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# Developing RT-qPCR Kit for Detection and Quantification of Hepatitis D Virus Unique Genome

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## Abstract

**BACKGROUND/AIMS:** Hepatitis D virus (HDV) is a significant global health problem identified in the 1970s in Hepatitis B virus (HBV) positive patients. HDV is classified under the Kolmioviridae family and has a unique single-stranded negative-sense circular RNA genome. Its replication relies on HBV surface antigens making HBV co-infection essential. HDV infection can occur either simultaneously with HBV or as a superinfection. This virus frequently leads to progressive liver disease, with approximately 70% of cases developing cirrhosis, highlighting the critical need for early diagnosis. This study aims to develop a high sensitivity and specificity quantitative reverse transcription polymerase chain reaction (RT-qPCR) (diagnostic kit for detecting HDV RNA in plasma samples. The newly developed kit is expected to provide reliable diagnosis and facilitate the early detection of HDV, thereby improving clinical outcomes and epidemiological surveillance.

**MATERIALS AND METHODS:** Following the RNA isolation step using the RN easy RNA Purification commercial kit (QIAGEN, Cat. No: 74104), the samples underwent a heat-shock protocol, 95 °C for 10 minutes, followed by rapid freezing at -20 °C to disrupt the secondary structure of the HDV RNA and enhance primer binding efficiency. The single-step RT-qPCR assay was carried out using a specific primer-probe set targeting conserved regions of the HDV genome, along with a human *ribosomal protein (RP)* gene as an internal control, to validate RNA extraction and the absence of sample degradation. RT-qPCR was performed using the QIAGEN Rotor-Gene Q-5plex device.

**RESULTS:** Developed RT-qPCR diagnostic kit successfully detected HDV RNA in all patient samples with high specificity. The fluorescence signals obtained from both the FAM (HDV target) and HEX (*RP* gene) channels confirmed the accurate amplification of the target regions. The kit was further validated using blind samples obtained from the Molecular Diagnosis and Quality Control Laboratory to ensure its clinical applicability and robustness.

**CONCLUSION:** The development of a novel HDV-specific RT-qPCR diagnostic kit provides a valuable tool for the early, accurate detection and quantification of HDV RNA in clinical samples. The kit's ability to offer rapid and reliable results, coupled with its high sensitivity and specificity, makes it an excellent candidate for widespread clinical use and epidemiological monitoring. Further validation studies are recommended to

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## Abstract

expand its application across diverse clinical settings and to evaluate its performance in different HDV genotypes. Furthermore, the in-house kit that was produced is thought to be a more affordable alternative to the current commercial kits, which might make it more accessible, particularly in low-income nations.

**Keywords:** Hepatitis delta, hepatitis delta virus, real time PCR, reverse transcriptase PCR

## INTRODUCTION

The global burden of hepatitis D virus (HDV)-related liver disease is significant, affecting an estimated 12 to 72 million people worldwide, with a high risk of severe liver complications such as cirrhosis and hepatocellular carcinoma.[1,2] Despite its severity, HDV remains underdiagnosed due to inadequate screening practices, particularly in populations co-infected with hepatitis B virus (HBV).[3,4] Recent studies indicate significant increases in reported HDV cases, particularly in regions including the United States (US) and parts of South America. Areas such as the Amazon basin and parts of Colombia show high HDV prevalence, exacerbated by poor healthcare access.[5,6]

There are several difficulties in the prevention of hepatitis D infections, including co-infection with HBV. Additionally, there is a lack of adequately effective and widespread processes for monitoring or detecting HDV, such as routine HDV antibody testing. There is also insufficient screening focused on high-risk groups. These groups include injection drug users, individuals with, multiple and/or unprotected sexual partners-particularly among men who have sex with men-and migrants from endemic regions. Furthermore, there is limited availability and access to treatment options.[1-4,7] There are currently no Food and Drug Administration (FDA)-approved drugs for the treatment of HDV in the US. However, bulevirtide is conditionally approved in the European Union and is under review with the US FDA. Current treatment strategies in many countries are based on the use of pegylatedinterferonalfa2a, which has limited efficacy.[3]

PCR-based diagnostics, particularly reverse transcription polymerase chain reaction (RT-qPCR) and (Droplet Digital Polymerase Chain Reaction), play a crucial role in monitoring HDV transmission and treatment outcomes. These methods enhance the detection and quantification of HDV-RNA, providing valuable insights into disease progression and therapeutic efficacy.[8,9] HDV has a circular and small RNA genome (1.7 kb) that exhibits high intramolecular base pairing, complicating amplification efforts.[10] However, there are at least eight known genotypes of HDV, each with distinct sequences, leading to variability in assay performance.[11] While PCR technologies significantly enhance the monitoring of HDV, challenges remain in the widespread implementation of these technologies, particularly in resource-limited settings where access to advanced diagnostic tools may be restricted. Our aim in this study was to develop an HDV-specific RT-qPCR diagnostic kit and position it as a valuable tool for the early and accurate detection and quantification of HDV RNA in clinical samples. Additionally, it is believed that the developed in-house kit offers a more cost-effective option compared to existing commercial kits, making it potentially more accessible, especially in low-income countries.

## MATERIALS AND METHODS

### Sample Collection and RNA Isolation

The evaluation panel included plasma samples obtained from 10 HDV positive patients from Kocaeli, Türkiye, that were referred to the laboratory previously and stored at -70 °C. RNA isolation from plasma samples was performed with the QIAGEN EZ1 automated sample purification device using the RNeasy RNA Purification commercial kit (QIAGEN, Cat. No:74104), according to the manufacturer's instructions. After isolation, to reduce the likelihood of natural internal base pairing being restored in the HDV genome, thermal shock was performed by incubating 10 µl of isolate at 95 °C for 10 minutes (min), followed by immediate cooling to -20 °C. The study received approval from Manisa Celal Bayar University Ethics Committee (approval number: 330, date: 19.09.2022) and Informed consent forms were signed by all participants and/or legal guardians

### HDV-specific RT-qPCR Diagnostic Kit Design

Sequence alignment of HDV strains (genotype 1-8) full-length genome was performed to locate conserved regions suitable for primer and probes. Primers and probes are designed for the HDV Ag) region.

Our forward primer (19bp) is positioned between 721 to 739 bp of the HDV genome, which corresponds to a palindromic sequence region. The reverse primer (20 bp) binds to the 1085-1104 bp region, located between the overlapping large hepatitis delta antigen (L-HDAg) and small hepatitis delta antigen regions encoded by ORF1. Our primers were designed to be diagnostically compatible with all 8 genotypes of HDV. For this purpose, SnapGene (GSL Biotech, available at snapgene.com) and the NCBI BLAST program were utilized. The sequences of the target gene were obtained from the HDVdb database.[12] The SnapGene tool was used for primer and probe design for HDV and human *ribosomal protein* (*RP*) genes as the internal control. Gene-specific probes were labelled with different fluorescent dyes. FAM for HDV L-HDAg region and HEX for the human *RP* gene were used. In silico specificity tests of designed primer and probe sequences were performed for 32 different viral and bacterial strains to avoid any possible cross-reaction during PCR. The PCR assay was performed using Takyon™ One-Step Low Rox Probe 5X MasterMix dTTP (Cat No: UF-LP5X-RT0501, Takyon™, Eurogentec Belgium) on the Rotor-Gene Q 5Plex Real-Time PCR (QIAGEN, Hilden, Germany). The optimized PCR reaction started with cDNA synthesis step at 55 °C for 40 min followed by an initial denaturation step at 95 °C for 15 min and 50 cycles of amplification consisting of denaturation at 95 °C for 20 s, at 62 °C for 60 s for annealing and fluorescent signal acquisition at FAM and HEX channels. The optimized reaction mix included 10 µL of one-step master mix, 0.4 µM final primer concentration, 0.2 µM final probe concentration, 6 µL of nucleic acid sample, and RNase/DNase-free dd$H_2O$ to a final volume of 20 µL.

### Diagnostic Performance, Amplification Efficiency and Analytical Sensitivity

Validation and performance experiments of the designed HDV detection kit were performed by testing 38 blind samples from Quality Control Program in Molecular Diagnosis (MOTAKK, Ankara, Türkiye). The commercially available Fluorion Real-Time PCR HDV 1.0 (Iontek, İstanbul, Türkiye) kit was used for the validation of the designed HDV detection kit; and the validation study was conducted with 10 plasma pooled samples. The limit of detection was tested by five replicates of serial dilutions, (17x101 to 17x105 copies per reaction) of the HDV RNA standard (HDV RNA Viral Load Quantitative & Qualitative Setup Kit, MOTAKK, Ankara, Türkiye). The amplification efficiency (Ex) was determined using the formula Ex = [10^ (-1/slope) - 1] ×100 for the HDV cDNA standard dilution series. A standard curve was generated using cycle threshold values of serial dilutions of the HDV standard.

## RESULTS

### Standardization of the RT-qPCR Conditions

The Multiplex RT-qPCR assay was optimized for the diagnosis of HDV infection, simultaneously targeting a conserved region of the HDV genome (L-HDAg gene region) and human *RP* gene, as the internal control. Upon primer and probe optimization, plasma samples were obtained from 10 patients previously diagnosed with HDV infection. RNA isolates obtained from plasma samples were tested with the RT-qPCR assay in optimized conditions. In HDV-positive samples and a positive control sample, RP (human internal control), and HDV target region were amplified simultaneously, forming sigmoidal curves. In the negative control reactions, $ddH_2O$ was used as the template, which led to no amplification line (Figure 1).

### Performance and Assay Validation

Thirty-eight blind samples from Quality Control Program in Molecular Diagnosis, (MOTAKK, Ankara, Türkiye) were analyzed using the developed kit. Among these, 22 samples tested positive and 16 tested negative. Results were confirmed by the quality control program. For assay validation, commercially available Fluorion Real-Time PCR HDV 1.0 (Iontek, İstanbul Türkiye) kit was used. Samples from 10 clinically confirmed HDV positive patients, 10 HDV negative patients, MOTAKK samples, and no template control samples were analyzed simultaneously with both kits following the manufacturer's instructions. The in-house kit exhibited 100% positive percent agreement with the commercially available diagnostic kit.

### RT-qPCR Efficiency and Limit-of-Detection

The standard curve analysis was performed to test the efficiency, sensitivity, and LOD of the assay. A dilution series was prepared with HDV RNA standard (HDV RNA Viral Load Quantitative & Qualitative Setup Kit, MOTAKK, Ankara, Türkiye) ranging from 1.7+ E1 to 1.7+ E5 copies per reaction. According to that,the LOD of the designed kit was established as 1.7+ E1 copy per reaction. Triplicate RT-qPCR analysis revealed that the results were consistent across technical replicates (Figure 2).

## DISCUSSION

The detection of HDV through PCR presents several significant challenges. These difficulties may stem from the unique characteristics of the virus and the limitations of current methodologies. The technical limitations of HDV PCR significantly impact the accuracy of results, primarily due to the inherent complexities of the HDV and the methodologies employed in testing. These limitations include viral heterogeneity, intra-host diversity, and challenges in assay standardization, which collectively contribute to variability in performance across different laboratories. HDV with a unique genome, exhibits at least eight genotypes, each with distinct genetic characteristics, complicating the design of universal assays.[11] On the other hand, the circular genome of HDV has high intramolecular base pairing, which can hinder effective amplification during PCR.[10] These unique genome features of HDV were considered challenges and were approached strategically during our development of the HDV RT-qPCR kit.

In the HDV PCR test kit development process, the methodology is particularly emphasized. Variability in RNA extraction methods and primer/probe design may lead to inconsistent results across different assays.[11] The lack of automation in some testing environments may increase the risk of human error, affecting reproducibility.[13] In our HDV RT-qPCR kit development studies, we focused on automation to minimize human error, to work with variables in a controlled manner, and to establish standard processes. Examples of these include RNA

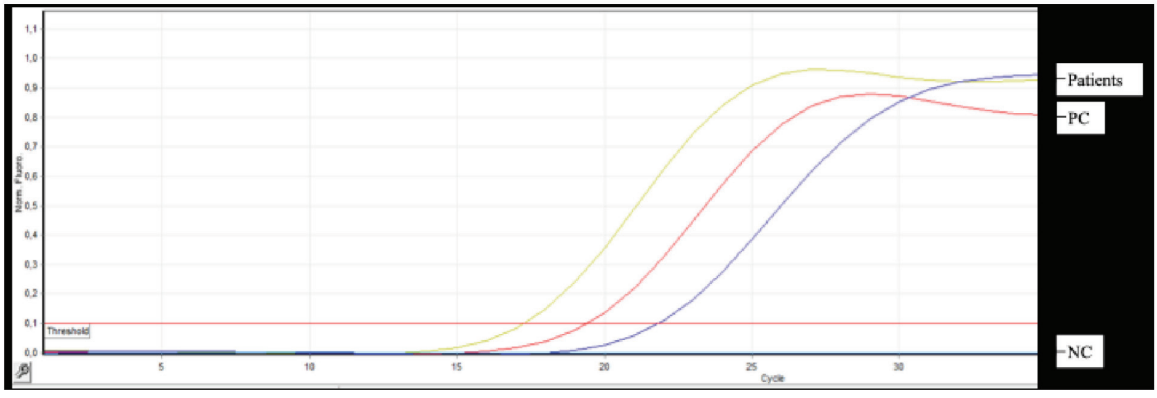


**Figure 1.** RT-qPCR amplification curve graphs of positive control (PC), no template control (NTC) and patient samples for HDV gene-specific region represented in the graph.

HDV: Hepatitis D virus.

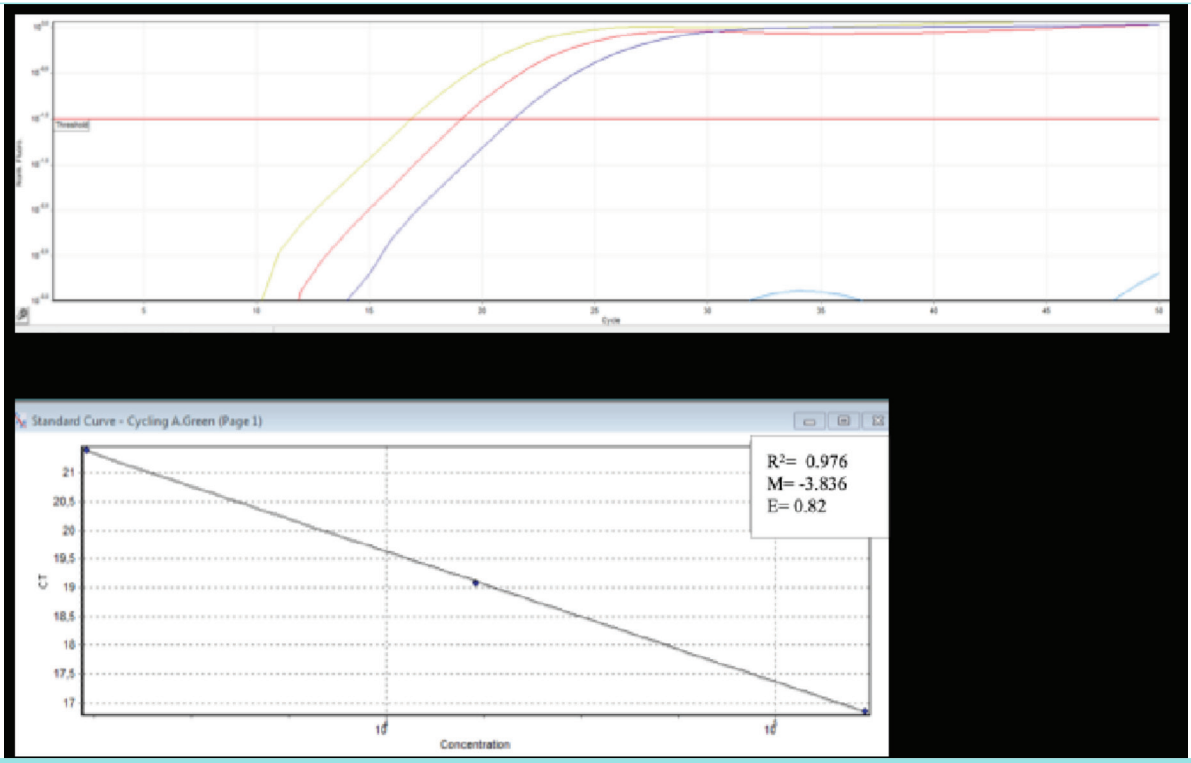


**Figure 2.** Standard curve analysis for multiplex RT-qPCR of *HDV-specific* gene primers. The reactions were carried out in triplicate. The amplification efficiency (E) is shown on the graph.

HDV: Hepatitis D virus.

extraction with EZ1 magnetic particle isolation, and PCR product extraction with Rotorgene Q 5plex thermal cycling. On the other hand, the lack of a universally accepted standard for HDV RNA quantification is a variable that may cause inconsistencies in test sensitivity and specificity.[11,14] In the current study, we tried to overcome this difficulty by using MOTAKK HDV RNA standards. In conclusion, HDV surveillance may need to be increased to better understand the international HDV incidence and etiology. The HDV-specific RT-qPCR diagnostic kit developed in this study may be a valuable tool for early, accurate, and quantitative detection of HDV RNA in clinical samples.

### Study Limitations

Although the HDV-specific RT-qPCR diagnostic kit developed in this study provides important advantages, the study has some limitations. Firstly, the performance of the kit was tested with a limited number of clinical samples. Verification studies with samples containing different HDV genotypes covering a wider geographical region are necessary to better assess the inter-genotype consistency and generalizability of the kit. This may affect the sensitivity of the test.

In addition, the cost of technologies such as the RNA isolation method used in the study and PCR devices may limit the applicability of the kit in low-resource laboratories. Therefore, it is important to explore more accessible and low-cost alternatives.

Finally, only MOTAKK HDV RNA standards were used in the study, and no comparative analysis was performed with international standards. This may affect the comparability of results between different laboratories. Future studies may address these limitations through validation with international standards and evaluation of the kit in further clinical settings.

## CONCLUSION

The specifically designed HDV qRT-PCR detection kit enables the detection of HDV's RNA for early, accurate, and quantitative results. Standardization and validation studies were made possible by comparing the results with a commercially available qRT-PCR kit, which shows 100% concordance. The PCR protocol was optimized because of the unique structure of the HDV genome and its limitations. Furthermore, the results of the designed kit's sensitivity and accuracy were consolidated using MOTAKK standards.

Despite the limitations of the current methods used in the diagnosis of HDV, this study has made significant progress in the molecular diagnosis of HDV with a standardized approach. The designed and developed kit not only improves clinical diagnostic processes but also provides a valuable tool for studies aimed at better understanding the international prevalence and etiology of HDV. In this context, the expansion and increasing accessibility of PCR-based technologies in HDV diagnosis and monitoring will contribute to global HDV control efforts. The cost-effectiveness of the developed kit, particularly in low-income countries, provides a significant contribution to healthcare services in these regions

## MAIN POINTS

- Due to the circular nature of the hepatitis D virus (HDV) genome, molecular diagnostic methods such as reverse transcription polymerase chain reaction face diagnostic challenges. This study aims to overcome these difficulties.
- In today's world, viral hepatitis has become increasingly significant due to large-scale migration.
- There are limited commercial kits available for HDV diagnosis. Therefore, our study focuses on addressing this gap by developing a reliable diagnostic approach.

## ETHICS

**Ethics Committee Approval:** The study received approval from Manisa Celal Bayar University Ethics Committee (approval number: 330, date: 19.09.2022).

**Informed Consent:** Informed consent forms were signed by all participants and/or legal guardians.

### Footnotes

### Authorship Contributions

Concept: K.G., H.S.V., T.Ş., M.S., S.A., H.K.E., F.Ç., G.A., G.T.D., M.K., Design: H.S.V., T.Ş., M.S., G.A., G.T.D., M.K., Data Collection and/or Processing: H.S.V., T.Ş., M.S., G.A., G.T.D., M.K., Analysis and/or Interpretation: K.G., H.S.V., T.Ş., M.S., S.A., H.K.E., F.Ç., G.A., G.T.D., M.K., Literature Search: S.A., H.K.E., F.Ç., Writing: H.S.V., T.Ş., M.S., G.A., G.T.D., M.K.

## DISCLOSURES

**Conflict of Interest:** No conflict of interest was declared by the authors.

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## REFERENCES

1. Negro F, Lok AS. Hepatitis D: A Review. JAMA. 2023; 330(24): 2376-87.
2. Terrault NA, Ghany MG. Enhanced screening for hepatitis d in the USA: overcoming the delta blues. Dig Dis Sci. 2021; 66(8): 2483-5.
3. Khattak A, Vongsavath T, Haque L, Narwan A, Gish RG. The forgotten virus, hepatitis d: a review of epidemiology, diagnosis, and current treatment strategies. J Clin Exp Hepatol. 2024; 14(5): 101395.
4. Tharwani A, Hamid S. Elimination of HDV: Epidemiologic implications and public health perspectives. Liver Int. 2023; 43(Suppl 1):101-7.
5. Fallon BS, Cooke EM, Hesterman MC, Norseth JS, Akhundjanov SB, Weller ML. A changing landscape: tracking and analysis of the international HDV epidemiology 1999-2020. PLOS Glob Public Health. 2023; 3(4): 0000790.
6. Vlachogiannakos J, Papatheodoridis GV. New epidemiology of hepatitis delta. Liver Int. 2020; 40(Suppl 1): 48-53.
7. Örmeci N, Erdem H. Changing trends in the epidemiology of delta virus infection. Viral Hepat J. 2023; 29(1): 1-9.
8. da Silva Queiroz JA, Roca TP, Souza RB, de Souza LFA, Passos-Silva AM, da Silva ALF, et al. Development of quantitative multiplex RT-qPCR one step assay for detection of hepatitis delta virus. Sci Rep. 2023; 13(1): 12073.
9. Olivero A, Rosso C, Ciancio A, Abate ML, Nicolosi A, Troshina G, et al. Clinical application of droplet digital PCR for hepatitis delta virus quantification. Biomedicines. 2022; 10(4): 792.
10. Pflüger LS, Volz T, Giersch K, Allweiss L, Dandri M, Lütgehetmann M. Method for Quantitative HDV RNA Detection: I, manual workflow (serum and Liver Tissue) and II, fully automated high throughput workflow for diagnostic Use. Methods Mol Biol. 2024; 2837: 171-84.
11. Wedemeyer H, Leus M, Battersby TR, Glenn J, Gordien E, Kamili S, et al. HDV RNA Assays Writing Group at the HBV Forum. HDV RNA assays: performance characteristics, clinical utility, and challenges. Hepatology. 2025; 81(2): 637-50.
12. HDVdb - Database Commons. (2020). Cncb.ac.cn. https://ngdc.cncb.ac.cn/databasecommons/database/id/7022 (Access date; 24 November 2024)
13. Pflüger LS, Nörz D, Volz T, Giersch K, Giese A, Goldmann N, Glebe D, Bockmann JH, Pfefferle S, Dandri M, Schulze Zur Wiesch J, Lütgehetmann M. Clinical establishment of a laboratory developed quantitative HDV PCR assay on the cobas6800 high-throughput system. JHEP Rep. 2021; 3(6): 100356.
14. Russo D, Malnati MS. Absolute quantification of viral DNA: the quest for perfection. Methods Mol Biol. 2014; 1160: 75-86.