EurobioPlex HDV

For **qualitative/quantitative** real-time RT-PCR

REF **EBX-071-25 / EBX-071-50 / EBX-071-100**

25/50/100 reactions

CE 0459 IVD

EBX-071 IFU- Version 1.01 of 02/05/2025

Validated on :

- CFX96™ Real Time PCR detection system (Bio-Rad) with analysis on CFX Manager version 3.1 (Bio-Rad)
- CFX® Opus 96 (Bio-Rad) with analysis on Bio-Rad CFX Maestro version 2.2(Bio-Rad)
- LightCycler®480 with analysis on LightCycler® 480 software v1.5 (Roche)
- QuantStudio™ 5 and QuantStudio™ 7 Pro (Applied Biosystems™ by ThermoFisher Scientific) with QuantStudio 6/7 Pro Real-Time PCR Systems Software v2.6.0 (Applied Biosystems™ by ThermoFisher Scientific)

Instructions for use
Available at www.eurobio-scientific.com

The Summary of Safety and Performance Characteristics (SSPC) will be made available by the Notified Body on EUDAMED once it is operational. It can also be obtained on request

Table of contents

1. General information

Hepatitis D is an infection caused by the hepatitis delta virus (HDV), discovered in the late 1970s by Mario Rizzetto. HDV is a satellite virus of hepatitis B (HBV). It is composed of a circular, pseudo-double-stranded RNA surrounded by the two isoforms of the delta protein (small or p24 and large or p27), forming the delta ribonucleoprotein which is enveloped by the HBV envelope proteins or AgHBs.

Because it is a satellite virus, HDV can only infect individuals infected with HBV. A distinction is made between co-infection, in which the two viruses HBV and HDV are transmitted together, and HDV superinfection in a chronic HBV carrier. All HBsAg-positive patients should therefore be systematically screened for HDV

HDV is considered to be the virus responsible for the most serious liver disease. It is more frequently responsible for acute, fulminant and often fatal hepatitis, and has a documented progression to chronicity, with major episodes of acute hepatic decompensation and accelerated progression to cirrhosis and hepatocellular carcinoma.

It is estimated that between 15 and 25 million people worldwide are infected with HDV, representing around 5% of HBV carriers.

HDV is characterised by very high genetic variability. Eight major genotypes (from 1 to 8) and several sub-genotypes have been described, with a characteristic geographical distribution: genotype 1 (HDV-1) is ubiquitous and is the most prevelant globally; HDV-2 and HDV-4 are prevelant in Asia; HDV-3, in the north of South America; and HDV-5 to HDV-8 in sub-Saharan Africa. However, due to migration, multiple genotypes are seen in each region.

Unlike HBV, HDV has no specific enzyme for replication of its genome, making it more difficult to develop an effective therapeutic strategy against this infection. This was based on the use of standard high-dose interferon alpha, especially in its pegylated form (IFN-PEG). New studies have shown that REP is safe, well tolerated and effective against HBV infection and HDV in combination with low-dose TDF and pegIFN in cirrhotic patients. Since 2020, the European Medicines Agency (EMA) has granted marketing authorisation for Bulevirtide, an analogue of the large HBsAg isoform, which blocks the binding of HBV and HDV to hepatocytes, thereby blocking their entry into the hepatocyte. New specific anti-HDV drugs have been developed and are currently being evaluated in several clinical trials, such as farnesylation inhibitors blocking the morphogenesis of delta virions and inhibitors of virus secretion.

The diagnosis of HDV infection is based on the detection of total or IgG anti-delta antibodies in the serum of HBsAg-positive patients. Viral replication must be assessed in all anti-delta antibody-positive patients. This is based on the detection and quantification of serum HDV-RNA viral load using real-time RT-PCR. Quantification of HDV viremia also enables the natural course of the infection and the efficacy of antiviral treatment to be monitored.

2. Purpose of the device

The **EurobioPlex HDV (Hepatitis Delta Virus**) device is a real-time RT-PCR amplification kit for the detection and quantification of HDV RNA in the blood of infected patients.

The test is indicated in all HBV-infected patients with positive HDV serology to determine the replicative or non-replicative nature of the infection and the level of HDV RNA viral load during the natural history of the disease or under treatment. The test is not intended for HDV screening for blood or organ.

RNA isolate is the starting material for the EurobioPlex HDV device. The user is responsible for extraction of RNA from source samples.

This amplification system has been validated on plasma and serum samples and on the endogenous internal control.

The EurobioPlex HDV test is an in vitro diagnostic medical device and must be used by qualified medical laboratory staff. It must not be recycled after use and must be disposed of in accordance with legislation on hazardous waste.

3. Symbols

	Reference
	Batch number
	Upper and lower storage temperature limits
	Expiry date
	Sufficient content for "N" reactions
	Manufacturer
	Date of manufacture
	*In vitro* diagnostic medical device
	Consult the instructions for use
	Attention
	Keep away from sunlight
	Do not use if packaging is damaged
	CE marked product

4. Principle

The EurobioPlex HDV device a quantitative ribonucleic acid (RNA) amplification test used to analyse the absence or presence and quantification of the hepatitis delta virus. The area of HDV amplified is part of the delta antigen. This gene region was selected because it is conserved across all 8 HDV genotypes.
An "endogenous human housekeeping gene" is used to control the quality of the sample and of the nucleic acids extracted and analysed, and to identify any inhibition of the RT-PCR amplification reaction.

The starting material used by the EurobioPlex HDV kit is RNA extracted from a sample of the patient's plasma or serum.

HDV RNA is detected using a FAM-labelled probe and the endogenous internal control using a Cy5-labelled probe (Table 1). During the elongation phase of RT-PCR, the probes emit a specific fluorescence in 'real time' following their hydrolysis. This is measured individually by an optical system and is proportional to the quantity of amplification products obtained.

Table1 Target detection by fluorophore

Targets	Fluorophore	Excitement	Broadcast
HDV	FAM	495 nm	515 nm
Endogenous internal control	Cy5	650 nm	670 nm

Equivalent channels on different PCR instruments :

- Channel FAM (ABI Systems, SmartCycler II, Mx Systems, Chromo4/CFX96, T-COR 8®-IVD), Channel 510 (LC 480), Channel Green (RotorGene),
- Channel Cy5 (ABI Systems, Mx Systems, Chromo4/CFX96, T-COR 8®-IVD), Channel Alexa647 (SmartCycler II), Channel 660 (LC 480), Channel Red (RotorGene)

Note 1: On LC480 instrument II: Apply colour compensation for FAM/Cy5 wavelengths (FAM: 483-533 / Cy5: 615-670).

The device is not automated. It is the user's responsibility to use equipment other than that approved, in which case performance is not guaranteed.

5. Kit components

The Eurobioplex HDV real-time RT-PCR kit is a ready-to-use kit containing the reagents and enzymes required for the specific detection and quantification of HDV RNA (Table 2).
The fluorescence emitted is measured individually by an optical system during PCR.
Detection of the amplified fragments is carried out by a fluorometer using the channels indicated in Table 1.

Table2 Kit components

Plug colour	Kit contents	25 reactions	50 reactions	100 reactions	Reconstitution
Red	Enzymes	156.25 µL	318.75 µL	637.5 µL	Ready to use
Transparent	Oligomix *	160 µL	315 µL	625 µL	Ready to use
White	Positive control CP	40 µL	80 µL	80 µL	Ready to use
Blue	Water = negative control (CN-H2O)	1000 µL	1000 µL	1000 µL	Ready to use
Yellow	5 HDV standards: S-1 to S-5**	50 µL of each	2 x 50 µL of each	2 x 50 µL of each	Ready to use

***Note*: centrifuge tubes well before use**

*Oligomix: contains primers and probes for the detection of HDV and for endogenous control.

** The EBX-071 kit contains quantification standards in 5 different concentrations (see section 10.3.2 Quantitative RT-PCR).

6. Conservation and storage

All reagents should be stored between -15°C and -22°C.
All reagents can be used up to the expiry date indicated on the kit label.

⚠ **The sensitivity of the test may be reduced if the kit components undergo multiple freeze/thaw cycles. The kit can be used after initial opening for a maximum of 3 freeze/thaw cycles.**

7. Equipment required but not supplied

- PSM II
- Real-time PCR
- Centrifuge for microtubes and microplates
- Vortex
- Plates or tubes for real-time PCR reaction
- Micropipettes
- DNAse-free and RNAse-free filter tips for micropipettes
- Sterile microtubes
- Gloves (talc-free)

8. Real-time PCR instrument

The EurobioPlex HDV kit has been developed and validated with the following real-time PCR machines:

- CFX96TM Real Time PCR detection system (Bio-Rad) with analysis on CFX Manager version 3.1 (Bio-Rad)
- CFX® Opus 96 (Bio-Rad) with analysis on Bio-Rad CFX Maestro version 2.2(Bio-Rad)
- LightCycler®480 with analysis on LightCycler® 480 software v1.5 (Roche)
- QuantStudio™ 5 and QuantStudio™ 7 Pro (Applied Biosystems™ by ThermoFisher Scientific) with QuantStudio 6/7 Pro Real-Time PCR Systems Software v2.6.0(Applied Biosystems™ by ThermoFisher Scientific)

9. Warnings and precautions

Read these instructions carefully before starting the protocol.

- This experiment must be carried out by medical biology laboratory technicians.
- Ensure that the instruments have been installed, calibrated and maintained in accordance with the manufacturer's recommendations.
- It is the user's responsibility to use equipment other than that approved, in which case performance is not guaranteed.
- Clinical samples should be considered as potentially infectious material and should be prepared in a laminar flow hood.
- This experiment must be carried out in accordance with good laboratory practice.
- Do not use this kit after the expiry date indicated.
- The kit is shipped in dry ice, and the kit components should arrive frozen. If one or more components arrive thawed, or if the tubes have been damaged in transit, contact Eurobio Scientific .
- Avoid freezing/thawing reagents, as this can reduce the sensitivity of the test.
- Once the reagents have thawed, centrifuge the tubes briefly before use.
- The use of ice or an ice pack is recommended in the event of long delays due, for example, to a large number of samples to be processed or high temperatures.
- It is recommended to define three distinct work areas: 1) RNA isolation, 2) Preparation of the reaction mixture, and 3) Amplification/Detection of the amplified products.
- The fluorescent probes contained in the oligomix are light sensitive. Any prolonged exposure of the oligomix to light should be limited to the technical time required to prepare the PCR plate.

- It is recommended that the positive control and the standard range are opened and handled separately from the biological samples to be tested and from the other kit components in order to avoid cross-contamination.
- Wear separate gowns and gloves (talc-free) in each work area.
- Pipettes, reagents and other working materials must not be moved between these areas.
- Particular care must be taken to maintain the purity of reagents and reaction mixtures.
- The endogenous internal control detects a cellular target present in all samples of human origin but not in the CN-H2O negative control supplied in the kit. The absence of a signal in the negative control prevents cross-contamination.
- Appropriate RNA preparation/extraction methods must be used for quality RNA production and RT-PCR application, in particular to avoid any risk of degradation by RNAses.
- Use filter tips for micropipettes, RNAse-free and DNAse-free.
- Do not pipette by mouth. Do not eat, drink or smoke in the laboratory.
- Avoid aerosols.
- The kit is not intended for single use. When reusing the device, it is necessary to follow the recommendations concerning authorised freeze-thaw cycles and precautions to prevent contamination of the reagents.
- The kit components must not be used separately (either with other reagents or with reagents from other batches).
- Reagents must be thawed carefully so as not to affect the performance of the device (at +2°C/+8°C or room temperature).
- The device is not automated. It is the user's responsibility to use equipment other than that approved, in which case performance is not guaranteed.

10. Protocol

10.1 Collecting samples

- Collect samples in sterile tubes containing EDTA anticoagulant to prepare plasma or without anticoagulant for serum.
- To prepare the plasma or serum (once the blood has coagulated), centrifuge the blood for 20 minutes at 800 - 1600 g and transfer the plasma or serum obtained into sterile tubes. If longer storage times are required, only the plasma and/or serum may be frozen from -20°C (maximum 1 week) to -80°C (recommended if stored for more than one week).

⚠ The use of heparin as an anticoagulant is prohibited.

- It is up to the user to control their own sample collection, transport, storage and extraction conditions to ensure that RNA extraction using appropriate systems produces quality RNA.
- Samples must be prepared and extracted immediately, or stored at +4°C for a maximum of 72 hours.
- **Effects of biochemical parameters**: very high concentrations of lipids (9 g/dL) or bilirubin (110 g/L) do not affect the results.

Table3 Storage recommendations before use

Recommendations for storage at room temperature before preparing EDTA plasma and serum from blood	Recommendations for storage at + 4°C before preparation of EDTA plasma and serum from blood
< 24 h	< 72 h

Recommended maximum storage times for plasma/serum samples before extraction*.	
< 2 h	Ambient temperature
< 5 days	+2°C/+8°C
Between 5 days and 1 month	-20°C
Between> 1 month and 12 months	< -70°C
Avoid refreezing	
Maximum storage recommendations for RNA samples	
12 months	<-70°c
Avoid refreezing	

Attention	
	- The user can refer to the recommendations issued by the World Health Organisation or the Haute Autorité de Santé for the correct storage of samples. - Extracted RNA should be stored at <-70°C to ensure stability. After one year's storage of RNAs at <-70°C, the Ct values obtained may increase. It is advisable to re-extract a biological sample stored for more than one year. It is advisable to limit the number of times RNAs are thawed to 3. - The transport of clinical samples is subject to local regulations for the transport of infectious agents. Human blood must always be considered infectious and appropriate protective procedures must be employed.

***Values** estimated on the basis of scientific articles and documents mentioned in the bibliography.

10.2 RNA extraction

It is the user's responsibility to ensure that the nucleic acid extraction system used is compatible with real-time RT-PCR technology. We recommend using virus RNA extraction methods suitable for blood samples, and referring to the instructions of the supplier of the extraction kit used.

In the Eurobioplex HDV kit, the endogenous internal control read on the Cy5 channel is already present in the clinical sample to be extracted. Its detection after amplification makes it possible to check the quality of the sample and extraction.

RNA extraction kits are available from several manufacturers. You can use your own extraction system or a suitable commercial system, referring to the manufacturer's instructions.

Several automated systems have been used and validated for RNA extraction using the Eurobioplex HDV kit:

- m2000 sp (Abbott)
- Nuclisens EasyMAG (Biomérieux)
- Cobas 4800 (Roche)
- QiaSymphony SP (Qiagen)

See section 13. Performance analysis: Comparison of different automated RNA extraction systems.

10.3 Real-time RT-PCR

General remark :

- Both the positive control and the standard range contain high concentrations of matrix. Handling must be carried out with care to avoid contamination.

- To check that the RT-PCR is working correctly, it is necessary to test the positive control, as well as the negative control (water supplied = CN-H2O) (see II-2/6 of the real-time RT-PCR protocol).

10.3.1 Qualitative RT-PCR

No standard range is performed. Only the positive CP control and the negative control are tested.

10.3.2 Quantitative RT-PCR

In addition to the negative and positive controls (PCR), a standard curve must be produced using the five ready-to-use HDV standard tubes S-1 to S-5. The standards do not need to be extracted.

In order to be able to correct any variation linked to experimentation, we recommend testing HDV standards S-1 to S-5 in *triplicate*.

The HDV standards (FAM channel) are supplied at concentrations in IU/5µL (table 4 below). It is up to each user to enter these concentrations into the real-time PCR software, taking into account the characteristics of their extraction system and the protocol used (equation 1). The software will display a standard curve and calculate the number of International Units per millilitre of plasma/serum (viral load in IU/mL) for the samples directly.

Table4 Concentrations of HDV standards

Standard-Tube N°.	***HDV* standard RNA [IU/5µL]**
S-1	3,50E+06
S-2	3,50E+04
S-3	3,50E+03
S-4	3,50E+02
S-5	3,50E+01

Equation1 : Calculation

$$\text{HDV standard value (table 4)} \times \frac{\text{Elution volume extraction}}{\text{Test volume serum or plasma for extraction}} \times \frac{1000}{\text{Eluate volume in the well reaction (5µL)}} = \textbf{HDV standard value in IU/mL} \text{ (For information in the software)}$$

It is recommended that you use the Paul Ehrlich Institute's HDV-RNA international standard (1st WHO international standard for HD virus RNA for NAT testing WHO IS PEI: PEI code 7657/12) to establish the performance of your extraction system if it is not one of the validated systems.

Procedure diagram :

1 - PREPARATION OF REACTION MIXTURE / MASTERMIX

Number of reactions	N+3
Enzyme mix	(N+3) x 6.25 µL
Water	(N+3) x 8.75 µL
Oligomix	(N+3) x 5 µL
Total volume Mastermix	(N+3) x 20 µL

2 - PREPARATION OF REACTIONS

Sample

20 µL Mastermix
+
5 µL RNA sample

Positive control(s)

Qualitative :
20 µL Mastermix
+
5µL of CP

Quantitative :
20 µL Mastermix
+
5µL from S-1 to S-5

Negative Control

20 µL Mastermix
+
5 µL Molecular biology water (CN-H2O)

3 - REAL-TIME PCR INSTRUMENT

Programme	Tempera-ture	Duration	Cycle(s)	
Reverse Transcrip-tion	45°C	10 min	1	-
Denaturation	95°C	3 min	1	-
Amplification	95°C	15 sec	40	-
	58°C	30 sec		Fluorescence acquisition

10.4 Detailed protocol

1) Check that the reagents are completely thawed. Homogenise the enzyme tubes and vortex the Oligomix, CP and standards for approximately 15 seconds, then centrifuge briefly. The use of a cold block throughout the protocol is strongly recommended for large batches.
2) Prepare the Mastermix as below, where N is the number of reactions (including positive and negative controls). Prepare enough Mastermix for at least N+3 reactions.

Table5 Mastermix

Number of reactions	N+3*
Enzymes	(N+3) x 6.25 µL
Water	(N+3) x 8.75 µL
Oligomix	(N+3) x 5 µL
Total volume Mastermix	(N+3) x 20 µL

**For small series (≤10): preparing for N+2 is sufficient.*

3) Homogenise the Mastermix prepared in 2) and centrifuge briefly.
4) Dispense 20 µL of Mastermix using a micropipette and filter tips into independent microplate tubes/wells.
5) Add 5 µL of extracted RNA sample.
6) At the same time, carry out the following checks:
   - Positive control(s) :
     - Qualitative test :
       - 20 µL of Mastermix + 5 µL of CP positive control
     - Quantitative test :
       - 20 µL of Mastermix + 5 µL of S-1 to S-5 for the standard HDV range
   - Negative control :
     - 20 µL Mastermix + 5 µL water supplied (CN-H2O)
7) Immediately seal the tubes or plates with adhesive film to avoid contamination.
8) Centrifuge briefly to collect the reaction mixture at the bottom of the tubes or microplate wells.
9) Run the following program on the real-time PCR instrument.

Table6 Programme

Programme	Temperature	Duration	Cycle(s)	
Reverse Transcription	45°C	10 min	1	-
Denaturation	95°C	3 min	1	-
Amplification	95°C	15 sec	40	-
	58°C	30 sec		Fluorescence acquisition

Note 1: On CFX96™ (Bio-Rad), run from version 1.6 or later, then analyse with version 3.1 (see § Experiment validation). **Be sure to use white PCR plates for proper reading in all channels**.
Note 2: On Applied Biosystems (ABI), do not use the "ROX" passive reference.

11. Validation of the experiment

Qualitative analysis

Table7 Validation of qualitative analysis

Positive control (PC)	
FAM	Ct < 30
CY5	Ct < 30
Negative control (CN)	
FAM	Ct not determined
CY5	Ct not determined

** No amplification but a weak fluorescence signal may be seen. In this case the threshold bar should be positioned just above the maximum signal of the negative control.*

Quantitative analysis

- Negative control (NC): no visible amplification on the FAM channel. On the CY5 channel, no amplification but it is possible to see a weak fluorescence signal. In this case, the threshold bar should be positioned just above the maximum signal of the negative control.

- HDV positive control (CP) :
  - PCR signal on CY5 channel < 30,
  - Viral load HDV (FAM): 35,000 IU/mL < CP < 350,000 IU/mL, i.e. 5 log IU/mL +/- 0.5 log.

- Values of HDV quantisation standards S-1 to S-5: The Ct value of standards S-1 to S-5 on the FAM channel must be +/-2 Ct of the value in the table below.

Table8 Ct values for HDV standards S-1 to S-5

Standard-Tube N°.	***HDV* standard RNA [IU/5µL]**	**FAM* Ct**	**FAM** Cp**
S-1	3,50E+06	11	13.5
S-2	3,50E+04	17,6	20,1
S-3	3,50E+03	20,9	23,4
S-4	3,50E+02	24,2	26,7
S-5	3,50E+01	27,5	30

*Ct for Biorad and AppliedBiosystem thermal cyclers
**Cp for Roche LC480II thermocycler

- PCR efficiency on the FAM channel should be between 90% and 110% and $R^2 \geq 0.98$ (equivalent to a "*Slope*" of between -3.6 and -3.1).

Note: A deviation from the standards may be observed (between 2 Ct and 3 Ct). It is then possible to exclude a maximum of 2 range points, if and only if the S-1 is between 9 and 13, the efficiency is between 90 and 110%, the slope is between -3.1 and -3.6, and the mean $R^2 \geq 0.98$.

Post-acquisition data analysis on a CFX96 PCR instrument (Bio-Rad) must be carried out using version 3.1 of the CFX Manager software (Bio-Rad). In order to upgrade to this version from a run launched on a previous version, please follow the procedure below: at the end of the run, the data file with the suffix .pcrd must be opened and processed with version 3.1 of the CFX Manager (Bio-Rad).

If the run was launched with the CFX Manager v1.6 software, for example, to open a data file with the CFX Manager v3.1 software, click on the CFX Manager v3.1 icon. The welcome screen appears.

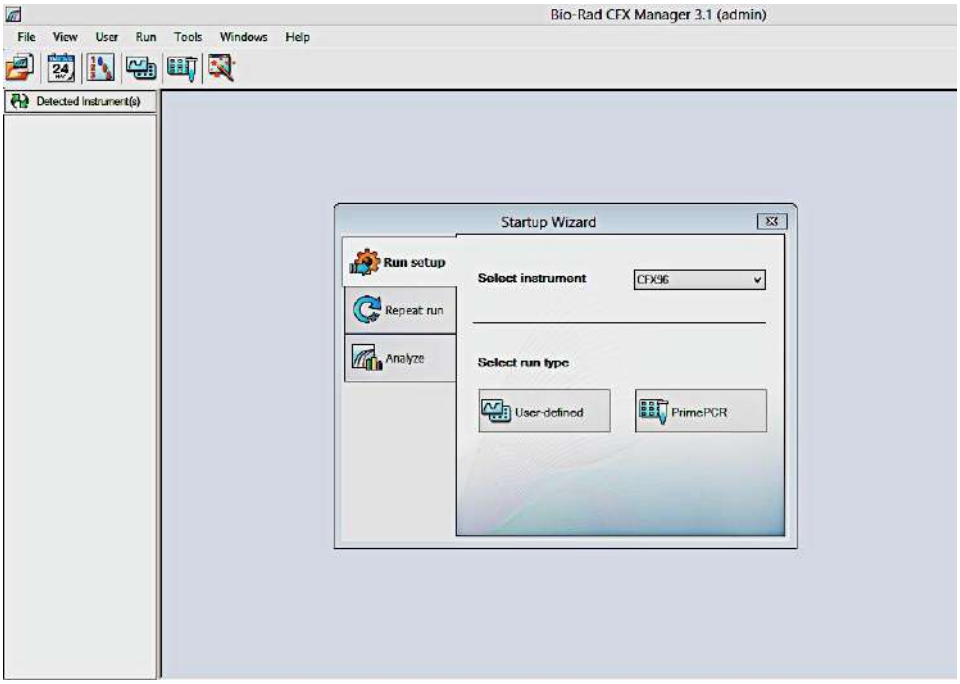


Figure 1

Click on "File" and select "Open" then "Data File".

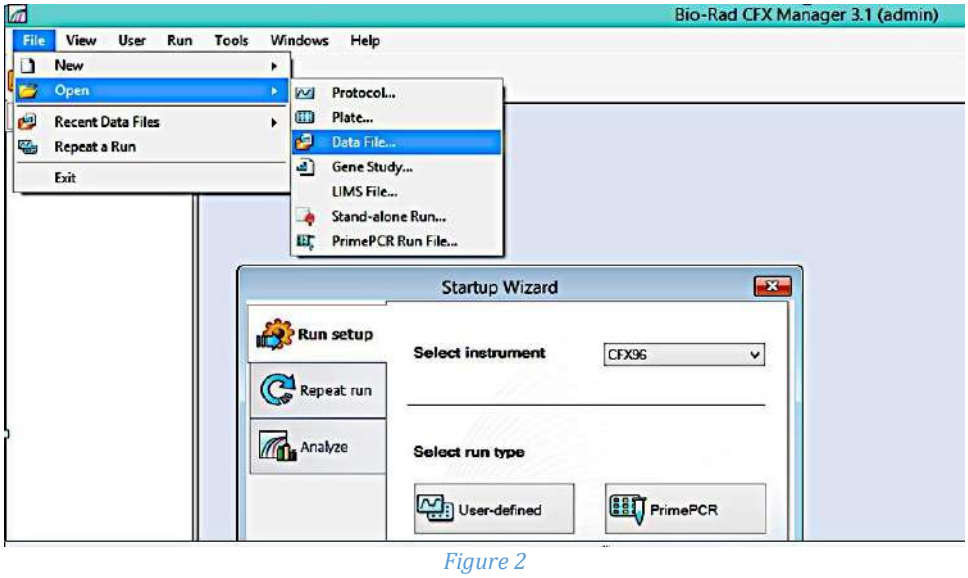


Figure 2

Select the file you wish to analyse and click "Open".

To optimise the run analysis, check the "Log Scale" box for each channel analysed. Then place the *threshold* bar above the background noise corresponding to the middle of the

exponential phase. The 2 channels of interest have different RFUs depending on which channel is being analysed, so the "Log Scale" option makes it easier to read and analyse the run.

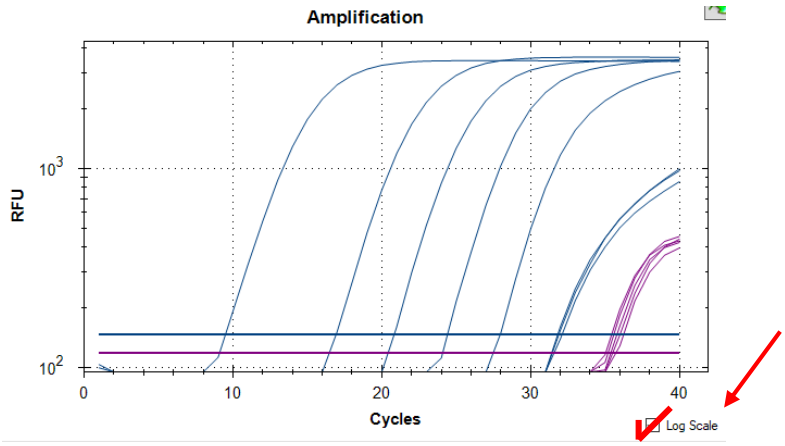


Figure 3

Once this stage has been completed, the analysis can begin for the 2 channels under consideration, **FAM** and **CY5**. **Do not apply the drift correction.**

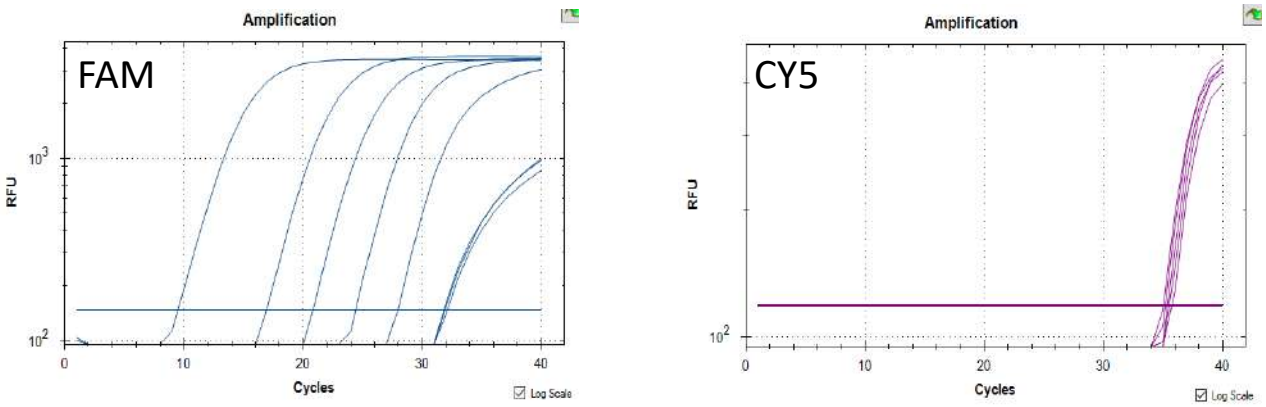


Figure 4

12. Data analysis and interpretation

Qualitative analysis
The results are analysed in the **FAM** and **Cy5** channels.

Table9 Interpretation of qualitative analysis

PCR signal		Validity of the test / comment
HDV (FAM)	IC (CY5)	
+	+/-	Valid / HDV positive
-	+	Valid/Negative for HDV
-	-	Invalid / NI

NI: not interpretable because of RT-PCR inhibition or extraction problems. No conclusion can be drawn. It is therefore recommended that a new sample be taken and/or the extraction repeated and/or the sample diluted 5 times.

Quantitative analysis
The results are analysed in the **FAM** and **Cy5** channels.

Table10 Detection and quantification of the HDV virus

PCR signal + : Ct/Cp less than 45 - No Ct/Cp		Validity of the test comment
HDV (FAM)	IC (CY5)	
+ CV > 50 IU/mL	+	Valid / Positive at HDV, Quantifiable viral load
+ CV < 50 IU/mL	+	Valid / Positive at HDV, Non-quantifiable viral load
+ CV > 50 IU/mL	-*	Valid / Positive at HDV, Estimation of quantification may be underestimated.
-	+	Valid / Negative for HDV
-	-	Invalid / NI

CV: Viral load
**If the AFM signal is positive (CV>50IU/mL) and the CY5 signal is negative, it is recommended that a new sample is taken and/or the extraction is repeated and/or the sample is diluted 5 times. The quantification estimate may be underestimated.*

NI: not interpretable because of RT-PCR inhibition or extraction problems. No conclusion can be drawn. It is therefore recommended that a new sample be taken and/or the extraction repeated and/or the sample diluted 5 times.

Limits of use and interpretation :

- All samples must be treated as potentially infected and local biosafety regulations must be followed.
- The interpretation of results must take into account the possibility of false negatives and false positives.
  False negatives may be due to :
  - Inappropriate collection of samples or incorrect storage,
  - Samples outside the viremia phase,
  - Unsuitable extraction conditions or the use of non-validated PCR instruments
  - An experiment that did not respect all the elements of these instructions for use.

  False positives may be due to :
  - Contamination due to mishandling of high-positive samples, the positive control or PCR amplification products,
  - Failure to follow the procedure described in these instructions for use, in particular to avoid any source of contamination.
- All results must be interpreted by medical staff within the patient's clinical context, history and symptoms.
- This test does not rule out the presence of pathogens other than the HDV virus.

13. Performance analysis

The Eurobioplex HDV kit has been evaluated in accordance with the Common Specifications (CS) for Class D *in vitro* diagnostic medical devices (Implementing Regulation EU 2022/1107) and in compliance with Regulation (EU) 2017/746 of the European Parliament.

Analytical sensitivity / Detection limit

The analytical sensitivity of the kit was determined by analysis of serial dilutions of the first WHO international standard for HDV RNA, PEI code 7657/12.

Table11 Detection Limit (LOD) 95% Probit on the various instruments.

Instrument	Detection limit (IU/mL)
CFX 96	32.7
CFX OPUS	52.4
LightCycler® 480	42.6
QuantStudio®7	21.3

Limit of quantification

The limit of quantification of the kit was determined by serial dilution analysis of the first WHO international standard for HDV RNA, PEI code 7657/12.

The Limit of Quantification (LOQ) of the kit is 50 IU/mL on CFX96, CFX Opus from Biorad, LC480 from Roche and QuantStudio 5, 6 and 7 pro from Thermo scientific.

Linearity

The linear range of the EurobioPlex HDV kit was determined by analysis of serial dilutions of the first WHO international standard for HDV RNA, PEI code 7657/12 from 64565.4 IU/mL (4.81 log IU/mL) to 29.5 IU/mL (1.47 log IU/mL). Each dilution was tested n=9 from 4.81 to 2.9 log IU/mL and n=15 from 2.42 to 1.47 log IU/mL).

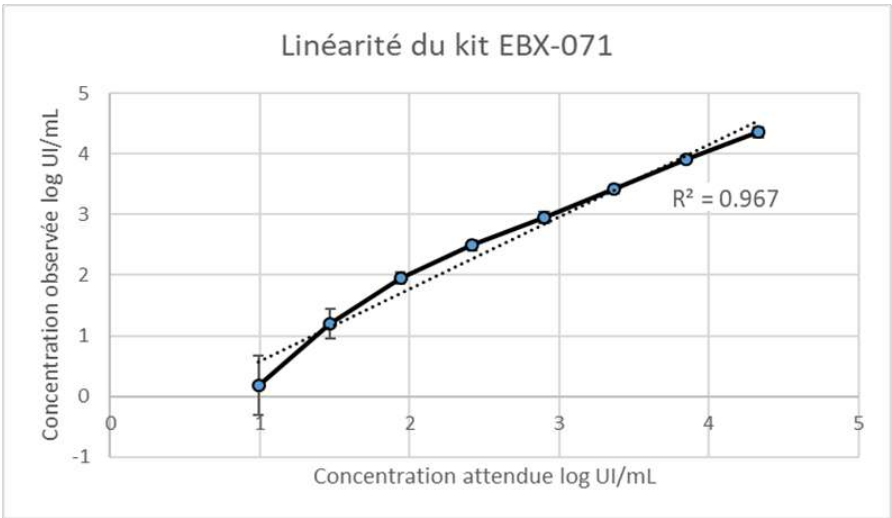


Figure5 Linearity

Specific

- **Detection and quantification of HDV genotypes**

The sensitivity of the *EurobioPlex HDV* kit was evaluated for all HDV genotypes. Serial dilutions to 1/10th of eight samples of genotypes HDV-1 to HDV-8 (excluding HDV-3, in fact genotype 3 could not be tested because it is an extremely rare genotype, for which we were unable to obtain samples, However, an in silico analysis shows that we can detect it using our EBX-071 kit), which had been estimated at around 5 logUI/mL using the *EurobioPlex HDV q-RT-PCR* kit *(EBX-004*), were quantified in duplicate or triplicate.

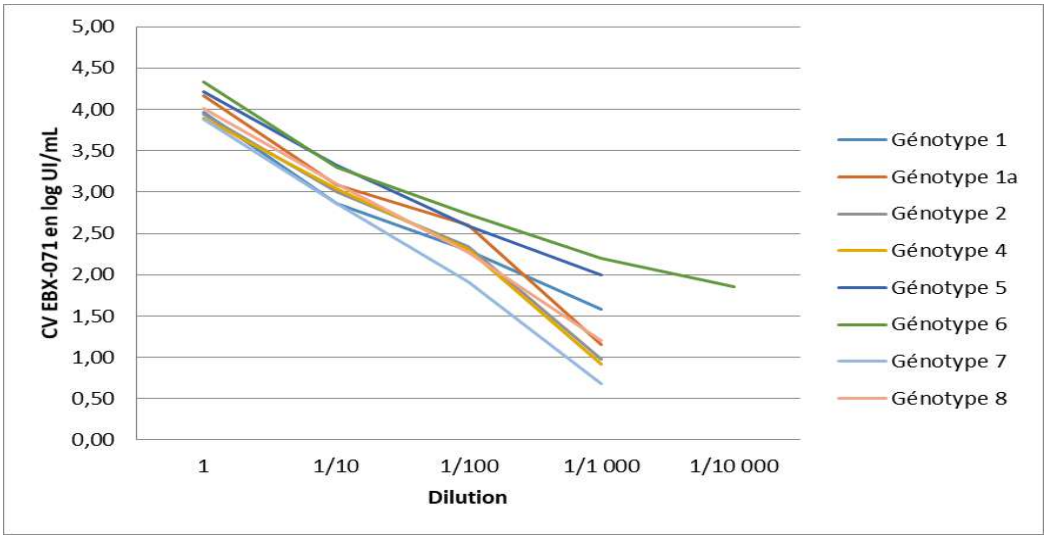


Figure6 HDV genotype specificity

- **Analytical and diagnostic specificity**

Diagnostic specificity: 100 blood donor samples, negative for HBsAg (Architect, Abbott) and negative for HDV serology (total anti-HDV Ac, Liaison XL, Diasorin) were tested. No amplification (FAM channel) was observed.

Table12 Diagnostic specificity

n=100		*EBX-071*	
		Positive	Negative
EBX-004	Positive	**0**	0
	Negative	0	**100**

HDV diagnostic specificity: 100/100*100= **99.99%.**

Analytical specificity: 100 samples negative for HDV RNA and 50 samples positive for HDV RNA were tested.

Table13 Analytical specificity

n=150		*EBX-071*	
		Positive	Negative
EBX-004	Positive	**50**	0
	Negative	0	**100**

HDV analytical sensitivity: 50/50*100= **99.99%**
HDV analytical specificity: 100/100*100= **99.99%**
HDV concordance: 150/150*100=100%.

- **Cross-reactivity linked to related viruses**

Of 25 samples tested (HDV Total Acids negative, but viremic (positive viral load and/or positive IgM) for HAV (n=5), HBV (n=5), HCV (n=5), HEV (n=5) or HIV (n=5)), no false positives were found with the *EurobioPlex HDV* kit.

- **Non-specific cross-reactivity linked to endogenous and exogenous interfering substances**

A sample of known HDV viral load was diluted in 10 serum samples with triglyceride levels between 5g/L and 9g/L (n=5) or bilirubin levels between 60mg/L and 110 mg/L (n=5). The viral load was quantified on these reconstituted samples using the *EurobioPlex HDV* kit, and compared with the expected value (3.53 logUI/mL) for a sample diluted in plasma without interference. Under the conditions tested, no interference was detected.

In addition, a study was carried out on different haemoglobin concentrations in the samples. 3 haemoglobin concentrations were tested: 0.1 g/L, 0.05g/L and 0.01 g/L, on 2 different samples,

at 2 different concentrations (one of which was close to the DRL). Under the conditions tested, no interference was detected.

Finally, Bulévirtide was used to test exogenous interference. This was tested at a concentration of 0.4 mg/L in the samples. Under the conditions tested, no interference was detected.

Sensitivity

The real-life sensitivity of the *EurobioPlex EBX-071* kit was evaluated using 101 samples initially assayed with the *EurobioPlex EBX-004* kit between September 2021 and April 2023 and selected for their low viral load. All samples had a viral load of less than 3.30 log IU/mL (with the EBX-004 kit), and 72.3% of values were between the theoretical LLOD (100 IU/mL) and the theoretical LLOQ (1000 IU/mL) of the *EurobioPlex EBX-004* kit.

Table14 Sensitivity

n=101		*EBX-071*	
		Positive	Negative
EBX-004	Positive	**100**	1*
	Negative	0	**0**

*During the first run, 5 samples with a low load on EBX-004 were found to be negative on EBX-071. This gave the EBX-071 test a sensitivity of 95%. However, during a second run only 1 sample remained negative on EBX-071. We will keep this result as it can be explained by the degradation of samples dating from December 2022.

HDV sensitivity: 100/101*100 = **99%.**

Precision

The accuracy of the kit was measured on dilution series of the first WHO international standard for HDV RNA, PEI code 7657/12 at 2 loading levels (3.76 log IU/mL and 2.76 log IU/mL).

True value of HDV standard diluted 1:100 = 3.76 log IU/mL

Bias (%) = ((3.56-3.76)/3.76) *100 = **-5.**

True value of HDV standard diluted 1:1000 = 2.76 log IU/mL

Bias (%) = ((2.76-2.76)/2.76) *100 =

Fidelity

Repeatability on the first WHO international standard for HDV RNA, PEI code 7657/12 (n=24):

Table15 Repeatability

	FAM (HDV)
Mean CV intra-batch WHO 1/100 (%)	0,33
Mean CV intra-batch WHO 1/1000 (%)	0,46

CV: Coefficient of Variation

Reproducibility on the first WHO international standard for HDV RNA, PEI code 7657/12 (n=120):

Table16 Reproducibility

	FAM (HDV)
Average inter-batch CV WHO 1/100 (%)	4,23
Mean inter-batch CV WHO 1/1000 (%)	2,02

CV: Coefficient of Variation

Comparison of viral loads in plasma and serum

The *EurobioPlex HDV* viral load results for the 50 HDV RNA positive samples are presented below. The median difference between serum and plasma samples was 0.17 logUI/mL.

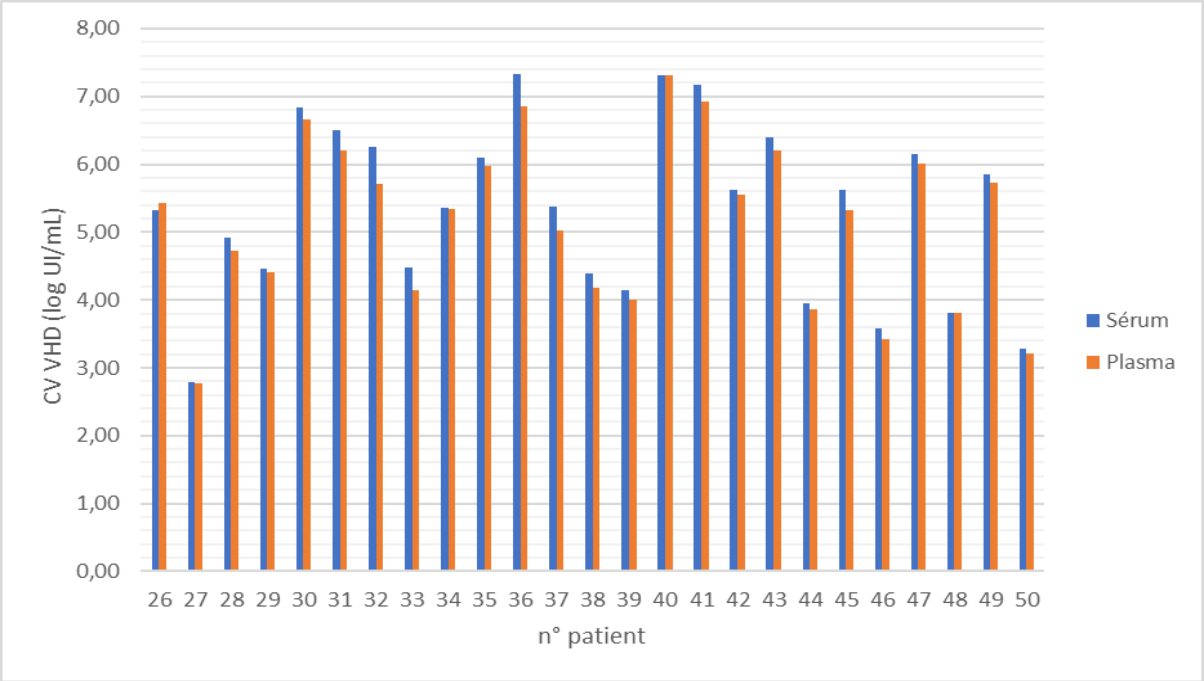


Figure7 comparison of viral loads in plasma and serum

Comparison of different RNA extraction systems

The comparison of 4 automatic RNA extractors was carried out on a panel of 24 samples of various genotypes and viral loads. The reference extractor was the m2000 sp (Abbott). The results were compared with those obtained using 3 other automatic extractors: the EasyMag (Biomérieux), the QiaSymphony SP (Qiagen) and the Cobas 4800 (Roche).

Plasma viral loads were calculated taking into account the data specific to each extraction method (volume of test sample and volume of eluate).

Table 17

	m2000	EasyMag	QiaSymphony	Cobas
Test drive	500 µL	500 µL	400 µL	650 µL
Eluat	70 µL	70 µL	60 µL	20 µL

The results obtained show an average difference in quantification of 0.48 log IU/mL, 0.23 log IU/mL and -0.09 log IU/mL between extraction with m2000sp and extractions on QiaSymphonySP, Cobas 4800 and EasyMag respectively.

Table 18

	Difference in quantification (logIU/mL) m2000 vs.		
	EasyMag	**QiaSym-phony**	**Cobas**
Average	-0,09	0,48	0,23
Minimum	-0,80	-0,51	-0,47
Maximum	0,24	1,49	0,48

Match between the EBX-004 kit and the EBX-071 kit

- **EBX-004/ EBX-071 correction factor: quantitative concordance**

Quantitative concordance: 561 samples were tested in comparison using the two kits. Of these 561 samples, 251 were positive and 310 negative. Of the 251 positive samples, 173 had a CV log IU/ml > 3. These 173 samples were used to establish concordance of quantification between EBX-004 and EBX-071.

The Bland-Altman diagram below shows the difference between the two kits. For viral loads greater than 3 logUI/mL with the *EurobioPlex EBX-004* kit, there was an average difference of 0.89 logUI/mL between the EBX-004 kit and the EBX-071 kit.

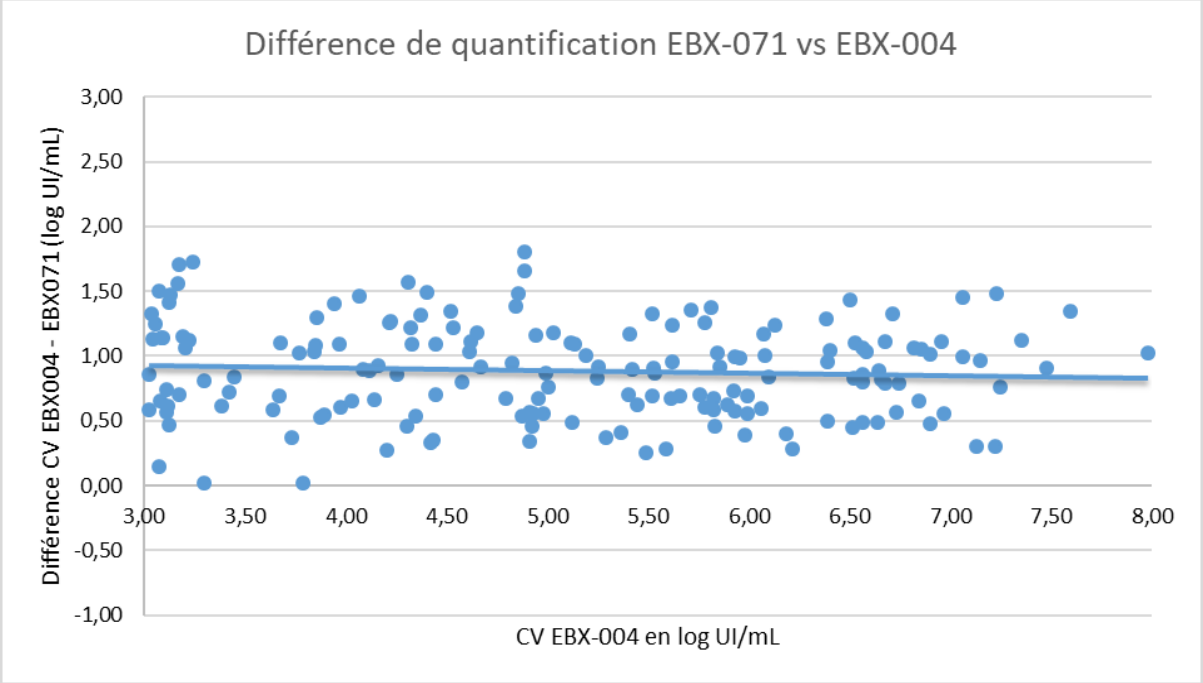


Figure8 EBX-004 and EBX-071 concordance

- **Sensitivity, specificity and overall qualitative agreement of the EBX-071 kit compared with the EBX-004 kit**

Sensitivity, specificity and overall qualitative agreement: 561 samples were tested in comparison using the two kits. Of these 561 samples, 251 were positive and 310 negative. Of the 251 positive samples, 173 had a CV log IU/ml > 3. These 173 samples were used to establish the concordance of quantification between EBX-004 and EBX-071.

n=561		*EBX-071*	
		Positive	Negative
EBX-004	Positive	**250**	1*
	Negative	1	**309**

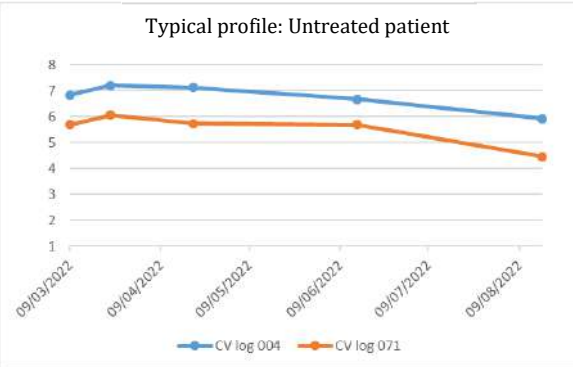
* During the first run, 5 samples with a low load on EBX-004 were found to be negative on EBX-071. This gave the EBX-071 test a sensitivity of 98%. However, during a second run, only 1 sample remained negative on EBX-071. We will keep this result because it can be explained by the degradation of samples dating from December 2022.

Overall HDV sensitivity compared to EBX-004: 250/251*100= **99.6**
Overall specificity of HDV compared with EBX-004: 309/310*100= **99.7%.**
Overall qualitative concordance HDV compared to EBX-004: 559/561*100= **99.6**

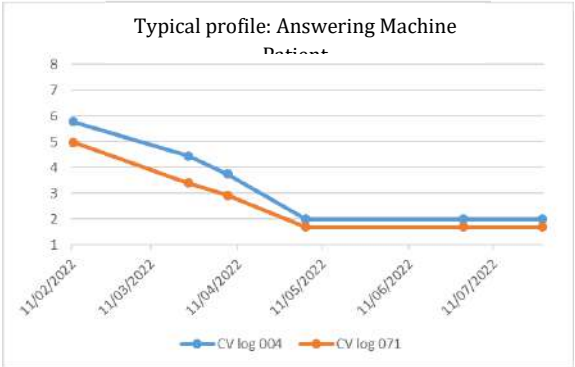
Longitudinal follow-up EBX-004 vs EBX-071

Longitudinal monitoring of the VHD viral load of 11 patients was carried out with 5 to 7 samples per patient. The patients selected had different treatment histories: 2 were untreated, 5 were responders (treated with bulevirtide (BLV), pegylated interferon (pegIFN) + bulevirtide or REP-2139), 1 patient was a non-responder on pegIFN and 3 patients had a responder-relapsed profile to their treatment.

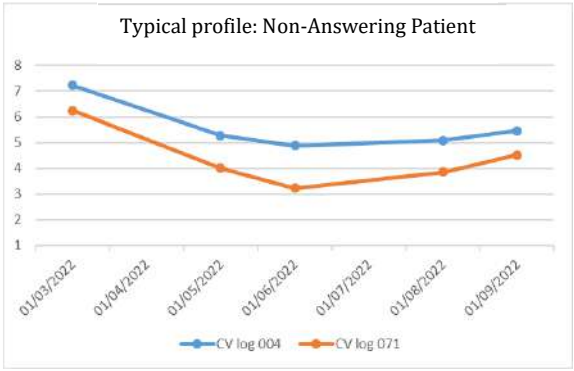
Of the 62 samples, all the results were qualitatively concordant. 14 were undetectable with both RT-qPCR kits. The figures below show that all the viral load monitoring curves for each patient were parallel and within the expected range (difference of 1.10 log IU/mL for positive values).



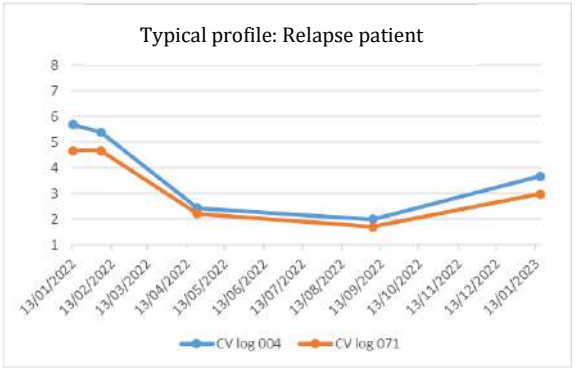
Nom	Date pvt	CV log 004	CV log 071	différence
Patient Non traité	09/03/2022	6.83	5.70	1.13
Patient Non traité	23/03/2022	7.2	6.07	1.13
Patient Non traité	20/04/2022	7.12	5.74	1.38
Patient Non traité	15/06/2022	6.67	5.69	0.98
Patient Non traité	17/08/2022	5.93	4.46	1.47



Nom	Date pvt	CV log 004	CV log 071	différence
Patient répondeur	11/02/2022	5.78	4.99	0.79
Patient répondeur	24/03/2022	4.45	3.41	1.04
Patient répondeur	07/04/2022	3.75	2.93	0.82
Patient répondeur	05/05/2022	<2,00	<1,70	ND
Patient répondeur	30/06/2022	<2,00	<1,70	ND
Patient répondeur	28/07/2022	<2,00	<1,70	ND



Nom	Date pvt	CV log 004	CV log 071	différence
Patient non répondeur	22/03/2022	7.22	6.24	0.98
Patient non répondeur	13/05/2022	5.27	4.00	1.27
Patient non répondeur	10/06/2022	4.89	3.23	1.66
Patient non répondeur	05/08/2022	5.08	3.85	1.23
Patient non répondeur	30/09/2022	5.46	4.53	0.93



Nom	Date pvt	CV log 004	CV log 071	différence
Patient rechuteur	13/01/2022	5.68	4.66	1.02
Patient rechuteur	04/02/2022	5.39	4.67	0.72
Patient rechuteur	21/04/2022	2.44	2.22	0.22
Patient rechuteur	06/09/2022	<2,00	<1,70	ND
Patient rechuteur	16/01/2023	3.67	2.98	0.69

Figure 9 Longitudinal follow-up of typical profiles of VHD+ patients

The blue curve represents the LOG of the viral load (LOG CV in IU/mL), obtained with the reference of the EBX-004 kit, the orange curve the LOG of the viral load (LOG CV in IU/mL), obtained with the reference of the EBX-071 kit. Follow-up was carried out over periods ranging from 6 to 12 months. The patients studied are representative of the affected population, with 4 typical profiles represented:

- Typical untreated patient: The LOG CV (in log IU/mL) does not change significantly over time, but remains at a significantly high level.
- Typical responder patient (treated with bulevirtide (BLV), pegylated interferon (pegIFN) + bulevirtide or REP-2139): The LOG CV (in log IU/mL) decreases significantly over time.
- Typical non-responder treated patient: The LOG CV (in log IU/mL) does not change significantly over time.
- Typical relapser: The CV LOG (in log IU/mL) increased significantly at the end of the study, after an initial fall in the CV LOG (in log IU/mL) during the first few months.

We can also see that all the results are correlated between the two kits tested, with an average difference of 1.10 Log IU/mL (see diagram below).

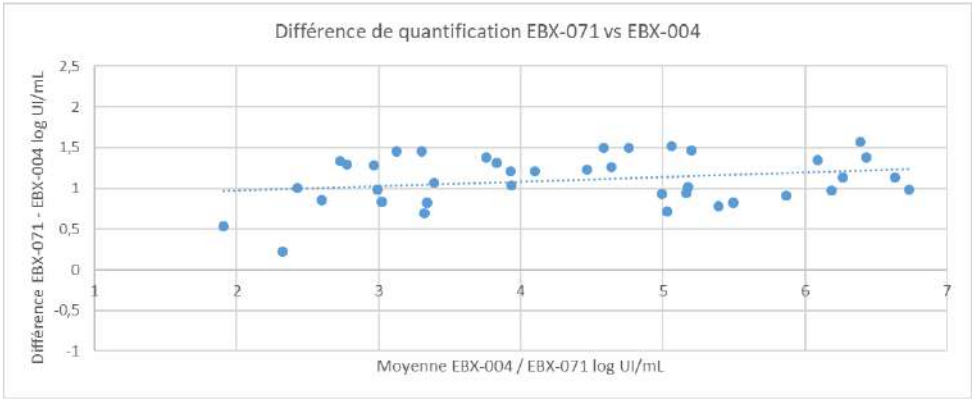


Figure 10 Bland-Altmann diagram

The performance of the EurobioPlex HDV kit (reference EBX-071) was studied on 62 samples taken from the medical follow-up of 11 patients with varied clinical profiles. This study was carried out in comparison with the previous version of the Eurobio kit (EurobioPlex HDV qRT-PCR, reference EBX-004).
The tests carried out enabled us to demonstrate that the longitudinal monitoring of viral load was similar with the two kits. It should be noted that the difference between the values obtained with the references of the EBX-004 and EBX-071 kits is close to that presented in the main study. As demonstrated in the main study, the difference is constant and does not vary as a function of viral load. This "mismatch" will have important consequences for the clinical follow-up of patients, particularly those receiving antiviral treatment, but is intended to improve the overall harmonisation of results.

Finally, the tests carried out enabled us to demonstrate that the longitudinal monitoring of viral load was similar with the two kits.

14. Quality control

In line with Eurobio's ISO EN 13485-certified quality management system, each batch of EurobioPlex HDV is tested to predefined specifications to ensure consistent product quality.

15. Waste disposal

Dispose of all waste in accordance with legislation on DASRI.

16. Incident report

Any incident occurring in connection with the device shall be notified to EUROBIO SCIENTIFIC as well as to the competent authority of the country where the incident occurred.

17. Technical assistance

For assistance with our products, please contact our technical support team.

Eurobio Scientific customer service can be contacted by e-mail at adv@eurobio-scientific.com or by telephone on +33 (0)1.69.79.64.80.

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