ROBOSCREEN DIAGNOSTICS

Instructions for Use

RoboGene HDV RNA Quantification Kit 3.0

Rev. 2 RUO_ 11 / 2024

Research Use Only

Order No.:

847-0207650096-RUO	96 reactions
847-0207650032-RUO	32 reactions

Manufacturer:

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IFU RoboGene HDV RNA Quantification Kit 3.0 Rev 2-RUO 11 / 2024

Contents

1 Introduction

1.1 Intended use

RoboGene HDV RNA Quantification Kit 3.0 is a real-time PCR kit for quantification and detection of Hepatitis D Virus (HDV) RNA in EDTA- and citrate-plasma or serum samples.

For research use only. Not for use in diagnostic procedures.

CONSULT INSTRUCTIONS FOR USE
The Instructions for use 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 for use.

1.2 Pathogen information

Hepatitis delta virus is a pathogenic human virus whose RNA genome and replication cycle resembles those of plant viroids but encodes a single nuclear phosphoprotein, hepatitis delta antigen (HDAg), which is required for virus replication. It is associated with Hepatitis B virus and causes disease only in conjunction with this particular virus [1].

Chronic hepatitis D virus (HDV) infection is associated with an increased risk of developing liver cirrhosis and hepatic complications, including the development of hepatocellular carcinoma (HCC) [2–9].

1.3 Technical assistance

If you have any questions or problems regarding any aspects of the RoboGene HDV RNA Quantification Kit 3.0 please do not hesitate to contact our technical support team. For technical assistance please contact us at the manufacturer site as shown inside the cover of the Instructions for use.

1.4 Reporting of Incidents

In case of any serious incidents that have occurred in relation to RoboGene HDV RNA Quantification Kit 3.0, please inform us.

1.5 Symbols and Abbreviations

For easy reference and orientation, the IFU uses the following warning and information symbols:

Symbol	Information
	**REF** Catalogue number.
 N	**Content** Contains number of determinations as indicated.
	**Storage temperature** Store at temperatures between upper and lower limits as indicated.
	**Consult instructions for use** This information must be observed to avoid improper use of the kit.
	**Used by** Expiry date. The product is to be used by the indicated date.
	**Lot number** The lot number of the kit.
	**Manufactured by** Contact information of the manufacturer.
	**For single use only** Single use only. Do not use the product twice.
	**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
HBV	Hepatitis B Virus
HDAg	Hepatitis Delta Antigen
HDV	Hepatitis Delta Virus
IC	Internal Control
IFU	Instruction For Use
IU	International Units
NA	Nucleic Acid
NAT	Nucleic Acid Test
NAT-System	NA-extraction kit in combination with the RNA quantification kit
NTC	Non-template control
PEI	Paul-Ehrlich-Institut, Langen, Germany
RM	Reagent Mix
rxn	Reactions
RT	Real-time
SD	Standard deviation
WHO	World Health Organization

2 Test description and principle

2.1 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-specific primers in combination with a fluorescence-labelled oligonucleotide probe, complementary to the desired target sequence. In the presence of target the probe hybridizes with its target-complementary sequence. The Taq DNA polymerase from the RT PCR Enzyme LYO possesses a 5`→ 3` exonuclease activity that cleaves the probe 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.

2.2 Explanation of the test

RoboGene HDV RNA Quantification Kit 3.0 is a real-time PCR amplification test for quantification and detection of HDV RNA in plasma or serum samples. The assay is able to quantify all eight genotypes of HDV [10], by applying primers and probes specific for a subsequence of the Hepatitis delta antigen (HDAg). Determination of specimen concentrations is performed by amplification of the included quantification standards in parallel.

A synthetic internal control is integrated to monitor the whole procedure from RNA extraction to the real-time PCR. Thus, the risk of false-negative results is significantly reduced. Amplification of HDV- and IC-RNA in samples and standards is measured independently at different wavelengths due to probes labelled with different fluorescent reporter dyes. HDV RNA is detected in the FAM channel.

For detection of IC RNA, the kit provides two options depending on the set up of the real-time PCR system allowing detection in Yakima Yellow/VIC/JOE or Cy5 channel.

RoboGene HDV RNA Quantification Kit 3.0 is intended for use in combination with real-time PCR systems listed in **chapter 6** and viral nucleic acid extraction kits listed in **chapter 7** of this IFU. Nucleic acid extraction must be performed strictly according to manufacturer's instructions.

2.3 Restrictions

RoboGene HDV RNA Quantification Kit 3.0 is validated for use of EDTA- or citrate-plasma and serum as sample material. Heparinized plasma must be excluded from analysis. If other than the recommended sample types are used incorrect results may be obtained.

Lipaemic samples must be excluded from analysis. This is substantiated by the results of the performance evaluation revealing an inhibitory effect for highly lipaemic samples containing 1000-2000 mg/dl of lipids.

Safety and performance of RoboGene HDV RNA Quantification Kit 3.0 cannot be guaranteed if there are any deviations from the instructions in this IFU.

Do not use expired components or mix components from different kit lots.

The kit shall only be handled by educated staff in a laboratory environment!

3 Safety precautions

NOTE

Read this chapter carefully to guarantee your own safety and a trouble-free operation.

Follow all the safety instructions explained in the IFU, as well as all given messages and information.

Plasma and serum samples must be considered as potentially infectious. Thus, always wear lab coat and gloves.

Always use clean and nuclease-free equipment.

Purification of nucleic acid, PCR reagent assembly and amplification should be performed in different rooms.

Be careful while pipetting specimen material to avoid carry-over contamination.

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

Maintain your laboratory equipment regularly, e.g., calibration of thermal mixer temperature.

For single use only

This kit is made for single use only!

4 Performance assessment

Validation of the RoboGene HDV RNA Quantification Kit 3.0 was performed in combination with INSTANT Virus RNA/DNA Kit (manual nucleic acid extraction kit) and INSTANT Virus RNA/DNA Kit – FX 2.0 (automated nucleic acid extraction kit) and real-time PCR-systems CFX96 Touch, CFX96 Dx ORM, CFX Opus 96 Dx, QuantStudio 5, QuantStudio 5 Dx, QuantStudio 7 flex, LightCycler 480II, qTOWER^3 and Rotor-Gene 3000.

NOTE

The Quantification standard (HDV/IC STD 1-4) of RoboGene HDV RNA Quantification Kit 3.0 is calibrated against the 1st World Health Organization International Standard for Hepatitis D Virus RNA for Nucleic Acid Amplification Techniques (NAT)-Based Assays (PEI code 7657/12).

4.1 Analytical sensitivity

The analytical sensitivity of RoboGene HDV RNA Quantification Kit 3.0 was determined by analyzing dilution series of the WHO Reference Material HDV RNA (PEI code 7657/12). The determined limits of detection for five real-time PCR-systems (CFX96 Touch, QuantStudio 5, LightCycler 480II, qTOWER^3, Rotor-Gene 3000) are summarized below in Table 1a for INSTANT Virus RNA/DNA Kit (manual NA extraction) and Table 1b for INSTANT Virus RNA/DNA Kit – FX 2.0 (automated NA extraction).

Table 1a: Determined PCR-system specific limit of detection and confidence interval using manual NA-extraction INSTANT Virus RNA/DNA Kit

Real-time PCR-system	Limit of detection [IU/ml]	95 % confidence interval	
		Lower [IU/ml]	Upper [IU/ml]
CFX96 Real-Time PCR Detection Systems (CFX)	0.82	0.70	0.94
QuantStudio™ real-time PCR systems (QS)	0.48	0.44	0.52
LightCycler® 480 II (LC)	0.55	0.50	0.60
qTOWER^3 / iris (qT)	1.39	1.00	1.78
Rotor-Gene 3000/ 6000/ Q (RG)	0.63	0.56	0.70
all PCR-systems included	0.97	0.87	1.07

Table 1b: Determined PCR-system specific limit of detection and confidence interval using automated NA-extraction INSTANT Virus RNA/DNA Kit – FX 2.0

Real-time PCR-system	Limit of detection [IU/ml]	95 % confidence interval	
		Lower [IU/ml]	Upper [IU/ml]
CFX96 Real-Time PCR Detection Systems (CFX)	5.87	4.34	7.40
QuantStudio™ real-time PCR systems (QS)	6.23	4.56	7.91
LightCycler® 480 II (LC)	5.86	4.25	7.46
qTOWER^3 / iris (qT)	9.15	7.58	10.72
Rotor-Gene 3000/ 6000/ Q (RG)	4.84	3.64	6.04
all PCR-systems included	6.80	5.90	7.70

The detection limit was calculated by PROBIT analysis of at least 24 replicates of each dilution of reference material on each real-time PCR-system with confidence of 95 % (see Figure 1).

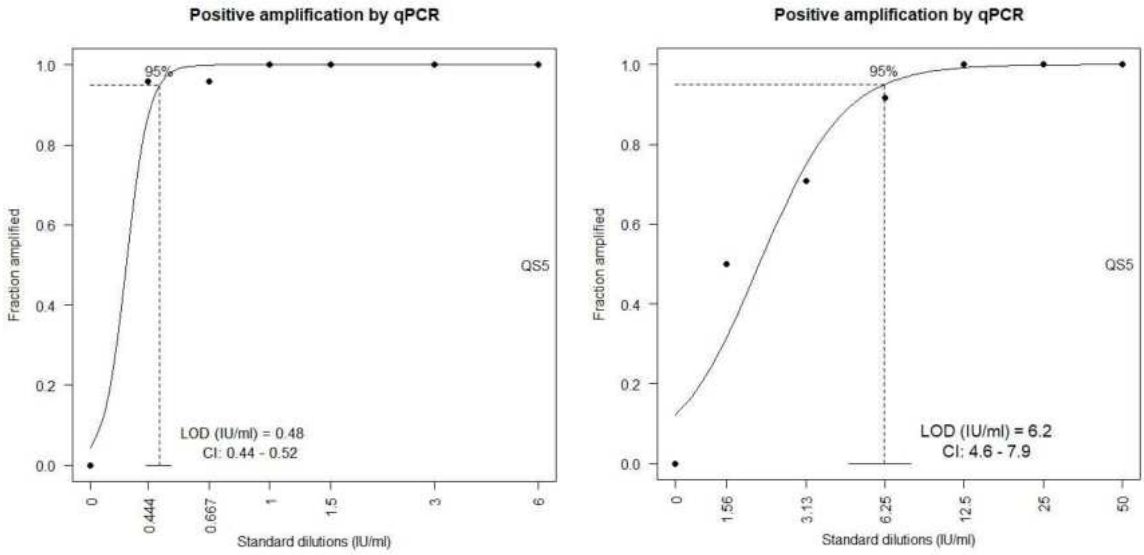

Figure 1: PROBIT analysis for the determination of real-time PCR-system specific limits of detection (LOD) and 95 % confidence interval (CI) shown exemplarily for QuantStudio 5 (QS5). Manual NA extraction (left chart) and automated NA extraction (right chart). Concentrations are given in IU/ml.

4.2 Genotype detection and quantification

Specificity of RoboGene HDV RNA Quantification Kit 3.0 for detection and quantification of known HDV genotypes/subtypes was evaluated at the French National Reference Laboratory for Hepatitis Delta (Dr. Emmanuel Gordien, Bobigny, France). The genotype panel included the most prevalent HDV genotypes (1, 1a, 2, 4, 5, 6, 7 and 8).

One-log dilution series of all specimens were prepared, and nucleic acids were extracted from 400 µl sample volume using manual and automated NA extraction. Efficiency of quantification of the test was proven to only vary within a range of ±9 % for all HDV-genotypes compared to a reference product.

Additionally, a genotype panel consisting of 22 HDV-positive samples including genotype 1, 1a, 2, 4, 5, 6, 7, 8 and two HDV-negative samples were tested. Comparison of the quantification results obtained with various commercial products verifies an accurate genotype detection of the test [15]. All results and the respective ±0.5 $\log_{10}$ IU/ml interval of the mean values are shown in figure 2.

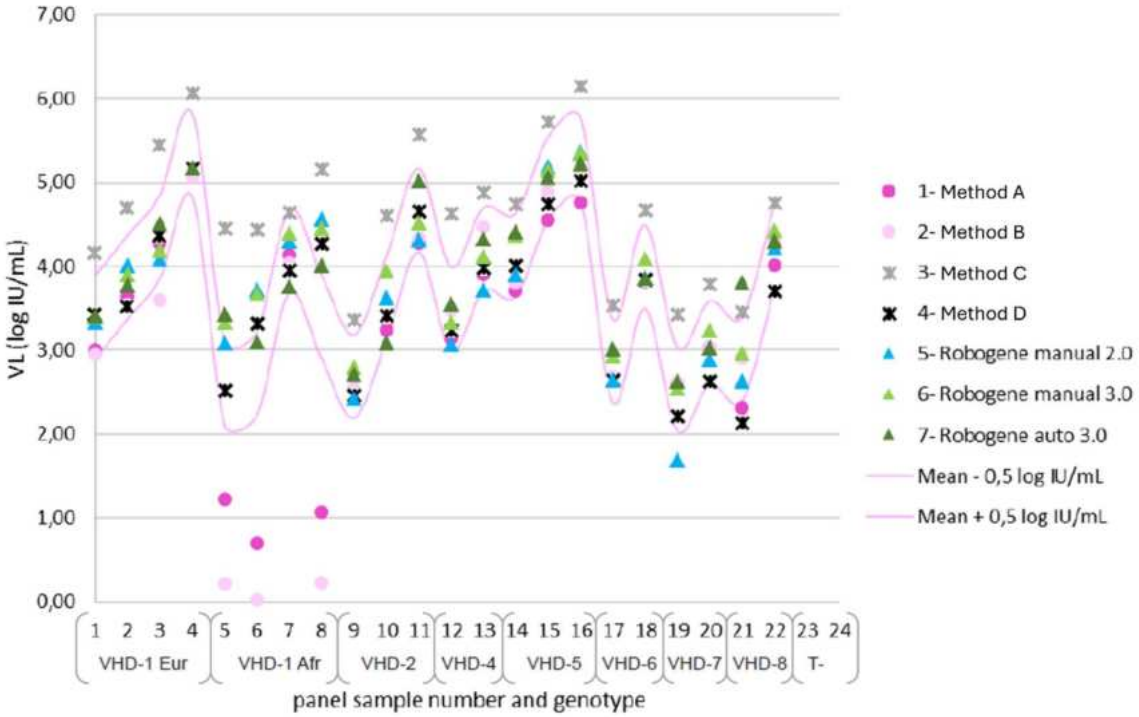

Figure 2: Shown are the quantification results of the pan genomic panel for HDV [15] obtained with the RoboGene HDV RNA Quantification Kit 3.0 (green) and five other commercial assays. Quantification results (determined with QuantStudio 7 flex) are separately indicated for each of the used NAT-systems. Also indicated are the ±0.5 $\log_{10}$ IU/ml acceptance intervals individually calculated for each of the 7 participants.

The rare genotype HDV-3 prevalent in South America was evaluated by using synthetic RNA specific for this HDV-genotype.

A dilution series of the synthetic HDV-3 RNA was directly quantified with RoboGene HDV RNA Quantification Kit 3.0 without NA-extraction using five real-time PCR-systems (CFX96 Touch, QuantStudio 5, LightCycler 480II, qTOWER^3, Rotor-Gene 3000) and compared to results of RoboGene HDV RNA Quantification Kit 2.0.

The quantification efficiency of the test was proven with a variation of only 6 % for genotype HDV-3 compared to RoboGene HDV RNA Quantification Kit 2.0.

4.3 Linear range

Linear range of the quantification of HDV RNA was determined by analyzing dilution series of synthetic HDV RNA ranging from 1.7 to $1.7x10^{10}$ IU/ml (manual NA extraction) and 12 to $1.2x10^{11}$ IU/ml (automated NA extraction) respectively. Each dilution was quantified with n=4. The results verify that linear quantification of the test ranges over 8 log_{10} -steps. Data and limits of quantification are shown in figures 3a and 3b.

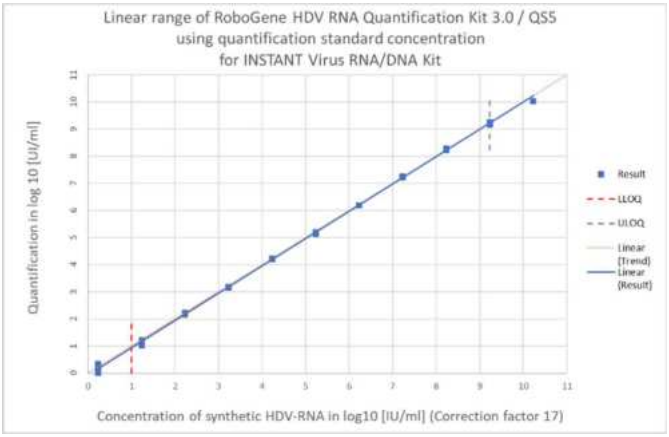

Figure 3a: Linear range of quantification of the HDV RNA Quantification Kit 3.0 applying standard concentrations for manual NA extraction, exemplarily for QuantStudio 5 (QS5).

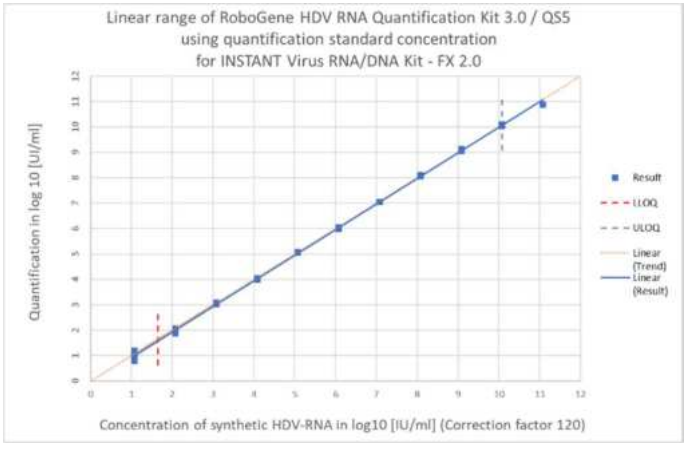

Figure 3b: Linear range of quantification of the HDV RNA Quantification Kit 3.0 applying standard concentrations for automated NA extraction, exemplarily for QuantStudio 5 (QS5).

Dilution series of characterized native HDV RNA positive sample material with concentrations ranging from 2.5 to $2x10^6$ IU/ml were quantified by the test in combination with manual and automated NA extraction. Each dilution was extracted with n=2 per extraction and quantified with n=1 using five different real-time PCR-systems (CFX96 Touch, QuantStudio 5, LightCycler 480II, qTOWER^3, Rotor-Gene 3000). Data and charts of statistical analysis are shown in Figure 4.

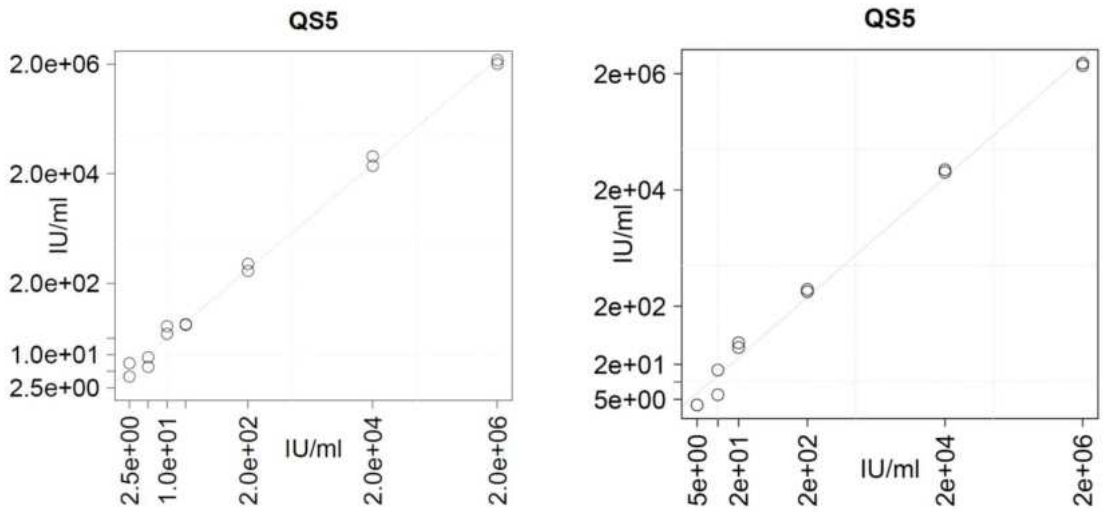

Figure 4: Results of RoboGene HDV RNA Quantification Kit 3.0 in combination with manual NA extraction (left chart) and automated NA extraction (right chart), exemplarily for QuantStudio 5 (QS5). Scatter plot with regression line.

The statistical analysis of data sets was carried out according to CLSI guideline EP06-A [13] and CLSI guideline EP17-A2 [14] to determine the limits of quantification (LOQ). Due to the high comparability of the results of all five real-time PCR-systems, the quantification limits essentially depend on the NA extraction used. The lower- and upper limits of quantification (LLOQ and ULOQ) are summarized in Table 2.

Table 2: Determined limits of quantification, NA extraction specific

NA extraction kit	Lower limit of quantification [IU/ml]	Upper limit of quantification [IU/ml]
INSTANT Virus RNA/DNA Kit (manual)	10.0	$1.7x10^9$
INSTANT Virus RNA/DNA Kit – FX 2.0 (automated)	44.0	$1.2x10^{10}$

Individual quantification results below the quantification limit may be plausible but are correlated with a higher variation. To increase the precision of quantification of samples with low viral load analysis of 3 replicates is recommended.

4.4 Analytical specificity

Analysis of 10 DNA-virus positive, non-HDV samples (HSV1, HSV2, PVB19, EBV, HPV16, HPV18, HCMV, HBV) and two RNA-virus positive, non-HDV samples (HIV, HCV) of human origin confirmed 100 % analytical specificity of RoboGene HDV RNA Quantification Kit 3.0 (see Table 3).

Table 3: Results of 12 DNA- or RNA-virus positive, non-HDV samples including negative and positive control samples. Concentration of human pathogen viruses ranged between $8x10^3$ and $2x10^6$ IU/ml.

Sample	HDV RNA detected	IC detected and valid
HDV positive control (n = 1)	1/1	1/1
Virus negative control (n = 1)	0/1	1/1
HSV type 1 (n = 1)	0/1	1/1
HSV type 2 (n = 1)	0/1	1/1
PVB19 (n = 2)	0/2	2/2
EBV (n = 1)	0/1	1/1
HPV16 (n = 1)	0/1	1/1
HPV18 (n = 1)	0/1	1/1
HCMV (n = 2)	0/2	2/2
HIV (n = 1)	0/1	1/1
HCV (n = 1)	0/1	1/1
HBV (n = 1)	0/1	1/1

4.5 Accuracy, as a result of trueness and precision

The analysis of accuracy, trueness and precision of HDV RNA quantification was performed using the two NAT-systems consisting of RoboGene HDV RNA Quantification Kit 3.0 in combination with INSTANT Virus RNA/DNA Kit (manual) and in combination with INSTANT Virus RNA/DNA Kit – FX 2.0 (automated).

A 5-step dilution series of a native HDV RNA positive sample covering 4 $\log_{10}$ steps were extracted with n=3 per dilution and NA extraction (manual and automated) followed by quantification on 3 different days. Each day a different kit lot of the test was used to quantify the extracts.

The quantifications were carried out in combination with five different real-time PCR-systems (CFX96 Touch, QuantStudio 5, LightCycler 480II, qTOWER^3, Rotor-Gene 3000). One result had to be excluded from the analysis due to a not valid IC. A total of 449 quantification results were obtained (225 and 224 per NAT-system).

Accuracy

All 225 quantification results of the assay in combination with the manual NA extraction showed reliable accuracy within the ±0.6 log_{10} IU/ml acceptance interval for all tested concentrations (see Table 4a).

Table 4a: Results of accuracy analysis (manual NA extraction)

Dilution	Target HDV RNA concentration [IU/ml]	Number of quantifications	Number of accepted quantifications within ±0.6 log10 IU/ml
1	100,000	45	45
2	10,000	45	45
3	1,000	45	45
4	100	45	45
5	10	45	45

The 224 quantification results of the assay in combination with automated NA extraction showed reliable accuracy within the ±0.6 log_{10} IU/ml acceptance interval for all tested concentrations of dilution 1 to 4. Solely 10 out of the 44 quantifications of dilution 5 (10 IU/ml) were outside the acceptance interval. As the concentration of these samples is below the limit of quantification (< LLOQ), these results are acceptable, see Table 4b.

Table 4b: Results of accuracy analysis (automated NA extraction)

Dilution	Target HDV RNA concentration [IU/ml]	Number of quantifications	Number of accepted quantifications within ±0.6 log10 IU/ml
1	100,000	45	45
2	10,000	45	45
3	1,000	45	45
4	100	45	45
5	10	44	34

Trueness

The quantification results of the assay in combination with manual NA extraction showed reliable trueness within the ±0.5 log_{10} IU/ml acceptance interval for all tested conditions. The data and the ±0.5 log_{10} IU/ml acceptance interval (red dashed line) are shown in figure 5a.

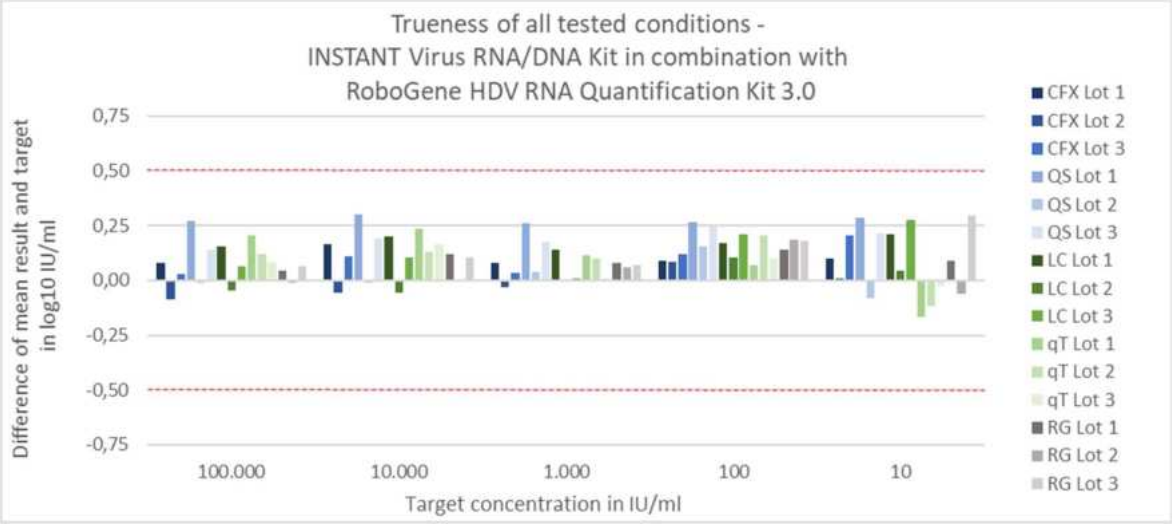

Figure 5a: Results of the trueness analysis of the assay in combination with manual NA extraction. The bar chart shows the difference between the target concentration and the mean value of the results obtained for the respective experimental condition (5 dilutions, 5 real-time PCR-systems and 3 kit lots).

The quantification results of the assay in combination with automated NA extraction showed reliable trueness within the ±0.5 log_{10} IU/ml acceptance interval for all tested conditions. Solely one result below LLOQ (condition: 10 IU/ml quantified with LightCycler 480II using kit lot 3) is outside the acceptance interval. All data and ±0.5 log_{10} IU/ml acceptance interval (red dashed line) are shown in figure 5b.

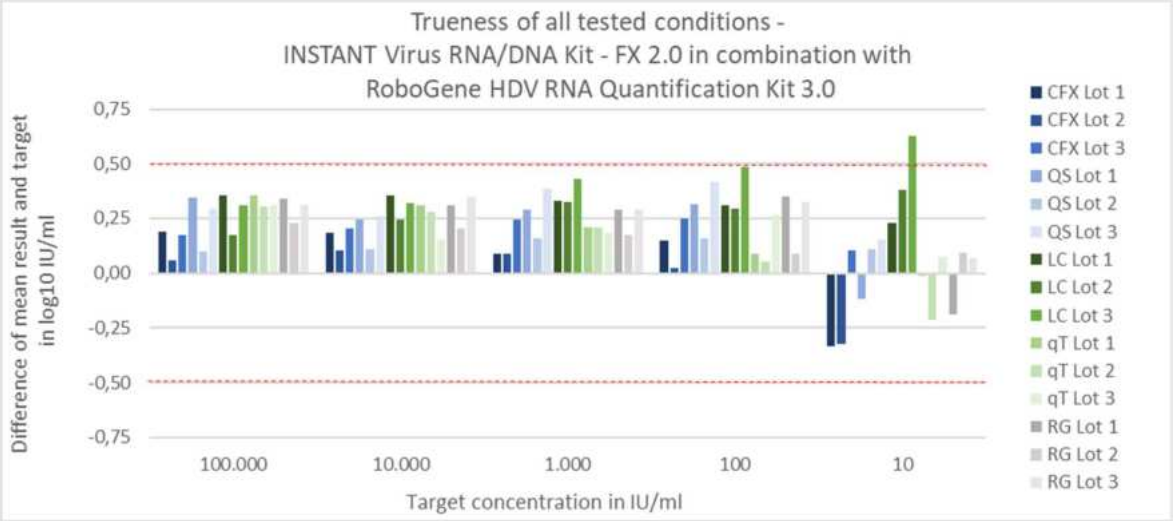

Figure 5b: Results of the trueness analysis of the assay in combination with automated NA extraction. The bar chart shows the difference between the target concentration and the mean value of the results obtained for the respective experimental condition (5 dilutions, 5 real-time PCR-systems and 3 kit lots).

Precision

The intra- and inter-assay precision were evaluated by assessing the standard deviation (SD) of the obtained quantification results. As limit of acceptance SD ≤ 0.17 $\log_{10}$ IU/ml was used (3-sigma-rule: 99.7% probability that concentration differences ≥ 1 $\log_{10}$ IU/ml show no overlap in quantification results).

Analysis of intra-assay precision of the test in combination with manual NA extraction proved standard deviations between **0.02 and 0.12 $\log_{10}$ IU/ml** of all tested concentrations. Analysis of intra-assay precision of the test in combination with automated NA extraction proved standard deviations between **0.04 and 0.12 $\log_{10}$ IU/ml** of tested concentrations between 100 and 100,000 IU/ml. The required precision is not achieved for dilution 5 (10 IU/ml). This is acceptable because the concentration was below the lower limit of quantification (< LLOQ). The data and the 0.17 $\log_{10}$ IU/ml limit of acceptance (red dashed line) are shown in figures 6a and 6b.

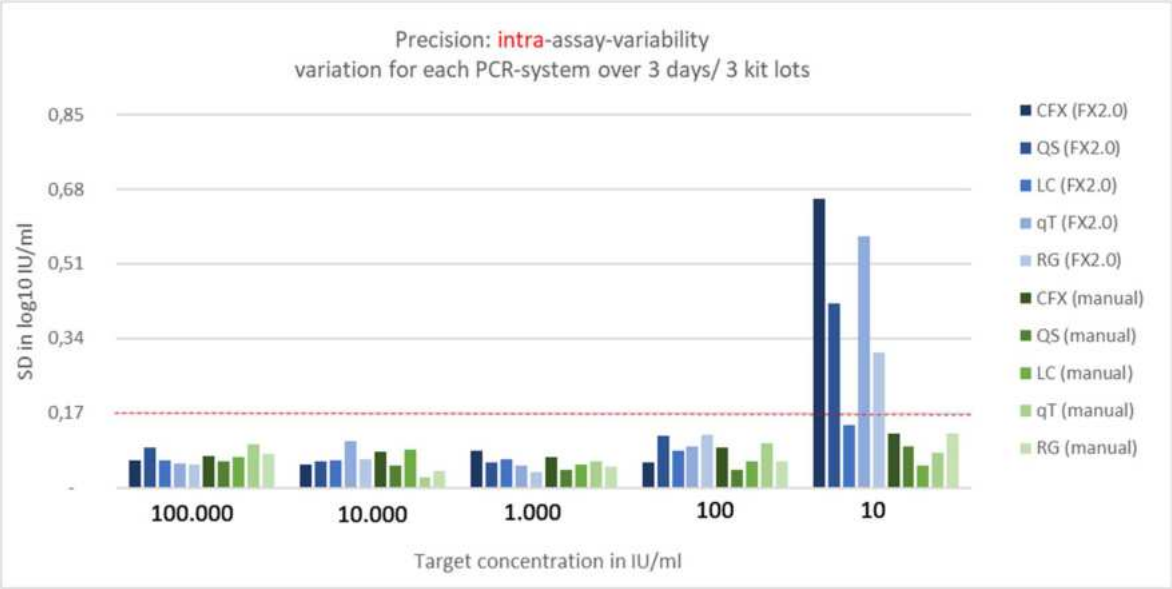

Figure 6a: Results of intra-assay precision analysis of the assay in combination with manual and automated NA extraction. The bar chart shows mean of SD for each real-time PCR-system over 3 days/ 3 kit lots.

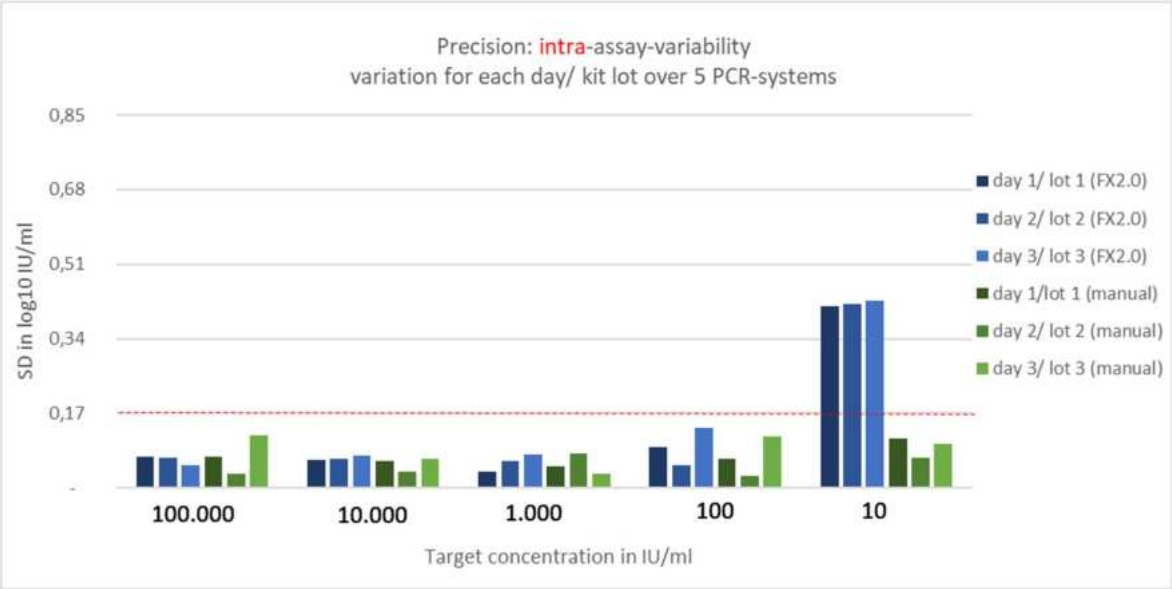

Figure 6b: Results of intra-assay precision analysis of the assay in combination with manual and automated NA extraction. The bar chart shows mean of SD for each day/ kit lot over 5 real-time PCR-systems.

Analysis of inter-assay precision of the test in combination with manual NA extraction proved standard deviations between **0.01 and 0.19 log_{10} IU/ml** of all tested concentrations. SD of mean for dilution 5 (10 IU/ml = LLOQ) using QuantStudio 5 is 0.02 log_{10} IU/ml and for day/kit lot1 0.01 log_{10} IU/ml slightly above limit of acceptance.
Analysis of inter-assay precision of the test in combination with automated NA extraction proved standard deviations between **0.01 and 0.14 log_{10} IU/ml** of tested concentrations between 100 and 100,000 IU/ml. The required precision is not achieved for dilution 5 (10 IU/ml). This is acceptable because the concentration was below the lower limit of quantification (< LLOQ). The data and the 0.17 log_{10} IU/ml limit of acceptance (red dashed line) are shown in figures 7a and 7b.

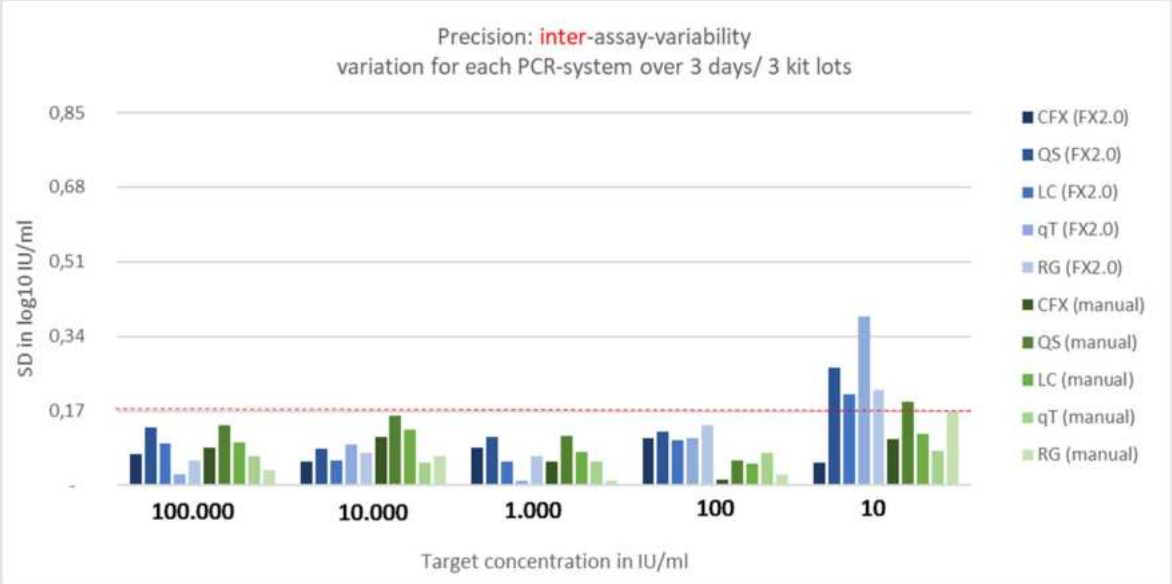

Figure 7a: Results of inter-assay precision analysis of the assay in combination with manual and automated NA extraction. The bar chart shows SD of mean for each real-time PCR-system over 3 days/ 3 kit lots.

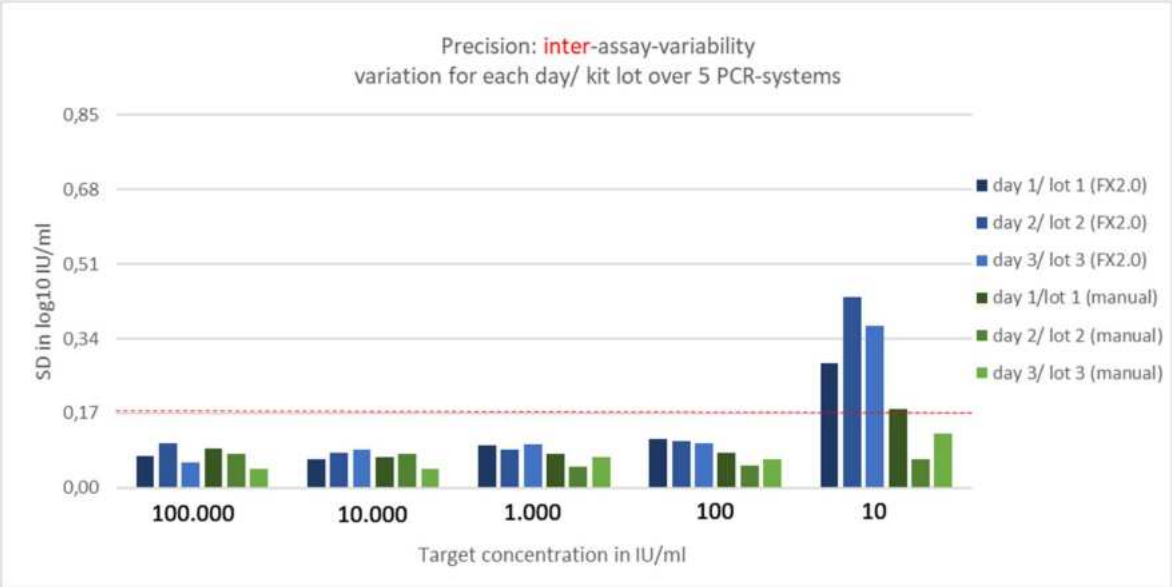

Figure 7b: Results of inter-assay precision analysis of the assay in combination with manual and automated NA extraction. The bar chart shows SD of mean for each day/kit lot over 5 real-time PCR-systems.

4.6 Failure rate of the whole system

Analysis of whole system failure rate of RoboGene HDV RNA Quantification Kit 3.0 was carried out in combination with INSTANT Virus RNA/DNA Kit (manual) and INSTANT Virus RNA/DNA Kit – FX 2.0 (automated). A total of 110 samples (manual) and 112 samples (automated), containing WHO reference material diluted with HDV RNA negative plasma to 3 IU/ml (manual) and 21 IU/ml (automated) were analyzed. Indicated HDV RNA concentrations represent the 3-fold 95 % cut-off values of the two NAT-systems. For measurement five real-time PCR-systems were used (CFX96 Touch, QuantStudio 5, LightCycler 480II, qTOWER^3, Rotor-Gene 3000).

In the analysis whole system failure rates between 0.00 and 0.91 % were determined. A total of 1110 measurements were performed and 1108 of them showed true-positive results leading to a **failure rate of 0.18 %**. Results are shown in Tables 5a and 5b.

Table 5a: Results of failure rate analysis (manual NA extraction)

	(+) Results	Failure rate
CFX96 Real-Time PCR Detection Systems		
HDV RNA (FAM)	109/110	0.91 %
IC (VIC/ Cy5)	110/110	
QuantStudio™ real-time PCR systems		
HDV RNA (FAM)	110/110	0.00 %
IC (VIC / Cy5)	110/110	
LightCycler® 480II		
HDV RNA (FAM)	110/110	0.00 %
IC (YY/ Cy5)	110/110	
qTOWER3 / iris		
HDV RNA (FAM)	110/110	0.00 %
IC (YY/ Cy5)	110/110	
Rotor-Gene 3000/ 6000/ Q		
HDV RNA (FAM)	110/110	0.00 %
IC (YY/ Cy5)	110/110	
all PCR-systems included		
HDV RNA (FAM)	549/550	0.18 %
IC (Cy5)	555/550	

Table 5b: Results of failure rate analysis (automated NA extraction)

	(+) Results	Failure rate
CFX96 Real-Time PCR Detection Systems		
HDV RNA (FAM)	112/112	0.00 %
IC (VIC/ Cy5)	112/112	
QuantStudio™ real-time PCR systems		
HDV RNA (FAM)	112/112	0.00 %
IC (VIC / Cy5)	112/112	
LightCycler® 480II		
HDV RNA (FAM)	112/112	0.00 %
IC (YY/ Cy5)	112/112	
qTOWER3 / iris		
HDV RNA (FAM)	111/112	0.89 %
IC (YY/ Cy5)	112/112	
Rotor-Gene 3000/ 6000/ Q		
HDV RNA (FAM)	112/112	0.00 %
IC (YY/ Cy5)	112/112	
all PCR-systems included		
HDV RNA (FAM)	559/560	0.18 %
IC (Cy5)	560/560	

5 Kit components, storage and stability

RoboGene HDV RNA Quantification Kit 3.0 is available in 2 configurations with 32 or 96 applications as summarized in the table below.

Table 6: Kit configurations

		Σ 32	Σ 96
REF		847-0207650032-RUO	847-0207650096-RUO
IC [1]		Internal Control Lyophilized 1 Tube	Internal Control Lyophilized 3 Tubes
HDV/IC STD 1 – 4 [2]		Quantification standard Lyophilized 4 Strips	Quantification standard Lyophilized 4 Strips
HDV/IC RM [3]		Reagent Mix Vacuum dried 1 Tube	Reagent Mix Vacuum dried 2 Tubes
PCR grade H_2O [4]		Water 1.5 ml 1 Tube	Water 1.5 ml 2 Tubes
RT PCR Enzyme LYO [5]		Enzyme Mix Lyophilized 1 Tube	Enzyme Mix Lyophilized 3 Tubes
Reconstitution Buffer [6]		Enzyme Buffer 0.26 ml 1 Tube	Enzyme Buffer 0.26 ml 3 Tubes

STORAGE CONDITIONS

RoboGene HDV RNA Quantification Kit 3.0 is shipped at ambient temperature. After arrival store the kit at -40°C to -15°C. The kit is stable until the expiry date when stored under these conditions.

IMPORTANT

1 The **IC** should be dissolved in **PCR grade H_2O** shortly before use. Remaining dissolved **IC** can be stored at -40°C to -15°C for up to 60 days. Repeated freezing and thawing up to 5 times is possible.

2 The **HDV/IC STD 1 – 4** should be dissolved in **PCR grade H_2O** shortly before use. Storage of dissolved **HDV/IC STD 1 – 4** is not possible.

3 The **HDV/IC RM** should be dissolved in **PCR grade H_2O** shortly before use. Remaining dissolved **HDV/IC RM** can be stored at -40°C to -15°C for up to 60 days.
Repeated freezing and thawing up to 5 times is possible.
Always protect from light!

4 Repeated freezing and thawing of **PCR grade H_2O** is possible.

5 The **RT PCR Enzyme LYO** should be dissolved in **Reconstitution Buffer** shortly before use. Remaining dissolved **RT PCR Enzyme LYO** can be stored at -40°C to -15°C for up to 120 days.
Repeated freezing and thawing up to 5 times is possible.

6 Repeated freezing and thawing of **Reconstitution Buffer** is possible.

6 Necessary laboratory equipment

RoboGene HDV RNA Quantification Kit 3.0 is intended for use in combination with real-time PCR-systems listed in Table 7.

Table 7: Real-time PCR-systems

Real-time PCR-systems	Manufacturer
CFX96 Real-Time PCR Detection Systems	Bio-Rad
QuantStudio™ real-time PCR Systems	Thermo Fisher Scientific
LightCycler® 480 II	Roche
qTOWER^3 / qTOWER iris	Analytik Jena
Rotor Gene 3000 / 6000 / Q	Corbett Research / Qiagen

Table 8: Common laboratory equipment

Common equipment	
Centrifuges	Micro centrifuge
	Plate centrifuge
Mixer	Thermal mixer
	Vortex mixer
Pipettes	Adjustable pipettes suitable for a volume range of 1 to 1000 µl

7 Consumables not included in the kit

RoboGene HDV RNA Quantification Kit 3.0 is intended for use in combination with kits for nucleic acid extraction listed in Table 9.

Table 9: Kits for nucleic acid extraction

Kits for nucleic acid extraction	Manufacturer	Order number
INSTANT Virus RNA/DNA Kit	Roboscreen GmbH	847-0259200602 / 847-0259200603
INSTANT Virus RNA/DNA Kit – FX 2.0	Roboscreen GmbH	847-0259200908-RUO

Table 10: Recommended PCR consumables and ordering information.

Real-time PCR system	PCR consumable	Manufacturer / Order number
qTOWER³ / qTOWER iris, CFX96 Real-Time PCR Detection Systems, QuantStudio™ real-time PCR Systems (96-Well 0.1-mL Block)	96 Well PCR Plate 0.2 ml, Full skirt, white	Sarstedt / 721980010
	Optical sealing foil	Azenta / 4ti-0565
LightCycler® 480 II	LightCycler® 480 Multiwell Plate 96, white	Roche / 04729692001
	LightCycler® 480 Sealing Foil	Roche / 04729757001
QuantStudio™ real-time PCR Systems (96-Well 0.2-mL Block)	96 Well PCR Plate 0.3 ml, Half skirt, white	Sarstedt / 721979010
	Optical sealing foil	Azenta / 4ti-0565
Rotor-Gene 3000/ 6000/ Q	Strip Tubes and Caps 0.1 ml	Qiagen / 981103

Table 11: Recommended control material

Control material	Manufacturer	Order number	Intention
1st WHO International Standard for Hepatitis D Virus RNA for NAT testing	Paul-Erlich-Institute	PEI code 7657/12	Prove of recovery (evaluation not mandatory)
human plasma or serum, positive for HDV RNA (high viral load)	e.g., Cerba Xpert or in-house sample stock	upon request	Positive control (dilute for higher aliquot number)
human plasma or serum, negative for HDV RNA and/or HBV DNA	e.g., in.vent Diagnostika GmbH or in-house sample stock	NDPO 4.2.1.1	Negative control and diluent

Table 12: Consumables

Consumables
1.5 ml tubes
2.0 ml tubes
Sterile and PCR grade pipette tips with aerosol-barrier, suitable for a volume range of 1 to 1000 µl

8 Procedure

8.1 Collection and handling of samples

Collect 5-10 ml of whole blood with standard specimen collection tubes.

- **Preparation of plasma**: EDTA or citrate anticoagulant must be used; heparin is non-applicable due to its inhibitory effect on PCR.

  The cells must be separated from plasma by centrifugation at 1,000–2,000 x g for 10 minutes [11].

- **Preparation of serum:** after collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature for up to 30 minutes. Separate the clot by centrifugation at 1,000-2,000 x g for 10 minutes [11].

8.2 Storage conditions for plasma and serum

- Store serum or plasma at 2-8 °C. Test within 5 days [11].
- Plasma or serum samples may also be stored deeply frozen at -70 °C to -20 °C. Test within several months depending on the storage temperature. Avoid repeated freezing and thawing [12].

8.3 HDV RNA purification from samples

RoboGene HDV RNA Quantification Kit 3.0 has been validated in combination with a manual and an automated method for viral nucleic acid extraction.

Use ‚**protocol 2: Isolation using IC Spiking Tube**' of the INSTANT Virus RNA/DNA Kit for manual NA extraction and follow the IFU thoroughly. This requires a sample volume of 400 µl.

For automated nucleic acid extraction from 400 µl sample volume use INSTANT Virus RNA/DNA Kit - FX 2.0. Perform nucleic acid extraction steps according to the IFU.

8.4 Internal Control

RoboGene HDV RNA Quantification Kit 3.0 includes the **IC**, containing lyophilized internal control RNA and carrier nucleic acid.

Adding reconstituted **IC** to samples prior to nucleic acid extraction allows to control the whole procedure and to detect false-negative results.

To judge the correctness of the result, the Ct value obtained from the internal control should not exceed the limits summarized in chapter 11.2 (Criteria for run validation).

8.5 General procedure of quantitative analysis

Quantification standard **HDV/IC STD 1 – 4** is a 4-well strip stably coated with four defined amounts of synthetic HDV RNA. The standards are calibrated against the 1st WHO International Standard for HDV RNA reference material (7657/12) obtained from the German Federal Agency for Sera and Vaccines (PEI). Therefore, the standard values are given in IU/ml and can be calculated directly from the reverence curve without the need of further subsequent conversion by an equation.

NOTE

Please note that the values for the standards and thus also the quantification of the HDV RNA depend on the NA extraction used in combination with the RoboGene HDV RNA Quantification Kit 3.0.

Results are only valid when one of the validated viral nucleic acid extraction kits is used in combination with one of the Real-Time PCR systems and consumables indicated in chapters 6 and 7.

9 Protocol

9.1 Preparation of Internal Control

NOTE

RoboGene HDV RNA Quantification Kit 3.0 has been validated in combination with nucleic acid extraction kits INSTANT Virus RNA/DNA Kit and INSTANT Virus RNA/DNA Kit - FX 2.0. The internal control IC is a component of RoboGene HDV RNA Quantification Kit 3.0. Prepare the IC according to the instructions below and extract the IC nucleic acid following the instructions of the used nucleic acid extraction kit.

1. Centrifuge the **IC** briefly at full speed to collect the lyophilized **IC** at 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 to collect solution at the bottom of the tube.
3. Incubate at 37° C for 5 min using a thermal mixer (800-1000 rpm), mix by vortexing briefly followed by brief centrifugation to collect solution at the bottom of the tube.

9.2 Application of the IC during extraction of viral NA

1. Add **10 µl** of resuspended **IC per 400 µl sample volume** to the Lysis Solution of the corresponding extraction kit.
2. Follow instructions given in the IFU of the extraction kit. When using INSTANT Virus RNA/DNA Kit apply '**Protocol 2: Isolation using IC Spiking Tube**'. For INSTANT Virus RNA/DNA Kit – FX 2.0 use corresponding applications in **Application Studio extract**.
3. The **elution volume** used for both extraction kits in combination with RoboGene HDV RNA Quantification Kit 3.0 is **60 µl**.

9.3 Preparation of 25x Reagent Mix

1. Centrifuge **HDV/IC RM** briefly at full speed to collect the dried reagent mix at the bottom of the tube.
2. Add **53 µl PCR grade H_2O** to **HDV/IC RM**; close the tube, mix by brief vortexing followed by brief centrifugation to collect the solution at the bottom of the tube.
3. Incubate at 37° C for 5 min using a thermal mixer (800 - 1,000 rpm), mix by brief vortexing followed by brief centrifugation to collect the solution at the bottom of the tube.

9.4 Preparation of RT PCR Enzyme LYO

1. Centrifuge the **RT PCR Enzyme LYO** and **Reconstitution Buffer** briefly at full speed to collect the lyophilized enzyme and the buffer at the bottom of the tube.
2. Add **240 µl** of **Reconstitution Buffer** to **RT PCR Enzyme LYO**; close the tube, mix by gently inverting several times until lyophilized enzyme is dissolved. Centrifugate briefly to collect the solution at the bottom of the tube.

9.5 Preparation of 1x Master Mix

1. Prepare the 1x Master Mix according to the following table in a 1.5 ml or 2.0 ml tube.
2. Mix by vortexing for at least 10 s followed by brief centrifugation to collect the solution at the bottom of the tube.

Table 13: Composition of 1x Master Mix per reaction

Component	Volume for 1x rxn [µl]	Final concentration
PCR grade H_2O	7.75	-
RT PCR Enzyme LYO Enzyme Mix, 4x	6.25	1x
HDV/IC RM Reagent Mix, 25x	1.00	1x
Total	15.00	-

9.6 Preparation of Quantification Standards

1. Open the sealing foil of standard strip **HDV/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 **HDV/IC STD 1 - 4**; mix by pipetting up and down several times.

NOTE

Use a new pipette tip for each standard, to avoid carry over contamination.

It is important to mix quantification standards **HDV/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 master mix.

9.7 Preparation of reaction mixtures

1. Place real-time PCR device-specific PCR consumables (see chapter 7) onto a suitable ice-cold rack.

2. Add **15 µl of 1x Master Mix** to all wells intended for sample analysis, NTCs and quantification standards.

3. Add **10 µl PCR grade H_2O** to wells that serve as **NTC**.
Add **10 µl of resuspended HDV/IC STD 1 - 4** to all wells that serve as quantification standards. Add quantification standards in the right order. Make sure all reaction mixtures to be mixed properly. Mix by pipetting up and down several times.

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

4. Add **10 µl eluate** from the nucleic acid extraction to the respective sample wells pre-filled with 1x Master Mix. Make sure all reaction mixtures to be mixed properly. Mix by pipetting up and down several times.

NOTE
Do not exceed a final reaction volume of 25 µl.
Use a new pipette tip for each standard and sample for transfer into the master mix, to avoid carry over contamination.

5. Seal the PCR consumables with recommended optical sealing foil. Centrifuge PCR plates for 1 min at 1,000 rpm to collect the PCR mix at the bottom of each well (not necessary for Rotor-Gene™ tubes).

6. Program the applied real-time PCR system as indicated in chapter 10 and start the program.

10 PCR thermal profile and data acquisition

NOTE

The essential in-run standard curve provides run validation criteria slope and R^2 value (see chapter 11.2).

Never use external standard curves for quantification.

Table 14: Real-time PCR protocol

Step	Cycle	Profile	Temperature	Time
1	1	Reverse transcription	50 °C	15 min
2	1	Taq activation	95 °C	2 min
3	45	Denaturation	95 °C	10 sec
		Annealing/ Elongation*	60 °C	30 sec

* Data acquisition via fluorescence detection

General settings:

- Detection channel:
  FAM (Target: HDV RNA)
  Yakima Yellow/ VIC/ JOE (IC)
  Cy5 (IC)
- Ramping Rate [°C/sec] for each step on highest setting, if adjustable

Further settings for qTOWER^3/ qTOWER iris:

- the following presetting is recommended:
  Open new project -> Scan -> Gain: for FAM = 4; for VIC/JOE = 3; for Cy5 = 5

Further settings for LightCycler® 480II:

- an additional cooling step is recommended:
  40 °C for 30 sec at the end of the protocol

Further settings for Rotor-Gene 3000/ 6000/ Q:

- the following presetting is recommended:
  Open new project -> Confirm Profile -> Gain Calibration (Data acquisition step) -> Perform Calibration Before 1st Acquisition (all detection channels)

ATTENTION

Due to diversity of RT-PCR systems including configuration of detection channels, the kit contains an internal control allowing detection of the IC signal by detection channels YY/ VIC/ JOE or Cy5.

Crosstalk between FAM- and YY/ VIC/ JOE- signals and detection channels is a known phenomenon of some RT-PCR systems with older software versions not further defined. To avoid crosstalk, use Color Compensation for FAM and YY/ VIC/ JOE of the corresponding software, if available (see chapter 11.1).

11 Data analysis

Each RNA amplification is associated with generation of a fluorescence signal measurable in FAM channel (for HDV RNA) and in YY/VIC/JOE and/ or Cy5 channel (for IC) resulting in a sigmoid growth curve (linear scale).

The data analysis is performed according to manufacturer's instructions of the real-time PCR instrument using the respective software. Check all settings of the RT-PCR system for analysis (see chapter 11.1).

Check the obtained data to ensure that the run is valid and to interpret results (see chapter 11.2).

11.1 Settings for data analysis

HDV RNA quantification standard concentrations

HDV 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 HDV RNA concentration is expressed in IU/ml. The following two tables list the concentrations of HDV RNA quantification standards in case of using the INSTANT Virus RNA/DNA Kit (see Table 15) or INSTANT Virus RNA/DNA Kit - FX 2.0 (see Table 16).

Table 15: HDV RNA quantification standard concentrations using INSTANT Virus RNA/DNA Kit for manual NA extraction

HDV/IC STD 1 - 4	HDV RNA [IU/ml]
1	17,000,000
2	170,000
3	1,700
4	170

Table 16: HDV RNA quantification standard concentrations using INSTANT Virus RNA/DNA Kit – FX 2.0 for automated NA extraction

HDV/IC STD 1 - 4	HDV RNA [IU/ml]
1	120,000,000
2	1,200,000
3	12,000
4	1,200

Threshold setting

The setting of thresholds may markedly influence Ct values. Nevertheless, the quantification is in the linear range of logarithmic scaled amplification curves only slightly influenced by the setting of the threshold.

In general, it is recommended to set the threshold in the linear range of logarithmic scaled amplification curves but above the baseline noise of NTC and negative control (see Figure 8).

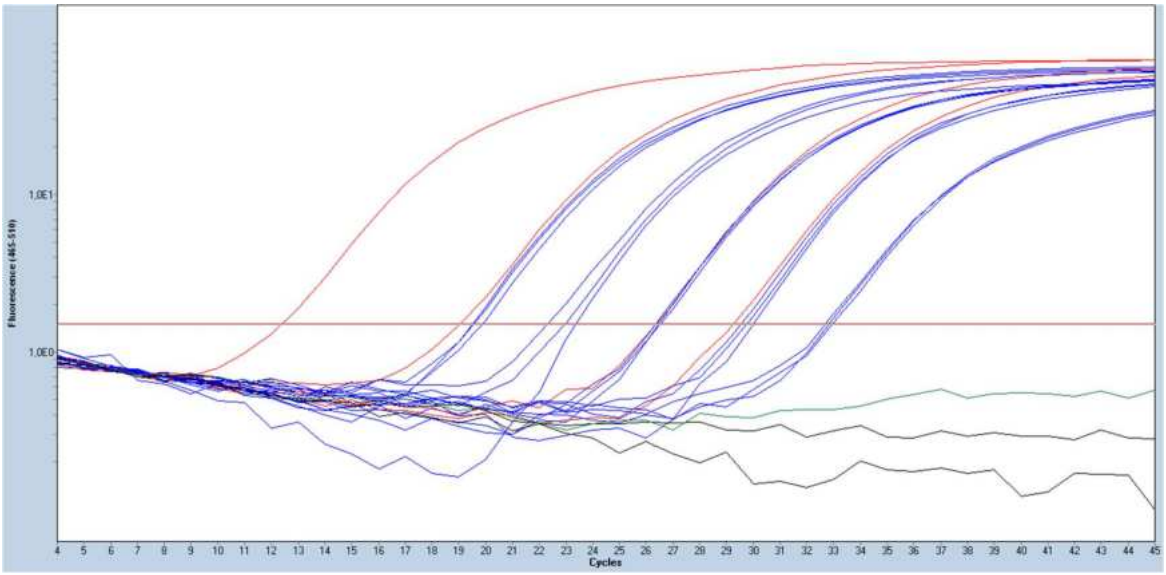

Figure 8: logarithmic scaled amplification curves and threshold (red line), red graph (HDV/IC STD 1-4), black graph (NTC), green graph (negative control), blue graph (samples)

Recommendations for threshold setting on validated real-time PCR systems are shown in Table 17.

Table 17: RT-PCR system specific threshold settings

RT-PCR system	FAM channel	YY/ VIC/ JOE channel	Cy5 channel
CFX96 Real-Time PCR Detection Systems	400	150	100
QuantStudio™ real-time PCR systems	250,000 (0.25)	60,000 (0.06)	60,000 (0.06)
LightCycler® 480II	≥ 1	≥ 1	≥ 0.5
qTOWER^3/ qTOWER iris	5	5	5
Rotor-Gene 3000/ 6000/ Q	0.03	0.02	0.02

Further settings for qTOWER^3/ qTOWER iris:
- Analysis Type: Analysis -> Absolute quantification
- Color Compensation: Settings -> Scan -> Standard1

Further settings for Light Cycler® 480II:
- Analysis Type: Abs Quant/ Fit Points
- Color Compensation (In Database) for FAM and VIC channel
- First cycle: 5 to 8
- Noiseband: FAM ≥ 1; YY/ VIC/ JOE ≥ 1; Cy5 ≥ 0.5
- Fit points: 3 to 4

Further settings for Rotor-Gene 3000/ 6000/ Q:
- Analysis -> Quantitation -> Dynamic Tube
- Analysis -> Quantitation -> Slope Correct for IC-detection channels, for FAM-channel optional, depending on confirmation of run validation criteria

NOTE

It is important to maintain the RT-PCR system so that the settings are efficient. If the recommended settings are not applicable, please contact technical service.

11.2 Run validation and interpretation

Criteria for run validation

Criteria for run validation are the slope and R^2 value of the standard curve (see Table 18).

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 Table 19 and Table 20).

If slope and/or R^2 are out of range due to a single standard Ct-value outlying the regression curve, the aberrant quantification standard can be excluded. This is appropriate, as three quantification standards are sufficient for correct quantification of HDV RNA and verification of a valid test result.

Table 18: Criteria for run validation

Parameter	Acceptance range/ limit
Slope and R^2 of quantification standard curve	
Range of slope	-3.10 to -3.60
linear regression coefficient R^2	0.98 to 1.00 (Not applicable to LightCycler®480 analysis)
Expected Ct values for IC of the quantification standards, samples negative- and positive for HDV RNA (dependent on the set of threshold value, see above)	
YY/ VIC/ JOE and Cy5	≤ 38

Table 19: Guidance Ct values of the quantification standards qTOWER³, LightCycler® 480II and CFX96 Touch Real-Time PCR Detection System

HDV/IC STD 1 - 4	Expected increment between Ct values	qTOWER³/iris		LightCycler® 480II		CFX96 Real-Time PCR Detection System	
		mean	range	mean	range	mean	range
1	-	14.04	13.80-14.29	15.13	14.08-16.08	13.97	13.27-14.57
2	1 to 2 + ~ 6.64	20.43	20.20-20.72	21.95	21.11-23.69	20.59	19.99-22.29
3	2 to 3 + ~ 6.64	27.32	27.09-27.73	28.73	27.63-30.90	27.33	26.43-29.38
4	3 to 4 + ~ 3.32	30.58	30.03-31.10	31.92	31.44-32.83	30.62	29.70-31.22

Table 20: Guidance Ct values of the quantification standards QuantStudio™ real-time PCR systems and Rotor-Gene 3000/6000/Q

HDV/IC STD 1 - 4	Expected increment between Ct values	QuantStudio™ real-time PCR systems		Rotor-Gene 3000/6000/Q	
		mean	range	mean	range
1		13.62	13.21-14.25	11.45	11.20-11.81
2	1 to 2 + ~ 6.64	20.26	20.01-20.53	18.22	18.05-18.44
3	2 to 3 + ~ 6.64	26.96	26.70-27.41	24.84	24.47-25.10
4	3 to 4 + ~ 3.32	29.91	28.56-30.49	27.93	27.71-28.35

Interpretation of results

If the run is valid continue with the interpretation of measured samples as shown in table below.

Table 21: Interpretation of results

FAM channel x = Ct value	YY/ VIC/ JOE or Cy5 channel x ≤ 38 Ct	Interpretation
Interpretation of detection results		
x	x	Valid - detection of HDV RNA and IC Sample positive for HDV RNA.
x	-	Not valid - detection of HDV RNA Quantification may be incorrect, repeat.
-	x	Valid - detection of IC Sample negative for HDV RNA.
-	-	Not valid - no detection of HDV RNA and IC Sample may be positive for HDV RNA, repeat.
Interpretation of quantification results		
-	x	No Ct-value, HDV RNA cannot be detected. HDV RNA not present or below limit of detection. Indication: “HDV RNA not detected”
< LLOQ (including < LOD)	x	Below lower limit of quantification of test, HDV RNA cannot be quantified accurately. Indication: “HDV RNA detected; result < LLOQ”
> ULOQ	x	Above upper limit of quantification of test, HDV RNA cannot be quantified accurate. Indication: “HDV RNA detected; result > ULOQ”
≥ LLOQ ≤ ULOQ	x	In linear range of quantification of test, HDV RNA can be quantified accurately. Indication: “HDV RNA detected; result”

NOTE

For validated limits of detection and quantification of the assay in combination with other products please refer to chapter 4 (Performance Assessment).

12 Troubleshooting

Problem / probable cause	Comments and suggestions
No signal at all	
■ Fluorescence measurement not activated	Read the user guide of the real-time PCR System. Activate fluorescence measurement at end of elongation.
■ False channels selected	Select FAM channel for HDV RNA and YY/VIC/JOE or Cy5 channel for IC.
■ Incorrect thermal profile/ protocol	Check and adjust 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 HDV-negative sample RNA	
■ Incorrect cycling profile/ protocol or analysis setting	Check and adjust instrument settings, including data analysis, 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 or lipemic)	Request for fresh serum, EDTA- or Citrate-plasma.

▪ 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 <-> FAM)	Analyze using color compensation. 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 components	Read IFU, check storage conditions, prepare components new.
▪ Storage conditions did not comply with instructions, expiry date of detection kit is exceeded	Check storage conditions and expiry date.
▪ Ct-value of standards could not be aligned to the correct HDV RNA concentration	Check if standards contained in HDV/IC STD 1 – 4 are pipetted into the PCR consumable in the right order.
Different amplification behavior of sample HDV 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.
▪ Incorrect sample material	Use recommended sample type.
FAM signal for HDV-negative samples / NTC recorded	
▪ Contamination with HDV RNA or RNA amplicons	Repeat NA extraction and PCR with new reagents; decontaminate instruments and workspace. Increase number of NTC and negative controls when repeating.

If you have any further questions, please contact our technical service.

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14 Document Revision

Document Revision Documentation		
Rev.2	November 2024	■ Reconstitution Buffer: colour of screw cap was changed from orange to yellow. ■ Kit configuration with 96 applications: 1 tube of RT PCR Enzyme LYO and 1 tube of Reconstitution Buffer each for 96 rxn were replaced by 3 tubes of RT PCR Enzyme LYO and 3 tubes of Reconstitution Buffer each for 32 rxn. ■ General editorial changes