LightMix® Kit *HDV*

Cat.-No. 40-0449-16 **Protocol for the LightCycler® 480 II Instrument included**

Kit with reagents for the detection of the Hepatitis D Virus (*HDV)* genotype 1 using the Roche Diagnostics LightCycler® 2.0 and 480 Instruments.

Lyophilized mix of primers and probes for a total of 96 reactions with a final volume of 20 µl each. **Store protected from light at room temperature (18-25°C), do N OT freeze!**

Instructions for use with the LightCycler® 2.0 Instruments see pages 4-5.
Instructions for use with the LightCycler® 480 Instruments see pages 6-7.

1. Introduction

HDV (Hepatitis D Virus) infections of humans appear only together with a Hepatitis B infection (HBV). Coinfection with *HDV* results in more severe complications than HBV alone. The virus consists of a negative sense, single-stranded circular RNA packed in the HBsAg envelope protein from HBV, explaining the linkage to this virus. Vaccination against HBV prevents also *HDV* infections.

HDV is in some Mediterranean countries, in Rumania and Arabian countries endemic but also found in Africa and South America.

HDV produces two specific delta antigens which are used for the serological detection of the virus. Also most published PCR assays target the HDV delta antigene gene [1-3].

The LightMix® Kit *HDV* provides a fast, easy and accurate system to identify *HDV* genotype 1 in a nucleic acid extract. A control amplification reaction acts as Internal Control (IC).

This LightMix® Kit is tested on the LightCycler® 2.0 / 480 II Instruments with Roche Diagnostics 'LightCycler® FastStart DNA Master HybProbe'. A 1-step RT PCR procedure was not tested.

2. Description

A 113 bp long fragment of the *HDV* delta antigen gene is amplified with specific primers. The resulting PCR fragment is analyzed with hybridization probes labeled with LightCycler® Red 640 (detected in channel 640).

The PCR reaction is monitored by an additional PCR product of 143 bp, formed from the IC. This control does not interfere with the *HDV* specific reactions. The amplification will usually fail in the presence of higher concentrated *HDV c*DNA samples (1,000 copies or higher) while displaying an amplification signal in negative and low-concentrated samples. The hybridization probes are labeled with LightCycler® Red 677. Detection is recorded in channel 705.

The IC is supplied separately to allow running the assay in the presence or absence of the IC.

The use of a color compensation file generated with the Roche Diagnostics 'LightCycler® Multicolor Demo Set' is a prerequisite to run the duplex reaction.

The supplied standard row allows to determine the linear range of the reaction and to estimate the quantity of the target sequence in unknown samples.

3. Set contents

6 Vials with blue caps containing premixed lyophilized primers and probes for 16 PCR reactions each of *HDV.*
6 Vials with white caps containing premixed lyophilized primers and probes for 16 PCR reactions each of the internal positive control (IC).
1 Vial with colorless caps containing the IC-DNA.
1 Standard row with 6 lyophilized cloned plasmid standards of *HDV* from 10^1 to 10^6 target equivalents per reaction.
1 Sealing foil for the standard row.

4. Additional reagents and items required

Roche Diagnostics:

LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
LightCycler® Multicolor Demo Set	Cat.-No. 03 624 854001
High Pure Viral Nucleic Acid Kit	Cat.-No. 11 858 874 001
Transcriptor First Strand cDNA Synthesis Kit	Cat.-No. 04 379 012 001
LightCycler® Capillaries (20 µl)	Cat.-No. 04 929 292 001
LightCycler® 480 Multiwell Plate 384, white (LightCycler® 480 Instruments)	Cat.-No. 04 729 749 001
or LightCycler® 480 Multiwell Plate 96, white (LightCycler® 480 Instruments)	Cat.-No. 04 729 692 001

5. Product characteristics

PCR results are obtained within 50 minutes (50 cycles and melting curve) with the LightCycler® 2.0 Instruments and within 80 minutes (50 cycles and melting curve) with the LightCycler® 480 Instruments.

Sensitivity
These reagents detect 10 copies of *HDV* cDNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler 2.0 / 480 Instruments (in an exemplary system, using cloned targets as reference).

Measuring range
The linear measuring range of the assay is 10^1 to 10^6 copies of *HDV* cDNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler® 2.0 / 480 Instruments.

Storage and Stability
- Lyophilized reagents are stable for at least 3 months after shipment if stored protected from light at room temperature (18-25°C).
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 5 days if stored protected from light and refrigerated (4°C).

6. Experimental protocol

The following procedure was developed for use with the LightCycler® 2.0 / 480 Instruments.
Start programming before preparing the solutions. See the LightCycler® Instrument operator's manual for details.

Sample material: Use aqueous nucleic acid preparations (e.g. Roche Diagnostics 'High Pure Viral Nucleic Acid Kit' combined with Roche Diagnostics 'Transcriptor First Strand cDNA Synthesis Kit').

Negative control: Always run at least one no-template control (NTC) - replace the template DNA with water.

Positive control: Run a positive control - replace the template DNA with the provided control DNA.
For quantification always use a fresh solution prepared from the provided standard row (single use only).

6.1. Preparation of parameter-specific reagents and reagents for the IC (16 reactions):

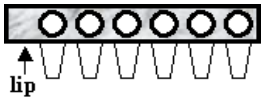
One reagent vial with a **blue** cap contains all primers and probes to run 16 LightCycler® reactions for *HDV*.
One reagent vial with a **white** cap contains all primers and probes to run 16 LightCycler® reactions for the IC.

Add 66 µl PCR-grade water to each reagent vial, mix the solution (vortex) and spin down.

►**Use 4 µl** reagent for a 20 µl PCR reaction.

This solution is stable at least five days when stored refrigerated at 4°C. Avoid prolonged exposure t o light.

6.2. Preparation of the standard row



The target DNA is provided in 6 different quantities to yield from 10^1 to 10^6 target molecules in 5 µl once resolved. Start with the lowest concentrated standard (first tube from the extended lip). Use the pipette tip to punch a hole through the sealing foil. **Add 40 µl** PCR-grade water to each vial of the row. Mix the target DNA by pipetting the solution up and down 10 times.

►**Use 5 µl** standard for a 20 µl PCR reaction.

This standard solution is not long-term stable and will lose sensitivity under prolonged storage. Use only fresh prepared solutions as quantification references. Older solutions can be used as a qualitative standard (positive control). After adding the target DNA to the LightCycler® reaction mix use the provided sealing foil to close the vials in order to avoid changes in the concentration due to evaporation.
Please note that opening these vials may cause contaminations of the work-space (aerosol).

6.3. Preparation of the IC DNA

Add 100 µl PCR-grade water to the vial with a colorless cap. Mix the IC DNA by pipetting the solution up and down 10 times.

►Use 1 µl IC DNA for a 20 µl PCR reaction.

This solution is stable at least five days when stored refrigerated at 4°C, for long term storage free ze at -20°C. Avoid repeated freezing thawing cycles.
Please note that opening these vials may cause contaminations of the work-space (aerosol).

6.4. Preparation of the LightCycler® reaction mix

In a cooled reaction tube, prepare the reaction mix by multiplying each volume for a single reaction by the number of reactions to be cycled plus one additional reaction.

For use with the Roche FastStart Master	
Single reaction	**Component**
1.6 µl	water, PCR-grade (colorless cap, provided with the Roche Master kit)
2.4 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)
4.0 µl	**reagent** mix (parameter specific reagents containing primers and probes, see **6.1.**)
4.0 µl	**IC** mix (IC reagents containing primers and probes, see **6.1.**)
1.0 µl	**IC** DNA, (see **6.3**)
2.0 µl	Roche Master (red cap, for preparation see Roche manual)
15.0 µl	**Volume of reaction mix**

To include the internal positive control **add 4 µl** of the IC reagent per reaction
To run the assay without the internal positive control substitute the µl of IC components with 4 µl PCR-grade water.

Mix gently, spin down and **transfer 15 µl** each of the reaction mix to a LightCycler® capillary.

Add 5 µl of sample or standard to each capillary for a final reaction volume of 20 µl.

Start run.

7. LightCycler® 2.0 Instrument

7.1. Programming

The protocol consists of four program steps

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification of the target DNA
- 3: Melting: melting curve analysis for identification of the PCR product derived from the target DNA
- 4: Cooling: cooling the instrument

Program Step:	Denaturation	Cycling			Melting				Cooling
Parameter									
Analysis Mode	None	Quantification mode			Melting Curves mode				None
Cycles	1	50			1				1
Target [℃]	95	95	62	72	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:05	00:00:15	00:00:05	00:00:20	00:00:30	00:00:00	00:00:30
Ramp Rate [℃/s]	20	20	20	20	20	20	20	0.2	20
Sec Target [℃]	-	-	55	-	-	-	-	-	-
Step Size [℃]	-	-	0.5	-	-	-	-	-	-
Step Delay (Cycles)	-	-	1	-	-	-	-	-	-
Acquisition Mode	None	None	Single	None	None	None	None	Cont	None

(Melting not relevant for detection)

7.2. Data Analysis

Switch the color compensation mode on. If this mode is not enabled run the color compensation program. Follow the instructions in the manual of the Roche Diagnostics 'LightCycler® Multicolor Demo Set'.

Perform data analysis, as described in the LightCycler® Instrument operator's manual.
We recommend using the Second Derivative Maximum method (Automated (F'' max)). The cycle number of the Crossing Point (CP) of each sample is calculated automatically. The Fit Points method is more-error prone due to the user's influence.

View *HDV* data in channel 640, Quantification mode. The negative control (NTC) must show no signal.

If the internal positive control is used, view *HDV* data in channel 640 Quantification mode, and the IC data in channel 705, Quantification mode. The negative control and the low-concentrated *HDV* DNA samples (10 to 1,000 copies) should show an amplification curve for the IC with a CP at approximately cycle 28.

The provided standard row of cloned and purified DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn of *HDV* should have CPs between cycles 16 and 33 (CPs calculated with Second Derivative Maximum method).

7.3. Sample Data – typical results

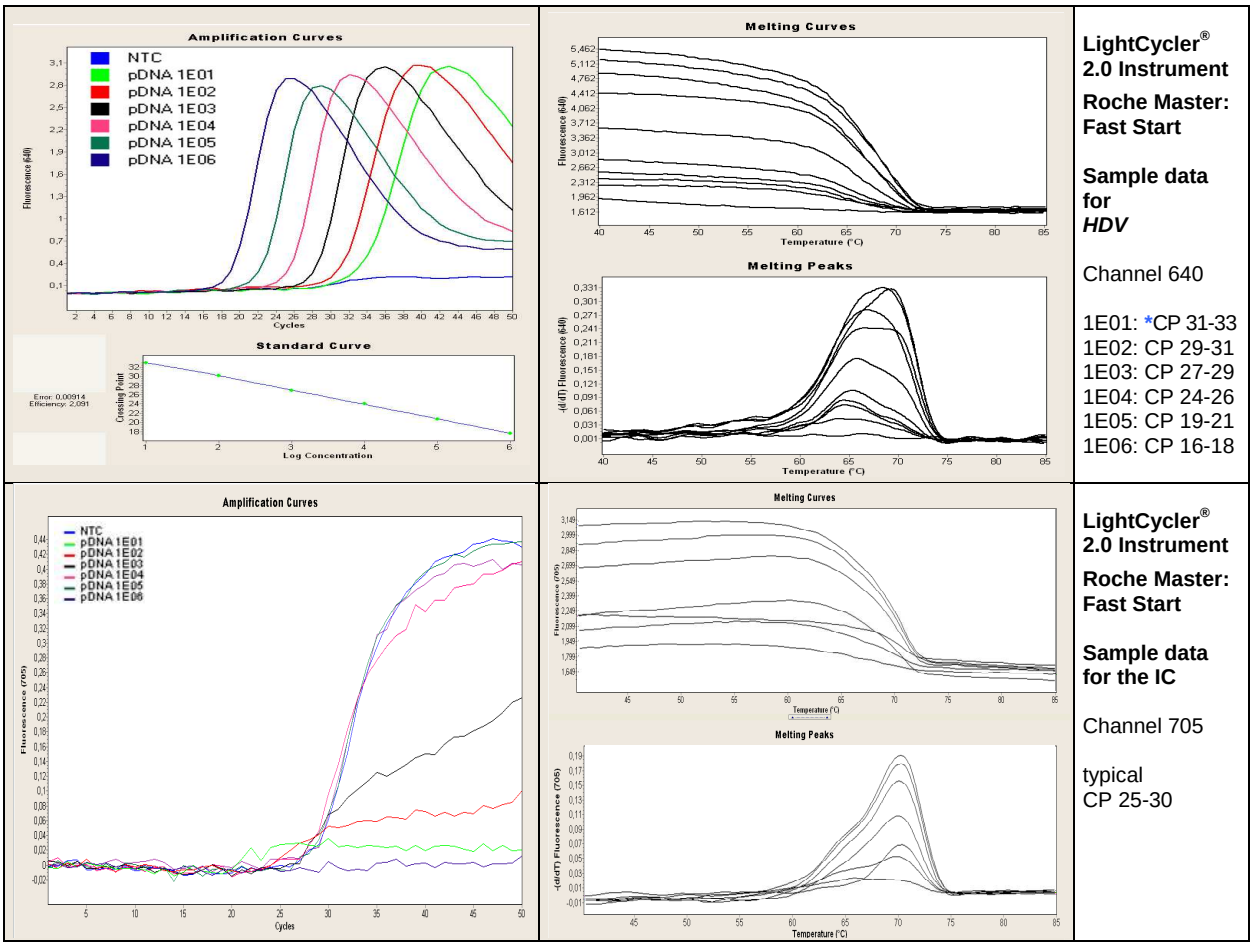


Fig.1. Sample data for the *HDV* detection system.

Upper panels: Data from LightCycler® 2.0 Instrument. Left panel channel 640 quantification mode (Second Derivative Maximum) with calibration curve for *HDV*. Right panel channel 640 melting analysis for *HDV* (not relevant for detection).
Lower panels: Data from LightCycler® 2.0 Instrument. Left panel channel 705 quantification mode (Second Derivative Maximum) for the IC. Right panel channel 705 melting analysis for the IC (not relevant for detection).

* **Note:** Fluorescence levels depend on instrument settings and may vary. The characteristics of the curve (shape and trend of fluorescence levels) should be similar to the curve in the manual. The fluorescent curves over cycles (quantification mode) must be smooth and not zigzag shaped. The CP values will vary from instrument to instrument by up to 2 cycles, while the distance between two dilution steps should be relative constant (delta CP). The CP values described in this manual (chart text) have been obtained using the supplied standard row; the entire set of curves can be horizontal shifted.

7.4. Interpretation of data

HDV **(sample)**	**HDV (positive control)**	**IC (sample)**	**NTC**	**Result**
no amplification	amplification signal	detectable	negative	Negative
amplification signal	amplification signal	not relevant	negative	Positive
no amplification	amplification signal	not detectable	not relevant	PCR failure, repeat experiment
not relevant	no amplification	not relevant	not relevant	PCR failure, repeat experiment
not relevant	not relevant	not relevant	positive	Contamination, repeat experiment

Tab. 3. Typical analysis results (LightCycler® 2.0 Instrument, Roche Master: Fast Start)

8. LightCycler® 480 Instruments

8.1. Programming

The protocol consists of four program steps

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification of the target DNA
- 3: Melting: melting curve analysis for identification of the PCR product derived from the target DNA
- 4: Cooling: cooling the instrument

Detection Format:
LightCycler® 480 Instrument: 483-533, 483-640, 498-670
LightCycler® 480 II Instrument: 465-510, 498-640, 498-660

Program Step:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	50			1			1
Target [°C]	95	95	62	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:05	00:00:15	00:00:30	00:02:00	00:00:00	00:00:30
Ramp Rate [°C/s] **96**	4.4	4.4	2.2	4.4	4.4	1.5	-	1.5
Ramp Rate [°C/s] **384**	4.6	4.6	2.4	4.6	4.6	2.0	-	2.0
Sec Target [°C]	-	-	55	-	-	-	-	-
Step Size [°C]	-	-	0.5	-	-	-	-	-
Step Delay (Cycles)	-	-	1	-	-	-	-	-
Acquisition Mode	None	None	Single	None	None	None	Continuous	None
Acquisitions [per °C]	-	-	-	-	-	-	3*	-

(Melting not relevant for detection)

8.2. Data Analysis

Note: For use on LightCycler® 480 Instrument select Filter Combination 483-533 instead of Filter Combination 465-510, Filter Combination 483-640 instead of Filter Combination 498-640 and Filter Combination 483-670 instead of Filter Combination 498-660 for detection.

Switch the color compensation mode on. If this mode is not enabled run the color compensation program. Follow the instructions in the manual of the Roche Diagnostics 'LightCycler® Multicolor Demo Set'.

Perform data analysis, as described in the LightCycler® Instrument operator's manual.
We recommend using the Second Derivative Maximum method (Automated (F" max)). The cycle number of the Crossing Point (CP) of each sample is calculated automatically. The Fit Points method is more-error prone due to the user's influence.

View *HDV* data with Filter Combination 498-640. The negative control (NTC) must show no signal.

The provided standard row of cloned and purified DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn of *HDV* should have CPs between cycles 17 and 34 (CPs calculated with Second Derivative Maximum method).

If the internal control is used, view IC data with Filter Combination 498-660, Quantification mode. The negative control and the low-concentrated *HDV* DNA samples (10 to 1,000 copies) should show an amplification curve for the IC with a CP at approximately cycle 25-28.

8.3. Sample Data – typical results

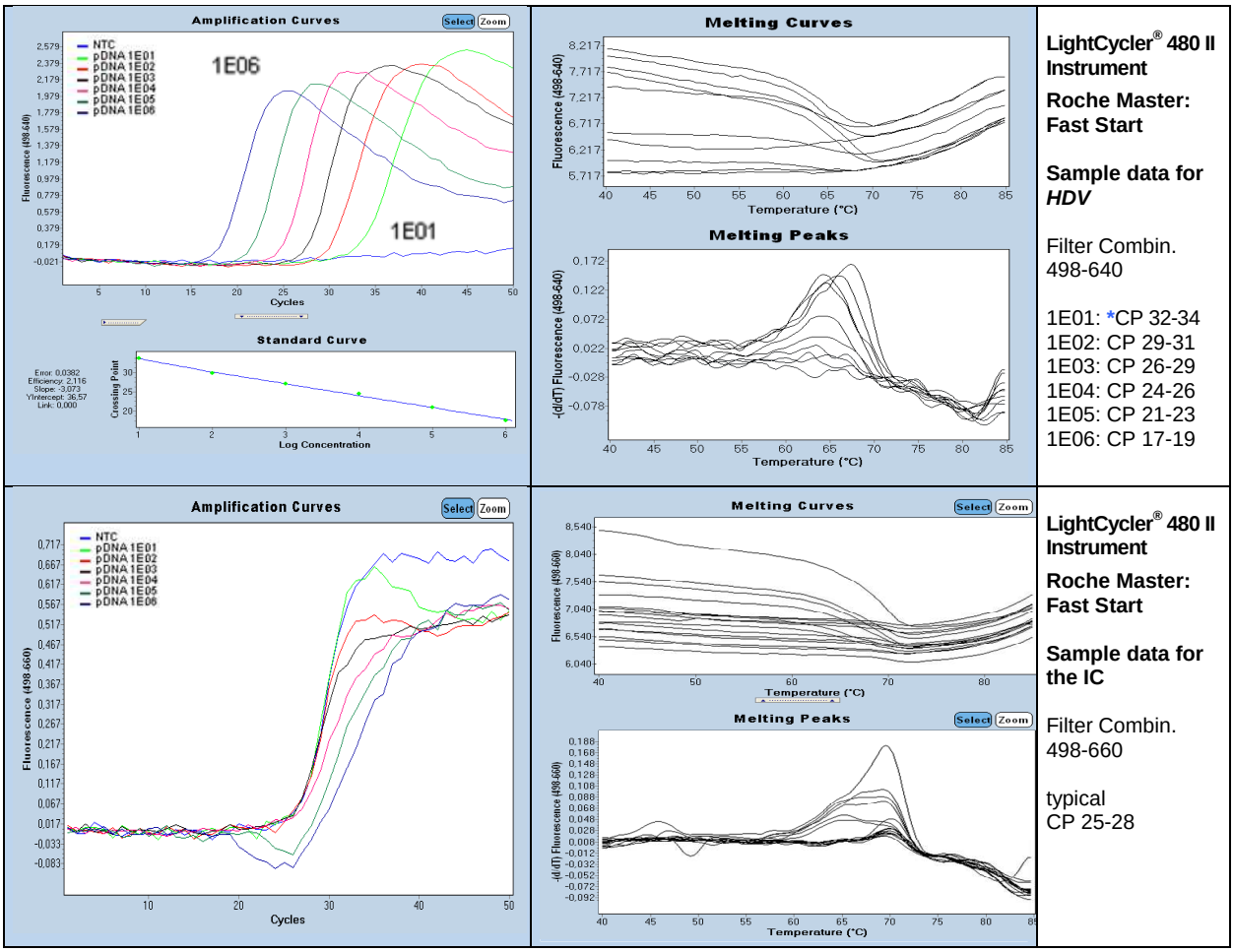


Fig.2. Sample data for the *HDV* detection system.

Upper panels: Data from LightCycler® 480 II Instrument. Left panel Filter Combination 498-640 quantification mode (Second Derivative Maximum) with amplification curves for *HDV*. Right panel Filter Combination 498-640 melting analysis for *HDV* (not relevant for detection)
Lower panels: Data from LightCycler® 480 II Instrument. Left panel Filter Combination 498-660 quantification mode (Second Derivative Maximum) for the IC. Right panel Filter Combination 498-660 melting analysis for the IC (not relevant for detection).

* **Note:** Fluorescence levels depend on instrument settings and may vary. The characteristics of the curve (shape and trend of fluorescence levels) should be similar to the curve in the manual. The fluorescent curves over cycles (quantification mode) must be smooth and not zigzag shaped. The CP values will vary from instrument to instrument by up to 2 cycles, while the distance between two dilution steps should be relative constant (delta CP). The CP values described in this manual (chart text) have been obtained using the supplied standard row; the entire set of curves can be horizontal shifted.

8.4. Interpretation of data

HDV (sample)	HDV (positive control)	IC (sample)	NTC	Result
no amplification	amplification signal	detectable	negative	Negative
amplification signal	amplification signal	not relevant	negative	Positive
no amplification	amplification signal	not detectable	not relevant	PCR failure, repeat experiment
not relevant	no amplification	not relevant	not relevant	PCR failure, repeat experiment
not relevant	not relevant	not relevant	positive	Contamination, repeat experiment

Tab. 5. Typical analysis results (LightCycler® 480 II Instrument, Roche Master: Fast Start)

9. Version History

V080709	Instructions for the LightCycler® 2.0 Instrument
V100819	Note to LightCycler® 1.x software version
V110310	Protocol for LightCycler® 480 Instruments and Version History
V110328	Restriction to detect HDV genotype 1 only
V110804	Change of color of hybridization probes, now 677. Read out in channel 705

10. References

[1] 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

[2] Quantification 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 et al., JCM 43.5.2363-2369 (2005)

[3] Establishment of a Novel Quantitative Hepatitis D Virus (HDV) RNA Assay Using the Cobas TaqMan Platform To Study HDV RNA Kinetics. Mederacke et al., JCM (2010)

These reagents were developed and manufactured by TIB MOLBIOL GmbH, Berlin, Germany.
LightCycler® hybridization probes produced under license from Roche Diagnostics GmbH.