit Profile

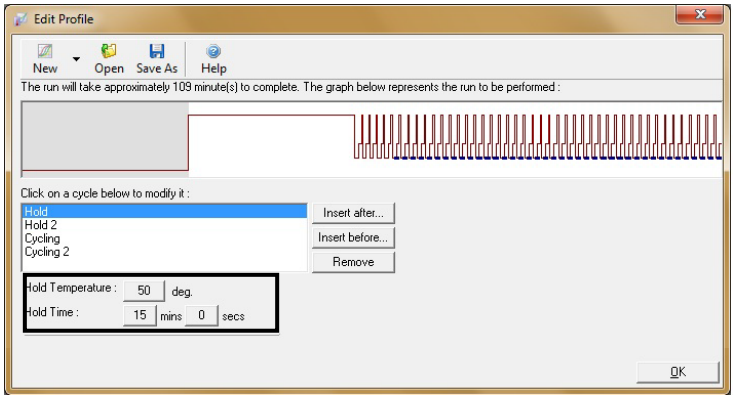


Figure 5. Hold: Reverse transcription step

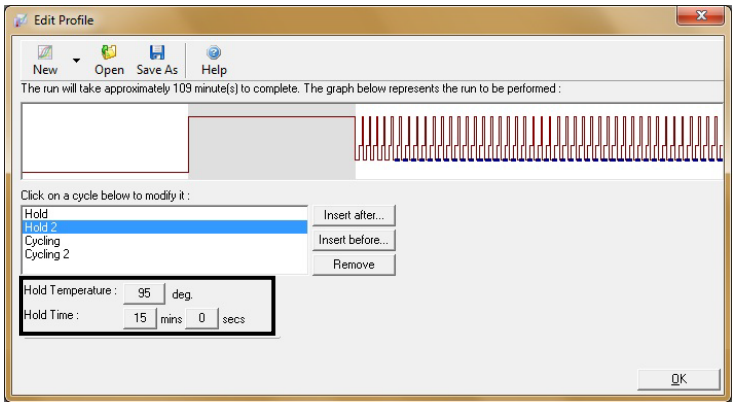


Figure 6. Hold 2: Enzyme activation step

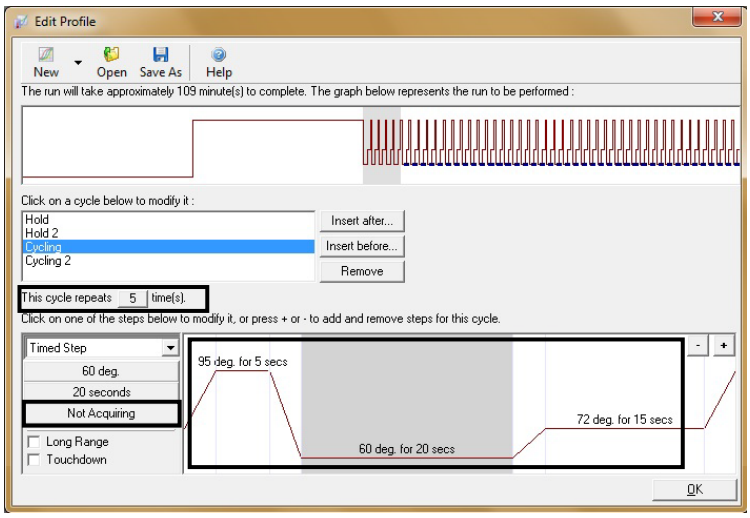


Figure 7. Cycling: 1st Amplification Step

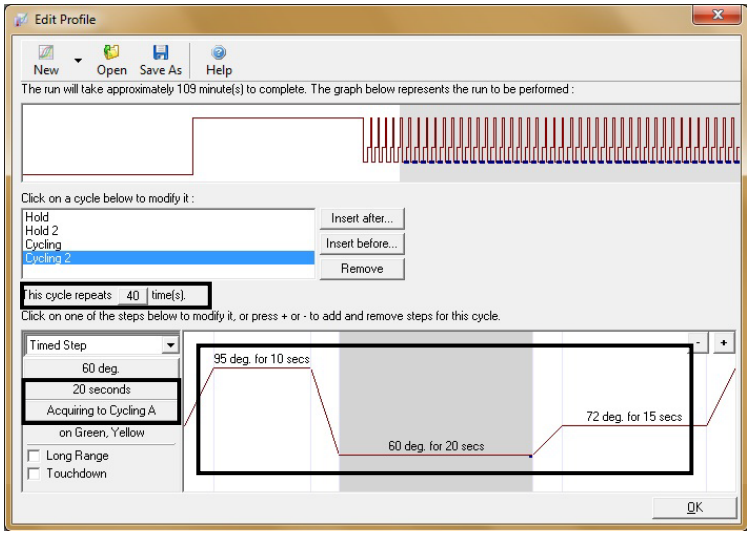


Figure 8. Cycling: 2nd Amplification and Detection Step

6. Click “Gain Optimisation” in the “New Run Wizard” menu and set the following parameters:

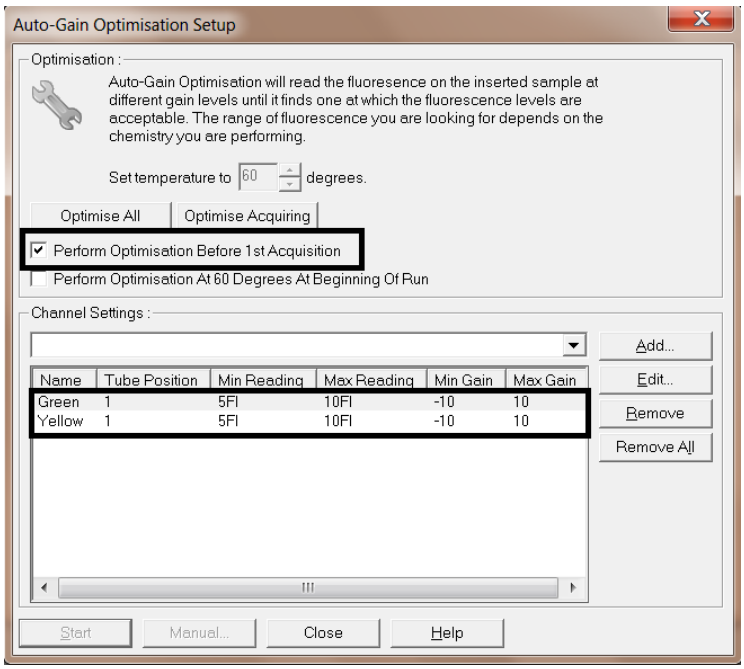


Figure 9. Gain Optimisation

7. Place PCR tubes carefully onto RotorGene™ 6000/Q rotor, close lid and start run.

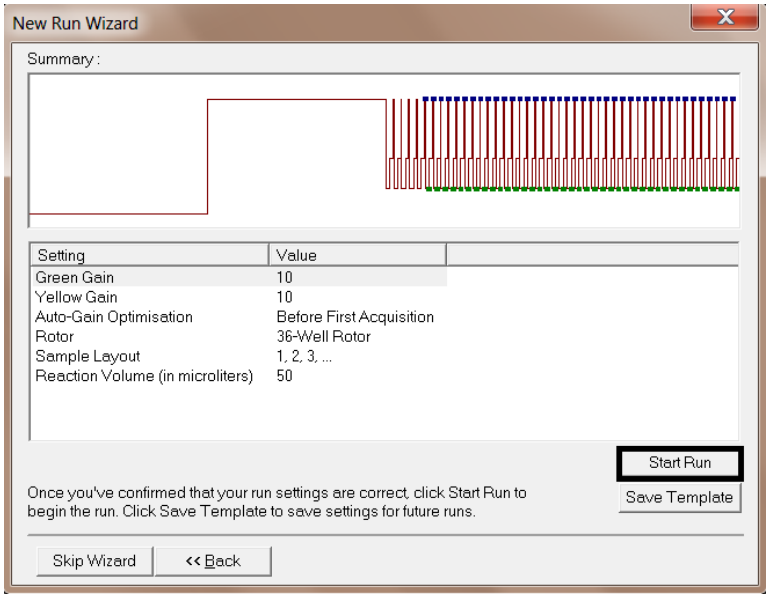
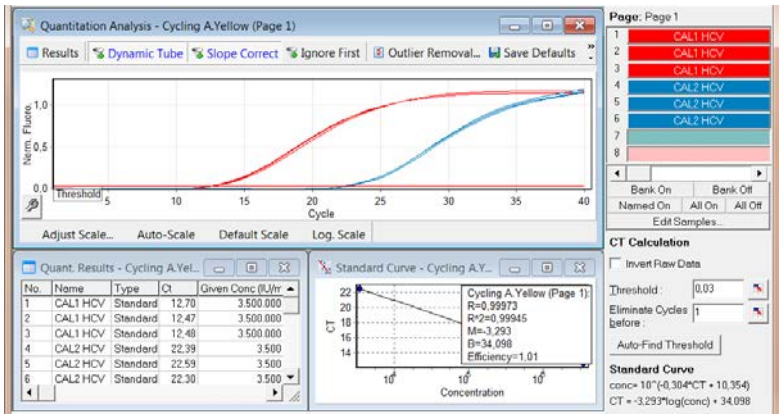
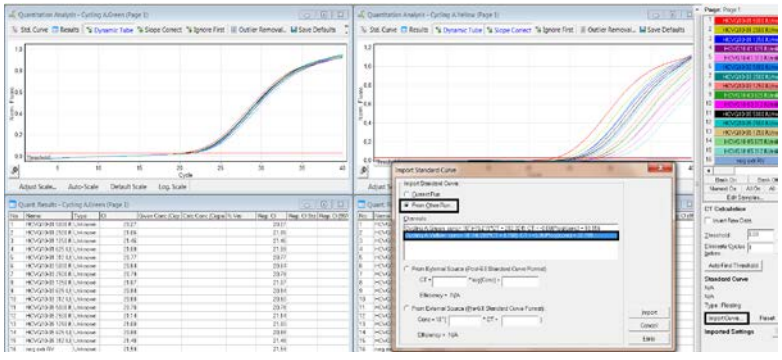


Figure 10. Starting the run.

8. Enter sample positions and names (“unknown” for samples, “Negative Control” and “Positive Control” for controls). If the kit calibration run is performing, use name “standard” for CAL1 and CAL2 and enter the concentrations of the Quantitative Standards reported on HCV Real-TM Quant Dx Data Card.



9. User must import the calibration experiment with Standard Curves to subsequent experiments with clinical samples to have quantitative results.To do this click Import Curve, select From Other Run option, choose the Calibration file to be imported and Import Joe/Yellow Channel.



Data Analysis

IC amplification analysis (*Cycling A.Green (Fam*)

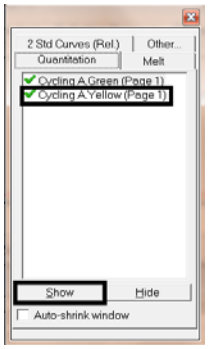
1. Press *Analysis* and then select *Quantitation* → *Cycling A.Green* (*Cycling A.Fam*) → *Show*

2. Turn off the automatic option *Threshold.*
3. Activate the *Dynamic tube* and *Slope Correct* buttons in the main window menu (Quantitation analysis).

4. In the table of results (*Quantitation Analysis*) select *More settings* and set *NTC Threshold* to 10%
5. Select *Threshold*: 0,03
6. In the table of results (*Quantitation Results*) appear the values of Ct (Threshold cycle) which should be ≤ 30 (see Internal Control)of the IC (Fam/Green channel).

HCV amplification analysis (*Cycling A.Yellow(Joe)*

1. Press *Analysis* and then select *Quantitation* → *Cycling A.Yellow* (*Cycling A.Joe)* → *Show*



2. Turn off the automatic option *Threshold.*

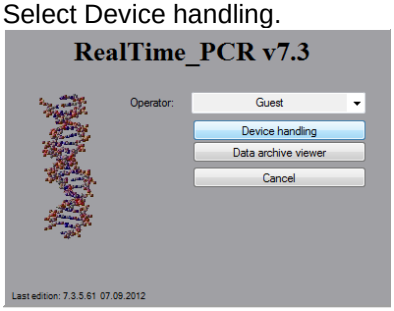
3. Press the buttons *Dynamic Tube*, *Slope Correct*

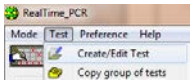
4. In the table of results (*Quantitation Analysis*) select *More settings* and set *NTC Threshold* to 10%.
5. Select *Threshold*: 0,03
6. In the table of results (*Quantitation Analysis*) appear the values of Ct (Threshold cycle).
7. In the new window *Quantitation Results* appear values of HCV RNA IU/ml.

PROTOCOL FOR SaCycler-96® Real Time PCR system

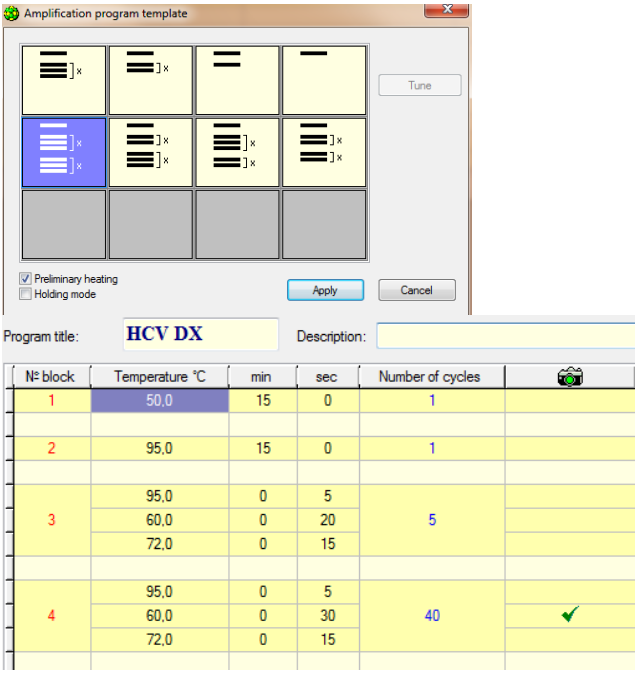
Recommended settings:

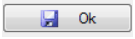
1. Double click on the RealTime_PCR software icon 
2. Select Device handling.

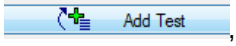


3. Select the plugged device and click “Connect”.
4. Select from Menu “Test -> Create/Edit test”  . Click “Create new test” button  and enter name “HCV Real-TM Quant DX”, click OK.
5. Select: Analysis Type “Multiplex detection” and Method “Threshold”, Standard Quantity “2” and copy “3” (total 6 standards), 2 positive controls, 1 negative control, FAM (IC) and R6G (s), 50 µl of Mixture volume:

6. Under “Amplification Program”, click  to create a new program.
7. Select “Preliminary heating” and choose the template below. Click Apply, then program the instrument as follows.

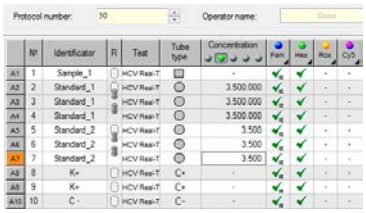


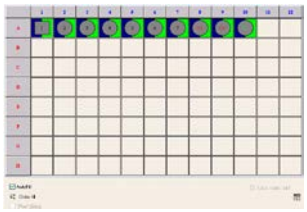
8. Click  and save the amplification program file in a convenient folder. Then click OK in the previous window.

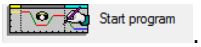
9. Click add test button  , then select “HCV Real-TM Quant DX” from dropdown menu Test, indicate the number of samples and click first Add, then OK.

10. Use the default Autofill positioning of PCR tubes into the plate or the Free Filling to chose manually the position of the tubes. In the “Concentration” column, click the green circle 

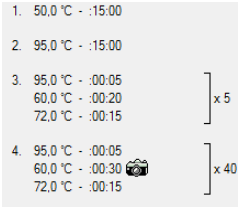
and insert the HCV standard values reported in the Data Card (see example below).

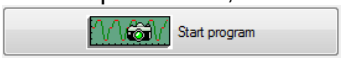




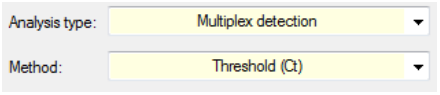
11. Clicking Apply will bring to the Start Program window 

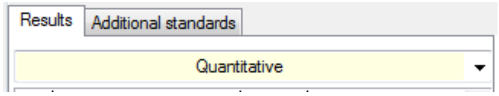
.
12. The thermalcycling program will be the following:



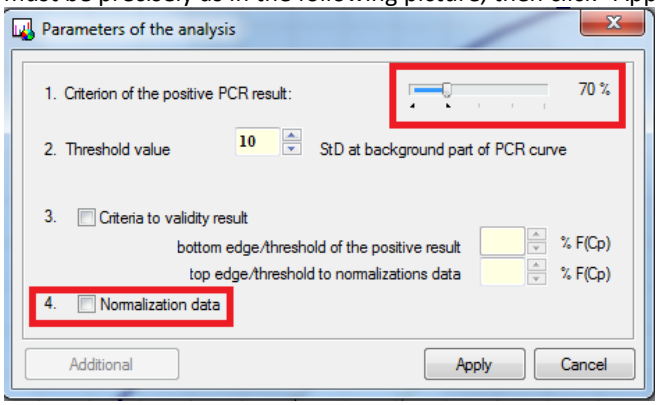
13. Click Open block, insert PCR tubes into the instrument, click Close block and then click 

to start the PCR amplification program.
14. After the run is complete click OK to interpret the results.
15. Select analysis type “Multiplex detection”, “Threshold (Ct)” method and “Quantitative” from results table.





16. Click on the icon for changing the parameter of data analysis 
, a new window will show up. The settings must be precisely as in the following picture, then click “Apply”:



Set **70%** as “Criterion of the positive PCR result”; “Normalization data” checkbox must be **deselected.**

17. Adjust the threshold line using mouse drag and drop. Suggested values for Threshold line are: 40 for HEX channel, 25 for FAM channel (slightly adapt threshold level according to your result curves).
18. Viral load results will appear in the “Results” column for HEX channel. Final results are already expressed in IU/ml.
19. To import standard curve from other run, click on “Additional standards” tab and select the run file containing standards. Then come back to “Results” tab to see viral load results.

NOTE: for IVD version of SaCycler, since the HCV Real-TM DX protocol is already inserted in the PC supplied with the instrument, ignore points 4-8 and just follow instructions from point 9.

TROUBLESHOOTING

1. Weak (Ct > 30) or exceeding the established range signal (see Internal Control)of the IC (Fam/Green 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. The correlation coefficient R^2 is less than 0.9: retesting of all samples is required.
3. The calculated concentrations of CONTROL 1 and/or CONTROL 1are different from given control concentrations, reported in the Data Sheet: retesting of all samples is required.
4. Any signal on the Joe/HEX/Yellow 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.
5. 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.

REFERENCES

1. World Health Organization. Hepatitis C—Global prevalence (update). WklyEpidemiol Rec, 1999, 74, 425–427.
2. Alter, H. 1999.Discovery of non-A, non-B hepatitis and identification of its etiology. Am. J. Med.107 (Suppl.6B):16S–20S.
3. Bendinelli, M., M. Pistello, F. Maggi, and M. Vatteroni. 2000. Blood-borne hepatitis viruses: hepatitis B, C, D, and G viruses and TT virus, p.306–337. In S.Specter, R.L. Hodinka, and S.A. Young (ed.), Clinical virology manual, 3rd ed. ASM Press, Washington, D.C.
4. Choo, Q.-L., G. Kuo, A.J. Weiner, L.R. Overby, D.W. Bradley, and M. Houghton. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244: 359–362.
5. Lauer, G.M., and B.D. Walker. 2001. Hepatitis C virus infection .N. Engl. J. Med. 345: 41–52
6. De Medina M, Schiff ER. Hepatitis C: diagnostic assays. Sem Liver Dis, 1995, 1, 33–40
7. Kao, J.-H., M.-Y. Lai, Y.-T. Hwang, P.-M. Yang, P.-J. Chen, J.-C. Sheu, T.-H. Wang, H.-C. Hsu, and D.-S. Chen. 1996. Chronic hepatitisC without anti-hepatitis C antibodies by second generation assay: a clinicopathologic study and demonstration of the usefulness of a third generation assay. Dig. Dis. Sci. 41: 161–165.
8. Schiff ER, de Medina M, Kahn RS. New perspectives in the diagnosis of hepatitis C. Sem Liver Dis, 1999, 19, 3–15.
9. Lok ASF, Gunaratnam NT. Diagnosis of hepatitis C. Hepatology, 1997, 26(Suppl 1), 48–56S.
10. Pawlotsky J M , Bouvier-Alias M, Hezode C, Darthuy F, Remire J, Dhumeaux D. Standardization of hepatitis C virus RNA quantification. Hepatology. 2000; 32: 654-659.
11. Manns MP, McHutchisonJG,GordonSC,etal.Peginterferonalfa-2bplusribavirincompared with interferonalfa-2bplusribavirinforinitialtreatmentofchronichepatitisC:a randomized trial.Lancet.2001;358:958-965
12. Weiland O, Braconier JH, Fryden A, Norkrans G, Reichard O, Uhnoo I. Influence of pretreatment factors on outcome of interferon-alpha treatment of patients with chronic hepatitis C. Scand J Infect Dis. 1999; 31: 115-118.
13. Fried MW, Shiffman ML, Reddy KR,et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection.NEngl J Med. 2002; 347:975-982.
14. Jensen DM, Morgan TR, Marcellin P, et al. Early identification of HCV genotype 1 patients responding to 24weeks peginterferon alpha-2a (40kd)/ribavirin therapy. Hepatology. 2006; 43:954-960
15. Davis GL, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferonalfa-2b plus ribavirin in patients with chronic hepatitis C. Hepatology. 2003; 38:645-652
16. Terrault NA, Pawlotsky JM, McHutchisonJ,et al. Clinical utility of viral load measurements in individuals with chronic hepatitis C infection on antiviral therapy. J Viral Hepat. 2005; 12:465-472

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