A novel real-time RT-PCR test kit for detection and quantification of hepatitis D virus (HDV)

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1 Introduction

Hepatitis D virus (HDV) is an enveloped satellite virus with a circular negative-sense ssRNA genome causing liver disease in both acute and chronic forms. Since hepatitis B virus (HBV) envelope proteins are required for HDV packaging, HDV cannot propagate without HBV infection. Therefore, HDV infection cannot occur in the absence of HBV. Approximately 15 million people are co-infected with HDV and HBV worldwide. In >95% of the cases the infection is acute and self-limited. But the co-infection of HBV with HDV causes more severe disease than HBV mono-infection. If the infection with HDV occurs after the infection with HBV (superinfection) the course of the disease and liver damage is more severe. In general, co-infection with HDV leads to a ten-fold higher mortality in patients than infections with HBV only. HDV is transmitted by contact with blood and/or body fluids of infected persons. Even though there is no HDV vaccine, protection can be achieved by HBV vaccination. Currently HDV is grouped in eight clades with a different distribution pattern worldwide. Since HDV infections are emerging, there is a desperate need for reliable assays to detect and monitor the abundance of HDV.

The developed RealStar® HDV RT-PCR Kit 1.0 allows the detection and quantification of HDV RNA of all eight clades.*

2 Methods

The RealStar® HDV RT-PCR Kit 1.0 detects and quantifies HDV RNA. It contains an Internal Control to monitor the efficiency of the nucleic acid extraction process and possible inhibitory effects during PCR. Probes specific for HDV RNA are labelled with the fluorophore FAM™. The probe specific for the Internal Control (IC) is labelled with the fluorophore JOE™. All experiments were performed using the CFX96™ Deep Well Real-Time PCR Detection System (Bio-Rad).

The four Quantification Standards of the RealStar® HDV RT-PCR Kit 1.0 were calibrated against the 1st World Health Organization International Standard for Hepatitis D Virus RNA for Nucleic Acid Amplification Techniques (NAT)-Based Assays (HDV WHO Standard).

The analytical sensitivity (Limit of Detection: LoD) is defined as the concentration (IU/µl) of HDV specific RNA molecules that can be detected with a positivity rate of ≥ 95%. The analytic LoD was determined using half-logarithmic dilutions ($3.16x10^{-5}$ IU/µl to $1x10^{0}$ IU/µl) of a quantified HDV specific *in vitro* transcript (IVT).

The analytical specificity of the assay was evaluated by testing 30 HDV-negative human plasma samples and different pathogens found in human plasma or causing similar symptoms as HDV.

The linear range was determined by testing a logarithmic dilution series of $4x10^{-3}$ to $4x10^{5}$ IU/µl (n=7) of HDV IVT.

In order to verify the detection of all eight HDV clades (Reactivity), IVTs including the target sequence of the corresponding reference sequences were synthesized and tested with the RealStar® HDV RT-PCR Kit 1.0.

RNA from 40 pretested HDV-positive clinical plasma and serum samples was extracted using either the QIAamp® Viral RNA Mini Kit or the AltoStar® Purification Kit 1.5 and tested with the RealStar® HDV RT-PCR Kit 1.0. In addition, eight HDV-positive serum samples were tested with the RealStar® HDV RT-PCR Kit 1.0 and the quantitative results were compared to the results from the UniversitätsSpital Zürich (USZ). The use of samples tested at the USZ underwent signed patients' approval.

3 Results

Reactivity

The RealStar® HDV RT-PCR Kit 1.0 detects all eight clades of HDV (see Figure 1).

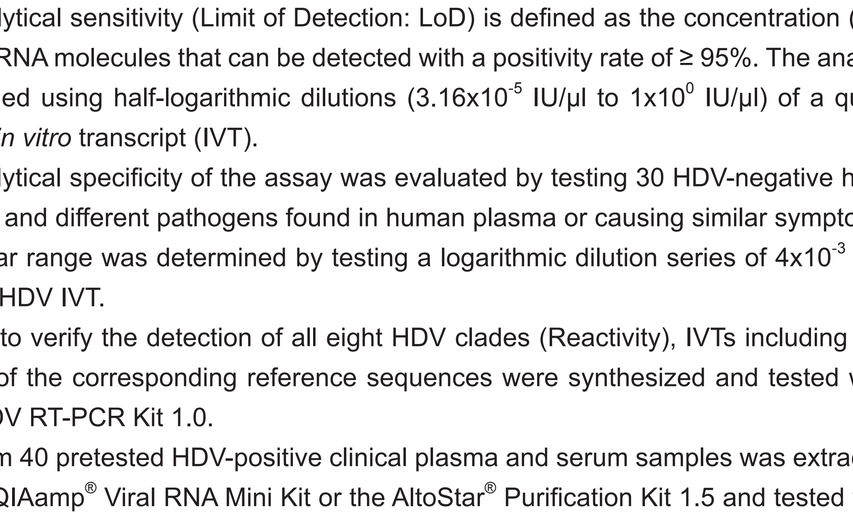

Figure 1: Amplification of HDV clades 1-8 IVTs (FAM). $1x10^{4}$ cop/µl of clade 1 (red), clade 2 (cyan), clade 3 (purple), clade 4 (gray), clade 5 (dark green), clade 6 (light green), clade 7 (brown) and clade 8 (black) were tested in duplicates. All clades were detected.

Analytical sensitivity

The LoD of the RealStar® HDV RT-PCR Kit 1.0 was determined to be 9.48 $x10^{-3}$ IU/µl.

Table 1: Test results of different pathogens with the RealStar® HDV RT-PCR Kit 1.0.

Pathogen	HDV	Internal Control
HBV	Negative	Valid
HAV	Negative	Valid
HCV	Negative	Valid
HEV	Negative	Valid
HBV	Negative	Valid
EBV	Negative	Valid
CMV	Negative	Valid
HSV-1	Negative	Valid
HSV-2	Negative	Valid
Mumps virus	Negative	Valid
HAdV	Negative	Valid
Entamoeba histolytica	Negative	Valid

Analytical specificity

30 HDV-negative human plasma samples were tested negative using the RealStar® HDV RT-PCR Kit 1.0. Testing of the RealStar® HDV RT-PCR Kit 1.0 with high concentrations of DNA/RNA of different pathogens listed in Table 1 showed no cross-reactivity.

Quantification standards and standard curve

The Quantification Standards of the RealStar® HDV RT-PCR Kit 1.0 (see Figure 2) were calibrated against the HDV WHO standard (see Figure 3).

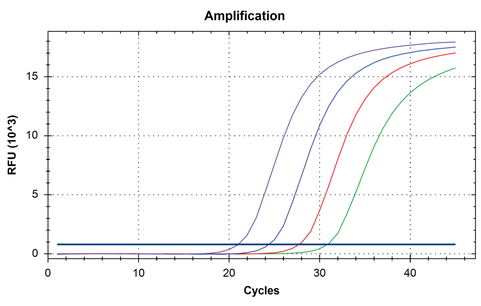

Figure 2: Amplification of Quantification Standards QS1 ($1x10^{3}$ IU/µl, purple), QS2 ($1x10^{2}$ IU/µl, blue), QS3 ($1x10^{1}$ IU/µl, red) and QS4 ($1x10^{0}$ IU/µl, green) (FAM).

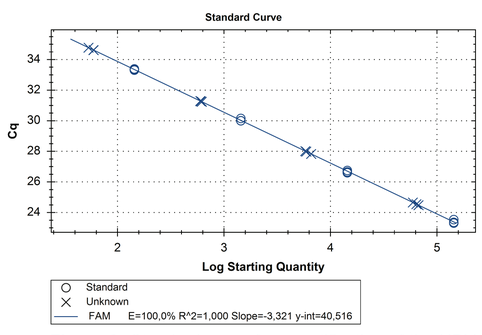

Figure 3: Standard curve for quantification of the HDV IVT using the HDV WHO standard. A dilution series of $1x10^{1}$ to $1x10^{4}$ cop/µl of the HDV IVT (crosses) was calibrated in IU/µl using a dilution series of $1.15x10^{1}$ to $1.15x10^{4}$ IU/ml of the HDV WHO Standard (circles). The input to output ratio during NA extraction was considered in the calculation of the standard curve.

Linear range and Internal Control

Using linear regression analysis the linear range of the RealStar® HDV RT-PCR Kit 1.0 was determined to be $4x10^{-2}$ to $4x10^{5}$ IU/µl (see Figure 4). For monitoring of the efficiency of the nucleic acid extraction process and possible PCR inhibitory effects, the Internal Control is added during the extraction (see Figure 5).

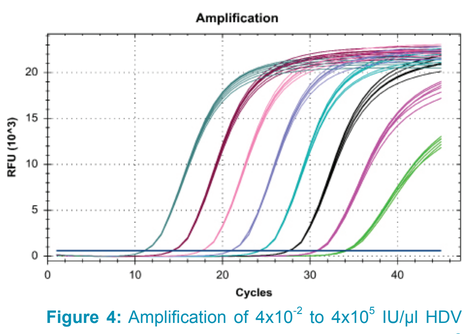

Figure 4: Amplification of $4x10^{-2}$ to $4x10^{5}$ IU/µl HDV IVT (FAM) depicting the linear range of the RealStar® HDV RT-PCR Kit 1.0.

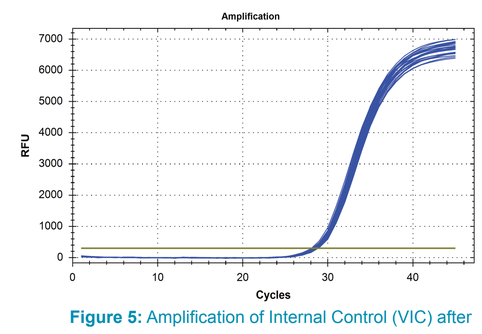

Figure 5: Amplification of Internal Control (VIC) after nucleic acid extraction of 30 HDV-negative plasma samples.

Retrospective analysis and HDV quantification of HDV-positive human plasma and serum samples

All 40 samples previously tested positive for HDV were tested positive with the RealStar® HDV RT-PCR Kit 1.0 (see Figure 6).

All eight serum samples from patients at the UniversitätsSpital Zürich previously tested positive for HDV were tested positive with the RealStar® HDV RT-PCR Kit 1.0 and the quantification results differed by less than 0.3 log (see Table 2).

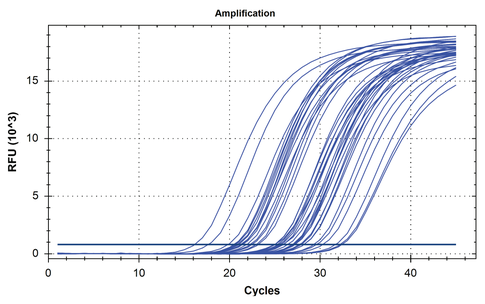

Figure 6: Amplification of 40 HDV-positive plasma and serum samples (FAM). All samples were tested positive with the developed RealStar® HDV RT-PCR Kit 1.0.

Table 2: Comparison of quantification results at altona Diagnostics GmbH and UniversitätsSpital Zürich.

Sample	Quantification result altona Diagnostics GmbH (IU/ml)	Quantification result UniversitätsSpital Zürich (IU/ml)	Log difference UniversitätsSpital Zürich/altona Diagnostics GmbH
1	$6.89x10^{5}$	$8.63x10^{5}$	0.10
2	$6.87x10^{5}$	$1.35x10^{6}$	0.29
3	$4.44x10^{6}$	$3.31x10^{6}$	-0.13
4	$1.03x10^{5}$	$1.05x10^{5}$	0.01
5	$9.67x10^{5}$	$1.03x10^{6}$	0.03
6	$1.06x10^{7}$	$1.07x10^{7}$	0.003
7	$2.92x10^{5}$	$2.18x10^{5}$	-0.13
8	$1.96x10^{2}$	$1.86x10^{2}$	-0.02

4 Conclusion

The RealStar® HDV RT-PCR Kit 1.0 provides a reliable, highly specific and sensitive real-time RT-PCR based assay for the detection and quantification of hepatitis D virus RNA.

References

Rizzetto, M. Hepatitis D: Thirty years after. J Hepatol 2009 May;50(5):1043-1050.

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