PrimerDesign ™ Ltd

Quantification of Hepatitis Delta Virus genomes

Large HD antigen

For general laboratory and research use only

Advanced Kit

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Introduction to Hepatitis Delta Virus

Hepatitis Delta Virus (HDV) is a single-stranded RNA virus of the *Deltavirus* genus and is responsible for causing Hepatitis D. The circular RNA genome is 1679 bp in length of which about 70% is self complimentary and folds up upon itself forming a rod-like structure. The genome encodes two ribozymes that catalyse site-specific genome cleavage. After cleavage, these shorter RNA sequences arrange into circular forms. The RNA also encodes two proteins known as the small and large delta antigens that play differing roles in genome replication.

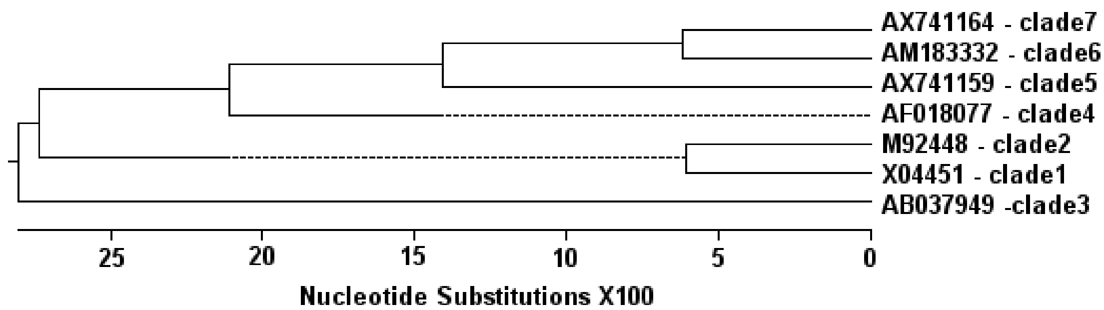
HDV is a subviral satellite and can only replicate in the presence of Hepatitis B Virus (HBV). HDV redirects HBV assembly to package the HDV genome along with multiple copies of the delta antigen, creating a viral particle with a diameter of about 38nm.
After infecting the host liver cells, the viral RNA is replicated in the cell nucleus using the host polymerase. The small delta protein which is comprised of 195 amino acids is essential in this replication process. The large delta protein which has an additional 19 amino acids, is inhibitory to the replication process. After genome replication, the envelope proteins from HBV assemble the new viral particle. This includes the large envelope protein which via the preS1 domain interacts with HBV receptors and spread the virus to the next host cell. Individuals who are infected with HDV and HBV simultaneously are said to have a co-infection, while HDV infection in an individual with a current or previous HBV infection is a super-infection. Vaccinations against HBV also provide vaccination against HDV as it invades new host cells via the same pathway as HBV.

Co-infection results in acute hepatitis of both type B and D with an incubation period of between 3 and 7 weeks. The infection results in fatigue, lethargy and nausea, lasting up to 1 week until the signs of jaundice appear. If the infection is self-limiting then symptoms desist.
Super-infection results in severe, acute hepatitis which may progress to chronic type D hepatitis. In acute infections, symptoms are often severe with rapid onset, and may result in cirrhosis. These fulminant infections are relatively uncommon but can lead to hepatic encephalopathy presenting with personality changes, disturbances in sleep, confusion, somnolence and coma. The mortality rate of fulminant acute hepatitis D can reach 80%. The infection can progress to cirrhosis over a number of years and does so in 60-70% of chronic sufferers. At this stage the only treatment for the infection is liver transplant. Hepatocellular carcinoma can also be found in individuals with chronic HDV infection and is thought to be associated with cirrhosis.

Specificity

The *PrimerDesign™ Quantification Kit for Hepatitis D virus (HDV)* Genomes is designed for the *in vitro* quantification of all HDV clades 1-7. The primers and probe have 100% homology with all reference sequences included in the phylogenic tree below (fig.1). The primer and probes are also 95% homologous to the recently identified clade 8 sequences and are therefore predicted to also to detect this clade with high efficiency. However, due to the inherent instability of RNA viral genomes, it is not possible guarantee quantification of all clinical isolates.

Phylogentic tree of clade members with 100% homology to the primers and probe:



Kit Contents

- **Pathogen specific primer/probe mix (150 reactions BROWN)**
- **Pathogen positive control template (for Standard curve RED)**
- **Internal extraction control RNA (150 reactions BLUE)**
- **Internal extraction control primer/probe mix (150 reactions BROWN)**
- **Endogenous ACTB primer/probe mix (150 reactions BROWN)**
- **Internal extraction control/Pathogen/ACTB RT primer mix (150 reactions GREEN)**
- **RNAse/DNAse free water**

Reagents and equipment to be supplied by the user

- **Real-Time PCR Instrument**
- **Mastermix or Mastermix components**
  This kit is designed to work well with all commercially available Mastermixes. However, we recommend the use of PrimerDesign 2x *Precision*™ Mastermix.
- **Pipettors and Tips**
- **Vortex and centrifuge**
- **Thin walled 1.5 ml PCR reaction tubes**

Kit storage and stability

This kit is stable at room temperature but should be stored at -20°C on arrival. Once the lyophilized components have been re-suspended unnecessary repeated freeze/thawing should be avoided. Under these conditions reagents are stable for six months from date of purchase.

Suitable sample material

All kinds of sample material suited for PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration, and RNA/DNA integrity (An internal PCR control is supplied to test for non specific PCR inhibitors). Always run at least one negative control with the samples. To prepare a negative-control, replace the template RNA sample with RNAse/DNAse free water.

Dynamic range of test

Under optimal PCR conditions PrimerDesign pathogen detection kits have very high priming efficiencies of >95% and can detect between $1X10^8$ and $1X10^2$ copies of target template.

Principles of the test

Real-time PCR

A pathogen specific primer and probe mix is provided and the target sequence can be detected through the **FAM** channel.

The primer and probe mix provided exploits the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the pathogen DNA/cDNA. A fluorogenic probe, is included in the same reaction mixture which consists of an oligonucleotide labeled with a 5`-reporter dye and a downstream, 3`-quencher, During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of real-time PCR platforms.

Positive control

For copy number determination, and as a positive control for the PCR set up, the kit contains positive control template. This can be used to generate a standard curve of pathogen copy number / CT value. Alternatively the positive control can be used at a single dilution for a qualitative analysis of the samples. Each time the kit is used, at least one positive control reaction must be included on the run. A positive result indicates that the primers and probes for quantification of the target pathogen gene are working properly in your particular experimental scenario. If a negative result is obtained the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false positive results. This can be achieved by handling this component in a Post PCR environment.

Negative control

To confirm absence of contamination, a negative control reaction should be included every time the kit is used. For this reaction, the RNAse/DNAse free water should be used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run. If a positive result is obtained the results should be ignored and the test samples repeated. Possible sources or contamination should first be explored and removed.

Internal RNA extraction control

When performing RNA extraction, it is often advantageous to have an exogenous source of RNA template that is spiked into the lysis buffer. This control RNA is then co-purified with the sample RNA and can be detected as a positive control for the extraction process. Successful co-purification and real-time PCR for the control RNA also indicates that PCR inhibitors are not present at a high concentration.

A separate RT primer mix and a real-time PCR primer/probe mix are supplied with this kit to detect the exogenous RNA using real-time PCR. The PCR primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control cDNA does not interfere with detection of the pathogen target cDNA even when present at low copy number. The Internal control is detected through the **VIC** channel and gives a CT value of 26+/-3.

Endogenous ACTB control

To confirm extraction of a valid biological template a primer and probe mix is included to detect the Actin Beta (ACTB) gene. Detection of ACTB is through the FAM channel and it is **NOT** therefore possible to perform a multiplex for ACTB and the pathogen primers. A poor ACTB signal may indicate that the sample did not contain sufficient human material.

Carry-over prevention using UNG (optional)

Carry over contamination between PCR reactions can be prevented by including uracil-N-glycosylase (UNG) in the reaction mix. Some commercial mastermix preparations contain UNG or alternatively it can be added as a separate component. UNG can only prevent carry over from PCR reactions that include deoxyuridine triphosphate (dUTP) in the original PCR reaction. PrimerDesign recommend the application of 0.2 U UNG per assay with a 15 minute incubation step at 37°C prior to amplification. The heat-labile UNG is then inactivated during the Taq polymerase activation step (95°C for 10 minutes).

Bench side Protocol

To minimize the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR cabinet. Filter tips are recommended for all pipetting steps.

1. **Pulse-spin each tube in a centrifuge before opening.**
   This will ensure lyophilised primer and probe mix is in the base of the tube and is not spilt upon opening the tube.

2. **Reconstitute the kit components according to the table below**
   To ensure complete resuspension, vortex each tube thoroughly, allow to stand for 5 minutes and vortex again before use.

Component	Volume
Pre-PCR box	
Pathogen Primer/Probe mix (**BROWN**)	165 µl
Internal extraction control RNA (**BLUE**)	600 µl
Internal extraction control primer/probe mix (**BROWN**)	165 µl
Internal extraction control/Pathogen/ACTB RT primer mix (**GREEN)**	165 µl
Endogenous ACTB primer/probe mix (**BROWN**)	165 µl
Post PCR bottle	
Positive Control Template (**RED**) *	500 µl

* This component contains high copy number template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.

RNA extraction

The internal extraction control RNA can be added either to the RNA lysis/extraction buffer or to the RNA sample once it has been resuspended in lysis buffer.

DO NOT add the internal extraction control RNA directly to the unprocessed biological sample as this will lead to degradation and a loss in signal strength.

1. **Add 4µl of the Internal extraction control RNA (BLUE) to each sample in RNA lysis/extraction buffer**

2. **Complete RNA extraction according to the manufactures protocols**

Reverse Transcription

The protocols below are for PrimerDesign *Precision*™ Reverse Transcription kit. If you are using an alternative RT kit then follow the manufacturer's instruction.

1. **For each RNA sample prepare a reaction mix according to the table below:**

Component	1 reaction
RNA template	X µl
Internal extraction control/Pathogen/ACTB RT primer mix (**GREEN)**	1.0 µl
dNTP mix 10mM of each (**ORANGE**)	1.0 µl
RNAse/DNAse free water (**WHITE**)	X µl
Final volume	**10 µl**

2. **Heat samples to 65°C for 5 minutes.**
   This can be achieved using a thermostatically controlled "hot block" or heated water bath. Do not heat the RNA to a higher temperature as this can lead to RNA degradation.

3. **Immediately cool the tubes in an ice water bath.**
   It is important that the samples are transferred directly from 65°C to the ice. Do not allow the samples to cool prior to cooling on ice.

4. **For each RNA sample prepare a reaction mix according to the table below:**

Component	1 reaction
MMLV 5Xbuffer (**CLEAR**)	4.0 µl
RNA/DNAse free water (**WHITE**)	5.2 µl
MMLV enzyme (**CLEAR**)	0.8 µl
Final volume	**10 µl**

5. **Add 10µl of this mix to each of the samples on ice.**

6. **Apply a lid to each sample, mix tubes by briefly vortexing and incubate at 42°C for 60 minutes.**
   This can be achieved using a thermostatically controlled "hot-block" or heated water bath

7. **Store cDNA samples at -20°C until use.**

Real-time PCR detection

1. **Prepare a reaction mix according to the tables below:**
   Include sufficient reactions for the standard curve wells (8 samples in duplicate) and also the negative control.

   Pathogen detection mix

Component	1 reaction
2X Precision™ Mastermix	10 µl
Pathogen primer/probe mix (**BROWN**)	1.0 µl
Internal extraction control primer/probe mix (**BROWN**)	1.0 µl
RNAse/DNAse Free water	3.0 µl
Final volume	**15 µl**

   Endogenous ACTB detection mix (Optional)

Component	1 reaction
2X Precision™ Mastermix	10 µl
Endogenous ACTB primer/probe mix (**BROWN**)	1.0 µl
RNAse/DNAse Free water	4.0 µl
Final volume	**15 µl**

2. **Pipette 15µl of this mix into each well according to your real-time PCR experimental plate set up.**

3. **Prepare sample cDNA templates for each of your samples (suggested concentration 5ng/µl) in RNAse/DNAse free water.**
   If the concentration of RNA that was used to make the cDNA is not known, then dilute your RT reaction mix 1:20 (10µl of sample DNA and 90µl of water)

4. **Pipette 5µl of diluted cDNA template into each well, according to your experimental plate set up.**
   For negative control wells use 5µl of RNAse/DNAse free water. The final volume in each well is 20µl

5. Preparation of standard curve dilution series

1) Pipette 900µl of RNAse/DNAse free water into 7 tubes and label 2-8
2) Pipette 100µl of Positive Control Template (**RED**) into tube 2
3) Vortex thoroughly
4) Change pipette tip and pipette 100µl from tube 2 into tube 3
5) Vortex thoroughly

Repeat steps 4 and 5 to complete the dilution series

Standard Curve	Copy Number
Tube 1 Positive control (**RED**)	$2X10^7$ per µl
Tube 2	$2X10^6$ per µl
Tube 3	$2X10^5$ per µl
Tube 4	$2X10^4$ per µl
Tube 5	$2X10^3$ per µl
Tube 6	$2X10^2$ per µl
Tube 7	20 per µl
Tube 8	2 per µl

6. Pipette 5µl of standard template into each well, according to your experimental plate set up.

The final volume in each well is 20µl

Amplification Protocol

Amplification conditions using PrimerDesign 2X *Precision*™ MasterMix.

	Step	Time	Temp
	UNG treatment (if required) **	15 mins	37°C
	Enzyme Activation (if required)***	10 mins	95°C
50 cycles	Denaturation	10s	95°C
	DATA COLLECTION*	60s	60°C

* Fluorogenic data for the control DNA should be collected during this step through the FAM and VIC channels
** Required if your mastermix includes UNG to prevent PCR carryover contamination
*** Not all Mastermixes require this enzyme activation step. Follow the manufactures instructions for your mastermix.

Interpretation of Results

Pathogen	Internal control	Negative control	Positive control	Interpretation
+ve	+ve	-ve	+ve	+ve
+ve	-ve	-ve	+ve	+ve
-ve	+ve	-ve	+ve	-ve
-ve	-ve	-ve	-ve	Exp Fail
+ve	+ve	+ve	+ve	Exp Fail

Internal PCR control

When used according to the above protocols, assuming a 100% extraction efficiency, that 1:4 of extracted RNA is used in the RT reaction and that 1:20 of the RT reaction is used in an individual well, then a CT value of 26 is expected. However this can vary significantly depending on the extraction efficiency, the quantity of RNA added to the RT and PCR reaction and the individual machine settings. CT values of 26±3 are within the normal range. When amplifying a pathogen sample with high genome copy number, the internal extraction control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result.

Endogenous ACTB control

The signal obtained from the ACTB primer and probe set will vary according to the amount of biological material present in a given sample.

Notices and disclaimers

During the warranty period PrimerDesign pathogen detection kits allow precise and reproducible data recovery combined with excellent sensitivity. For data obtained by violation to the general GLP guidelines and the manufacturer's recommendations the right to claim under guarantee is expired.

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