HDV RNA QUANTITATION (QT)

Quantitative Real-Time PCR for detection of HDV genome

4BShopLab

Rev. 9

HDV RNA

A. INTENDED USE

The **HDV Quantitation** Real-Time PCR kit coded **DRNA.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).**
DRNA.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 Thermacyclers ABI 7500 Sequence Detection System® (Software SDS version 1.3.1, Applied Biosystems™*) or MX3000P® (Software MxPro version 4.01, Stratagene™***) and CFX96 (Software CFX manager version 1.7, Biorad™**).

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** Biorad is a registered trademark.
***Stratagene is a registered trademark.

B. INTRODUCTION

Hepatits 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 depends on the hepatits 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 ribonucloproteins, referred to as the small hepatits delta antigen (sHDAg) and the large hepatits delta antigen (LHDAg). This production is related to the use different termination codons. The two form of protein have different functions: the sHDAg is required for HDV replication, whereas LHDAg inhibit 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 hepatits evolves to cirrhosis and hepatocellular carcinoma.
The current HDV treatment efficacy is disappointing. At this moment treatment relies on the long-term administration of high doses of αIFN but only 50% of patients respond and 40% of them relapse during 6 months following the end of the therapy. 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 hepatits D.

C. PRINCIPLE OF THE TEST

The DNA.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 retrotrascripted to cDNA and amplified using 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 DRNA.CE contains reagents for 50 tests.

Component	Labelling and Contents	DRNA.CE 50 Reactions
A **CODED: ALL/MM** **Colour code : blue**	Master mix	N° 2 vials / 0.4 ml
B **CODED: HDV/CB** **Colour code : yellow**	Lyophilised Primers/Probes	N° 2 vials (Dissolve with the volume of ALL/C indicated on the vial label)
REV **CODED: HDV/REV** **Colour code : white**	Lyophilised Retro transcription Primer	N° 2 vials (Dissolve with the volume of ALL/C indicated on the vial label)
C **CODED: ALL/C** **Colour code : red**	MG Water	N° 3 vials/1.5 ml
NTC **CODED: ALL/NTC** **Colour code : white**	Negative control	N° 1 vial /1.5 ml
STD Quantitation Standard ($1.0x10^4$ copies/µl) **CODED: HDV/STD** **Colour code : red**	Lyophilised Quantitative Standard	N° 4 vials (Dissolve with the volume of ALL/C indicated on the vial label)
I.C. **CODED: HDV/IC** **Colour code: green**	Lyophilized Internal Control	N° 2 vials (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, 100, 150 tests, as reported below **:**

Component A	n°1 vial/0.4 ml	n°4 vials/0.4 ml	n°6 vials/0.4 ml
Component B	n°1 vial	n°4 vials	n°6 vials
REV	n°1 vial	n°4 vials	n°6 vials
Component C	n°2 vial/1.5 ml	n°3 vials/1.5 ml	n°5vials/1.5 ml
NTC	n°1 vial/1.5ml	n°2 vials/1.5ml	n°3 vials/1.5ml
STD	n°2 vial	n°4 vials	n°6 vials
IC	n°1 vial	n°4 vials	n°6 vials
Pack. insert	n° 1	n° 1	n° 1
Number of tests	**25**	**100**	**150**
Code	**DRNA.CE.25**	**DRNA.CE.100**	**DRNA.CE.150**

E.STORAGE AND STABILITY

The kit DRNA.CE must be stored at +2…8 °C.

Once dissolved Component REV (coded HDV/REV) must be stock at -20°C.

Once dissolved Component REV (coded HDV/REV), Component B (coded HDV/CB) and Component IC (coded HDV/IC) are stable for 1 month at -20°C.

Once dissolved component STD (coded HDV/STD) is stable for 2 weeks 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 one defreezing is 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. Thermalcycler
11. Real-Time PCR Thermalcycler (*)
12. Absorbent paper tissues.
13. 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 them from strong light exposition
8. Pay **Special Attention** during the dissolution of the lyophilized Components taking care that the powder on the wall of the original component vial would be totally dissolved in the volume of the water used. The centrifugation step must be correctly applied and checked.
9. Incorrect dissolution of the lyophilized tube could compromise the result..
10. Avoid vibration of the bench surface where the test is undertaken
11. Upon receipt, store the kit at 2..8°C into a temperature controlled refrigerator or cold room.
12. Do not interchange components between different lots of the kits. It is recommended that components between two kits of the same lot should not be interchanged.
13. 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.
14. The kit due to the retrotranscription step is highly susceptible to cross-contaminations among reagents and samples avoid it using disposable plasticware, changing frequently the disposable gloves and cleaning frequently the working areas.
15. Avoid cross-contamination between samples by using disposable tips and changing them after each sample.
16. Avoid cross-contamination between kit reagents by using disposable tips and changing them between the use of each one.
17. Do not use the kit after the expiration date stated on the external container and internal (vials) labels.
18. 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.
19. Store and extract positive materials (specimens, controls and amplicons) separately from the other reagents and use a separate room for their handling
20. 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
21. Carry on all the working operations as quickly as possible maintaining the components on ice or in a cooling block.
22. Workflow in the laboratory must proceed in an unidirectional way, beginning in the Extraction Area and moving to the Amplification and Data Analysis Area. Do not return samples, equipment and reagents to the area where previous steps have been performed.
23. 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.
24. 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.
25. 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.
26. 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 venepuncture 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 hyperlipemic ("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 300 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 titre loss

I. PREPARATION OF COMPONENTS AND WARNINGS

Master Mix:

Component A. Ready to use. Mix well on vortex before use and briefly centrifuge to collect the whole volume.

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

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 (Cod: ALL/C) indicated on the vial label
- Keep it dissolve on the bench top for at least 15 min at room temperature (15°C<RT<25°C)
- Briefly vortex it avoiding the formation of foam
- Shortly spin to collect the whole volume

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

Retro-transcription Primer:

Component REV

- Dissolve homogenously the Lyophilized Component REV with the volume of Component C (Cod: ALL/C) indicated on the vial label
- Keep it dissolve on the bench top over-night at room temperature (15°C<RT<25°C) before the first use.
- Briefly vortex it avoiding the formation of foam
- Centrifuge the vial at 11000 rpm for 1 min to collect the whole volume

MG Water:

Component C. ready to use

NTC or Negative Control :

Component D. Ready to use.

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 Component HDV/ STD with the correct volume of Component C (Cod: ALL/C) as indicated on the vial label
- Keep it dissolve on the bench top for at least 15 min at room temperature (15°C<RT<25°C)
- Briefly vortex it avoiding the formation of foam
- Shortly spin to collect the whole volume
- Prepare 4 Nuclease Free tubes for the preparation of the Standard Curve
- Set up a 1:10 serial dilution in **Component C** (Cod: ALL/C) to obtain the standard curve as table below:

Standard curve preparation		
Name	**Procedure**	**copies/µl***
HDV/STD	**Add Volume of Component C (Code: ALL/C) as written on the vial label**	**10000 copies/µl**
STD 1	10 µl (STD) + 90 µl **Component C (Code: ALL/C)**	**1000 copies/µl**
STD 2	10 µl (STD 1) + 90 µl **Component C (Code: ALL/C)**	**100 copies/µl**
STD 3	10 µl (STD 2) + 90 µl **Component C (Code: ALL/C)**	**10 copies/µl**
STD 4	10 µl (STD 3) + 90 µl **Component C (Code: ALL/C)**	**1 copies/µl**

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

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

Internal Control:

Component I.C..

- Centrifuge the vial at 11000 rpm for 1 min
- Open carefully the vial cap avoiding powder dispersion
- Dissolve homogenously the Lyophilized Component IC with the volume of Component C (Cod: ALL/C) as indicated on the vial label
- Keep it dissolve on the bench top for at least 10 min at room temperature (15°C<RT<25°C)
- Briefly vortex it avoiding the formation of foam
- Shortly spin to collect the whole volume

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 DRNA.CE Kit is intended for the use in combination with QIAamp Viral RNA Code.52906 (QIAGEN).The end users must strictly follow the Instruction for use supplied by the manufacturer.
3. **Retro-transcription Step:** The DRNA.CE Kit is intended for the use in combination only with RNA Retro-transcription Kit, code RNART.HDV.CE (Dia.Pro srl).The end users must strictly follow the Instruction for use supplied by the manufacturer.
4. **Real-Time Thermalcyclers.** The DRNA.CE Kit is intended for the use in combination only with the Real Time Thermal cyclers ABI 7500 (Software SDS version 1.3.1, Applied Biosystems), and MX3000P (Software MxPro version 4.01, Stratagene), and CFX96 RTS, 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 on the bottom of the Lyophilized components vials is present a well formed aggregate. Check that no breakage occurred in transportation and no spillage of liquid is present inside the box.
3. Dissolve the Lyophilized Components with the appropriate amount of Component C (Cod: ALL/C) as described in the proper section.
4. Turn the Thermalcyclers on, check settings and be sure to use the right assay protocol.
5. Follow strickly the Instruments Manual supplied by the manufacturers for the correct setting of the Real-Time Thermalcyclers.
6. Check that the micropipettes are set to the required volume.
7. Check that all the other equipment is available and ready to use.
8. 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	QIAamp Viral RNA mini kit®	52906	Qiagen™

The RNA isolation must be carried out only according to the Manufacturer's instructions.

N.2 Viral RNA Retro-transcription to cDNA

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

Material	Description	Kit code	manufacturer
RNA	HDV RNA Retrotranscription kit	RNART.HDV.CE	Dia.Pro srl

The RNA retro transcription assay for HDV RNA must be carried out as following below:

RETROMIX Preparation

Number of Reactions		1	25
REAGENT A (RNART.CE)	MMULV	1 µl	25 µl
REAGENT B (RNART.CE)	Retro mix	9.5 µl	237.5 µl
REAGENT C (RNART.CE)	MB WATER	8,5 µl	212.5 µl
HDV/REV*	Specific Reverse Primer	1 µl	25 µl
Tot vol.		**20 µl**	**500 µl**

Important note: for the correct use of Component REV see section E and I

Preparation of Retrotranscription assay

- Prepare the required number of reaction tubes for the samples
- Considering 1 plus tube for negative control
- Thawing Retro mix shortly to use
- **WORK ON ICE**

Number of reaction	1
RETROMIX	20 µl
Sample (RNA) or NTC	10 µl
Tot. vol.	**30 µl**

The thermal profile for the retrotranscription reaction is reported in the table below:

Step	Cycle	Temp.	Time
1	1	42°C	60 min
2	1	99°C	10 min
3	1	4°C	inf.

At the end of the retro transcription assay the samples should be placed at 2°C….8°C no more than 1 hour or keep at -20°C.

N.3 Setting up of the reaction

DRNA.CE kit is intended to be used exclusively in combination with ABI 7500 (Software SDS version 1.3.1, Applied Biosystem) and MX3000P (Software MxPro version 4.01, Stratagene) and CFX96 software CFX manager version 1.7 (Biorad).

N.3.1 Preparing the 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 or triplicate;
- Include at least one tube for the NTC (negative control)
- Prepare the **Amplification Mix** for **Samples, NTC and standard curve** as table below:

Preparation of the Amplification Mix

Number of Reactions		1
A	Master mix	12,5 µl
B	Primers/probes	2 µl
I.C.	Internal Control	0,5 µl
Tot vol.		**15 µl**

N.3.2 Amplification procedure

- dispense 15 µl of the amplification mix in each reaction tube or microplate well
- add 10 µl of the **Samples, NTC and Standard curve** to the reaction tubes
- 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 Thermacycler Thermoblock Holder
- after the setting operation described in the section N5 (Instrument Programming) start the Thermoclycler run.

Important note: The Lyophilized Components after dissolution in component C (MG water) are stable no more the 3 hours kept on ice or at 2°C….8°C.
At the end of the working day discard adequately the material leftover of the STD Dilution Points.
The not used volume of Component B, REV, STD and IC can be split in aliquots. The aliquots must be thaw only one time.

scheme for preparation of PCR assay

Number of reactions	1
Amplification Mix	15 µl
Sample, NTC, Standard Curve	10 µl
Tot vol.	**25 µl**

N.5 Instrument programming

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

Important Note: *For Mx3000P set "Filter set gain settings" : ROX = x1, FAM = x8, VIC/JOE = x1. (see MxPro™ QPCR Software Instruction Manual, p.41)*

N.5.1 Thermal Profile

The thermal profile is reported in the table below:

Step	Cycle	Temp.	Time
1	1	50°C	2 min
1	1	95°C	15 min
2	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.5.2 Selection of the Detectors

Following the Instruction manuals of the Real-Time thermal cyclers suggested (ABI 7500, MX3000P 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	ROX	Non Fluorescent

Important Note:
Mx3000P thermalcycler: the fluorescence signal of the reporter VIC is detected by the filter/channel JOE

O. ASSAY SCHEME
Examples of dispensation scheme for Quantitative Analysis are reported below:

Microplate/Tubes

	1	2	3	.	.
A	**STD 1**	Sample 4			
B	**STD 2**	Sample 5			
C	**STD 3**	Sample 6			
D	**STD 4**	Sample 7			
E	NTC	Sample 8			
F	Sample 1	Sample 9			
G	Sample 2	Sample 10			
H	Sample 3	Sample 11			

Legenda: NTC = Negative Control STD 1,2,3,4 = HDV RNA Standard Curve, Sample 1,2,3 = Samples under evaluation.

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

Legenda: NTC = Negative Control STD 1,2,3,4 = HDV RNA Standard Curve, Sample 1,2,3 = Samples under evaluation.

P. INTERNAL QUALITY CONTROL

P.1 Pre- Analysis setting
Before starting the interpretation of the data:

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

baseline	
ABI ™ PRISM 7500SDS	Auto baseline
MX3000P® (Stratagene™)	Adaptive baseline Important note: *Do not ues Mx4000 v1.00 to v3.00 algorithm*
CFX96® RTS (Bio-Rad™)	Auto calculated Baseline

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

"Threshold"	FAM	VIC/JOE
ABI ™ PRISM 7500SDS	0.055	0.055
MX3000P® (Stratagene™)	0.055	0.045
CFX96® RTS (Bio-Rad™)	230	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 - STRATAGENE™ Mx3000P®	
Check	**Requirements**
NTC	Undetermined
STD 1	24 < Ct (Threshold Cycle) < 28
STD 2	27 < Ct (Threshold Cycle) < 31
STD 3	30 < Ct (Threshold Cycle) < 34
STD 4	34 < Ct (Threshold Cycle) < 38

BIORAD™ CFX96®	
Check	**Requirements**
NTC	Undetermined
STD 1	25 < Ct (Threshold Cycle) < 29
STD 2	28 < Ct (Threshold Cycle) < 32
STD 3	31 < Ct (Threshold Cycle) < 35
STD 4	35 < Ct (Threshold Cycle) < 39

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	-3.1 < Slope <-3.9

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.

For each positive samples detected by kit code DRNA.CE a correct Quantitation of the HDV viral load can be applied as reported in the table below:

Sample HDV run data (copies/µl)	HDV viral load (copies/µl)
Quantity > 1E+09	HDV viral load >1E+09
1E+00 ≤ Quantity ≤ 1.0E+09	***QUANTITATION***
Quantity < 1E+00	HDV viral load < 1E+00

IMPORTANT NOTE: *For samples quantitation refer to section R*

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

ABI™ PRISM® 7500SDS - BIORAD™ CFX96®	
Sample HDV data (IU/ml)	**HDV viral load (IU/ml)**
Quantity > 5.75E+05	HDV viral load > 5.75E+05
5.00E+01 ≤ Qty ≤ **5.75E+05**	***QUANTITATION***
Quantity < 5.00E+01	HDV viral load < 5.00E+01

STRATAGENE™ MX3000P®	
Sample HDV data (IU/ml)	**HDV viral load (IU/ml)**
Quantity > 5.75E+05	HDV viral load > 5.75E+05
2.50E+02 ≤ Qty ≤ **5.75E+05**	***QUANTITATION***
Quantity < 2.50E+02	HDV viral load < 2.50E+02

IMPORTANT NOTE: *For samples quantitation as copies/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 Retro transcription procedures have been executed correctly;
SAMPLE unknown	-	+	CORRECT RESULT *Negative*	
STD	+	+/-	CORRECT RESULT	***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.
STD	-	-	ATTENTION ! POSSIBILITY OF: Error in the	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure;

			pipetting or in the 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.
STD	-	+	ATTENTION ! POSSIBILITY OF: Error in the pipetting or in the procedure	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.
NTC	-	+	CORRECT RESULT	
NTC	+	+	ATTENTION ! POSSIBILITY OF: Contamination	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. 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 the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. 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 calibrator are treated like purified samples and it uses the same volume, 10µl.
The STD calibrators concentration is expressed as copies/µl.
The Viral Genome Concentration per mL for each patient specimen is calculated applying the following formula:

Results (copies/ml) = copies/µl (run data) x B x C x D x 1000

Here below a brief explanation of the "coefficients":

B= Value which express the dilution of the sample due to the Retrotranscription step.
10 µl of Extracted sample in 30 µl of the total reaction volume **= (30/10 = 3)**
C= Value which express the ratio between the volume of sample extracted and the eluted volume due to the Extraction Step **= (60 µl/140 µl = 0.43)**
D= A correction Factor calculated during the assay development **= 0.23**
"1000" = To obtain the viral load of HDV RNA genome in 1ml (copies/ml = copies/ul x 1000)

Results (copies/ml) = copies/µl x 3 x 0.43 x 0.23 x 1000

Results (copies/ml) = copies/µl x 300

On **ABI™ PRISM® 7500SDS** (Software SDS version 1.3.1, Applied Biosystem) and **STRATAGENE™ MX3000P®** (Software MxPro version 4.01, Stratagene) to convert samples viral load expressed in copies/ml to IU/ml the conversion factor is **2.36**

1 IU/ml = 2.36 copies/ml

Example:

Results (copies/ml) = 250 (run data) x 300

Results (copies/ml) = 75000 (7.5E+04)

Results (IU/ml) = 75000/2.36 = 31780 (3.2E+04)

On **BIORAD CFX96® RTS** to convert samples viral load expressed in copies/ml to IU/ml the conversion factor is **3.91**

1 IU/ml = 3.91 copies/ml

Example:

Results (copies/ml) = 414 (run data) x 300

Results (copies/ml) = 124200 (1.2E+05)

Results (IU/ml) = 124200/3.91 = 31765 (3.2E+04)

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 materials supplied by the Fondazione Ospedale Maggiore Policlinico Mangiagalli e Regina Elena, Milano, Italy.

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 **limit of quantitation:**
Limit of detection (LOD): it is the highest dilution of the analyte that is consistently detected. So that the LOD is the lowest **concentration** of the analyte that can be consistently detected.

Limit of quantitation: it is a lower and an upper limit detected in the quantitative molecular assay for precise and accurate quantitation of analyte .
In the quantitative molecular assay the **limit of detection (LOD)** it was be determined by testing serial dilution of standard curve constructed with known concentration of a plasmid carrying a fragment of the target marker. Analysis called **Probit,** based on 8 replicates of three standard points dilution tested in three different runs, should give positive result in 95% of them.
Limit of quantitation it was determined by measuring the **linearity** or **dynamic range.** The **linearity** (for quantitative assay) or **dynamic range** (for qualitative assay) it was determined by testing different concentrations of standard curve in a linearity experiment. Plotting the output values of standard curve against standard points concentrations they should result in a linear curve. This mean that exist a direct proportional between known concentration for single standard dilution and quantitation for each one.
In the kit code DRNA.CE the **LOD** it was determined by analysis of 24 replicates, 8 replicates for three times, of the three dilution of the standard curve .They can be detected in 100% of them. **Probit analysis** and it resulted is $0\log_{10}$ (1 copies/µl) .

The results are the following:

Detection Limit	
ABI™PRISM® 7500 SDS	**1 copies/ µl**
Stratagene™ MX3000P®	**1 copies/ µl**

This means that there is with the Instrument listed in the above table the 95% probability that 1 copies/ µl will be detected

S.1.1 Dynamic Range and Linearity
Linearity is the measure of the degree to which a standard curve approximate a straight line**.** The **linearity** or **dynamic range** is the span of standard points concentrations for which the final output value (Ct threshold cycle) of system is directly proportional to the concentration for each one.
The boundaries of the measuring range are the lower and upper limits of quantitation **(Limit of quantitation).**
In the kit code DRNA.CE a series of dilution of a DNA plasmid, produced in-house, whose concentration was determined by spectrophotometer, was prepared at defined copies/µl in appropriate buffer and then freeze-dry in order to produce, after dissolution in MB grade water, a curve of limiting dilutions. These dilutions were tested in the analytical system and their Ct (threshold cycle) determined.
The upper **limit of quantitation** is $9.00\log_{10}$ (10^9 copies/µl) and the lower limit of quantitation is $0.00\log_{10}$ (1 copies/µl) .
The dynamic range was also determined with serial dilution of 1st WHO International Standard for HDV (PEI code 7657/12).
For the kit DRNA.CE performed on ABI 7500 and on BioRad CFX96 the upper **limit of quantitation** is $5.76\log_{10}$ (5.75E+05 IU/ml) and the lower limit of quantitation is $1.70\log_{10}$ (5.00E+01 IU/ml).
For the kit DRNA.CE performed on Mx3000P the upper **limit of quantitation** is $5.76\log_{10}$ (5.75E+05 IU/ml) and the lower limit of quantitation is $2.40\log_{10}$ (2.50E+02 IU/ml).

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. Sera coming from patients suffering infections due to potential interfering organisms were obtained from Fondazione Ospedale Maggiore Policlinico Mangiagalli e Regina Elena Milano –Italy and tested

The results are reported in the following table:

Number of samples	Organism	Result
10	HBV (HDV Negative)	negative
10	HCV	negative
10	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%.

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 DRNA.CE this parameter was studied by examining HDV RNA positive sera samples in duplicates in the same run and then it was been calculated the percentage (%) of positive samples.
These samples were tested positive with the kit in use in the laboratory that provided these samples (Fondazione Ospedale Maggiore Policlinico Mangiagalli e Regina Elena, Milano –Italy).

HDV RNA Positive samples

TRUE POSITIVES	25
FALSE NEGATIVES	0
TOTAL SAMPLES	25
SENSITIVITY %	**100**

Moreover the Diagnostic Sensitivity of the assay was tested using a panel of sera coming from patients infected with HDV different Subtypes, gently provided from Fondazione Ospedale Maggiore Policlinico Mangiagalli e Regina Elena, Milano –Italy.

The results are shown here below:

Sample	HDV Subtype	Result of the Analysis
HDV001	1	Positive
HDV002	2	Positive
HDV003	3	Positive

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 DRNA.CE, **precision** was expressed as intra-assay variability and inter-assay variability. It were 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%) ≤ 10%.
Inter-Assay Coefficient Variation (CV%) ≤ 10%.

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 step 2 is 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.

U. BIBLIOGRAPHY

1. Hepatocellular carcinoma and infections with multiple hepatitis viruses.Brunetto MR, Oliveri F, Colombatto P, Bonino F. Princess Takamatsu Symp. 1995;25:61-6.
2. Hepatitis delta virus pathogenicity.Gowans EJ, Bonino F.Prog Clin Biol Res. 1993;382:125-30. Review.
3. A polymerase-chain reaction-based assay for serum HDV RNA.Negro F, Emerson SU, McRill C, Bonino F, Craxi A, Gerin JL, Miller RH, Purcell RH.Prog Clin Biol Res. 1991;364:179-84. No abstract available.
4. Quantitation of hepatitis delta virus RNA in serum by consensus real-time PCR indicates different patterns of virological response to interferon therapy in chronically infected patients.Le Gal F, Gordien E, Affolabi D, Hanslik T, Alloui C, Dény P, Gault E.J Clin Microbiol. 2005 May;43(5):2363-9.
5. Quantitation of the level of hepatitis delta virus RNA in serum, by real-time polymerase chain reaction--and its possible correlation with the clinical stage of liver disease.Yamashiro T, Nagayama K, Enomoto N, Watanabe H, Miyagi T, Nakasone H, Sakugawa H, Watanabe M.J Infect Dis. 2004 Apr 1;189(7):1151-7. Epub 2004 Mar 12.
6. Hepatitis B virus concentrations in serum determined by sensitive quantitative assays in patients with established chronic hepatitis delta virus infection.Sakugawa H, Nakasone H, Nakayoshi T, Kawakami Y, Yamashiro T, Maeshiro T, Kinjo F, Saito A, Zukeran H.J Med Virol. 2001 Nov;65(3):478-84.

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.

CE
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