ROBOSCREEN DIAGNOSTICS

Instructions for Use

RoboGene HCV RNA Quantification Kit 3.0

Rev. 6 _ 02 / 2022

CE 0483

IVD

Order No.:

847-0207610032	32 reactions
847-0207610096	96 reactions
847-0207610192	192 reactions

CE
0483

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Contents

1 Introduction

1.1 Intended use

The RoboGene HCV RNA Quantification Kit 3.0 is intended for real-time PCR quantification of Hepatitis C Virus (HCV) RNA in human EDTA- or citrate plasma and serum samples. For specimen purification the manual method (INSTANT Virus RNA/DNA Kit) as well as the automated method (INSTANT Virus RNA/DNA Kit - FX) is validated. For amplification and detection the RoboGene HCV RNA Quantification Kit 3.0 is validated on the following real-time PCR devices: qTOWER 2 & 3; CFX96; LightCycler® 480; 7500 Fast, Rotor-Gene® 3000/6000/Q; RealLine Cycler 48-4/48-5/96-4/96-5 and QuantStudio 5. The assay is purposed for the clinical management of patients with chronic HCV in conjunction with clinical presentation and other laboratory markers for HCV infection.

This test is intended to assess viral response to antiviral treatment as measured by changes in plasma and serum HCV RNA levels. Furthermore, in a course of antiviral therapy the probability of a sustained viral response can be judged.

The RoboGene HCV RNA Quantification Kit 3.0 is not intended for use as a screening test for the detection of HCV RNA in blood or blood products or as a diagnostic test to confirm the presence of HCV infection.

1.2 Pathogen information

Hepatitis C is a viral infection of the liver which had been referred to as parenterally transmitted "non A, non B hepatitis" until identification of the causative agent in 1989 [1]. Hepatitis C virus (HCV) infection accounts for the majority of post-transfusion and sporadic hepatitis. HCV is a single-stranded, positive sense RNA virus with a genome of approximately 9,700 nucleotides coding for 3,000 amino acids [2, 3]. There is a high frequency of progressive chronic hepatitis. The RNA genome contains highly conserved 5' and 3' untranslated regions that are used by most detection kits. Different HCV isolates show high sequence heterogeneity. Until today 8 genotypes and more than 90 subtypes have been classified [4, 5]. Genotype 1 is the most prevalent worldwide, followed by genotype 3 and 2. In Africa and the Arabian Peninsula genotype 4 is the most common. Genotype 3a is the most common e.g. in Pakistan [6, 7].

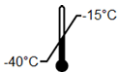
CONSULT INSTRUCTION FOR USE
This package insert must be read carefully prior to use. Given instructions must be followed accordingly. Reliability of results cannot be guaranteed if there are any deviations from the instructions in this package insert.

1.3 Technical assistance

If you have any questions or problems regarding any aspects of the RoboGene HCV RNA Quantification Kit 3.0 please do not hesitate to contact our technical support team which consists of experts with long-time experience in the field of molecular diagnostics. For technical assistance please contact us at the manufacturer site as shown inside the cover of the IFU.

1.4 Symbols and Abbreviations

For easy reference and orientation, the IFU uses the following warning and information symbols as well as the shown methodology:

Symbol	Information
	**REF** Catalogue number
	**Content** Contains sufficient reagents for <N> tests
	**Storage conditions**
	**Consult instructions for use** This information must be observed to avoid improper use of the kit and the kit components.
	**Use by**
	**Lot number** Lot number of the kit or component
	**IVD symbol** This kit is an *in vitro* diagnostic medical device
	**Manufactured by**
	Note / Attention Observe the notes marked in this way to ensure correct function of the device and to avoid operating errors for obtaining correct results.

The following abbreviations are used in the IFU:

Ct	Threshold cycle value
CV	Coefficient of variation
dNTP	2'-deoxynucleotide 5'-triphosphate
HCV	Hepatitis C Virus
IC	Internal Control
IFU	Instruction for use
IU	International Units
NTC	Non-template control
PEI	Paul-Ehrlich-Institut, Langen, Germany
WHO	World Health Organization

2 Safety precautions

NOTE
Read through this chapter carefully prior to guarantee your own safety and a trouble-free operation.
Follow all the safety instructions explained in the IFU, as well as all messages and information, which are shown.

Human plasma and serum samples have to be considered as potentially infectious. Thus, always wear lab coat and gloves.

Always use clean and nuclease-free equipment.

Set up of template preparation, PCR reagent assembly, amplification and detection should be performed in different rooms.

Please be careful while pipetting your specimen material to avoid carry-over contaminations.

Discard sample and assay waste according to your in-house safety regulations.

Please carry out regular maintenance of your equipment to ensure that the required temperatures, centrifugation speeds and mixing intensities (rpm) are maintained correctly.

ATTENTION
Do not eat or drink components of the kit!
The kit shall only be handled by educated personnel in a laboratory environment!

3 Test description and principle

3.1 Principle of the TaqMan® assay

TaqMan® real-time PCR is a highly sensitive assay that combines amplification with fluorescence-based online detection of the nucleic acid of interest (target, template). The assay is based on a conventional set of target and internal control-specific primers in combination with fluorescence-labelled oligonucleotide probes, complementary to the desired target sequences. In the presence of targets the probes hybridize with their target-complementary sequences. The Taq DNA polymerase from the RT PCR Enzyme possesses a 5`→ 3` exonuclease activity that hydrolyses the probes and displaces the fluorescent dye from the quencher. This event results in an increase of the fluorescence signal, which is directly proportional to the target amplification during each PCR cycle.

3.2 Explanation of the HCV RNA quantification test

The RoboGene HCV RNA Quantification Kit 3.0 is an amplification test for the quantification of HCV RNA in human plasma and serum samples. The assay is able to detect all known 8 genotypes of HCV [4, 5], by applying primers and probes specific for a subsequence of the 5' untranslated region of the viral genome. Quantification of specimens is performed by amplification of the included quantification standard strip in parallel.

A synthetic internal control is included to control the whole procedure from RNA extraction to the real-time PCR. Thus, the risk concerning false-negative results is drastically reduced, yielding in an increase of diagnostic correctness. Amplification of HCV RNA in samples and standards and of IC RNA is measured independently at different wavelengths due to probes labelled with different fluorescent reporter dyes. HCV RNA detection is performed in the FAM channel. For monitoring the Internal Control the kit provides

two options depending on the set up of the real-time PCR device and allows detection in Yakima Yellow/VIC/JOE or Cy5 channel.

Manual sample preparation should be conducted with the INSTANT Virus RNA/DNA Kit. RNA extraction must be performed strictly according to manufacturer's instructions using 'Protocol 2: Isolation of viral RNA/DNA from 400 µl of serum/plasma using IC Spiking Tube'.

Automated sample preparation should be conducted with the INSTANT Virus RNA/DNA Kit – FX in combination with the automated pipetting device "CyBio FeliX Basic Unit with Enclosure" together with the extraction add-on module "CyBio FeliX Extraction Set.

3.3 Restrictions

This test is validated for the usage with either human EDTA- or citrate plasma or serum. Heparinized plasma has to be excluded from analysis (see under point robustness of the test). Very high concentrations of lipids may act inhibitory on the quantification results. If other than the recommended sample types are used incorrect results may be obtained. The product is to be used only by personnel specially instructed and trained in *in vitro* diagnostics procedures. Strict compliance with the IFU is required for optimal PCR results. This kit may be used only with the mentioned real-time PCR devices and the recommended PCR consumables. Do not use expired components or mix with components from different lots.

4 Performance assessment

The RoboGene HCV RNA Quantification Kit 3.0 was validated according to the common technical specifications (CTS) for *in vitro* diagnostic medical devices (2002/364/EC) [8].

The validation of the RoboGene HCV RNA Quantification Kit 3.0 was executed with both purification methods, manual purification using the INSTANT Virus RNA/DNA Kit as well as the automated purification method using the INSTANT Virus RNA/DNA Kit - FX. With either method comparable results were achieved as shown in the Analytical Sensitivity.

4.1 Analytical Sensitivity

The analytical sensitivity of the RoboGene HCV RNA Quantification Kit 3.0 was determined by analyzing dilution series of the PEI Reference Material HCV RNA (#3443/04, genotype 1). Analytical sensitivities for used qPCR devices were determined as summarized below.

Table 1a: Determined device specific limits of detection and confidence intervals using manual nucleic acid purification.

qPCR device	Limit of detection (LOD) (IU/ml)	95 % confidence interval (IU/ml)	
qTOWER³	17.0	14.8	19.2
CFX96	13.6	11.6	15.5
LightCycler® 480	14.1	11.5	16.8
7500 Fast	20.2	17.1	23.2
Rotor-Gene® 6000	17.4	14.7	20.1

Table 1b: Determined device specific limits of detection and confidence intervals using automated nucleic acid purification

qPCR device	Limit of detection (LOD) (IU/ml)	95 % confidence interval (IU/ml)	
qTOWER³	11.7	9.8	13.7
CFX96	14.7	12.4	17.0
LightCycler® 480	13.2	11.2	15.1
Rotor-Gene® 3000	19.6	17.2	22.0
RealLine Cycler 48	26.5	22.6	30.5
RealLine Cycler 96	23.6	20.7	26.6
QuantStudio 5	19.7	17.4	22.0

Detection limit was calculated by PROBIT analysis of at least 24 replicates of each dilution of reference material on each qPCR device with confidence of 95 % (see Figure 1).

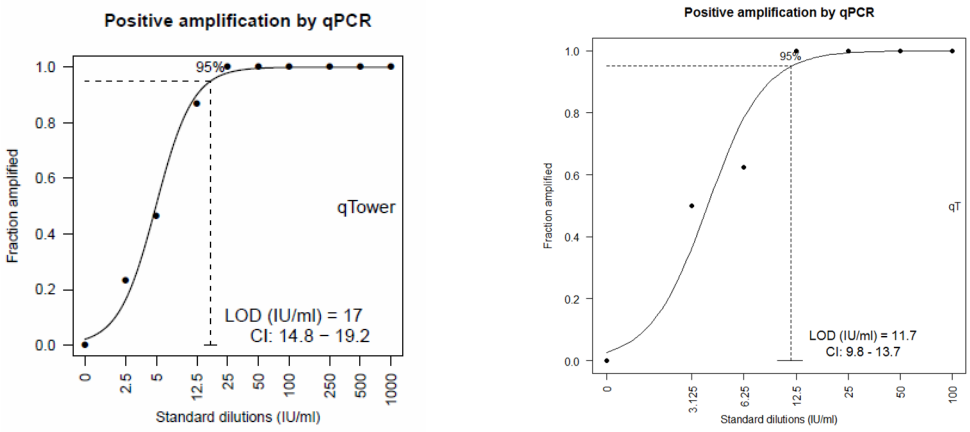


Figure 1: PROBIT analysis for the determination of PCR device specific limits of detection (LOD) with confidence of 95 % shown for an qTOWER³ device exemplarily for manual (left) and automated (FX) purification (right).

Individual values below the detection limit may be plausible but with a higher probability of error. To reduce this error probability 3 replicates of such samples are recommended.

4.2 Linear Range

The linear range for the quantification of HCV RNA was determined by analyzing dilution series of synthetic HCV RNA ranging from $4x10^{11}$ to $1x10^{1}$ IU/ml and of native sample material from $1x10^{7}$ to $1x10^{1}$ IU/ml. Experimental assessment was performed twice, quantifying all samples in triplicate using CFX96, qTOWER^3, LightCycler® 480, 7500 Fast and Rotor-Gene® 3000 real-time PCR devices.

Obtained quantification results covered a linear range over 9 log_{10} steps from 50 IU/ml to $4x10^{10}$ IU/ml.

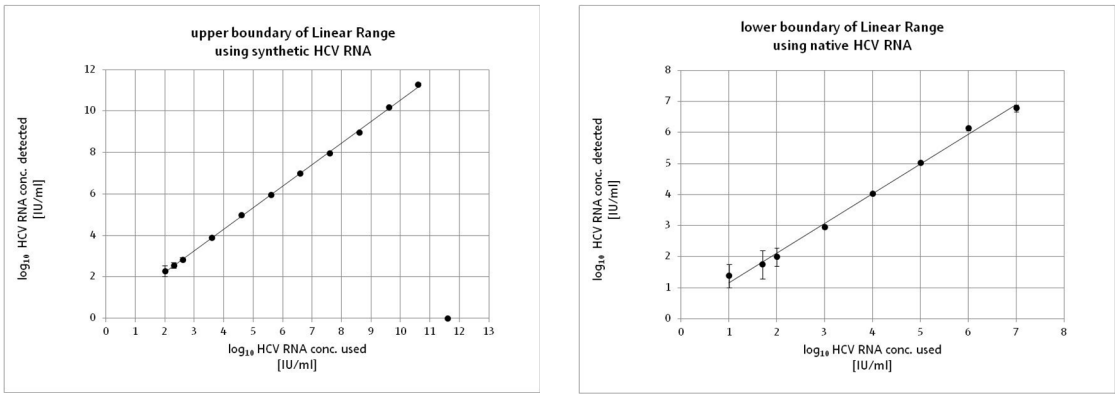


Figure 1: Boundaries of linear range exemplarily shown for CFX96 device. Mean quantification values are shown and variabilities are indicated by standard deviations. Highest synthetic HCV RNA concentration (11.6 log_{10} IU/ml) could not be detected.

4.3 Specificity

4.3.1 Genotype detection and quantification

The specificity of the RoboGene HCV RNA Quantification Kit 3.0 for the detection and quantification of known HCV genotypes/subtypes was tested using a genotype panel, provided by the University of Essen (Germany) containing HCV-1a, -1b, -2a, -2b, -2c, -2i, -3a, -4a, -5a and -6e samples.

Half-log dilution series of all specimens were prepared, and RNA was extracted using INSTANT Virus RNA/DNA Kit by means of starting sample volume of 400 µl.

All samples were quantified using RoboGene HCV RNA Quantification Kit 3.0 within the ± $\log_{10}$ acceptance interval of accuracy (see Figure 3) compared to the original quantification by means of Abbott RealTime HCV. All tested subtypes showed comparable quantification efficiencies.

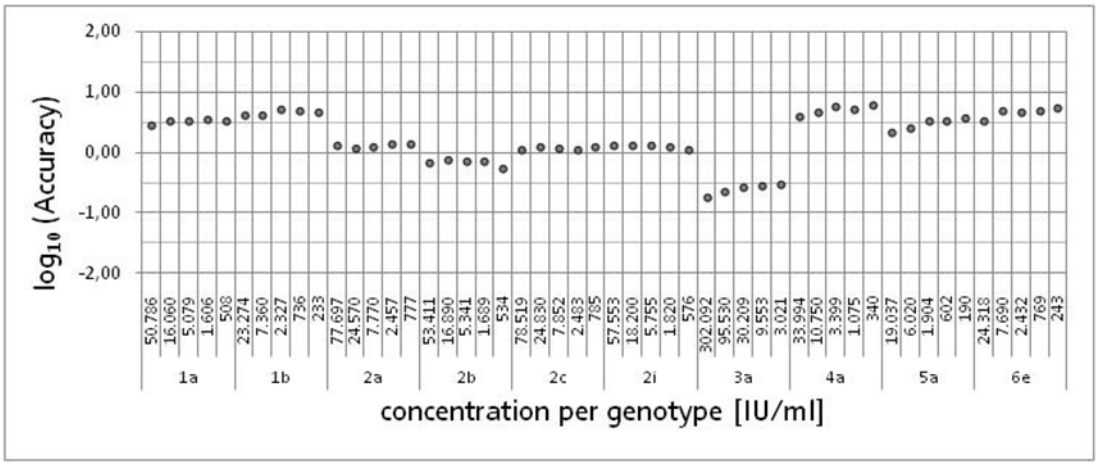


Figure 2: Accuracy plot for the quantification of dilution series of different HCV subtypes compared to the Abbott RealTime HCV.

The theoretical specificity for the detection of all other confirmed HCV genotypes and subtypes was proven by aligning the sequences of the underlying oligonucleotides against sequence data of respective subtype reference strains (data not shown).

4.3.2 Analytical specificity

Analysis of 10 non-HCV positive human Flavivirus samples (Zika virus, West Nile Virus I and II, Dengue virus genotypes 2-4) confirmed 100 % analytical specificity of RoboGene HCV RNA Quantification Kit 3.0 (see Table 2).

Table 2: Results of analysis of 10 non-HCV positive human Flavivirus samples.

Sample	HCV detection	IC detection
HCV positive control (n = 1)	1/1	1/1
HCV negative control (n = 1)	0/1	1/1
Zika virus (n = 4)	0/4	4/4
West Nile Virus (n = 3)	0/3	3/3
Dengue virus (n = 3)	0/3	3/3

4.3.3 Diagnostic specificity

Diagnostic specificity is expressed as a negative result in absence of the target. 100 patient samples tested negative for HCV RNA by Procleix Ultrio Assay were determined with the RoboGene HCV RNA Quantification Kit 3.0. All samples showed negative results for HCV RNA while positive for Internal Control RNA (see Table 3).

Table 3: Diagnostic Specificity

Sample	HCV detection	IC detection
HCV negative patient samples (n = 100)	0/100	100/100

The RoboGene HCV RNA Quantification Kit 3.0 had a perfect analytical and diagnostic specificity. None of the analyzed samples gave positive test results for HCV RNA.

4.4 Precision

The precision data represent the complete test procedure, i.e. plasma samples purified with the INSTANT Virus RNA/DNA Kit and quantified for HCV RNA using the RoboGene HCV RNA Quantification Kit 3.0.

Dilution series consisting of 3 different viral load levels were measured with 3 different lots of RoboGene HCV RNA Quantification Kit 3.0 on 3 different days and on 3 different real-time PCR devices (CFX96, qTOWER^3 and LightCycler® 480) for estimation of accuracy, intra-assay and inter-assay precision (see Table 4).

Table 4: Accuracy, intra-assay and inter-assay precision of the RoboGene HCV RNA Quantification Kit 3.0 – Precision data are shown in total

Given conc. [IU/ml]	Mean detected conc. [IU/ml]	Accuracy	$\log_{10}$ Accuracy	within acceptance interval *	Intra-Assay Precision		Inter-Assay Precision	
					SD [IU/ml]	CV [%]	SD [IU/ml]	CV [%]
25,000	26,303	1.05	0.02	yes	3,870	15	5,200	20
2,500	2,764	1.11	0.04	yes	373	13	565	20
250	237	0.95	-0.02	yes	55	23	79	33

*defined as ± 0.6 $\log_{10}$ of set point

4.5 Robustness

The robustness expresses the total failure rate of the RoboGene HCV RNA Quantification Kit 3.0 and was tested for the complete test procedure using the INSTANT Virus RNA/DNA kit for RNA extraction.

A total number of at least 104 samples, containing reference plasma diluted to 50 IU/ml (representing the 3-fold virus concentration of the 95 % cut-off value of the test) was analyzed on CFX96, qTOWER^3, LightCycler® 480, 7500 Fast and Rotor-Gene® 3000. Results of analysis are shown in Table 5a.

Table 5a: Results of failure rate study using RoboGene HCV RNA Quantification Kit 3.0

	(+) Results	Failure rate
CFX96		
HCV-RNA (FAM)	110/110	0 %
IC-RNA (VIC/ Cy5)	110/110	
qTOWER³		
HCV-RNA (FAM)	110/110	0 %
IC-RNA (YY/ CY5)	110/110	
LightCycler® 480		
HCV-RNA (FAM)	104/104	0 %
IC-RNA (VIC/ Cy5)	104/104	
7500 Fast		
HCV-RNA (FAM)	109/110	0.9 %
IC-RNA (VIC/ Cy5)	110/110	
Rotor-Gene® 3000		
HCV-RNA (FAM)	110/110	0 %
IC-RNA (JOE/ Cy5)	110/110	

Another 112 samples extracted with INSTANT Virus RNA/DNA Kit FX, containing reference plasma diluted to 50 IU/ml were analyzed with RealLine Cycler 48 / 96 as well as QuantStudio 5. Results of analysis are shown in Table 5b.

Table 5b: Results of failure rate study using RoboGene HCV RNA Quantification Kit 3.0

	(+) Results	Failure rate
RealLine Cycler 48		
HCV-RNA (FAM)	112/112	0 %
IC-RNA (Cy5)	112/112	
RealLine Cycler 96		
HCV-RNA (FAM)	111/112	0.9 %
IC-RNA (Cy5)	112/112	
QuantStudio 5		
HCV-RNA (FAM)	112/112	0 %
IC-RNA (YY/ Cy5)	112/112	

Amplification of HCV RNA using RoboGene HCV RNA Quantification Kit 3.0 could not be reduced by the addition of EDTA, citrate, bilirubin and hemoglobin.

Quantification results from samples with high concentrations of lipids might be decreased considerably. Thus, results obtained from lipaemic plasma or serum samples should be interpreted carefully. Heparinized plasma has to be excluded from analysis, because of its inhibitory effect on the activity of Taq polymerases.

The performance of the RoboGene HCV RNA Quantification Kit 3.0 during seroconversion was analyzed using pre seroconversion panels PHV 922, PHV 924 and PHV 925 obtained from SeraCare Life Sciences, Inc. (USA). HCV RNA quantification data were compared to the NAT assay used for initial viral load quantification of the respective seroconversion panel. Results are shown in Figure 4.

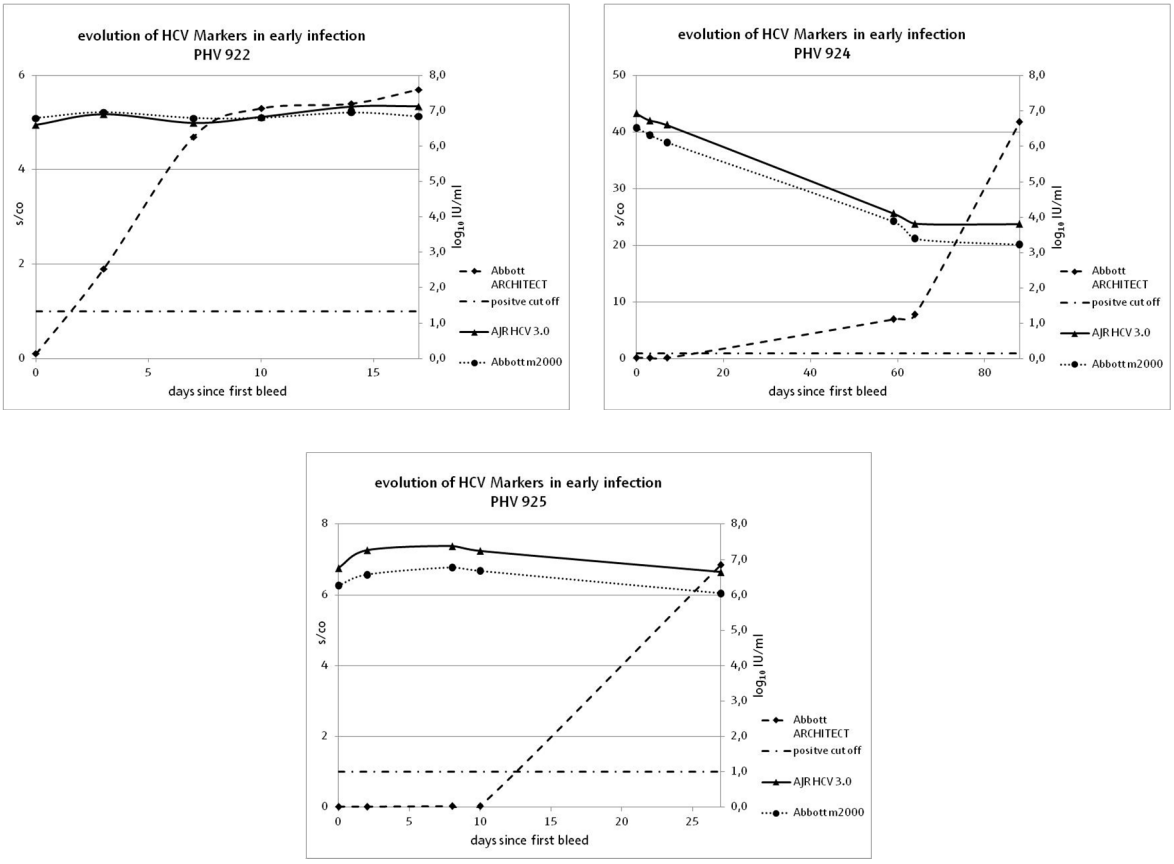


Figure 3: Performance of the RoboGene HCV RNA Quantification Kit 3.0 during seroconversion. HCV RNA concentrations were blotted against the initially measured antibody levels and the results of the Abbott m2000 assay for viral load quantification. Furthermore, HCV RNA concentrations were initially determined with the CAP/CTM assay (Roche) and Versant bDNA (Siemens) (data not shown).

In comparison to the initially applied NAT assays Abbott m2000, CAP/CTM assay (Roche) and Versant bDNA (Siemens) the RoboGene HCV RNA Quantification Kit 3.0 revealed a comparable performance.

4.6 Diagnostic Evaluation

The diagnostic sensitivity and linearity of the RoboGene HCV RNA Quantification Kit 3.0 were analyzed with 102 HCV RNA positive patient samples on CFX96, qTOWER^3, LightCycler® 480, 7500 Fast and Rotor-Gene® 3000/6000.

Quantitative data were compared with results obtained in advance with CE certified CAP/CTM assay (Roche) and CE certified in-house-

assay (MVZ Volkmann, Karlsruhe, Germany) performing linear regression and additional Deming regression and results are shown in Figure 5.

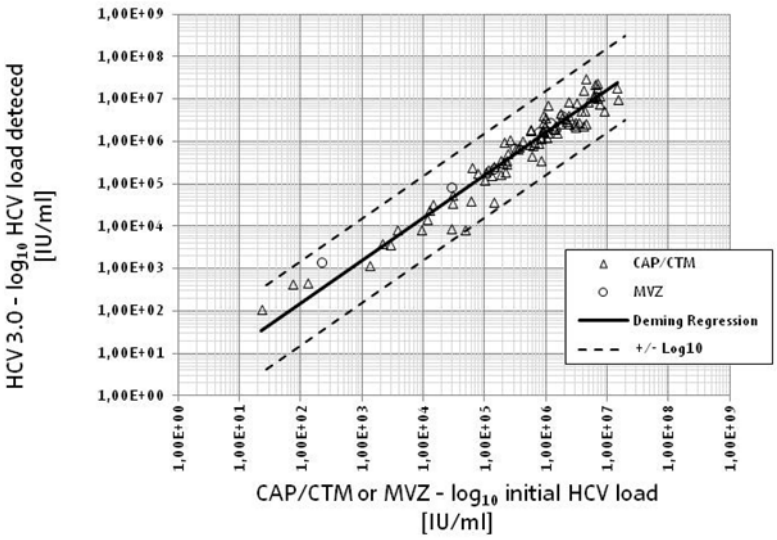


Figure 4: Diagnostic evaluation: comparison of the RoboGene HCV RNA Quantification Kit 3.0 with the CAP/CTM (Roche) assay and a certified in-house assay (MVZ KA). Data are shown exemplarily for the CFX96 quantification.

All samples were quantified within the ± log_{10} acceptance interval. Linear regression and Deming regression showed a high degree of correlation (see Table 6).

Table 6: Diagnostic evaluation - comparison of the RoboGene HCV RNA Quantification Kit 3.0 with CAP/CTM (Roche) assay and a certified in-house assay (MVZ KA).

	CFX96	**qTOWER³**	**7500 Fast**	**LightCycler® 480**	**Rotor-Gene® 6000**
Correlation	0.97	0.97	0.97	0.97	0.96
Out of ± log_{10}	0/102	0/102	0/102	0/102	0/64

NOTE

The quantification standards of the RoboGene HCV RNA Quantification Kit 3.0 are calibrated against the PEI HCV-RNA reference material (#3443/04) that itself was calibrated against the 1st WHO International Standard for HCV RNA (NIBSC 96/790).

It is known that quantifying several WHO standard generations with the same NAT may result in different concentrations, though the initial concentration is set to 100,000 IU/ml throughout all editions. Probably this artifact is caused during storage or shipping processes ("Update on the stability of HCV International Standards and a proposal for the 5th WHO International Standard for HCV", Fryer J.F., NIBSC).

Therefore we investigated the currently available 5th WHO International Standard for HCV RNA (NIBSC 14/150). Results revealed an overestimation of the standard (mean 5.27 $\log_{10}$ [IU/ml], SD 0.06 $\log_{10}$ [IU/ml]). This should be taken into account, when referencing the assay against the 5th WHO International Standard.

5 Kit components, storage and stability

Each kit contains two small inner boxes (1 and 2) and a small bag for storage of the following components:

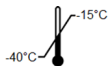
- box 1 for RT PCR Enzyme and IC
- box 2 for HCV/IC RM and PCR grade H_2O,
- bag for HCV/IC STD 1-4.

NOTE
RT PCR Enzyme has to be RE-PACKED to box 1 after arrival.

RoboGene HCV RNA Quantification Kit 3.0 is available in 3 sizes summarized in Table 7.

Table 7: Kit versions and components.

		Σ 32	Σ 96	Σ 192
REF		847-0207610032	847-0207610096	847-0207610192
IC [1]		IC Spiking Tube for 1 x 0.50 ml working solution	IC Spiking Tube for 3 x 0.50 ml working solution	IC Spiking Tube for 6 x 0.50 ml working solution
HCV/IC STD 1 - 4		4 Strips (4 x 4 wells)	4 Strips (4 x 4 wells)	4 Strips (4 x 4 wells)
HCV/IC RM[2]		Reagent Mix for 1 x 0.05 ml working solution	Reagent Mix for 2 x 0.05 ml working solution	Reagent Mix for 4 x 0.05 ml working solution
PCR grade H_2O[3]		1 x 1.5 ml	2 x 1.5 ml	4 x 1.5 ml
RT PCR Enzyme[4]		1 x 0.235 ml	1 x 0.660 ml	2 x 0.660 ml
IFU		1	1	1



STORAGE CONDITIONS

The RoboGene HCV RNA Quantification Kit 3.0 is shipped at room temperature, except the RT PCR Enzyme which is shipped on dry ice. After arrival store the RoboGene HCV RNA Quantification Kit 3.0 including the **RT PCR Enzyme** at -15 °C to -40 °C in the dark. The kit is stable until the expiry date when stored under these conditions.

IMPORTANT

[1] An appropriate amount of **IC** should be dissolved in **PCR grade H_2O** shortly before use. Remaining dissolved **IC** can be aliquoted properly and stored at -20 °C. Stored aliquots can be used up to 60 days. Repeated freezing and thawing up to 5 times is possible.

[2] An appropriate amount of Reagent Mix **HCV/IC RM** should be dissolved in **PCR grade H_2O** shortly before use. Remaining dissolved **HCV/IC RM** can be stored at -20 °C. Frozen **HCV/IC RM** can be used up to 60 days. Repeated freezing and thawing up to 5 times is possible. Always protect from light!

[3] Repeated freezing and thawing of **PCR grade H_2O** is possible.

[4] **RT PCR Enzyme** in general should be stored at -20 °C. Repeated freezing and thawing up to 5 times is possible. Nevertheless, **RT PCR Enzyme** should always be kept on ice-cold racks during usage.

6 Necessary laboratory equipment and additives

- HCV-positive control plasma (e.g. WHO International Standard for Hepatitis C Virus RNA for NAT testing or PEI Reference Preparation HCV RNA [PEI code #3443/04]). Provided quantification standards may be considered as positive control.
- HCV-negative control (e.g. human plasma or serum free of HCV RNA)
- For automated purification (Analytik Jena GmbH):
    - "CyBio FeliX Basic Unit with Enclosure"
    - „Laptop - english operation system" with software „Application Studio CyBio FeliX eXtract"
    - „CyBio FeliX Extraction Set"
- qTOWER 2 & 3 (Analytik Jena), CFX96 (Bio-Rad), LightCycler® 480 (Roche), 7500 Fast (Applied Biosystems), Rotor-Gene® 3000/6000/Q (Corbett Research/ Qiagen), RealLine 48-4/ 48-5/ 96-4/ 96-5 (Bioron), QuantStudio5 (Thermo)
- Real-time instrument specific software for data analysis and reporting
- Recommended real-time instrument specific PCR consumables (see table below)
- Suitable pipetting tools and sterile pipette aerosol-barrier tips
- Micro centrifuge
- Plate centrifuge
- Thermal mixer
- Vortex mixer
- 1.5 ml tubes
- 2.0 ml tubes
- Gloves, lab coat

Table 8: Recommended PCR consumables and ordering information.

Real-time PCR platform	PCR plastics	Sealing
qTOWER 2 & 3, CFX 96	96 Well PCR Plate 0.2 ml, fullskirt, white	Optical sealing foil
	Source: Analytik Jena Order number 844-70038-0	Source: Analytik Jena Order number 846-050-258
LightCycler® 480	LightCycler® 480 Multiwell Plate 96, white	LightCycler® 480 Sealing Foil
	Source: Roche Order number 04729692001	Source: Roche Order number 04729757001
7500 Fast	MicroAmp Fast Optical 96-Well Reaction Plate 0.1 ml (clear)	LightCycler® 480 Sealing Foil
	Source: Thermo Fisher Order number 4346907	Source: Roche Order number 04729757001
Rotor-Gene® 3000/6000/Q	Strip Tubes and Caps, 0.1 ml	-
	Source: Qiagen Order number 981103	-
RealLine Cycler 48 / 96 QuantStudio 5	96 x 0.2ml Transformer Netflex Plate White, High Profile	Axygen™ Microplate Sealing Film and Tapes
	Source: BIOplastics Order number B58709	Source: Axygen UC 500 Order number 14-222-873

7 Procedure

7.1 Collection and handling of clinical samples

- Collect 5-10 ml blood with standard specimen collection tubes.
- For plasma preferably EDTA or citrate anticoagulant has to be used; heparin is non-applicable, because of its inhibitory effect on PCR.
- Serum: after collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature for up to 30 minutes. Remove the clot by centrifuging at 1,000–2,000 x g for 10 minutes [9].
- Plasma: cells are removed from plasma by centrifugation for 10 minutes at 1,000–2,000 x g. Transfer to sterile tubes [9].
- Store serum and plasma at 2-8 °C. Test within 5 days [9].
- Plasma or serum samples may be stored deeply frozen for several months at -70 °C to -20 °C depending on the storage temperature. Avoid repeated freezing and thawing [10].

7.2 HCV RNA purification from clinical samples

The RoboGene HCV RNA Quantification Kit 3.0 has been validated with a manual and an automated purification method.

For manual purification method use the INSTANT Virus RNA/DNA Kit (Roboscreen GmbH, Order number: 847-0259200602 for 50 reactions; 847-0259200603 for 250 reactions). Perform the HCV RNA purification steps according to the respective IFU using 'Protocol 2: Isolation of viral RNA/DNA from 400 µl of serum/plasma using IC Spiking Tube'.

For automated purification method use the INSTANT Virus RNA/DNA Kit-FX (Roboscreen GmbH, Order number: 847-0259200902).

Perform the HCV RNA purification steps according to the respective IFU.

7.3 Internal RNA Control

The RoboGene HCV RNA Quantification Kit 3.0 contains the **IC** Spiking Tube stably coated with Internal Control nucleic acid and carrier nucleic acid.
Using **IC** together with the INSTANT Virus RNA/DNA Kit or INSTANT Virus RNA/DNA - FX always allows to control the whole procedure and to detect false-negatives due to failed extraction or excess of inhibitors within the sample. To judge the purification, the Ct value of the Internal Control purified together with HCV RNA negative or positive plasma should be in the instrument-specific ranges summarized in Table 15.

7.4 General procedure of quantitative analysis

The four quantification standards are provided as standard strips stably coated with defined amounts of synthetic HCV RNA. The standards are calibrated against the PEI reference material HCV RNA (#3443/04, calibrated against the 1st WHO International Standard for HCV RNA (NIBSC 96/790)). The standard values are given in IU/ml, i.e. the HCV RNA concentration of the analyzed sample may be directly calculated from the reference curve without the need for subsequent conversion by an equation.

NOTE
Please note that the given standard values, and thus quantification, are dependent on the RNA purification kit used together with the RoboGene HCV RNA Quantification Kit 3.0. Therefore quantification results are only valid when the INSTANT Virus RNA/DNA Kit or INSTANT Virus RNA/DNA Kit-FX in combination with indicated real-time PCR devices and device specific consumables were used.

8 Protocol

8.1 Preparation of Internal Control

NOTE
The RoboGene HCV RNA Quantification Kit 3.0 has been evaluated together with the INSTANT Virus RNA/DNA Kit as well as the INSTANT Virus RNA/DNA Kit - FX for nucleic acid extraction. The Internal Control is provided as **IC** Spiking Tube within the RoboGene HCV RNA Quantification Kit 3.0. Prepare the **IC** Tube according to the instructions below and extract RNA following the instructions of the INSTANT Virus RNA/DNA Kit or INSTANT Virus RNA/DNA Kit - FX.

1. Centrifuge the **IC** Spiking Tube tube briefly at full speed to collect the lyophilized **IC** on the bottom of the tube.
2. Add 520 µl **PCR grade H_2O** to the vial; close the tube, mix by vortexing briefly followed by brief centrifugation.
3. Incubate at 37 °C for 5 min using a shaking platform (800-1,000 rpm), mix by vortexing briefly followed by brief centrifugation.

8.2 Application of the dissolved IC for manual purification

1. Add 10 µl of resuspended IC per extraction reaction to the Lysis Solution of the corresponding INSTANT Virus RNA/DNA Kit.
2. Follow instructions of the extraction kit 'Protocol 2: Isolation of viral RNA/DNA from 400 µl of serum/plasma using IC Spiking Tube' purification.

8.3 Application of the dissolved IC for automated purification

Follow instructions of the INSTANT Virus RNA/DNA Kit - FX.

8.4 Preparation of 25x Reagent Mix

1. Centrifuge the **HCV/IC RM** briefly at full speed to collect the lyophilized Reagent Mix on the bottom of the tube.
2. Add 53 µl **PCR grade H_2O** to **HCV/IC RM**; close the tube, mix by brief vortexing followed by brief centrifugation.
3. Incubate at 37 °C for 5 min using a shaking platform (800-1,000 rpm), mix by brief vortexing followed by brief centrifugation.

8.5 Preparation of 1x Master Mix

1. Before setting up the Master Mix gently invert **RT PCR Enzym**e several times and centrifuge briefly.
2. Prepare the 1x Master Mix according to the following table. Mix by vortexing for at least 10 sec followed by brief centrifugation.

Table 9: Composition of 1x Master Mix per reaction.

Reagent	Volume for 1x rxn (µl)	Final concentration
PCR grade H_2O	7.75	-
HCV/IC RM Reagent Mix, 25x	1.00	1x
RT PCR Enzyme	6.25	1x
Total	15.00	

8.6 Preparation of Quantification Standards

1. Uncover standard strip **HCV/IC STD 1 -4** and place the strip onto a suitable ice-cold rack.
2. Add 25 µl **PCR grade H_2O** to each well of the quantification standard **HCV/IC STD 1 - 4**; mix by pipetting up and down several times.

NOTE
Be careful to use a new pipette tip for each standard to avoid carry over contamination.
It is important to mix quantification standards **HCV/IC STD 1 - 4** by pipetting up and down several times. Do not vortex the quantification standards!
Store quantification standard on ice or an ice-cold rack until introduction into PCR!

8.7 Preparation of reaction set

1. Place real-time PCR consumables (not provided) onto a suitable ice cold rack.
2. Add 15 µl 1x Master Mix to wells intended for sample quantification, NTCs and additional four wells for each of the quantification standard **HCV/IC STD 1 – 4**.
3. Add 10 µl **PCR grade H_2O** to wells that serve as NTC. Add 10 µl of resuspended **HCV/IC STD 1 - 4** to all wells that serve as quantification standards containing the 1x Master Mix. Do not exceed a final reaction volume of 25 µl. Make sure PCR grade H_2O and quantification standard solutions are mixed properly with the Master Mix.

NOTE
After usage discard the remaining solution of **HCV/IC STD 1-4**. To avoid contaminations we recommend to seal the quantification standard with a suitable cover (e.g. parafilm, not included in the kit).

4. Add 10 µl eluate from RNA isolation (INSTANT Virus RNA/DNA Kit or INSTANT Virus RNA/DNA Kit - FX) to the respective sample wells containing the 1x Master Mix. Do not exceed a final

reaction volume of 25 µl. Make sure master mix and eluate is mixed properly.

5. Cover the real-time PCR consumables. Centrifuge PCR plates for 1 min at 1,000 rpm to collect the PCR mix on the bottom of each well (not necessary for Rotor-Gene® tubes).
6. Program the applied real-time PCR platforms as indicated in Table 10 to 13 and start the program.

NOTE
The essential in-run standard curve provides run validation criteria slope and R2 value (see Table 16).
Never use external standard curves for quantification.

Table 10: PCR program for qTOWER 2 & 3 and CFX96.

Step	Cycle	Profile	Temperature	Time	Ramping
1	1	Reverse transcription	47 °C	15 min	5 °C/sec
2	1	Taq activation	95 °C	2 min	5 °C/sec
3	45	Denaturation	95 °C	15 sec	2.5 °C/sec
		Annealing/Elongation*	57 °C	1 min	5 °C/sec

* Data acquisition: Fluorescence Detection (FAM; Cy5) for qTOWER 2 & 3, (FAM; VIC/JOE and/ or Cy5) for CFX96

for qTOWER 2 & 3 the following device specific presetting are recommended: Open new project > Scan > Gain: for FAM = 4; for VIC/JOE = 3; for Cy5 = 5.

Table 11: PCR program for 7500 Fast and Rotor-Gene® 3000/6000/Q, RealLine Cycler 48 / 96

Step	Cycle	Profile	Temperature	Time	Ramping
1	1	Reverse transcription	47 °C	15 min	Not adjustable
2	1	Taq activation	95 °C	2 min	
3	45	Denaturation	95 °C	15 sec	
		Annealing/Elongation*	57 °C	1 min	

* Data acquisition: Fluorescence Detection (FAM; Cy5) for RealLine Cycler 48 / 96 and for 7500 Fast and Rotor-Gene® 3000/6000/Q (FAM; VIC/JOE and/ or Cy5)

Table 12: PCR program for QuantStudio 5.

Step	Cycle	Profile	Temperature	Time	Ramping
1	1	Reverse transcription	47 °C	15 min	3.16°C/sec
2	1	Taq activation	95 °C	2 min	3.16°C/sec
3	45	Denaturation	95 °C	15 sec	3.16°C/sec
		Annealing/Elongation*	57 °C	1 min	2.45°C/sec

* Data acquisition: Fluorescence Detection (FAM; VIC and/ or Cy5)

Table 13: PCR program for LightCycler® 480

Step	Cycle	Profile	Temperature	Time	Ramping
1	1	Reverse transcription	47 °C	15 min	4.4°C/sec
2	1	Taq activation	95 °C	2 min	4.4°C/sec
3	45	Denaturation	95 °C	15 sec	2.5°C/sec
		Annealing/Elongation*	57 °C	1 min	2.2°C/sec
4	1	Cooling	40 °C	30 sec	Max

* Data acquisition: Fluorescence Detection (FAM; VIC and/ or Cy5)

9 Data analysis

Each RNA amplification is associated with generation of a fluorescence signal measurable in FAM channel (for HCV RNA) and in YY/VIC/JOE or Cy5 channel (for IC) resulting in a sigmoid growth curve (log scale). The data analysis is performed according to manufacturer's instructions of the real-time PCR instrument using the respective software. Check the obtained data to ensure that the run is valid and to interpret results (Table 14).

Table 14: Interpretation of the results.

FAM channel	**YY/VIC/JOE or Cy5 channel**	**Interpretation**
Interpretation of detection results		
x	**x**	Sample valid - detection of sample HCV RNA
x	**-**	Sample invalid - repeat sample
-	**x**	Sample valid - only detection of IC, HCV RNA not detectable/ HCV negative sample
-	**-**	Sample invalid - no amplification/ detection at all, repeat sample
Interpretation of quantification results		
< LOD	**x**	Below lower limit of detection of the assay (e.g. 17 IU/ml for qTOWER^3). Three replicates of analysis are recommended to confirm positive result.
> 4x10^{10} IU/ml	**x**	Above upper limit of covered linear range of the assay (4 x 10^{10} IU/ml). Dilution of original sample is recommended.
< 50 IU/ml	**x**	Below lower limit of covered linear range of the assay (50 IU/ml). Three replicates of analysis are recommended to confirm quantification.

HCV RNA concentration of clinical specimens is determined based upon a standard curve resulting from analysis of the quantification standard strip and the Ct values of the respective samples. The HCV RNA concentration is expressed in IU/ml. Table 15 lists the concentrations of HCV RNA quantification standards in case of using the INSTANT Virus RNA/DNA Kit or INSTANT Virus RNA/DNA Kit-FX.

Table 15: HCV RNA quantification standard concentrations.

HCV/IC STD 1 - 4	HCV RNA [IU/ml]
1	40,000,000
2	400,000
3	4,000
4	400

NOTE

Setting of threshold may markedly influence Ct values. Recommendation for setting thresholds is shown below.

- qTOWER 2 & 3: FAM: 3.0 – 5.5; YY: 2.5 – 6.0; Cy5: 3.0 -5.5
- CFX96: FAM: 500 - 750; YY: 100 - 200; Cy5: 50 - 200
- LightCycler® 480:

Channel	Noiseband	Threshold	Fit Points
FAM	~ 1.1-8.0	~ 3.5-15.5	~ 4
YY	~0.7-2.0	~ 1.5-3.5	~ 3-4
Cy5	~ 0.5-1.4	~ 1.0-3.5	~ 4-5

- 7500 Fast: FAM: 0.15 – 0.23; YY: 0.03 - 0.05; Cy5: 0.03 – 0.07
- Rotor-Gene® 3000/6000/Q: FAM: 0.02 - 0.04; YY: 0.01 - 0.04; Cy5: 0.02 - 0.05
- RealLine Cycler 48 / 96: FAM: ~500; Cy5: ~700
- QuantStudio 5: FAM: ~250.000; YY: ~70.000; Cy5: ~110.000

Criteria for run validation are the slope and R^2 value of the standard curve (see Table 16). The ranges of expected Ct values of the standards refer to own validation data and should be used as guidelines for setting threshold values (see Tables 17 to 19).

If slope and/or R^2 are out of range (Table 16), one of the four quantification standards may be excluded (most outlying of regression line), as three quantification standards are sufficient for valid results.
In such case no right for warranty of the whole product may be deduced.

Table 16: Criteria for run validation.

Parameter	**qTOWER 2 & 3, CFX96, LightCycler® 480, 7500 Fast, Rotor-Gene® 3000/6000/Q, RealLine Cycler 48 / 96, QuantStudio 5**
Range of slope	-3.10 to -3.60
The linear regression coefficient (R^2) of the reference curve should be between 0.98 and 1.00 (not applicable to LightCycler®480 analysis).	
Expected Ct values for IC of the quantification standards (dependent on the set threshold value, see above)	
YY/VIC/JOE	≤ 40
Cy5	≤ 38
Expected Ct values for IC in HCV negative patient samples and HCV positive samples (dependent on the set threshold value, see above)	
YY/VIC/JOE	≤ 40
Cy5	≤ 38

Table 17: Guidance Ct values of the quantification standards on qTOWER 2 & 3, LightCycler® 480 and CFX 96.

HCV/IC STD 1 - 4	Expected increment between Ct values	qTOWER 2 & 3		LightCycler® 480		CFX96	
		mean	from – to	mean	from – to	mean	from – to
1		16.9	16.1 – 17.6	17.7	16.6 - 18.8	17.0	16.3 - 17.8
2	1 to 2 + ~ 6.64	23.3	22.5 - 24.1	24.4	23.2 - 25.6	23.7	22.9- 24.4
3	2 to 3 + ~ 6.64	29.7	28.7 - 30.6	31.1	29.9 - 32.3	30.3	29.5- 31.2
4	3 to 4 + ~ 3.32	33.3	31.8 - 34.7	34.6	33.0 - 36.2	33.7	32.6- 34.8

Table 18: Guidance Ct values of the quantification standard on 7500 Fast and Rotor-Gene® 3000/6000/Q.

HCV/IC STD 1 - 4	Expected increment between Ct values	7500 Fast		Rotor-Gene® 3000/6000/Q	
		mean	from – to	mean	from – to
1		16.9	16.2 - 17.6	15.4	14.3 - 16.4
2	1 to 2 + ~ 6.64	23.6	22.9 - 24.4	21.9	20.8 - 23.0
3	2 to 3 + ~ 6.64	30.3	29.5 - 31.2	28.8	26.9 - 30.8
4	3 to 4 + ~ 3.32	33.7	32.5 - 34.9	32.1	30.3 - 34.0

Table 19: Guidance Ct values of the quantification standards on RealLine Cycler 48 / 96 and Quant Studio 5.

HCV/IC STD 1 - 4	Expected increment between Ct values	RealLine Cycler 48		RealLine Cycler 96		Quant Studio 5	
		mean	from – to	mean	from – to	mean	from – to
1		15.1	14.4 – 15.5	15.4	14.2 – 16.2	15.5	14.3 – 16.9
2	1 to 2 + ~ 6.64	21.8	21.1 – 23.5	22.3	21.0 – 22.9	22.4	21.3 – 23.9
3	2 to 3 + ~ 6.64	28.3	27.4 – 29.0	29.0	27.9 – 29.8	29.2	28.1 – 30.7
4	3 to 4 + ~ 3.32	31.8	31.4 – 32.5	32.4	31.2 – 33.4	32.5	31.1 – 34.0

10 Troubleshooting

Problem / probable cause	Comments and suggestions
No signal at all	
▪ Fluorescence measurement not activated	Read the user guide of the real-time PCR device.
▪ False channels selected	Select FAM channel for HCV RNA and YY/VIC/JOE or Cy5 channel for IC.
▪ Incorrect cycling program	Check instrument settings, repeat run.
▪ Incorrect application of the kit	Read instruction for use.
▪ Storage conditions did not comply with instructions, expiry date of detection kit is exceeded	Check storage conditions and expiry date.
Low fluorescence signal recorded for both target and IC, target copy number underestimated	
▪ Target RNA degraded	Use RNase free consumables and reagents, store RNA on ice. Read instruction for use of the extraction kit.
▪ Optical lenses contaminated (Rotor-Gene®)	See chapter “Maintenance” of respective instrument brochure, alternatively clean lense once per month using absolute isopropanol and cotton swabs.
▪ Thermal block and/or optics polluted (96-well block format)	See chapter “Maintenance” of respective instrument brochure, alternatively fill each well with isopropanol, incubate 10 min at 50 °C, remove isopropanol and rinse with H_2O.
No or weak signal for IC in HCV-negative sample RNA	
▪ Incorrect cycling program	Check instrument settings, repeat run.
▪ Excess of inhibitors in the sample/ loss of RNA during extraction	Use the recommended extraction kit and follow exactly manufacturer’s instructions.

▪ Incorrect sample material (e.g. heparinized plasma)	Request for fresh EDTA- or citrate plasma or serum.
▪ Storage conditions did not comply with instructions, expiry date of detection kit is exceeded	Check storage conditions and expiry date.
Unexpectedly low Ct values for IC particularly with high standards or high viral load samples	
▪ Cross talk between target and IC recording channels (especially YY/VIC/JOE)	Calibrate instrument using pure fluorescence dyes or repeat run using Cy5 channel for IC detection.
Non-sigmoidal growth curves of quantification standards, unacceptable high deviation of Ct from expected values	
▪ Frequent freezing/thawing or incorrect storage of dissolved reagent mix	Read IFU, check storage conditions, prepare new reagent mix.
▪ Storage conditions did not comply with instructions, expiry date of detection kit is exceeded	Check storage conditions and expiry date.
Different amplification behavior of sample HCV RNA and standards, non-parallel growth curves in exponential phase of reaction	
▪ Excess of inhibitors in the sample	Use the recommended extraction kit, follow exactly the manufacturer's instructions; consult attending doctor for patient medication.
▪ Incorrect sample material	Use recommended sample type.
FAM signal for HCV-negative samples / NTC recorded	
▪ Contamination with HCV RNA or RNA amplicons	Repeat extraction and/or PCR with new reagents; decontaminate instruments and work space.

If you have any further questions which are not answered, please contact our technical service.

11 References

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12 For your notes