Instructions for Use
Life Science Kits & Assays

RoboGene® HDV RNA Quantification Kit 2.0

analytikjena
An Endress+Hauser Company

Order No.:
Regular profile tubes 0.2 ml (clear)
for use with Rotor-Gene™ 3000/6000/Q
847-0207400542 96 reactions
847-0207400544 32 reactions
Low profile strips 0.1 ml (white)
for use with 7500 Fast/ LightCycler® 480
847-0207400582 96 reactions
847-0207400584 32 reactions

CE
0483

Publication No.: Manual_qHDV-TM2_e_rev.5

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Contents

1 Introduction

1.1 Intended use

The RoboGene® HDV RNA Quantification Kit 2.0 is intended for real-time PCR quantification of Hepatitis D Virus (HDV) RNA in human EDTA plasma or serum samples using the INSTANT Virus RNA/DNA Kit (Analytik Jena). Two kit versions are available: low profile strips 0.1 ml (white) for LightCycler® 480 and 7500 Fast real-time PCR systems and regular profile tubes 0.2 ml (clear) for application on Rotor-Gene™ 3000/6000/Q, respectively. The assay is purposed for the clinical management of patients with chronic HDV infections in conjunction with symptoms and other laboratory markers of the disease.

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

The RoboGene® HDV RNA Quantification Kit 2.0 is not intended for use as a screening test for the detection of HDV RNA in blood or blood products or as a diagnostic test to confirm the presence of HDV infection.

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]. Superinfection by HDV leads to acute hepatitis and causes progression to liver cirrhosis in a significant proportion of HBsAg carriers [2, 3]. Thus, treatment of HBV infections and immunization against HBV antagonizes infection with HDV.

CONSULT INSTRUCTION FOR USE

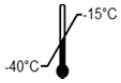
This package insert must be read carefully prior to use. Package insert instructions must be followed accordingly. Reliability of results cannot be guaranteed if there are any deviations from the instructions in this package insert.

1.3 Technical assistance

If you have any questions or problems regarding any aspects of the RoboGene® HDV RNA Quantification Kit 2.0 please do not hesitate to contact our technical support team which consists of scientists with long-time experience in the field of molecular diagnosis. For technical assistance please contact us at the manufacturer site as shown inside the cover of the IFU.

1.4 Notes on the use of this instructions for use

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**
	**For single use only**
	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 D Virus
IC	Internal Control
IFU	Instruction For Use
IU	International Units
LPW	Low profile strips 0.1 ml (white)
NTC	Non-template control
PEI	Paul-Ehrlich-Institut, Langen, Germany
RG	Rotor-Gene instruments
SD	Standard deviation
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 or 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.

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

ATTENTION!
Don’t 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-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 Mix FS 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.

3.2 Explanation of the HDV RNA quantification test

The RoboGene® HDV RNA Quantification Kit 2.0 is an amplification test for quantification of HDV RNA in human plasma or serum samples. The assay is able to detect all eight genotypes of HDV [4], by applying probes and primers specific for a subsequence of the Hepatitis delta antigen (HDag). Determination of specimen concentrations 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 increase of diagnostic correctness. Amplification of HDV RNA in samples and standards and of IC RNA is measured independently at different wavelengths due to probes labelled with different fluorescent reporter dyes (HDV RNA: FAM, IC RNA: Yakima Yellow).

Manual sample preparation should be conducted with the “INSTANT Virus RNA/DNA Kit” (Analytik Jena). RNA extraction must be performed strictly according to manufacturer’s instructions using ‘Protocol 3: Isolation of vi-

ral RNA/DNA from 400 µl of serum/plasma using IC Spiking Tube (modified)‘.

3.3 Restrictions

All reagents may exclusively be used for in vitro diagnostic applications. This test is validated for the usage together with either human plasma or serum. Plasma or serum with heparin has to be excluded from analysis (see under point robustness of the test). If other than the recommended sample types are used incorrect results may be obtained. The product is to be used by personnel specially instructed and trained in the in vitro diagnostics procedures (DIN EN ISO 18113) only. Strict compliance with the instruction for use is required for optimal PCR results. The product may be used only with the mentioned real-time PCR instruments. Do not use expired components or mix with components from different lots.

4 Performance assessment

The RoboGene® HDV RNA Quantification Kit 2.0 was evaluated according to the common technical specifications (CTS) for in vitro diagnostic medical devices (2002/364/EC) [5].

4.1 Analytical sensitivity

The analytical sensitivity of the kit was determined by analyzing dilutions of the 1st WHO International Standard for HDV RNA, genotype 1 (#7657/12, provided by PEI). Using the "INSTANT Virus RNA/DNA Kit" (starting sample volume 400 µl of plasma) the following analytical sensitivities were detected (see Table 1).

Table 1: Limit of Detection (LOD) using different instruments

Instrument	Detection limit [IU/ml]
7500 Fast	6
Rotor-Gene™ 3000	8
LightCycler® 480	14

The limit of detection means that from the statistical point of view at least 95% of samples containing this HDV concentration are correctly detected at a probability error of 5%. Individual values below the detection limit may be plausible but with a high probability of error. To reduce this error probability 3 replicates of such samples are recommended.

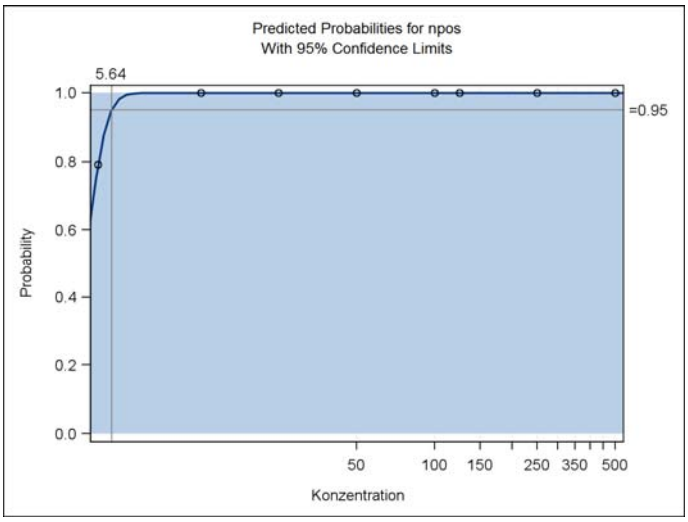


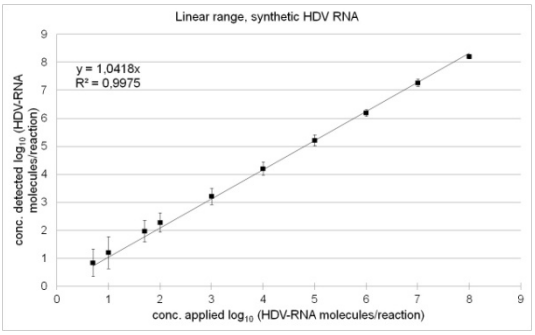
Figure 1: Analytical sensitivity of the RoboGene® HDV RNA Quantification Kit 2.0 is subject to the real-time PCR instrument and purification kit in use. Exemplarily a probit analysis on 7500 Fast is shown. Concentrations are given in IU/ml.

4.2 Linear range

The linear range of the RoboGene® HDV RNA Quantification Kit 2.0 was determined by analyzing a dilution series of the synthetic HDV quantification standard ranging from 5 to 1 x 10^9 synthetic HDV copies per run. Each dilution was tested in replicates on Rotor-Gene™ 3000, 7500 Fast and LightCycler® 480 (n=10 for each concentration). For clinical samples testing access for well characterized high viral loaded samples was limited. Therefore dilution series of a patient sample, tested positive for HDV RNA were tested in replicates on Rotor-Gene™ 3000, 7500 Fast and LightCycler® 480 (n=12 for each concentration).

The linearity of the assay was >8 logs as determined using results of synthetic HDV standard by a linear regression of the $\log_{10}$ calculated with the $\log_{10}$ nominal concentrations for all used real-time PCR instruments (see exemplarily on 7500 Fast in Figure 2a). Furthermore the linear range was determined using native patient material between 10 and 10^6 IU/ml resulted in comparable standard curve parameters and linearity (see Figure 2b).

a)



b)

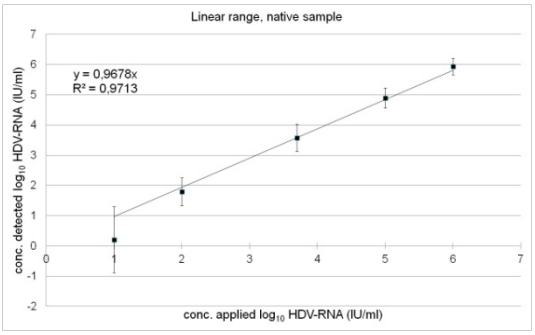


Figure 2: Linearity of the RoboGene® HDV RNA Quantification Kit 2.0 on 7500 Fast using synthetic HDV quantification standard (a) and native sample material positive for HDV RNA (b).

4.3 Specificity

HDV genotype detection and quantification

The performance of the RoboGene® HDV RNA Quantification Kit 2.0 regarding HDV genotype detection and quantification was assessed at the French National Reference Laboratory for HBV, HCV and HDV (Dr. Emmanuel Gordien, Bobigny, France) and at the Fundação de Medicina Tropical (FMT-HVD, Dr. Wornei Braga, Manaus, Brazil).

Analyzed samples covered all known genotypes: HDV-1, -2 and 4-8 (FNRL, 1 sample each) and HDV-3 (FMT-HVD, 2 samples), respectively. Specimens were processed and quantified, using 400 µl sample material.

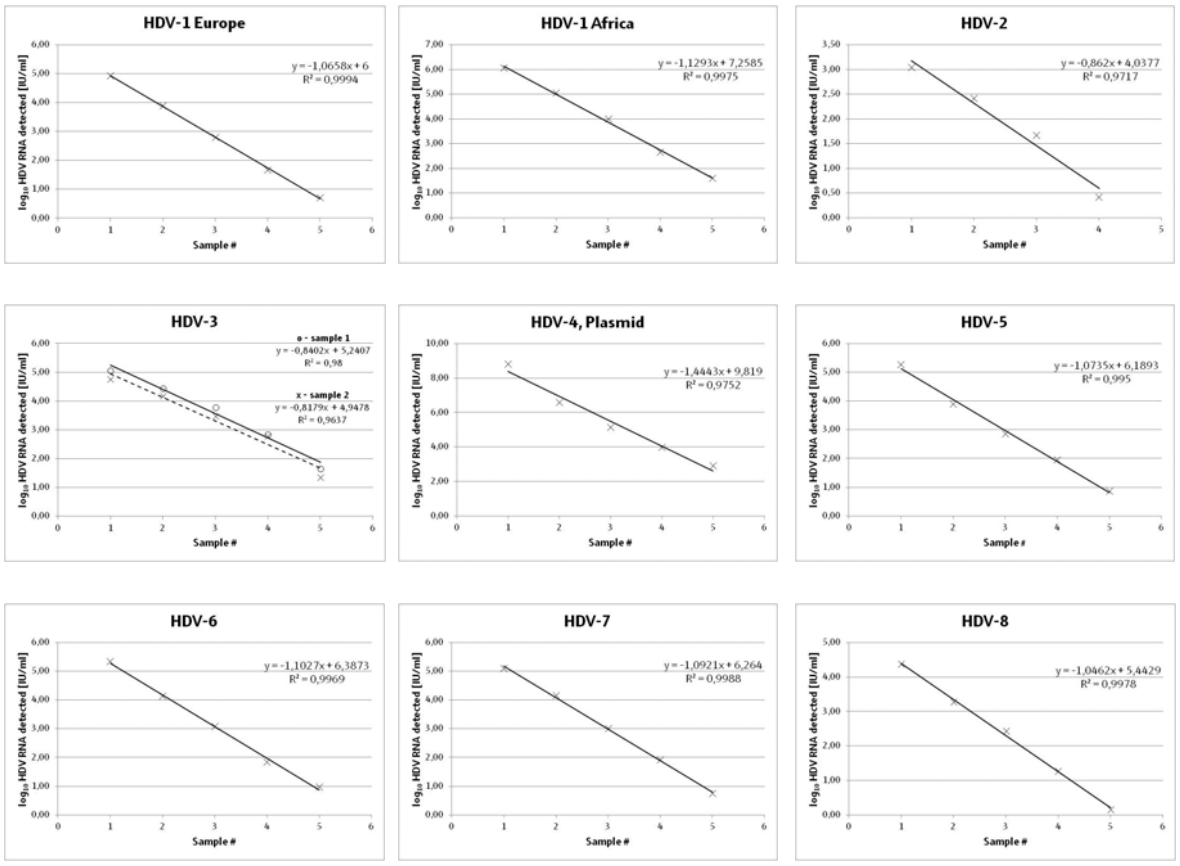


Figure 3: Genotype detection and quantification of HDV genotype dilutions using RoboGene® HDV RNA Quantification Kit 2.0 with 7500 Fast instrument. Samples # 1 represent neat specimens. All other represent log scale dilutions of the same sample, except for HDV-3. Here only half-log dilutions were assessed. Please note, HDV-4 performance was determined using a plasmid construct due to the lack of HDV-4 positive specimens.

All analyzed HDV genotypes were detected correctly. Samples were quantified linearily, down to the limit of detection (6 IU/ml = 0.8$\log_{10}$). Hence the RoboGene® HDV RNA Quantification Kit 2.0 was much more sensitive than in comparison to the FNRL in-house-assay (data not shown).

Diagnostic and analytical specificity

By scientific definition HDV replication is strictly dependent on the presence of HBV i.e., HBV-negative samples are HDV-negative, too. Thus plasma samples, tested negative for HBV using the Cobas® TaqMan® HBV Kit, were analyzed to determine the diagnostic specificity of the RoboGene® HDV RNA Quantification Kit 2.0, which is expressed as negative result in absence of the target. (see Table 2).

Table 2: Diagnostic specificity of the RoboGene® HDV RNA Quantification Kit 2.0

Analyzed samples	HDV RNA positive	IC-RNA positive
HDV negative plasma (n=100)	0	100

The analytical specificity was evaluated by analyzing 20 non-HDV-positive specimens. No HDV RNA (FAM) signal should be detected in these specimens (see Table 3).

Table 3: Pathogen samples used for analysis of analytical specificity

Control group	HDV RNA (FAM)	IC RNA (VIC/ JOE)
Hepatitis B virus (HBV), n=10	0/10	10/10
Hepatitis C virus (HCV), n=10	0/10	10/10

The RoboGene® HDV RNA Quantification Kit 2.0 had a perfect analytical and diagnostic specificity. None of the analyzed samples gave positive test results for HDV 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 HDV RNA using the RoboGene® HDV RNA Quantification Kit 2.0. Dilution series consisting of 3 different viral load levels, i.e. low (25 IU/ml), intermediate (2.5 x 10^3 IU/ml), and high (2.5 x 10^5 IU/ml) were measured.

Table 4: Inter-assay variability of the RoboGene® HDV RNA Quantification Kit 2.0

Factor	HDV RNA	S.D. in log	% CV	Within acceptance interval*
Different experiments	high	4.66	19.1	yes
	intermediate	2.69	22.2	yes
	low	0.69	20.6	yes
Different days of measurement	high	4.72	18.3	yes
	intermediate	2.60	13.5	yes
	low	1.11	35.3	yes
Different lots	high	4.48	7.5	yes
	intermediate	2.70	11.2	yes
	low	1.18	27.0	yes
Different laboratories	high	4.85	30.8	yes
	intermediate	2.56	14.3	yes
	low	0.92	37.8	yes
Different lots in different laboratories	high	4.90	33.5	yes
	intermediate	2.94	28.6	yes
	low	0.59	14.3	yes
Different days of measurement in different laboratories	high	4.75	24.3	yes
	intermediate	3.09	39.0	yes
	low	1.19	48.8	yes

* defined as +/- 0.5 log of set point

NOTE

The % CV of the Intra-assay variability in very low viral load concentrations (close to the limit of detection), normally expected at the end of an antiviral therapy, may be unacceptably high. In case of doubtful results compare the respective quantification outcome with earlier findings and repeat the analysis.

4.5 Robustness

The robustness expresses the total failure rate of the RoboGene® HDV RNA Quantification Kit 2.0 and was tested for the complete test procedure using the INSTANT Virus RNA/DNA kit. Reference plasma diluted to the 3-fold virus concentration of the 95% cut-off value of the test was analyzed on 7500 Fast, Rotor-Gene™ 3000 and LightCycler® 480 (Table 5).

Table 5: Robustness of the RoboGene® HDV RNA Quantification Kit 2.0

	Replicates	(+) Results	Failure rate
7500 Fast			0 %
HDV (FAM)	122	122	
IC-RNA (VIC)	122	122	
Rotor-Gene™ 3000			0 %
HDV (FAM)	72	72	
IC-RNA (JOE)	72	72	
LightCycler® 480			0 %
HDV (FAM)	72	72	
IC-RNA (VIC)	72	72	

Amplification of HDV RNA using RoboGene® HDV RNA Quantification Kit 2.0 could not be reduced by the addition of EDTA, citrate, bilirubin and haemoglobin.

Quantification results from lipaemic samples might be decreased slightly: Validation data show a possible influence of lipids on the amplification reaction. Thus results obtained from lipaemic plasma or serum samples should be interpreted carefully. Heparinised plasma or serum has to be excluded from analysis, because of its inhibitory effect on the activity of Taq polymerases.

4.6 Diagnostic evaluation

The diagnostic sensitivity and linearity of the RoboGene® HDV RNA Quantification Kit 2.0 were analyzed with 109 HDV RNA positive patient samples. Quantitative data were compared with results obtained at the "MVZ Volkmann und Kollegen" (Karlsruhe, Germany) applying a certified in-house-assay. The linear regression analysis showed that the results are in the linear range and exhibit a high degree of correlation (Figure 4).

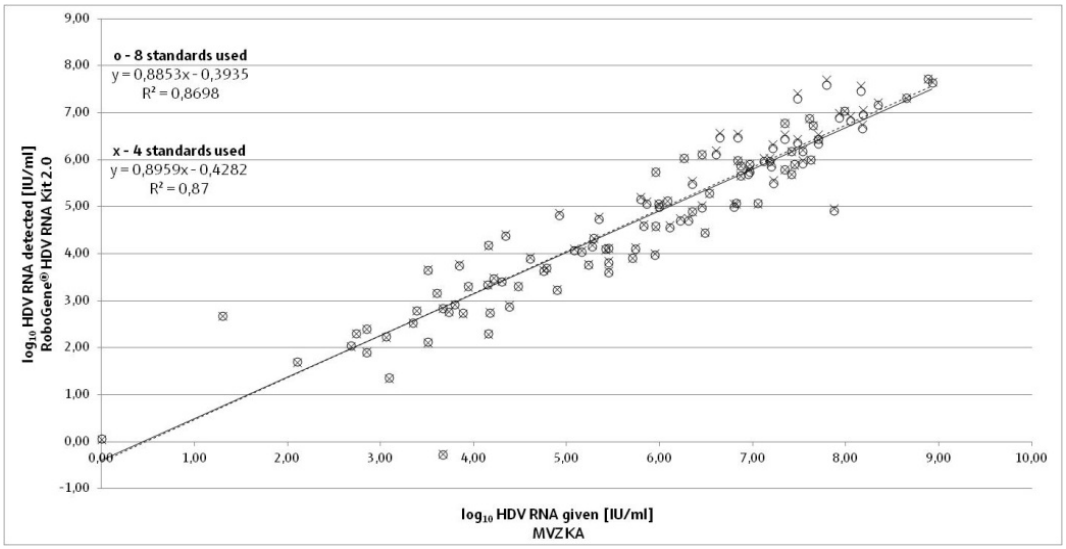


Figure 4: Diagnostic evaluation: comparison of the RoboGene® HDV RNA Quantification Kit 2.0 (sample purification with the INSTANT Virus RNA/DNA Kit) with a certified in-house assay (MVZ KA). The correlation of quantitative results from both tests (n=109) using 4 and 8 quantification standards (comparison new vs. old kit version) from the RoboGene® Kit, respectively, was analyzed by linear regression. The new kit version (4 standards) is potent to generate comparable quantification results. The equations of the respective regression lines are included in the figure.

By consideration of the method of inherent imprecision as well as the variability of individual data points (Deming regression analysis) no significant statistical differences between both methods were detected (data not shown).

5 Kit components, storage and stability

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

- Box 1 for RT PCR Enzyme Mix FS,
- Box 2 for IC RNA, HDV/IC RM and PCR grade water RNA,
- Bags for HDV/IC STD 1-4 and Sample RNA Strips/ Tubes.

NOTE
RT PCR Enzyme Mix FS must be re-packed to box 1 after arrival.

RoboGene® HDV RNA Quantification Kit 2.0 is available in 2 sizes summarized in tables 6 - 8.

Table 6: Kit versions and general components

	Σ 32	Σ 96
REF low profile strips 0.1 ml (white)	847-0207400584	847-0207400582
REF regular profile tubes 0.2 ml (clear)	847-0207400544	847-0207400542
IC RNA[1] 	IC Spiking Tube for 1 x 0.50 ml working solution	IC Spiking Tube for 2 x 0.50 ml working solution
HDV/IC RM[2] 	Reagent Mix for 1 x 0.05 ml working solution	Reagent Mix for 2 x 0.05 ml working solution
PCR grade water RNA[3] 	1 x 1.5 ml	2 x 1.5 ml
RT PCR Enzyme Mix FS[4] 	1 x 0.235 ml	1 x 0.660 ml
IFU	1	1

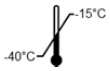
Table 7: Kit components for application to LightCycler® 480 and 7500 Fast using low profile strips 0.1 ml (white)

Component	Σ 32	Σ 96	Description
HDV Sample Strips LPW	4 Strips (4 x 8 Tubes)	12 Strips (12 x 8 Tubes)	Sample strips coated with amplification enhancer
HDV/IC STD 1-4 LPW	4 Strips (4 x 4 Tubes)	4 Strips (4 x 4 Tubes)	Quantification standard coated with HDV RNA, IC RNA and amplification enhancer
Optical Tape	1	2	Optical Tape

Table 8: Kit components for application to Rotor-Gene™ 3000/6000/Q using regular profile tubes 0.2 ml (clear)

Component	Σ 32	Σ 96	Description
HDV Sample Tubes RG	28 Tubes	92 Tubes	Sample tubes coated with amplification enhancer
HDV/IC STD 1-4 RG	4 Strips (4 x 4 Tubes)	4 Strips (4 x 4 Tubes)	Quantification standard coated with HDV RNA, IC RNA and amplification enhancer

STORAGE CONDITIONS



The RoboGene® HDV RNA Quantification Kit 2.0 is shipped at room temperature, except the **RT PCR Enzyme Mix FS**, which is shipped on dry ice. After arrival store the RoboGene® HDV RNA Quantification Kit 2.0 including the **RT PCR Enzyme Mix FS** at -40 C to -15°C in the dark. The kit is stable until the expiry date when stored under these conditions.

IMPORTANT

[1] An appropriate amount of **IC RNA** should be dissolved in **PCR grade water RNA** shortly before use. Remaining dissolved **IC RNA** 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 **HDV/IC RM** should be dissolved in **PCR grade water RNA** shortly before use. Remaining dissolved **HDV/IC RM** can be stored at 2 - 8°C up to 60 days. Always protect from light!

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

[4] **RT PCR Enzyme Mix FS** in general should be stored at -20°C. Repeated freezing and thawing for up to ten times has no negative effect on the performance of the enzyme. Nevertheless, **RT PCR Enzyme Mix FS** should always be kept on ice-cold racks during usage.

FOR SINGLE USE ONLY!

This kit is made for single use only!

6 Necessary laboratory equipment and additives

- HDV-positive control plasma (e.g. 1st WHO International Standard for Hepatitis D Virus RNA for NAT testing [PEI code 7657/12]). Provided quantification standards HDV/IC STD 1-4 LPW / HDV/IC STD 1-4 RG may be considered as positive control
- HDV-negative control (e.g. human plasma or serum free of HDV RNA)
- 7500 Fast (Applied Biosystems), LightCycler® 480 (Roche) or Rotor-Gene™ 3000/6000/Q (Corbett Research/ Qiagen)
- Real-time instrument specific software for data analysis and reporting
- 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
- Applicator for Optical Tape, using kit version “low profile strips 0.1 ml (white)”
- Precision plate holder for tube strips (for application to 7500 Fast Real Time PCR System)
- Adapter plate for tube strips (for application to LightCycler® 480)
- Gloves, lab coat

7 Procedure

7.1 Collection and handling of clinical samples

- Collect 5-10 ml blood with standard specimen collection tubes.
- Preferably EDTA (red cap, Sarstedt or equivalent manufacturer) anti-coagulant has to be used; heparin is non-applicable, because of its inhibitory effect on PCR.
- Store whole blood at 2-25 °C not longer than 6 hours, centrifuge for 20 min at 800-1,600 g to separate plasma or serum from blood cells and transfer to sterile tubes (e.g. Eppendorf).
- Plasma or serum samples may be transported at room temperature, do not exceed the time 6 hours after blood collection.
- 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!

7.2 HDV RNA purification from clinical samples

The RoboGene® HDV RNA Quantification Kit 2.0 has been validated together with the “INSTANT Virus RNA/DNA Kit” (Analytik Jena, Order number: 847-0259200602 for 50 reactions; 847-0259200603 for 250 reactions). Perform the HDV RNA purification steps according to the instructions of the respective IFU using ‘Protocol 3: Isolation of viral RNA/DNA from 400 µl of serum/plasma using IC Spiking Tube (modified)’.

NOTE
The lysis temperature of 70°C stated in the isolation protocol is crucial for an optimal HDV RNA extraction. Make sure the temperature of your thermal mixer is calibrated properly and the correct adapter for your lysis tube is mounted. We recommend using 2.0 ml tubes (not included).

7.3 Internal RNA Control

The RoboGene® HDV RNA Quantification Kit 2.0 contains the IC Spiking Tube **IC RNA** stably coated with internal control RNA and carrier nucleic acid.
Using **IC RNA** together with the INSTANT Virus RNA/DNA Kit 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 **IC RNA** purified together with HDV RNA negative plasma should be in the instrument-specific ranges summarized in Table 16.

7.4 General procedure of quantitative analysis

The quantification standards are provided as ready-to-use standard strips which are stably coated with defined amounts of HDV standard RNA. The standards are calibrated against the 1st WHO HDV RNA reference material obtained from the German Federal Agency for Sera and Vaccines (PEI). The standard values are given in IU/ml, i.e. the HDV 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 standard values are dependent on the RNA purification kit used together with the RoboGene® HDV RNA Quantification Kit 2.0 as well as the consumables necessary for respective Real-Time PCR platform. Quantification results are only valid when the INSTANT Virus RNA/DNA Kit is used in combination with one of the indicated Real-Time PCR devices and device specific consumables.

8 Protocol

8.1 Preparation of Internal Control

NOTE
The RoboGene® HDV RNA Quantification Kit 2.0 has been evaluated together with the INSTANT Virus RNA/DNA Kit for nucleic acid extraction. The Internal Control is provided as IC Spiking Tube **IC RNA** within in the RoboGene® HDV RNA Quantification Kit 2.0. Prepare the **IC RNA** Tube according to the instructions below and extract RNA following the instructions of the INSTANT Virus RNA/DNA Kit.

1. Centrifuge the provided IC Spiking Tube **IC RNA** briefly at full speed to collect the lyophilized IC RNA on the bottom of the tube. Add 520 µl **PCR grade water RNA** to the vial; close the tube, mix by brief vortexing followed by brief centrifugation at full speed.
2. Incubate at 37°C for 5 min using a thermal mixer (800 - 1,000 rpm), mix by vortexing briefly followed by brief centrifugation at full speed.
3. Add 10 µl of resuspended **IC RNA** per extraction reaction to the Lysis Solution of the corresponding INSTANT Virus RNA/DNA Kit (Analytik Jena), mix by brief vortexing.
4. Follow instructions of the extraction kit 'Protocol 3: Isolation of viral RNA/DNA from 400 µl of serum/plasma using IC Spiking Tube (modified)'. Be aware of the correct lysis temperature (70°C).

8.2 Preparation of 25x Reagent Mix

1. Centrifuge the **HDV/IC RM** briefly at full speed to collect the lyophilized Reagent Mix on the bottom of the tube.
2. Add 53 µl **PCR grade water RNA** to **HDV/IC RM**; close the tube, mix by brief vortexing followed by brief centrifugation at full speed.
3. Incubate at 37°C for 20 min using a thermal mixer (800 - 1,000 rpm), mix by brief vortexing followed by brief centrifugation at full speed.

8.3 Preparation of 1x Master Mix

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

Table 9: Composition of 1x Master Mix per reaction

Reagent	Volume for 1x rxn (µl)	Final concentration
PCR grade water RNA	12.75	-
HDV/IC RM Reagent Mix, 25x	1	1x
RT PCR Enzyme Mix FS	6.25	1x
Total	20	

3. Place **HDV Sample Strips LPW** or **HDV Sample Tubes RG** and quantification standard **HDV/IC STD 1-4 LPW** or **HDV/IC STD 1-4 RG** onto a suitable ice-cold rack.
4. Add 20 µl 1x master mix to sample tubes and each tube of quantification standard **HDV/IC STD 1 – 4.**
5. Add 5 µl **PCR grade water RNA** to wells that serve as NTC and to all quantification standards containing the 1x master mix. Do not exceed a final reaction volume of 25 µl.
6. Add 5 µl of eluate from RNA isolation (INSTANT Virus RNA/DNA Kit) to the respective sample wells containing the 1x master mix. Do not exceed a final reaction volume of 25 µl.
7. Cover the Real-Time PCR consumables. Make sure master mix and eluate are mixed properly. Centrifuge PCR strips LPW for 1 min at 1,000 rpm to collect the PCR mix on the bottom of each well (not necessary for Rotor-Gene-Tubes).

8. Program the applied Real-Time PCR platforms as indicated in table 10 to 12 below and start the program.

Table 10: PCR program for LightCycler ® 480

Step	Cycle	Profile	Temperature	Time	Ramping
1	1	Reverse transcription	55 °C	15 min	3.5 °C/sec
2	1	Taq activation	95 °C	2 min	3.5 °C/sec
3	45	Denaturation	95 °C	15 sec	3.5 °C/sec
		Annealing/Elongation*	60 °C	1 min	2.2 °C/sec
4	1	Cooling	40 °C	0:30 min	2.2 °C/sec
* Data acquisition: Fluorescence Detection (FAM; VIC)					

Table 11: PCR program for 7500 Fast and Rotor-Gene™ 3000

Step	Cycle	Profile	Temperature	Time	Ramping
1	1	Reverse transcription	55 °C	15 min	Max
2	1	Taq activation	95 °C	2 min	Max
3	45	Denaturation	95 °C	15 sec	Max
		Annealing/Elongation*	60 °C	1 min	Max
* Data acquisition: Fluorescence Detection (FAM; VIC/JOE)					

Table 12: PCR program for Rotor-Gene™ 6000/Q

Step	Cycle	Profile	Temperature	Time	Ramping
1	1	Reverse transcription	55 °C	15 min	Max
2	1	Taq activation	95 °C	2 min	Max
3	45	Denaturation	95 °C	15 sec	Max
		Annealing/Elongation*	60 °C	1:30 min	Max
* Data acquisition: Fluorescence Detection (FAM; JOE)					

9 Data analysis

Each RNA amplification is associated with generation of a fluorescence signal measurable in FAM channel (for HDV RNA) and in VIC/JOE channel (for IC RNA) 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 (see Table 13).

Table 13: Interpretation of results

FAM channel	VIC/ JOE channel	Interpretation
Interpretation of detection results		
x	x	valid, detection of sample HDV RNA
x	-	valid, detection of sample HDV RNA
-	x	valid, only detection of IC RNA, HDV RNA not detectable/ HDV negative sample
-	-	invalid, no amplification/detection at all, repeat sample
Interpretation of quantification results		
< LOD	x	Below lower limit of detection of test (e.g. 6 IU/ml for 7500 Fast). Three replicates of analysis are recommended to confirm positive result.
x (> 4·x 10^9 IU/ml)	x	Above upper limit of covered linear range of the assay (4 x 10^9 IU/ml). Dilution of original sample is recommended.

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. Tables 14 and 15 list the concentrations of HDV RNA quantification standards in case of using the INSTANT Virus RNA/DNA Kit. Table 17 and 18 list the expected Ct values of the standards on the corresponding Real-Time PCR platforms.

Table 14: HDV RNA quantification standard concentrations (7500 Fast or LightCycler® 480)

HDV/IC STD 1 - 4	HDV RNA [IU/ml]
1	20,000,000
2	200,000
3	2,000
4	200

Table 15: HDV RNA quantification standard concentrations (Rotor-Gene™ 3000/6000/Q)

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

NOTE

Setting of threshold may markedly influence Ct values. Recommendations for manual threshold settings are shown below.

7500 Fast: FAM: ≥ 0.2, VIC: ≥ 0.04

- Further settings: auto baseline

Rotor-Gene™ 3000/6000/Q: FAM: ≥ 0.02, JOE: ≥ 0.012

- Further settings: Dynamic tube: Yes; Slope correction: Yes; Ignore first: 4; No template control threshold: 5%

LightCycler® 480:

Channel	Noiseband	Threshold	Fit Points
FAM	≥ 1.8	≥ 5.0	~ 2 - 4
VIC	≥ 1.8	≥ 2.5	~ 2 - 4

- Further settings: Analysis Type: Abs Quant/ Fit Points, Color Compensation (In Database) for FAM and VIC channel

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 and 18). In case one of the four quantification standards is out of range as defined in table 17 and 18 calculation of standard curves is still possible and quantification is still valid, if standard curve parameters are in range (see table16). In such case no right for warranty of the whole product may be deduced.

Table 16: Criteria for run validation

Parameter	7500 Fast, LightCycler® 480, Rotor-Gene 3000/6000/Q
Ranges 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 LightCycler480® analysis).	

Expected Ct values for IC RNA of the quantification standards (dependent on the set threshold value, see above)	
VIC/JOE	≤ 39
Expected Ct values for IC RNA in HDV negative and HDV positive patient samples (dependent on the set threshold value, see above)	
VIC/JOE	≤ 39

Table 17: Guidance Ct values of the quantification standard on 7500 Fast and LightCycler® 480

HDV/IC STD 1 - 4.	Expected increment between Ct values	7500 Fast		LightCycler® 480	
		mean	from – to	mean	from – to
1		13.3	12.2 – 14.4	13.6	11.8 – 15.5
2	1 to 2 + ~ 6.64	20.3	19.1 – 21.6	20.5	18.7 – 22.3
3	2 to 3 + ~ 6.64	27.3	25.9 – 28.6	27.5	25.4 – 29.6
4	3 to 4 + ~ 3.32	30.8	29.0 – 32.6	31.3	29.1 – 33.5

Table 18: Guidance Ct values of the quantification standard on Rotor-Gene™ 3000/6000/Q

HDV/IC STD 1 - 4.	Expected increment between Ct values	Rotor-Gene™ 3000/6000/Q	
		mean	from – to
1		10.7	9.5 - 11.9
2	1 to 2 + ~ 6.64	17.4	16.0 - 18.8
3	2 to 3 + ~ 6.64	24.4	22.9 - 25.9
4	3 to 4 + ~ 3.32	28.1	26.4 - 29.9

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 HDV RNA and VIC/JOE channel for IC RNA.
■ 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 RNA in HDV-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 Citrat 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 RNA particularly with high standards or high viral load samples	
▪ Cross talk between target and IC recording channels (especially VIC/JOE)	Calibrate instrument using pure fluorescence dyes
Non-sigmoidal growth curves of quantification standards, unacceptable high deviation of Ct from expected values	
▪ 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 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; consult attending doctor for patient medication.
▪ Incorrect sample material	Use recommended sample type.
FAM signal for HDV-negative samples / NTC recorded	
▪ Contamination with HDV 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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