



Hepatitis D Virus (HDV)

REF: CL49K

Detection of Hepatitis D virus (HDV) by PCR of large HD antigen region

INTRODUCTION AND PURPOSE OF USE

The Hepatitis D virus (HDV) system is a qualitative test that allows the amplification of large HD antigen region of HDV genome.

The Procedure allows the detection by a double amplification according to the "nested" method. The retrotranscription and a first specific amplification of the large HD antigen region is performed using the red tube master mix, followed by a second amplification (blue tube master mix) specific to the region of the gene indicated above, starting from the first amplification products.

The amplification product, obtained from a double amplification using the nested method, is a 221 bp fragment and is detected by electrophoresis on (pre-cast) PCR CheckIT gel containing ethidium bromide.

CONTENT

The kit contains enough reagents to perform 48 amplification tests

	Quantity	Description
R1	48 tubes	HDV retrotranscription and 1st PCR master mix 36 µl of 1 st amplification gel (HDV upstream primer, dNTPs, Tris-HCl, KCl, MgCl ₂ , Triton X-100, Gel, Sodium Azide 0,05%) and 11 µl of retrotranscription gel (HDV downstream primer, dNTPs, Tris-HCl, KCl, MgCl ₂ , DTT, RNAsin, Gel, Sodium Azide 0,05%)
R7	48 tubes	HDV nested PCR master mix 47 µl of nested amplification solution (HDV-2 upstream primer, HDV-2 downstream primer dNTPs, Tris-HCl, KCl, MgCl ₂ , Triton X-100, Sodium Azide 0,05%)
R2	1 x 30 µl	Positive control Cloned DNA corresponding to the large HD antigen region
R3	1 x 30 µl	Negative control
R4	1 x 250 µl	Loading dye Gel-loading solution
R5	1 x 60 µl	100 bp ladder Containing 10 bands, with dimensions included between 100 bp and 1000 bp; the distance between two adjacent bands is 100 bp
R6	1 x 30 ml	TAE Tris-Acetate-EDTA buffer for electrophoresis run (concentrate)
R8	1 x 270 µl	Mix enzymes (in a separate package - store at -20°C) Mix of AMV retrotranscription 3,3 U/µl and Taq polymerase 0,67 U/µl
R9	1 x 90 µl	Taq polymerase 2U/µl (in a separate package - store at -20°C)
	5	Gels PCR CheckIT pre-cast with EtBr

MATERIALS AND STRUMENTATION REQUIRED BUT NOT SUPPLIED

Disposable latex powder-free gloves or similar material;
Bench microcentrifuge (12,000 - 14,000 rpm);
Micropipettes and Sterile tips with aerosol filter;
Thermal cycler;
Horizontal electrophoresis unit and power supply for electrophoresis; U.V. transilluminator and camera for gel recording.

ACCESSORY PRODUCTS

EX01 – Viral RNA Extraction from Plasma or Serum

The kit allows the viral RNA extraction from Human samples. The kit contains enough reagents to perform the RNA extraction from 50 samples.

SAMPLES AND STORAGE

The Hepatitis D virus (HDV) system must be used with extracted RNA from the following biological samples: Plasma or Serum. The samples collected must be shipped and stored at +2 - +8°C and used within 3 days from the collected data.

Store the sample at -20°C if used after 3 days.

PRECAUTIONS USE

This kit is for research use only.

Keep the Fast PCR Master Mix on ice while in use and return to -20°C immediately after the use.

At all times follow Good Laboratory Practice (GLP) guidelines.

Wear protective clothing such as laboratory coats and disposable gloves while assaying samples.

Avoid any contact between hands and eyes or nose during specimens collection and testing.

Handle and dispose of all used materials into appropriate bio-hazard waste containers. It should be discarded according to local law.

Keep separated the extraction, the reagents preparation and products detection area.

Never pipette solutions by mouth.

Amplification products must be handled in such a way to reduce dispersion into the environment as much as possible, in order to avoid the possibility of contamination.

Avoid the air bubbles during the master mix dispensing. Eliminate them before starting amplification.

Wash hands carefully after handling samples and reagents.

Do not mix reagents from different lots.

It is not infectious and hazardous for the health (see Material Safety data Sheet – MSDS).

Do not eat, drink or smoke in the area where specimens and kit reagents are handled.

Read carefully the instructions notice before using this test.

Do not use beyond the expiration date which appears on the package label.

Do not use a test from a damaged protective wrapper.

LIMIT OF THE METHOD

The extreme sensitivity of gene amplification may cause false positive due to cross-contamination between samples and controls. Therefore, you should:

- physically separate all the products and reagents used for amplification reactions from those used for other reactions, as well as from post-amplification products,
- use tips with filters to prevent cross-contamination between samples,
- use disposable gloves and often change them,

- carefully open test tubes to prevent aerosol formation,
- close every test tube before opening another one.

The proper functioning of the amplification mix depends on the correct collection, correct transportation, correct storage and correct preparation of a biological sample.

As with any diagnostic device, the results obtained with this product must be interpreted in consideration of all the clinical data and other laboratory tests done on the patient.

As with any diagnostic device, there is a residual risk of obtaining invalid results, false positives and false negatives with this product.

STORAGE AND STABILITY

Store the Mix enzymes and the Taq Polymerase at -20°C, while the rest of the kit can be stored at +2+8°C.

An intact and well stored product has a stability of 6 months from the date of production.

Do not use beyond the expiration date which appears on the package label.

ANALYTICAL PROCEDURE

Retrotranscription and first amplification in a single step:

1. prepare a number of vials corresponding to the number of samples to be tested, plus a positive control and a negative control
2. distribute to each test tube 3 µl of each of the RNA extracted
3. add to the positive control test tube 3 µl of the solution "positive control"
4. add to the negative control test tube 3 µl of the solution "negative control"
5. distribute in all test tubes 3 µl of enzymes (contained in vial "Mix Enzymes")
6. put the test tubes in the thermal cycler and run the following programme:

cycles	denaturation	annealing	extension
1			42° C 30 min
1	95° C 5 min		
30	95° C 20 sec	55° C 20 sec	72° C 30 sec

The method is validated on thermal cyclers **Applied Biosystem** mod. 2700 and **Veriti, Eppendorf, Techne** mod. Progene, **Biorad** Genecycler.

Nested amplification:

1. warm, in the thermal cycler, the vials used for the 1st amplification for 10 seconds at 90°C, then hold at 55°C to melt the gel
2. prepare a number of vials for the nested amplification corresponding to the number of vials used for the 1st amplification and distribute 1 µl of Taq polymerase
3. add to each tube 3 µl of the solution contained in the relevant test tube used for the 1st amplification
4. put the test tubes in the thermal cycler and run the following programme:
5. put the test tubes in the thermal cycler and run the following programme:

cycles	denaturation	annealing	extension
1	95° C 5 min		
30	95° C 20 sec	55° C 20 sec	72° C 30 sec

The method is validated on thermal cyclers **Applied Biosystem** mod. 2700 and **Veriti, Eppendorf, Techne** mod. Progene, **Biorad** Genecycler.

Electrophoresis buffer preparation:

Dilute the TAE buffer in 2 litres of distilled water.

Detection:

1. remove the test tubes from the thermal cycler.
2. move 10 µl of each amplification control specimen to an empty test tube.
3. move 10 µl of 100 bp ladder to an empty test tube
4. add 3 µl of loading solution to each test tube and carefully mix.
5. remove the pre-cast gel from its protection bag (cut the bag along three sides to prevent damages to the gel matrix). Do not touch the gel without gloves because it contains EtBr
6. immerse the pre-cast gel in the electrophoresis unit, after filling it with electrophoresis buffer.
7. slowly distribute the solution prepared during step 4 to the bottom of the gel wells.
8. run electrophoresis at 120 Volts for 15-30 minutes according to your apparatus.
9. put the gel on a transilluminator. Remove the plastic support on which it is stratified using a nylon-thread and take a picture of it. It is possible to take a picture without removing the plastic support, you must lean the gel on the transilluminator with the plastic support face up because it is not UV transparent.

INTERPRETATION OF RESULTS

The use of positive and negative control in each amplification session allows to verify the correct functioning of the amplification mix and the absence of any contamination.

Make sure that the negative control does not show a band identical to that appearing in the positive control to exclude any possibility of contamination or a false positive. The session is invalid and must be repeated from the amplification phase.

Ensure the correct amplification of the positive control represented by the 221 bp specific bands presence.

If the specific amplification product is absent in the positive control reaction, this means that problems have occurred during the amplification phase (inefficient or absent amplification) which may cause false negatives. The session is invalid and must be repeated from the amplification phase.

The appearance in the test samples of the 221 bp bands, verifiable by direct comparison with the positive control indicates the presence of HDV. In addition to the specific band of 221 bp it is possible to detect, in high positive sample, a band of 273 bp corresponding at the first amplification product.

Low molecular weight bands, which can appear in all samples, are produced by unincorporated primers. Such are not significant for the interpretation of results.

PERFORMANCES

Analytical sensitivity:

For the purposes of this evaluation is considered as analytical sensitivity the highest serum dilution (title) to which a positive sample can be subjected without the system losing

the ability to detect it as positive. The analytical sensitivity of the system was assessed by analyzing plasmid DNA (pTZ-HDV), quantified by spectrophotometric analysis, containing the genomic region of interest in serial dilutions from 100,000 copies to 1 copy/reaction.

Cone	1 cp	10 cps	100 cps	1,000 cps	1x10 ⁵ cps	1x10 ⁶ cps
Results	-	+	+	+	+	+

The Analytical sensitivity ≤ 10 copies reaction

Clinical sensitivity:

For the purposes of this evaluation is considered as clinical sensitivity the ability to determine true positives on the totality of positive samples screened. The analysis was performed on samples positive for HDV and the test was performed following the instructions in the package insert. Positive samples were confirmed with another system on the market. Results obtained showed a clinical sensitivity of 99.5%.

Analytical specificity:

The specificity of the test is guaranteed by the use of specific primers for the determination of HDV. The alignment analysis of the regions chosen for the hybridization of specific primers for HDV with sequences available in database of the large HD antigen gene shows their conservation, the absence of significant mutations and the complete specificity for the target analyzed.

INTERFERENCE

Verify that the extracted RNA from the sample base there are no mucoproteins and hemoglobin in order to exclude possible inhibition in the PCR reaction. Interference due to contaminants can be detected by spectrophotometric analysis and ratio of data obtained at 260 nm (absorption maximum of nucleic acids) and 280 nm (absorption maximum of proteins). A pure RNA should have a ratio of about 1.8.

QUALITY CONTROL

It's suggested to include a negative and a positive sample tested already before or reference material, as an internal quality control of each session of extraction, amplification and detection.

TROUBLESHOOTING

Amplification signal in the negative control reaction









Possible causes	Solutions
Error displacing the RNA extract.	<ul style="list-style-type: none"> - Use only micropipettes and Sterile tips with aerosol filter - Wear protective clothing such as laboratory coats and disposable gloves while assaying samples - Clean surfaces and instruments with aqueous detergents, wash lab coats, replace test tubes and tips in use - Use the positive control as the last sample - Use the negative control as the first sample
Contamination of the amplification mixes	<ul style="list-style-type: none"> - Don't dispense the amplification mix with the some pipettes used in the extraction or detection area - Use only micropipettes and Sterile tips with aerosol filter - Clean surfaces and instruments with aqueous detergents, wash lab coats, replace test tubes and tips in use - Close the amplification mix and return it at +2+8°C before open the others sample tubes
Contamination of the preparation area for amplification reaction	<ul style="list-style-type: none"> - Clean surfaces and instruments with aqueous detergents, wash lab coats, replace test tubes and tips in use

Failure or incorrect amplification of positive control

Possible causes	Solutions
Possible error in the dispensation of the positive control	<ul style="list-style-type: none"> - Dispense carefully the positive control - Check the calibration of micropipettes used - Verify that the amount of positive control used corresponds to the method described in the Analytical Procedure (3µl)
Positive Control Degradation	<ul style="list-style-type: none"> - Test the mixture through a new batch of positive control
Wrong heat protocol	<ul style="list-style-type: none"> - Check the thermal cycler setting
The reaction mix is maintained at room temperature for too long	<ul style="list-style-type: none"> - Keep the amplification mix on ice during the use and store it at +2+8°C immediately after finishing the preparation of test samples
Error during the dispensation of the amplification product in the the detection phase	<ul style="list-style-type: none"> - Be careful when loading on the gel of amplified

Aspecific bands in the sample amplification reaction

Possible causes	Solutions
Wrong heat protocol	<ul style="list-style-type: none"> - Check the thermal cycler setting
Interfering Presence	<ul style="list-style-type: none"> - Repeat the extraction step carefully
Excess of extracted RNA in the reaction	<ul style="list-style-type: none"> - Evaluate the RNA concentration

	In vitro diagnostic device
	Read the instruction's manual
	Range of temperature
	Use within (dd/mm/yyyy: year-month)
	Lot (xxxx)
	Code
	Manufacturer
	Contains sufficient for <n> tests



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