HDV ONESTEP
Quantitation (QT)

Quantitative Real -Time RT-PCR for detection of HDV genome

-for “in vitro” diagnostic use only-

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REF. HDVONEQT.CE
25/50/100 Tests

HDV ONESTEP (QT)

A. INTENDED USE

The **HDV ONESTEP Quantitation (QT)** Real-Time PCR kit coded **HDVONEQT.CE** is intended for the quantitative detection of Hepatitis D Virus RNA in human sample (plasma, serum) with a simultaneous control of the amplification reaction through an **Internal Control (IC).**

HDVONEQT.CE assay was standardised against the 1st WHO International Standard for Hepatitis D Virus RNA (PEI code 7657/12) to express samples concentration also in International Unit (IU/ml).

The kit has been adapted for the use on the Real-Time Thermal-cyclers ABI 7500 Sequence Detection System® (Software SDS version 1.3.1, Applied Biosystems™*) and CFX96 RTS (Software CFX manager version 1.7, Biorad™**).

* Applied Biosystems is a registered trademark and ABI PRISM® is a trademark of Applera Corporation or its subsidiaries in the US and/or certain other countries.
** Biorad is a registered trademark.

B. INTRODUCTION

Hepatitis delta virus (HDV) is a defective RNA virus which can only infected patient with acute or chronic hepatitis B virus. For this reason, virion assembly and propagation depend on the hepatitis B virus.

The HDV genome is a circular, single stranded RNA molecule of about 1700 bp and is strongly base-paired. The genome encodes two different ribonucleoproteins, referred to as the small hepatitis delta antigen (sHDAg) and the large hepatitis delta antigen (LHDAg). This production is related to the use different termination codons. The two forms of protein have different functions: the sHDAg is required for HDV replication, whereas LHDAg inhibits HDV replication and is required for virion formation. HDV infection can cause severe liver diseases, with fulminant hepatitis occurring more often than for HBV alone and with a higher chronicity rate in case of superinfection. In many case, chronic delta hepatitis evolves to cirrhosis and hepatocellular carcinoma.

The diagnosis of HDV infection usually based on the detection of specific anti-HDV antibodies and the presence of anti IgM reflecting on going virus replication. HDV replication is most efficiently evaluated by the detection of HDV RNA, in serum or liver, by real time PCR assay. Moreover this technology shows great potential for the diagnosis and monitoring of chronic hepatitis D.

C. PRINCIPLE OF THE TEST

The HDVONEQT.CE Kit is based on a Real Time chemistry which uses specific Primers and Probes.

HDV RNA, recovered from the biological sample under investigation through an extraction step, is retro-transcripted and amplified using a RT- Real Time amplification system. The amplified product is detected and quantified, against the standard curve using a fluorescent reporter dye probe specific for a HDV unique genomic sequence.

Internal Control (IC) serves as an amplification control for each individually processed specimen aiming to the identification of reaction inhibitors.

An external standard curve is supplied allowing the determination of the viral load.

D. COMPONENTS

The standard format of the product code HDVONEQT.CE contains reagents for 50 tests.

Component	Labelling and Contents	HDVONEQT.CE 50 Reactions
A **CODED: ALL/MM-8** **COLOR CODE: BLUE**	5x Master mix	N° 2 vials (Dissolve with the volume of ALL/RB indicated on the vial label)
RB **CODED: ALL/RB** **COLOR CODE: BLUE**	Master Mix Reconstitution Buffer	N° 2 vials/0.1 ml
B **CODED: HDVONE/CB** **COLOR CODE: YELLOW**	Lyophilized Primers/Probes for HDV ribozyme coding sequence	N°2 vials (Dissolve with the volume of ALL/C indicated on the vial label)
C **CODED: ALL/C** **COLOR CODE: RED**	MG Water	N°3 vials /1.5 ml
NTC **CODED: ALL/NTC** **COLOR CODE: WHITE**	Negative Control	N°1 vial /1.5 ml
STD Quantitation Standard ($5.0x10^4$copies/µl) **CODED: HDVONE/STD** **COLOR CODE: RED**	Lyophilised Quantitative Standard	N°2 vials (Dissolve with the volume of ALL/C indicated on the vial label)
I.C. Internal Control **CODED: ALL/IC** **COLOR CODE: GREEN**	Lyophilised Internal Control	N° 1 vial (Dissolve with the volume of ALL/C indicated on the vial label)
Package Insert	Instruction for Use	N° 1

Important note: Upon request, Dia.Pro can supply reagents for 25 and 100 tests, as reported below**:**

Component A	n°1 vial	n°4 vials
Component RB	n°1 vial/0.1 ml	n°4 vials/0.1 ml
Component B	n°1 vial	n°4 vials
Component C	n°2 vials/1.5 ml	n°5 vials/1.5 ml
NTC	n°1 vial/1.5 ml	n°1 vials/1.5 ml
Component STD	n°1 vial	n°4 vials
Component I.C.	n°1 vial	n°2 vials
Pack. insert	n°1	n°1
Number of tests	**25**	**100**
Code	**HDVONEQT.CE.25**	**HDVONEQT.CE.100**

E. STORAGE AND STABILITY

The kit coded HDVONEQT.CE should be store until 12 months at +2°C/+25°C. Labels on the device clearly indicate the expiration date and the storage temperature of each component.

Once dissolved the lyophilized components are stable for 1 month at -20°C. If the components are to be used only intermittently, they should be frozen in aliquots, repeated thawing and freezing should be avoided.

Only two thawing processes are allowed.

F. MATERIALS REQUIRED BUT NOT PROVIDED

1. Calibrated Micropipettes (0,5<volume<1000 μl)
2. RNA extraction kit
3. MG EtOH
4. Thermal Block
5. Microcentrifuge
6. Tube racks
7. Sterile filtered tips with aerosol barrier
8. 0,2 ml Microtubes recommended from the Real-Time PCR instruments manufacturers
9. Disposable gloves, powder-free
10. Real-Time PCR Thermalcycler (*)
11. Absorbent paper tissues.
12. Vortex or similar mixing tools.

(*) Attention: A valid calibration of the pure dyes (Pure Spectra Component File) and of the background (Background Component File) must be done at least once a year.

G. WARNINGS AND PRECAUTIONS

1. The kit has to be used by skilled and properly trained technical personnel only, under the supervision of a medical doctor responsible of the laboratory.
2. The technical personnel must be deeply trained in the use of Real-Time thermalcyclers, in the manipulation of Molecular Biology reagents and skilled in the Real-Time PCR amplification protocols.
3. The kit has to be used in a laboratory certified and qualified by the national authority in that field (Ministry of Health or similar entity) to carry out this type of analysis.
4. All the personnel involved in performing the assay have to wear protective laboratory clothes, talc-free gloves and glasses. The use of any sharp (needles) or cutting (blades) devices should be avoided. All the personnel involved should be trained in biosafety procedures, as recommended by the Center for Disease Control, Atlanta, U.S. and reported in the National Institute of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
5. All the personnel involved in sample handling should be vaccinated for HBV and HAV, for which vaccines are available, safe and effective.
6. The laboratory environment should be controlled so as to avoid contaminants such as dust or air-born microbial agents, when opening kit vials and the Components and when performing the test.
7. Component A and B are light sensitive. Protect it from strong light exposition
8. Avoid vibration of the bench surface where the test is undertaken
9. Upon receipt, store the kit at +2°C/+25°C into a temperature-controlled area.
10. Do not interchange components between different lots of kits. It is recommended that components between two kits of the same lot should not be interchanged.
11. Check that the reagents are clear and do not contain visible heavy particles or aggregates. If not, advise the laboratory supervisor to initiate the necessary procedures for kit replacement.
12. Avoid cross-contamination between samples by using disposable tips and changing them after each sample.
13. Avoid cross-contamination between kit reagents by using disposable tips and changing them between the use of each one.
14. Do not use the kit after the expiration date stated on the external container.
15. Treat all specimens as potentially infective. All human plasma/serum specimens should be handled at Biosafety Level 2, as recommended by the Center for Disease Control, Atlanta, U.S. in compliance with what reported in the Institutes of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
16. Store and extract positive materials (specimens, controls and amplicons) separately from the other reagents and use a separate room for their handling
17. Dissolve the lyophilised reagents with the correct amount, stated in the labels, with Molecular Grade water (Component C Coded: ALL/C) supplied in the kit.
18. Carry on all the working operations as quickly as possible.
19. The laboratory workflow must proceed in an unidirectional way, beginning in the Extraction Area and moving to the Amplification and Data Analysis Areas. Do not return samples, equipment and reagents to the area where previous steps have been performed.
20. The use of disposable plastic-ware is recommended in the preparation of the liquid components or in transferring components into automated workstations, in order to avoid cross contamination.
21. Waste produced during the use of the kit has to be discarded in compliance with national directives and laws concerning laboratory waste of chemical and biological substances. In particular, liquid waste generated from the washing procedure, from residuals of controls and from samples has to be treated as potentially infective material and inactivated before waste. Do not put in contact the extraction waste with blench.
22. Accidental spills from samples and operations have to be adsorbed with paper tissues soaked with household bleach and then with water. Tissues should then be discarded in proper containers designated for laboratory/hospital waste.
23. Other waste materials generated from the use of the kit (example: tips used for samples) should be handled as potentially infective and disposed according to national directives and laws concerning laboratory wastes.

H. SPECIMEN: PREPARATION AND RECOMMENDATIONS

1.Blood is drawn aseptically by venipuncture and plasma or serum is prepared using standard techniques of preparation of samples for clinical laboratory analysis.
2. No influence has been observed in the preparation of the sample with citrate, EDTA.
Attention: Heparin (≥10 IU/ml) affects the PCR reactions. Samples, which has been collected in tubes containing heparin as an anticoagulant should not be used. Also, samples of heparinised patients must not be used.
3. Avoid any addition of preservatives to samples.
4. Samples have to be clearly identified with codes or names in order to avoid misinterpretation of results. When the kit is used for the screening of blood units, bar code labeling and electronic reading is strongly recommended.
5. Haemolysed (red) and visibly hyperlipidemic ("milky") samples have to be discarded as they could generate false results. Samples containing residues of fibrin or heavy particles or microbial filaments and bodies should be discarded as they could give rise to false results.
6. Plasma and sera, if not used immediately, must be aliquoted and stored at -20°..-80°C after collection. Samples can be stored frozen at 20°..-80°C for several months. Any frozen samples should not be frozen/thawed more than once as this may affect the test result.
7.The plasma samples for RNA extraction must be collected according to the common laboratory procedures, transported and stored at +2/+8°C for a maximum period of 4 hours. The plasma samples can be stored frozen at -20°C for a maximum period of 30 days or -70°C for long periods
8.We recommend you, for optimal storage samples, to split them in several aliquots (minimum volume 200 μl) and store

them frozen at -20°C for a maximum period of 30 days or -70°C for long periods. Avoid repeated freezing/thawing cycles

9. When using frozen samples, thaw the samples just immediately before the extraction in order to avoid possible causes of nucleic acid degradation.

10. The whole peripheral blood samples for RNA extraction must be collected in EDTA according to laboratory devices, transported and stored at +2°C/+8°C for a maximum period of 3 days. Do not freeze the whole peripheral blood samples to avoid cell lysis and viral titer loss

I. PREPARATION OF COMPONENTS AND WARNINGS

Master Mix:

Component A. Dissolve homogenously the Lyophilized Component A with the volume of Component RB (Code: ALL/RB) indicated on the vial label.

WARNING: Component A is light sensitive. Protect it from strong light exposition

Reconstitution Buffer:

Component RB. Ready to use. Briefly centrifuge to collect the whole volume.

Primers/Probes:

Component B.

- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized Component B with the volume of Component C (Code: ALL/C) indicated on the vial label.
- Keep it dissolve on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex

WARNING: Component B is light sensitive. Protect it from strong light exposition.

MG Water:

Component C. Ready to use.

Negative Control:

NTC. Ready to use.

Internal Control:

I.C.

- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized I.C. with the volume of Component C (Code: ALL/C) indicated on the vial label.
- Keep it dissolve on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex

Standard Curve:

Component STD.

- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized STD with the volume of Component C (Code: ALL/C) indicated on the vial label
- Keep it dissolve on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex
- Prepare 4 Nuclease Free tubes for the preparation of the Standard Curve
- Set up an STD 1:10 serial dilution in Component C (Code: ALL/C) to obtain the standard curve points as described in the table below:

	Standard curve preparation	
STD	**Calibrator 50000 copies/ µl**	Add the Volume of Component C (Code: ALL/C) as written on the vial label
STD 1	**5000 copies/ µl**	10 µl (STD) + 90 µl Component C (Code: ALL/C)
STD 2	**500 copies/ µl**	10 µl (STD 1) + 90 µl Component C (Code: ALL/C)
STD 3	**50 copies/ µl**	10 µl (STD 2) + 90 µl Component C (Code: ALL/C)
STD 4	**5 copies/ µl**	10 µl (STD 3) + 90 µl Component C (Code: ALL/C)

L. INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT

1. **Micropipettes** have to be calibrated to deliver the correct volume required by the assay and must be submitted to regular decontamination (household alcohol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample. They should also be regularly maintained in order to show a precision of 1% and a trueness of +/-5%. Decontamination of spills or residues of kit components should also be carried out regularly.
2. **Extraction Device:** The HDVONEQT.CE Kit is intended for the use in combination with the manual extraction kit NucleoSpin Virus (Macherey-Nagel) and InnuPREP Virus DNA/RNA (AJ Innuscreen). The end users must strictly follow the Instruction for use supplied by the manufacturers.
3. **Real-Time Thermal-cyclers.** The HDVONEQT.CE Kit is intended for the use in combination with the Real Time Thermal cyclers ABI 7500 (Software SDS version 1.3.1, Applied Biosystems), and and CFX96 Real-Time System, Software CFX manager version 1.7 (Biorad™)
   The end users must strictly follow the Instruments Instruction for use supplied by the manufacturers.

M. PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the kit printed on the external label of the kit box. Do not use if expired.
2. Check that the liquid components are not contaminated by naked-eye visible particles or aggregates. Check that no breakage occurred in transportation and no spillage of liquid is present inside the box.
3. Turn the Thermal-cyclers on, check settings and be sure to use the right assay protocol.
4. Follow strictly the Instruments Manual supplied by the manufacturers for the correct setting of the Real-Time Thermal-cyclers.
5. Check that the micropipettes are set to the required volume.
6. Check that all the other equipment is available and ready to use.
7. In case of problems, do not proceed further with the test and advise the supervisor.

N. ASSAY PROCEDURE
The assay has to be carried out according to what reported here below.

N.1 Viral RNA extraction

The extraction step of the HDV genomic RNA has to be carried out exclusively in combination with the following kit:

Material	Description	Kit code	Manufacturer
Serum/Plasma	Nucleospin Virus kit	740983	Macherey-Nagel
Serum/Plasma	InnuPREP Virus DNA/RNA Kit	845-KS-4800nnn	AJ Innuscreen

The RNA isolation must be carried out only according to the manufacturer Instruction Manual (MN™, AnalitikJena).

Important Note: The following volumes have to be strictly used in the extraction procedures

Description	Sample volume µl	Elution volume µl
NucleoSpin Virus	200	100
InnuPREP Virus DNA/RNA Kit	200	100

The RNA extracted from the samples, not used in the run, has to be stored frozen (-20°C….-80°C).

Important note: *The IC of the* HDVONEQT.CE Kit *can be used in the isolation procedure as extraction control.*
The Internal Control Ct value is used to evaluate if the extraction procedure has been performed correctly (see section Q).

For this application
- add **2 µl of I.C.** at the sample mixture, after the thermal incubation step (AJ: 70°C x 10min; MN: RT x 3min) and proceed following the instruction manual supplied by the manufacturer of the Extraction Kit.

N.2 Setting up of the reaction

HDVONEQT.CE kit is intended to be used exclusively in combination with ABI 7500 (Software SDS version 1.3.1, Applied Biosystem) and CFX96 RTS (software CFX manager version 1.7, Biorad).

N.2.1 Preparing the RT-PCR

Important: *An example of dispensation scheme is reported in Section O. Please, refer to it before starting to read the instructions here below.*
- Prepare the components as described in Section I;
- Prepare the required number of reaction tubes or a 96-well reaction plate for the samples under evaluation and for the Standard curve (prepared as described in section I).

Important note: *Use only optical tubes or microplates suggested by the Real-Time thermalcyclers manufacturers.*

- Consider that the samples, if possible, should be tested in duplicate;
- Include at least one tube for the NTC (negative control)
- Prepare the **OneStep Mix** for **Samples, NTC and standard curve** as reported in table below:

Preparation of the OneStep Mix

(I.C. as Amplification control)

Number of Reactions		1	x12
A	5x Master Mix	4,0 µl	48 µl
B	Primers/Probe	2,0 µl	24 µl
I.C.	Internal Control	0,5 µl	6 µl
C	MG Water	3,5 µl	42 µl
Tot vol.		**10,0 µl**	**120 µl**

If the Internal Control was added during the RNA isolation procedure, prepare the **OneStep Mix** for **Samples, NTC and standard curve**, as reported in table below*:*

Preparation of the OneStep Mix

(I.C. as Extraction/Amplification control)

Number of Reactions		1	x12
A	5x Master Mix	4,0 µl	48 µl
B	Primers/Probe	2,0 µl	24 µl
C	MG Water	4,0 µl	48 µl
Tot vol.		**10,0 µl**	**120 µl**

- dispense **10 µl** of the OneStep Mix in each reaction tube or microplate well
- add **10 µl** of the **Samples, NTC and Standard curve** to the reaction tubes

scheme for preparation of PCR assay

Number of reactions	1
OneStep Mix	10 µl
Sample, NTC, Standard Curve	10 µl
Tot vol.	**20 µl**

- close strictly the tubes
- centrifuge briefly the reaction tube at 2000 rpm
- don't leave the reaction tube at room temperature (RT) for more than 30 min and at light exposure (cover the tubes)
- load the tubes in the Real-Time Thermal-cycler Thermoblock Holder
- after the setting operation described in the section N3 (Instrument Programming) start the Thermal-cycler run.

N.3 Instrument programming

For programming the instrument refer to the Instrumentation Instruction Manual provided by the manufacturers.

N.3.1 **RT-PCR Thermal Profile**

The thermal profile is reported in the table below:

Step	Cycle	Temp.	Time
1	1	49°C	30 min
2	1	95°C	10 min
3	45	95°C	15 sec
		60°C(*)	1 min

IMPORTANT NOTE :(*) step for the real time data collection

Warning*: keep attention to set up the Real-Time Thermalcycler with correct Thermal Profile following the instruments manual supplied by the manufacturer*

N.3.2 Selection of the Detectors

Following the Instruction manuals of the Real-Time thermal cyclers suggested (ABI 7500 and CFX96) select the Detectors reported in the table here below:

Detection	Reporter	Quencher
HDV	FAM	Non Fluorescent
Internal Control (I.C.)	VIC/JOE	Non Fluorescent
Passive Reference (only on ABI7500)	ROX	Non Fluorescent

***Warning**: keep attention to set up the Real-Time Thermalcycler with correct Thermal Profile settings following the instruments manual supplied by the manufacturer*

O. ASSAY SCHEME

Examples of dispensation scheme for Quantitative Analysis are reported below:

Microplate/Tubes

	1	2	3	.	.
A	**STD 1**	Sample 1			
B	**STD 2**	Sample 2			
C	**STD 3**	Sample 3			
D	**STD 4**	Sample 4			
E	NTC	Sample 5			
F					
G					
H					

Legend: NTC = Negative Control STD 1,2,3,4 = HDV Standard Curve, Sample 1-5= Samples under evaluation.

P. INTERNAL QUALITY CONTROL

P.1 Pre- Analysis setting

Before starting the analysis:

- Set the **"Baseline"** (the background fluorescence level) as reported in the table below:

"Baseline"	
ABI™PRISM® 7500 SDS	**Auto Baseline**
BIORAD™ CFX96®	**Auto Calculated Baseline**

- Set manually the FAM/JOE/VIC fluorescence **"Threshold"**

Fluorescence "Threshold"	FAM	VIC/JOE
ABI™PRISM® 7500 SDS	0.20	0.08
BIORAD™ CFX96®	300	150

P.2 Data Analysis

A check is carried out on the STD calibrators any time the kit is used in order to verify whether their Ct values are as expected and reported in the tables below:

ABI™PRISM® 7500 SDS	
Check	**Requirements**
STD 1	18.0 < Ct (Threshold Cycle) < 21.0

BIORAD™ CFX96®	
Check	**Requirements**
STD 1	19.0 < Ct (Threshold Cycle) < 22.0

Moreover, the Slope and R^2 values are checked in order to verify the quality of the run. The following requirements must be fulfilled.

Check FAM	Requirements
Slope	-3.9 < Slope < -3.1

Check FAM	Requirements
Efficiency	$R^2 > 0.98$

Q. INTERPRETATION OF THE RESULTS AND TROUBLESHOOTING

For each samples FAM fluorescence (positive/negative Ct value) and Internal Control JOE/VIC fluorescence are assumed to validate HDV detection as described in the table below:

HDV FAM	Internal control VIC	Assay result
SAMPLE POSITIVE	25 < Ct < 40	CORRECT
	Ct > 40 or undetermined	CORRECT*
SAMPLE NEGATIVE	25 < Ct < 40	CORRECT
	Ct > 40 or undetermined	INVALID**

(*) High initial concentration of cDNA of HDV in the sample (positive FAM) can lead to REDUCE or ABSENT Fluorescence Signal of Internal Control IC due to the reagent Competition
(**) In this case problems have occurred during the amplification step (inefficient or absent amplification) or during the extraction step (presence of inhibitors) which may lead to incorrect result and false negatives. It need to be repeated from the extraction of a new sample.

On the bases of the results obtained for the standardization of HDVONEQT.CE on the 1st WHO International Standard for Hepatitis D Virus RNA (PEI code 7657/12) a correct Quantitation of the HDV viral load can be applied as reported in the table below:

NucleoSpin Virus kit	
ABI™ PRISM® 7500SDS - BIORAD™ CFX96®	
HDV viral load (IU/ml)	
Quantity > **1.0E+05**	HDV POS > ULOQ
2.5E+02 ≤ Qty ≤ **1.0E+05**	***QUANTITATION (IU/ml)***
Quantity < **2.5E+02**	HDV POS < LLOQ

ULOQ= Upper Limit of Quantification
LLOQ= Lower Limit of Quantification

InnuPREP Virus DNA/RNA kit	
ABI™ PRISM® 7500SDS - BIORAD™ CFX96®	
HDV viral load (IU/ml)	
Quantity > **5.75E+05**	HDV POS > ULOQ
2.50E+02 ≤ Qty ≤ **5.75E+05**	***QUANTITATION (IU/ml)***
Quantity < **2.50E+02**	HDV POS < LLOQ

ULOQ= Upper Limit of Quantification
LLOQ= Lower Limit of Quantification

***IMPORTANT NOTE:** For samples quantitation as IU/ml refer to section R*

The result obtained with this product must be interpreted with consideration of clinical presentation and other laboratory markers inherent to the patient.
The following results are possible.

Troubleshooting table

	FAM	VIC	Result	CHECK
SAMPLE unknown	+	+/-	CORRECT RESULT *Positive*	***IMPORTANT***: High Initial concentration of HDV RNA (Positive FAM Signal) can lead to REDUCED or ABSENT Fluorescent Signal of Internal Control I.C. due to the reagents Competition.
SAMPLE unknown	-	-	ATTENTION ! POSSIBILITY OF: Inhibition, error in the procedure or no functioning of the Instruments	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. That the selected detection dyes are corrected FAM for the HDV detection and VIC/JOE for the I.C. detection; 4. that the Analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly; 6. that no potential PCR inhibitors have been contaminated the tube 7. That the Extraction and RT -PCR procedures have been executed correctly;
SAMPLE unknown	-	+	CORRECT RESULT *Negative*	
STD	+	+/-	CORRECT RESULT	Negative JOE/VIC signal is correct only if I.C. was used as extraction control
STD	-	-	ATTENTION ! POSSIBILITY OF: Error in the pipetting or in the procedure	1. that no mistake has been done in the assay procedure; 2. That the selected detection dyes are corrected FAM for the HDV detection and VIC/JOE for the I.C. detection; 3. that the Analysis has been run with the correct Instrument settings; 4. that the kit has been stored correctly; 5. that no potential PCR inhibitors contaminated the tube.
STD	-	+	ATTENTION ! POSSIBILITY OF: Error in the pipetting or in the procedure	1. that no mistake has been done in the assay procedure; 2. That the selected detection dyes are corrected FAM for the HDV detection and VIC/JOE for the I.C. detection; 3. that the Analysis has been run with the correct Instrument settings; 4. that the kit has been stored correctly;
NTC	-	+	CORRECT RESULT	
NTC	+	+	ATTENTION ! POSSIBILITY OF: Contamination	1. that no mistake has been done in the assay procedure; 2. That the work space and Instruments are decontaminated at regular intervals; 4.that the kit has been stored correctly;
NTC	+	-	ATTENTION ! POSSIBILITY OF: Contamination	1. that no mistake has been done in the assay procedure; 2. That the work space and Instruments are decontaminated at regular intervals; 4.that the kit has been stored correctly;

If the results of the test match the *CORRECT RESULT* requirements stated above, proceed to the next section.

If one of more of the problems described in the table above happen, after checking, report any residual problem to the supervisor for further actions.

R. QUANTITATION

The STD calibrators are treated as patient samples and the same volume, 10µl is used during the amplification step.
The STD calibrators concentration is expressed in copies/µl.
The **Viral Genome Concentration per mL** for each patient specimen is calculated applying the following formula:

$$\textbf{Results (copies/ml)} \equiv \frac{\textbf{copies/µl (run data) x Elution sample volume (µl)}}{\textbf{Sample Extraction volume (ml)}}$$

Example:

$$\text{Results (copies/ml)} \equiv \frac{500 \text{ (copie/µl)} \times 100 \text{ (µl)}}{0.2 \text{ (ml)}}$$

Results (copies/ml) ≡ 2.50E+05

To convert samples viral load expressed in copies/ml to IU/ml use the appropriate conversion factor as reported in the table below:

Extraction method	ABI™PRISM® 7500 BIORAD™ CFX96®	
	Conversion Factor	Result (IU/ml)*
NucleoSpin Virus kit	0.55	copies/ml ÷ 0.55
InnuPREP Virus DNA/RNA kit	0.53	copies/ml ÷ 0.53

*calibrated on 1st WHO International Standard (PEI code 7657/12)

Example:

Extraction method	ABI™PRISM® 7500 - BIORAD™ CFX96®		
	Result (copies/ml)	Conversion Factor	Result (IU/ml)
NucleoSpin Virus kit (MN)	2.50E+05	0.55	4.55E+05
InnuPREP Virus DNA/RNA kit	2.50E+05	0.53	4.72E+05

Important notes:

1. *Interpretation of results should be done under the supervision of the responsible of the laboratory to reduce the risk of judgment errors and misinterpretations.*
2. *When test results are transmitted from the laboratory to an informatics centre, attention has to be done to avoid erroneous data transfer.*

S. PERFORMANCES

Evaluation of Performances has been conducted in accordance to what reported in the Common Technical Specifications or CTS (art. 5, Chapter 3 of IVD Directive 98/79/EC).

The performance evaluation was carried out in DiaPro's laboratories on reference materials (PEI, QCMD) and on material supplied by the reference clinical laboratories.

S.1 ANALYTICAL SENSITIVITY

Analytical sensitivity of a quantitative molecular method refers to the smallest amount of the target marker that can be precisely detected.

In the context of the CTS it may be expressed as: **limit of detection** or **limits of quantitation:**

Limit of detection (LOD): it is the lowest amount of target that can be detected by the system with a stated probability.

For the NAT tests it is expressed as the smallest concentration of the **analyte** that tested in multiple repetitions gives a positive result.

The **limit of detection (LOD)** is determined by testing serial dilutions containing known concentrations of the analyte.

The **LOD** is the lowest concentration of analyte that can be consistently detected (e.g. in ≥ 95% of samples under routine laboratory conditions).

In the kit code HDVONEQT.CE the **LOD** has been determined by testing several serial dilution of the 1st International Standard for Hepatitis D virus RNA (PEI code 7657/12) extracted with both the validated manual extraction kit into borderline concentration.

Five dilution points has been tested in 8 replicates in three different runs (8 replicates x 3 runs) performed on ABI 7500 real-time instrument and CFX96 RTS. The result obtained from the 24 replicates, on each instrument considering also the extraction method used, were analized by a **Probit** analysis, to determine the detection limit at 95%.

The results of the PROBIT Analysis are the following:

LOD Limit of Detection (p=0.05)		
Extraction method	**ABI™ 7500SDS**	**BIORAD™CFX96®**
InnuPREP Virus DNA/RNA	66.71 IU/ml	75.43 IU/ml
NucleoSpin Virus	75.39 IU/ml	85.55 IU/ml

S.1.1 Limit of quantification

The **Limit of Quantitation** was determined by measuring the **linearity**, the **dynamic range** and the **reproducibility.**

The **Linearity** is the measure of the degree to which a curve approximates a straight line. It is expressed with the **SLOPE** value.

The **dynamic range** is the span of analyte concentrations for which the final output value (Ct threshold cycle) of the system is directly proportional to the analyte concentration, with acceptable trueness and precision.

The boundaries of the dynamic range are the lower and upper limits of quantitation **(Limit of quantitation).**

For the kit HDVONEQT.CE the Limit of quantitation was determined by testing, previous extraction, a serial dilution of the 1st International Standard for Hepatitis D virus RNA - genotype 1 strain of HDV (PEI code 7657/12) with known concentration (IU/ml).

On the basis of the results obtained with the HDVONEQT.CE kit on ABI 7500 and on BioRad CFX96 the Dynamic Ranges have been established as follow:

ABI™ PRISM® 7500SDS - BIORAD™ CFX96®	
InnuPREP Virus DNA/RNA	**NucleoSpin Virus**
2.50E+02 ≤ (IU/ml) ≤ **5.75E+05**	**2.50E+02** ≤ (IU/ml) ≤ **1.00E+05**
1.33E+02 ≤ (cp/ml) ≤ **3.05E+05**	**1.38E+02** ≤ (cp/ml) ≤ **5.50E+04**

Moreover the analytical dynamic range was tested on all of the HDV genome subtype using DNA plasmid vectors grouped by specific target sequence homology in reference to the HDV genotype 1 strain , whose concentration were determined by spectrophotometer.

The results are shown here below:

HDV genotype strain	Result of the Analysis
1 – 2 – 4 – 5	**5.0E-01** ≤ (cp/µl) ≤ **5.0E+04**
3 – 6 – 7 – 8	**5.0E-01** ≤ (cp/µl) ≤ **5.0E+04**

S.2 ANALITYCAL SPECIFICITY

Analytical specificity is the ability of a method to detect and quantify only the target marker.

The analytical specificity of HDV RNA assay has been studied as follow:

1. The primer/probe Set has been choose analysing the genome target sequence with an appropriate software (Primer Express v.3.0" supplied by Applied Biosystem Inc.).
2. The primer/probe Set and the target genome sequence has been controlled by the "BLAST" software, in order to check if any of the nucleotide sequences deposited in the worldwide genomic banks has any homology with HDV, and by the "ClustalX" software, in order to compare the genome target sequences of the different genotypes of HDV.
3. The specificity was improved through the selection of stringent reaction conditions.
4. Interfering sample supplied by NIBSC and Acrometrix were tested

The results are reported in the following table:

Number of samples	Organism	Result
4	HBV (HDV Negative)	negative
4	HCV	negative
4	HIV	negative

S.3 DIAGNOSTIC SPECIFICITY AND SENSITIVITY

S.3.1 Diagnostic Specificity

Diagnostic specificity is the probability that the device gives a negative result in the absence of the target marker. So that **true negative** sample is a specimen known to be negative for the target marker and correctly classified by the device

TRUE NEGATIVES	100
FALSE POSITIVES	0
TOTAL SAMPLES	100
SPECIFICITY %	**100**

On the basis of the results obtained Diagnostic Specificity of the system has been calculated ≥99.5%.

S.3.2 Diagnostic Sensitivity

Diagnostic sensitivity is the probability that the device gives a positive result in the presence of the target marker. So that **true positive** sample is a specimen known to be positive for the target marker and correctly classified by the device.
In the kit code HDVONEQT.CE this parameter was studied by by examining 12 HDV RNA positive samples and the QCMD Panel 2019 and 2021.

HDV RNA Positive samples

TRUE POSITIVES	27
FALSE NEGATIVES	0
TOTAL SAMPLES	27
SENSITIVITY %	**100**

On the basis of the results obtained Diagnostic Sensitivity of the system has been calculated in the 100%.

Diagnostic Sensitivity	100 %
Diagnostic Specificity	≥99.5 %

S.4 PRECISION

Precision shows the degree of the system's reliability. Every measurement procedure has an inherent random variation called "random error". Random error does not have a number value but it is determined by dispersion of measurement as standard deviation (DevST) and coefficient variation (CV%). Usually precision of an assay refers to the agreement between replicate measurements of the same material.
In the kit code HDVONEQT.CE, **precision** was expressed as intra-assay variability and inter-assay variability. It was tested in the same run (intra-assay) and in three different runs (inter-assay) with 4 standard points curve in duplicates.
Intra and inter-assay variability were then calculated.

In absence of an established parameters in the European IVD Directive CTS we have identified the following value of acceptability for the HDV RNA:
Intra-Assay Coefficient Variation (CV%) < 3%.
Inter-Assay Coefficient Variation (CV%) < 3%.

T. LIMITATIONS

The user of this kit is advised to carefully read and understand this package insert. Strict adherence to the protocol is necessary in order to obtain reliable test results. In particular, accurate sample and reagent pipetting,application of a correct workflow along with careful programming of thermocycling steps are essential for accurate and reproducible HDV RNA detection and quantitation.
The determination of HDV RNA in a patient sample has extensive medical, social, psychological and economic implications.
It is recommended that confidentiality, appropriate counselling and medical evaluation be considered as an essential aspect of the testing sequence.

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5.Symbols

LEGENDA			
REF	Product code		Storage temperature
IVD	In Vitro Diagnostic Device		See use instructions
LOT	Lot number		Manufacturer
	Expiry date		Number of tests
CE	CE conformity mark		Date of manufacturing

All the IVD Products manufactured by the company are under the control of a certified Quality Management System approved by an EC Notified Body. Each lot is submitted to a quality control and released into the market only if conforming with the EC technical specifications and acceptance criteria.

Manufacturer
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