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Research Article

Half Reaction Volume Optimization of Viral Load Real Time PCR: Lessons, Challenges, and Experience in A Resource Limited Setting

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Abstract

Quantitative polymerase chain reaction (qPCR) is a powerful tool for gene expression analysis.However, qPCR is expensive test however, optimizing the assay can be challenging, especiallywhen working with limited amounts of Nucleic acid. This study aimed to evaluate and optimizehalf reaction qPCR approach for the detection and quantitation of Hepatitis B, C and human CMV. **Methods:** The reaction efficiency using half volumes of the RT-qPCR assay were evaluated. Comparison and stratification of Ct values between standard and half reactions of Hepatitis B,Hepatitis C and CMV positive samples was evaluated. **Results:** The qPCR efficiencies of half reaction were 100.9 %, 101.2% and 105.7% of HepatitisB viral load, Hepatitis C viral load, CMV viral load respectively. The R2 for standard reactionwas found to be 0.98, 1 and 1 for all the three PCR assessed as compared to R2 half reactions which was 1. **Conclusions:** Quantitative polymerase chain reaction (qPCR) is a powerful tool for gene expression analysis. Utilization of half volumes of the RT-qPCR assay was optimized andvalidated in this article. We explored the benefits and considerations of this optimization strategy in Hepatitis B, Hepatitis C and CMV viral load assays. The use of the RT-qPCR half-reaction proved feasible and economic for the detection of the same.

Keywords: Quantitative polymerase chain reaction; Hepatitis B, C; human CMV; Standardcurve

Introduction

Quantitative PCR (qPCR) is a widely used molecular biology technique that allows researchers to measure the expression of genes in a sample. This powerful tool is based onthe polymerase chain reaction (PCR), which amplifies a specific DNA sequence. The first step in qPCR is to extract RNA from the sample of interest. RNA is then reverse transcribedinto complementary DNA (cDNA) using reverse transcriptase enzymes. The cDNA is then amplified using specific primers that target the gene of interest. During amplification, fluorescent probes bind to the amplified DNA, and their fluorescence is measured in real-time by a specialized instrument called a qPCR machine. The amount of fluorescence is directly proportional to the amount of DNA/RNA present in the sample, allowing researchers to measure gene expression levels accurately. Quantitative viral load assays are laboratory teststhat measure the viremia load in a patient's blood. These tests are commonly used to monitorthe progression of viral infections, such as HIV, hepatitis B, and hepatitis C. The results of these tests can help healthcare providers determine the effectiveness of treatment and adjust as needed. Various consensus guidelines about initiating treatment in patients have been formulated which includes HBeAg positivity, viral load measured by real time polymerase chain reaction, elevated transaminases, histological lesions, duration of CHB (age > 40 yearsis likely to be a surrogate marker of disease duration) and family history of HCC [2-5]. Similarly for the diagnostic strategy for chronic HCV infection is initial screening with an HCV antibody serological assay, followed by laboratory-based molecular viral load testing for HCV RNA, to confirm the presence of HCV viraemia and need for treatment [6,7]. Theadvent of pan-genotypic direct-acting antivirals has essentially rendered HCV genotype determination obsolete prior to treatment.

Human Cytomegalovirus (hCMV) is one of the most common pathogens that infect solid organ transplant (SOT) recipients [8]. The impact of hCMV on the outcome of SOT is enormous—the virus not only causes a highly morbid and potentially fatal illness but also indirectly influences other relevant outcomes, such as allograft rejection, risk of other opportunistic infections, and overall patient and allograft survival [8]. Because of the magnitude of its direct and indirect impacts, there have been extraordinary efforts aimed at defining strategies for its prevention and treatment. hCMV infection on transplant outcomes warrants efforts toward improving its prevention, diagnosis, and treatment. During the last 2 decades, significant breakthroughs in diagnostic virology have facilitated remarkable improvements in CMV disease management. During this period, CMV nucleic acid amplification testing (NAT) evolved to become one of the most performed tests in clinical virology laboratories. NAT provides a means for rapid and sensitive diagnosis of CMV infection in transplant recipients. Viral quantification also introduced several principles of CMV disease management. Specifically, viral load has been utilized (i) for prognostication of CMV disease, (ii) to guide preemptive therapy, (iii) to assess the efficacy of antiviral treatment, (iv) to guide the duration of treatment, and (v) to indicate the risk of clinical relapse or antiviral drug resistance.

Quantitative polymerase chain reaction (qPCR) is a powerful tool for gene expression analysis. It is widely used in research and clinical settings to quantify the amount of DNA/ RNA in a sample. However, optimizing the assay can be challenging, especially when working with limited amounts of Nucleic acid. One approach that has gained popularity is the use of half volumes of the RT-qPCR assay. In this article, we will explore the benefits and considerations of this optimization strategy.

To successfully optimize the qPCR assay using half volumes, there are some best practices to follow. First, it is important to perform a pilot experiment to determine the optimal reaction conditions for your specific samples and targets. This can include testing different annealing temperatures, cycle conditions, and reaction volumes. Additionally, it is important to use high-quality reagents and controls to ensure accurate and reproducible results. Finally, it is recommended to validate the optimized assay using a larger sample size or an independent method [with same principle or different principle] to confirm the results.

In conclusion, using half volumes of the qPCR assay can be a useful optimization strategy for gene expression analysis. It can save time and resources while improving accuracy and reproducibility. However, careful consideration and calibration are necessary to ensure optimal results. By following best practices and validating the assay, researchers can confidently use this approach in their work. This study aimed to optimize the qPCR method for the detection of Hepatitis B, Hepatitis C, CMV using only half of the total volume of reagents. In addition, we tested a panel of Hepatitis B, Hepatitis C and CMV positive and negative clinical samples using the standard assay in order to obtain a preliminary evaluation of clinical sensitivity and specificity of the half reaction.

Materials and Methods

The study was conducted at the Department of Microbiology, ESIC Medical College & Hospital (ESICMC&H), Faridabad, India. All methods were carried in accordance with relevant guidelines and regulations. A written informed consent was obtained from the participants. Approval for the study was obtained from the Institutional Review Board and Ethics Committee [Approval No:134X/11/13/2021-IEC/52].

Nucleic acid extraction

Nucleic acid extraction was done using TRUPCR® Total Viral Nucleic Acid Extraction Kit) as per the manufacturer's instruction with a starting serum volume of 500µl.

Real-Time HBV quantitation:

TRUPCR® HBV Viral Load Kit is an in vitro diagnostic test for the detection and quantification of HBV specific DNA in human EDTA plasma and serum. This method utilizes a fragment size of 134 base pairs (bp) in the HBV core gene. TRUPCR® HBV Qualitative Kit is an in vitro nucleic acid amplification assay for the Quantitative detection of HBV viral DNA from patient samples. In addition to the HBV DNA specific amplification and detection system the assay includes oligonucleotides for the amplification and detection of the IC (Internal Control). Probes specific for HBV DNA are labelled with the fluorophore FAM™. The probe specific for the IC is labelled with a fluorophore detectable in the VIC™ channel. The amplification conditions consisted of 94℃ for 3 min, 35 cycles of 94℃ for 30 sec, and 55℃ for 30 sec. This was followed by a final primer extension at 72℃ for 1 min using the TRUPCR® HBV Qualitative Kit. Briefly, to perform the standard RT-qPCR reaction, with 15µL of RT-qPCR mix containing 11µL of Master Mix, 2µL of combined primer/probe mix, 2µL of endogenous Internal control primer/probe mix, 15µL of nucleic acid extract. For the RT-qPCR half reaction, half of the volumes of the reagents listed above were utilized i.e., with 7.5µL of RT-qPCR mix containing 5.5µL of Master Mix, 1µL of combined primer/probe mix, 1µL of endogenous Internal control primer/probe mix, 7.5µL of nucleic acid extract. The analytical sensitivity of this assay is 2.5 IU/ml for TRUPCR with 95% detection limit with a linear range of 2.5 –1x108 IU/ml respectively for TRUPCR kits respectively.

Real-Time HCV quantitation:

TRUPCR® HCV Viral Load Kit is an in-vitro diagnostic test for the qualitative and quantification assessment of HCV RNA in human EDTA plasma and serum. This method utilizes a target mostly from the conserved region. In addition to the HCV RNA specific amplification and detection system the assay includes oligonucleotides for the amplification and detection of the IC (Internal Control). Probes specific for HCV DNA are labelled with the fluorophore FAM™. The probe specific for the IC is labelled with a fluorophore detectable in the VIC™ channel. The amplification conditions consisted of 55℃ for 10 min, 94℃ for 10 mins, 40 cycles of 94℃ for 15 secs, 56℃ for 45 sec and 72℃ for 15 sec. Briefly, to perform the standard RT-qPCR reaction, with 15µL of RT-qPCR mix containing 10µL of Master Mix, 4µL of combined primer/probe mix, 1µL of enzyme mix, 10µL of nucleic acid extract. For the RT-qPCR half reaction, half of the volumes of the reagents listed above were utilized i.e., with 7.5µL of RT-qPCR mix containing 5µL of Master Mix, 1µL of combined primer/probe mix, 0.5µL of enzyme mix, 5µL of nucleic acid extract. The analytical sensitivity of this assay is 20 IU/ml for TRUPCR with 95% detection limit with a linear range of 20–1x108 IU/ml respectively for TRUPCR kits respectively.

Real-Time CMV quantitation:

TRUPCR® CMV QT Kit is in vitro nucleic acid amplification assay for the detection and quantitation of Cytomegalovirus (CMV) specific DNA in human plasma. In this kit there are two independent reactions running in parallel in each tube: the first detects CMV (FAM channel), second detects internal control (IC) DNA (ROX channel) which allows excluding unreliable results. The amplification conditions consisted of 94℃ for 10 mins, 40 cycles of 94℃ for 15 secs, 58℃ for 45 sec and 72℃ for 15 sec. Briefly, to perform the standard RT- qPCR reaction, with 15µL of RT-qPCR mix containing 10µL of Master Mix, 3µL of combined primer/probe mix, 2µL of endogenous Internal control primer/probe mix, 10µL of nucleic acid extract. For the RT-qPCR half reaction, half of the volumes of the reagents listed above were utilized i.e., with 7.5µL of RT-qPCR mix containing 5µL of Master Mix, 1.5µL of combined primer/probe mix, 1µL of endogenous Internal control primer/probe mix, 5µL of nucleic acid extract. The analytical sensitivity of this assay is 50 IU/ml for TRUPCR with 95% detection limit with a linear range of 50–1.12x108 IU/ml respectively for TRUPCR kits respectively.

HBV/HCV/CMV Standards

The standards of TRUPCR HBV/HCV/CMV Viral Load Kit are assigned value in IU/µl after calibration of the TRUPCR HBV/HCV/CMV quantitation standards in congruence with the third WHO international standard for HBV (NIBSC code: 10/266)/ fifth WHO international standard for HCV (NIBSC code: 14/150)/ 1st WHO International Standard for Cytomegalo Virus for Nucleic acid amplification techniques (NIBSC code: 09/162). Quantification of standards are used to generate standard curves which helps in extrapolation of viral load data.

Standard curve to assess efficiency

The analytical sensitivity for HBV of the TRUPCR® HBV Viral Load Kit was determined by analyzing dilution series of 4th WHO International Standard for Hepatitis B Virus for Nucleic acid amplification techniques (NIBSC code: 10/266) and confirmed by WHO multiplex material (NIBSC Code: 14/198-XXX). The analytical sensitivity for HCV of the TRUPCR® HCV Viral Load Kit was determined by analyzing dilution series of 5th WHO International Standard for Hepatitis C Virus for Nucleic acid amplification techniques (NIBSC code: 14/150) and confirmed by WHO multiplex material (NIBSC Code:14/198-XXX). TRUPCR®

CMV Viral Load Kit was determined by analyzing dilution series of 1st WHO International Standard for Cytomegalo Virus for Nucleic acid amplification techniques (NIBSC code: 09/162). We determined the slope of the curve by linear regression and defined the required levels for PCR efficiency ([100 x 10(-1/slope) -1]) and linearity (R2) of each RT- qPCR target to be 90–110% and >0.95, respectively (7).

Evaluation of clinical specimens

A total of 384/202/78 clinical samples from different Hepatitis B/ Hepatitis C/CMV patients attending the ESIC Medical College & Hospital a tertiary care hospital was submitted to department of Microbiology. All samples were analyzed using both the standard and the half reaction protocols as described above. Template volume used was also reduced in proportion to the reaction volume.

Statistical analysis: All data were analyzed using STATA statistical software version 12.1 (Stata Corp LP, College Station, TX, USA).

Results

To determine concordance in standard and half reaction

The RT-qPCR half reaction assay was performed of 112 Hepatitis B, 98 Hepatitis C and 48 CMV clinical samples. All clinical samples were subjected to the standard RT-qPCR described by the manufacturer; of them, 87 tested positive for Hepatitis B, 58 tested positive for Hepatitis C, 43 tested positive for CMV. The assigned staff was blinded towards the results of standard reaction. Sample positivity was ascertained and compared between both standard and half reaction.100% concordance was observed on comparison. The positivity was same in comparison to half reaction, none of the specimens yielded discordant results i.e., none of the negative results yielded positive results.

The Ct values of standard reaction for hepatitis B, Hepatitis C and CMV varied from 13.7 to 36.8. The cut off value for all the three RT-qPCR was 37. The comparison of Ct values of half reaction with the standard reaction showed a slight decrease in Ct values from 0.6-1.2 in Hepatitis B, 0.5-1.15 in Hepatitis C and 0.6-1.15 in CMV.

Standard curve to assess efficiency

The reaction efficiency using half of the volume was evaluated for HBV, HCV and CMV Viral load in comparison to the standard RT-qPCR reaction by using a respective control. The qPCR efficiencies of half reaction were 100.9 %, 101.2% and 105.7% of Hepatitis B viral load, Hepatitis C viral load, CMV viral load respectively. Conversely, the qPCR for Hepatitis B/ Hepatitis C/CMV Viral load, the efficiency values were 103.1%, 101.9 % and 102.3% for standard reaction (Table 1). The R2 for standard reaction was found to be 0.98, 1 and 1 for all the three PCR assessed as compared to R2 half reactions which was 1.

Table 1: Comparison and stratification of Ct values between standard and half reactions of Hepatitis B viral load, Hepatitis C viral load and hCMV viral load positive samples.

Hepatitis B Viral Load			Hepatitis C Viral Load			hCMV Viral Load		
RT-qPCR Ct values	Half reaction qPCR Ct values	Standard reaction qPCR Ct values	RT-qPCR Ct values	Half reaction qPCR Ct values	Standard reaction qPCR Ct values	RT-qPCR Ct values	Half reaction qPCR Ct values	Standard reaction qPCR Ct values
≤20 [12]	0.5	1.4	≤20 [28]	0.4	1.2	≤20 [6]	0.6	1.1
20-25[32]	0.7	1.2	20-25[16]	0.7	1.1	20-25[5]	0.6	1.4
25-30[28]	0.5	0.9	25-30[11]	0.6	1.3	25-30[22]	0.7	1.2
≥30[15]	0.8	1.2	≥30[3]	0.5	0.9	≥30[10]	0.8	1.1

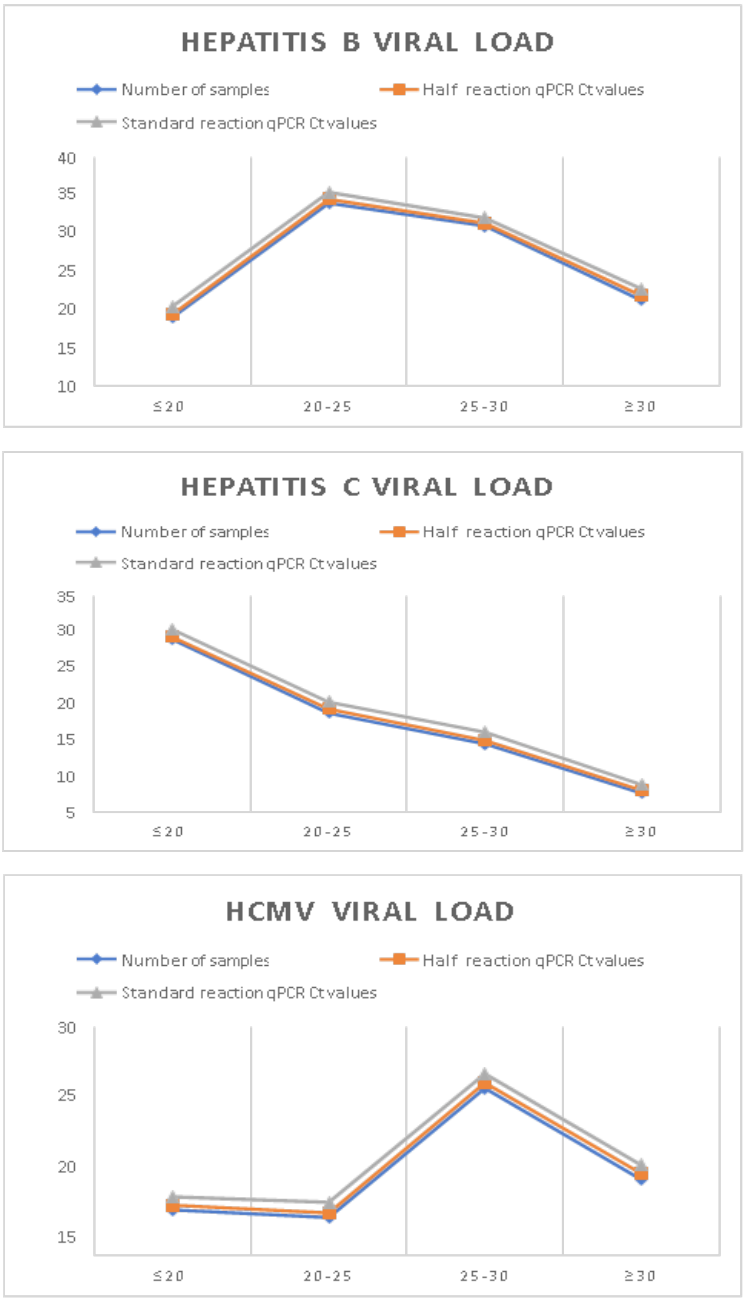

Figure 1: Diagrammatic representation and deviations of Ct values between standard and half reactions of Hepatitis B viral load, Hepatitis C viral load and hCMV viral load positive samples.

Discussion

Quantitative polymerase chain reaction (qPCR) is a powerful tool for gene expression analysis. It is widely used in research and clinical settings to quantify the amount of RNA in a sample. However, optimizing the assay can be challenging, especially when working with limited amounts of RNA. One approach that has gained popularity is the use of half volumes of the RT-qPCR assay. In this article, we will explore the benefits and considerations of this optimization strategy. The study was designed to evaluate the utilization of half reaction in Hepatitis B, Hepatitis C and CMV viral load assays. These half reaction assays can be utilized as a routine protocol

in diagnosis and management of the disease. The assessment of this technique would aid in saving consumables and reagents. The results demonstrated that half reaction efficiency was equivalent to that of the standard qPCR. The R2 for standard reaction was found to be 0.98, 1 and 1 for all the three PCR assessed as compared to R2 half reactions which was 1.

The positivity remained perpetual when both standard and half reaction were assessed, there was 100% concordance. The Ct values of the various viral load assays when compared between standard and half reaction revealed a slight decrease in half reactions in case of Hepatitis B, C and CMV.

Using half volumes of the RT-qPCR assay can save time and resources. It allows for more efficient use of reagents, reduces the amount of sample required, and can increase the throughput of the assay. Additionally, it can improve the accuracy and reproducibility of the results, as smaller reaction volumes are less prone to pipetting errors and variations in reaction conditions.

The present study optimized the RT-qPCR method for the detection and viral load assessment of Hepatitis B, C and CMV. The computation of data concedes that the half reaction confers better fulfilment in terms of performance. The half reaction optimisation presented a decrease of up to 5.5 cycle thresholds compared with standard RT-qPCR in a study for the detection of SARS-CoV-2 [9-10] The decrease in Ct values could be due to changes in the reaction kinetics. The reduced volume PCR method has been shown to be reliable and cost efficient in a study by Bessekri,et al where the 10 µL reaction proved more reliable than the 5 µL [11]. The reduced reaction volume in our study was 7.5 µL in each of the assay.

While using half volumes can be advantageous, there are some considerations to keep in mind. First, it is important to ensure that the quality and quantity of RNA is sufficient for the assay. If the RNA concentration is low, it may be necessary to increase the reaction volume to maintain sensitivity. Additionally, it is important to carefully calibrate the pipettes and use accurate measurements to avoid errors in the reaction setup. Finally, it may be necessary to adjust the annealing temperature or cycle conditions to optimize the assay for smaller reaction volumes.

To successfully optimize the RT-qPCR assay using half volumes, there are some best practices to follow. First, it is important to perform a pilot experiment to determine the optimal reaction conditions for your specific samples and targets. This can include testing different annealing temperatures, cycle conditions, and reaction volumes. Additionally, it is important to use high-quality reagents and controls to ensure accurate and reproducible results. Finally, it is recommended to validate the optimized assay using a larger sample size or an independent method to confirm the results.

In conclusion, using half volumes of the RT-qPCR assay can be a useful optimization strategy for gene expression analysis. It can save time and resources while improving accuracy and reproducibility. However, careful consideration and calibration are necessary to ensure optimal results. By following best practices and validating the assay, researchers can confidently use this approach in their work.

Disclosure: None to declare.

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