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Brief guide to RT-qPCR

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ABSTRACT

RNA quantification is crucial for understanding gene expression and regulation. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is a widely used technique for RNA quantification because of its practical and quantitative nature, sensitivity, and specificity. Here, we provide an overview of RT-qPCR, focusing on essential reagents, the importance of primer design, the detailed workflow, and data analysis methods. This guide will be useful for scientists who are unfamiliar with RT-qPCR, highlighting key considerations for successful RNA quantification.

Keywords: Reverse transcription-quantitative polymerase chain reaction, RNA quantification

INTRODUCTION

Quantification of RNA is fundamental for understanding gene expression and regulation, and for diagnosing and monitoring diseases (Artika et al., 2022; Ginzinger, 2002; Kim et al., 2022b). The evolution of RNA quantification techniques, including reverse transcription-quantitative polymerase chain reaction (RT-qPCR), RNA sequencing, northern blotting, and microarray, has advanced the capabilities of molecular biology research (Nonis et al., 2014, Yoo et al., 2022). Researchers choose RNA quantification techniques based on their needs for specific research, including available resources, transcriptome complexity, sensitivity, and specificity (Ding et al., 2023).

Among the aforementioned RNA quantification techniques, RT-qPCR has gained popularity because of its practicality, sensitivity, specificity, speed, and quantitative capability (Harshitha and Arunraj, 2021; Wagner, 2013). RT-qPCR enables quantitative measurement of the levels of mRNAs, small RNAs, and other noncoding RNAs (Chen et al., 2011; Ding et al., 2022; Kristensen et al., 2019). The accuracy and efficiency of RT-qPCR depend on the choice of reagents and the design of primers (Rodríguez et al., 2015). Proper analysis of RT-qPCR data is also crucial for comprehensive understanding of gene expression. Here, we provide a brief guide to RT-qPCR assays for scientists who are interested in performing molecular biological techniques (Fig. 1).

MAIN BODY

Reagents for RT-qPCR

Reverse Transcription Reagents

Reverse transcription (RT) is a process that converts RNAs to complementary DNAs (cDNAs) (Green and Sambrook., 2018). RT requires several reagents, including primers, reverse transcriptase, RNA templates, deoxynucleoside triphosphates (dNTPs), $MgCl_2$, and ribonuclease (RNase) inhibitors (Martín-Alonso et al., 2021; Taylor et al., 2010). The primers are crucial for initiating the synthesis of cDNAs from RNA templates. Gene-specific primers, oligo(dT) primers, and random primers are 3 common types of RT primers (Ginzinger, 2002). Gene-specific primers enhance the sensitivity and specificity of RT, enabling targeted gene expression analysis and detection of low-abundance transcripts (Wagner, 2013). Oligo(dT) primers are short sequences of thymidine nucleotides that bind to the poly(A) tail to reverse-transcribe mRNAs (Nam et al., 2002). Random primers are randomly generated oligonucleotide used to synthesize cDNAs originating from various RNAs, including non-poly(A) RNAs (Zucha et al., 2021). After primer binding to the RNA template, reverse transcriptase catalyzes cDNA synthesis by using dNTPs as the building blocks (Huber et al., 2023). Reverse transcriptase has ribonuclease H activity that degrades the RNA strand in the RNA-DNA hybrid to remove the RNA template during cDNA synthesis (Menéndez-Arias et al., 2017). $MgCl_2$ provides Mg^{2+}, an essential cofactor for reverse transcriptase activity (Goldschmidt et al., 2006). RNase inhibitors are also included in the RT reaction mixture to prevent degradation of template RNAs (Pasloske., 2001).

Quantitative Polymerase Chain Reaction Reagents

Quantitative polymerase chain reaction (qPCR) is a technique that amplifies and quantifies target DNAs (Singh and Roy-Chowdhuri, 2016). Using proper reagents is crucial for the accuracy and efficiency of qPCR (Svec et al., 2015). Essential

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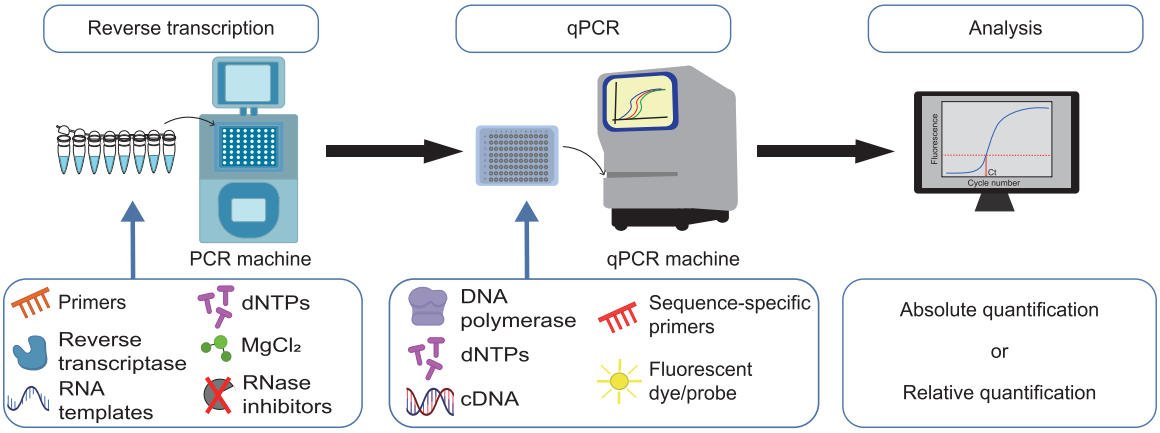

Fig. 1. Overview of reverse transcription-quantitative polymerase chain reaction (RT-qPCR) workflow. RT-qPCR consists of reverse transcription, qPCR, and analysis steps. Reverse transcription is a process that converts RNA templates into cDNA using primers, reverse transcriptase, RNA templates, dNTPs, $MgCl_2$, and ribonuclease (RNase) inhibitors. For the qPCR step, DNA polymerase, dNTPs, DNA template (cDNA), sequence-specific primers, and fluorescent dye/probe are used to amplify cDNA. The analysis strategies for RT-qPCR include absolute quantification or relative quantification. cDNA, complementary DNA; dNTPs, deoxynucleoside triphosphates.

reagents for qPCR are DNA polymerase, dNTPs, DNA templates, sequence-specific primers, and fluorescent dyes/probes (van Pelt-Verkuil et al., 2008). DNA polymerase synthesizes new DNA strands by adding dNTPs to the 3′ end of the annealed primers, using cDNA as a template to amplify DNA molecules (Singh et al., 2012). Designing sequence-specific primers is crucial for the effective amplification of target sequences (Rodríguez et al., 2015). To detect and quantify the amount of target DNA, fluorescent dyes and probes are required (Singh et al., 2012). Fluorescent dyes, such as SYBR Green (Applied Biosystems), bind to double-stranded DNA and emit fluorescence during DNA amplification (Dragan et al., 2012). Fluorescent probes, such as TaqMan probes (Applied Biosystems), are labeled oligonucleotides that hybridize to specific target sequences during PCR reaction (Tajadini et al., 2014). The 5′ exonuclease activity of Taq polymerase cleaves the probe by separating the fluorescent reporter from the quencher, resulting in an increase in fluorescence, proportional to the amount of the amplified product (Laws et al., 2001).

Primer Designs for RT-qPCR

Proper primer design is crucial for ensuring precise detection and quantification of target sequences in RT-qPCR reactions. Researchers need to choose oligos among gene-specific primers, oligo(dT) primers, and random primers based on the type of RNA being converted into cDNA in RT (Bustin et al., 2005). Gene-specific primers are typically 18 to 25 nucleotides in length and bind to unique regions of the target RNA (Lee et al., 2023a; Thornton and Basu, 2015). The general length of oligo (dT) primers is 12 to 18 nucleotides for binding to the poly(A) tail of most mRNAs without nonspecific binding (Nam et al., 2002). Random primers consist of a mixture of short random sequences, approximately 6 to 9 nucleotides in length (Adams, 2020).

For qPCR reactions, the specificity and efficiency of primers depend on critical elements such as target sequences and lengths, GC contents, and secondary structures (Bustin et al., 2020). First, primers are preferentially designed to span exon-exon junctions to avoid amplifying genomic DNA and to ensure specificity for cDNA (Padhi et al., 2020). The preferable distance between forward and reverse primers is 70 to 200 base pairs to ensure efficient qPCR (Diehl et al., 2022). Basic Local Alignment Search Tool from NCBI is a powerful primer design tool that ensures the specificity of the primers (Ye et al., 2012, https://blast.ncbi.nlm.nih.gov/Blast.cgi). The Integrated Genome Browser provides detailed visualization and analysis of the genomic landscape surrounding the target genes (https://www.bioviz.org/). Second, the optimal length of primers is 18 to 25 nucleotides, with a GC content of 40% to 60% for stable binding (Rodríguez et al., 2015). OligoAnalyzer from Integrated DNA Technologies is an example of a helpful tool for calculating melting temperature (T_m), GC content, and molecular weight of designed primers (https://sg.idtdna.com/pages/tools/oligoanalyzer). Third, researchers need to design primers to avoid sequences that form secondary structures because these structures interfere with the amplification process (Thornton and Basu, 2015). Primer3PLUS is an example of a useful tool for predicting the secondary structure formed by designed primers (https://www.primer3plus.com/).

The Process of RT-qPCR

Reverse Transcription

RT involves a 4-step process to convert RNA into cDNA: denaturing secondary structures of RNA, primer annealing, cDNA synthesis, and reaction termination. First, RNA templates extracted from biological samples are incubated at 65°C to 70°C for 5 to 10 minutes to denature secondary structures (Bachman, 2013; Lucas et al., 2023). Second, the RNA templates and primers are heated to allow primer annealing. Third, reverse transcriptase and reaction components, including dNTPs, RNase inhibitors, and $MgCl_2$, are incubated at the optimal temperature to synthesize cDNA (Gerard, 2018). This reaction

is usually performed at 37°C to 50°C for 30 to 60 minutes, depending on the optimal temperature for a specific reverse transcriptase (Chen et al., 2011). Fourth, the reaction is terminated by inactivating the reverse transcriptase via heating at 70°C to 85°C (Kadja et al., 2023).

Quantitative Polymerase Chain Reaction

qPCR involves 2 main steps to quantify the amount of a specific DNA sequence: reaction setup and thermal cycling. First, cDNA and the reaction mixture are aliquoted into a qPCR plate. The qPCR reaction mixture contains DNA polymerase, gene-specific primers, dNTPs, fluorescent dye or probe, and $MgCl_2$ (van Pelt-Verkuil et al., 2008). Second, during thermal cycling, the cDNA is denatured, annealed with primers, and extended with fluorescence measurement. The reaction begins with an initial denaturation step at 95°C to separate the cDNA strands (Hawkins and Guest, 2017). This is followed by cyclic reactions of denaturation (95°C), primer annealing (55-65°C), and extension by DNA polymerase (72°C) (Seo et al., 2023; Shao et al., 2023). During the extension phase, the fluorescent dye or probe binds to the newly synthesized DNA, and the fluorescence is measured in real time. This process is repeated for 30 to 40 cycles, with fluorescence data collected to quantify the amount of DNA amplified at each cycle (Chen et al., 2011).

The Analysis of RT-qPCR

Quantification of the target RNA is performed based on the fluorescence levels emitted by the labeled probes or dyes (Adams, 2020). The cycle threshold (Ct) value represents the number of cycles required for the fluorescent signal to surpass a defined threshold, indicating detectable amplification of the target RNA. Low Ct values indicate high RNA quantities, whereas high Ct values indicate low quantities (Ma et al., 2021). RNA quantification methods include absolute quantification and relative quantification (RQ) (Harshitha and Arunraj, 2021). Absolute quantification determines the exact amount of RNA in the sample by using standard curve using known concentrations of the target RNA (Lin et al., 2020). RQ provides a ratio of expression levels between samples by comparing the expression level of a target gene relative to that of reference genes (Arabkari et al., 2020; Habib et al., 2022). RQ requires normalizing the Ct values of a target gene against those of a reference gene to account for variability in RNA input and amplification efficiency (Lee et al., 2023a, 2023b; Rao et al., 2013). The RQ value indicates a gene expression level relative to a control sample (Hellemans and Vandesompele, 2014). The formula typically used is $RQ = 2^{-\Delta\Delta Ct}$ (Kim et al., 2022a; Rao et al., 2013). ΔCt is the difference between the Ct value of the target gene and the Ct value of the reference gene within the same sample (Kozera and Rapacz, 2013). $\Delta\Delta$Ct is then calculated by subtracting the ΔCt of the control from the ΔCt of the experimental sample. The RQ value helps understand how gene expression changes under different conditions, providing insights into RNA function and regulation.

Software tools, such as Bio-Rad CFX Manager Software (Taylor et al., 2010, https://www.gear-genomics.com/rdml-tools/) and StepOne (Applied Biosystems, https://stepone-software.software.informer.com/download/), are available for analyzing RT-qPCR data. Bio-Rad CFX Manager Software and StepOne analyze and visualize the results of RT-qPCR. These tools enable absolute quantification using the standard curve method and RQ using the $2^{-\Delta\Delta Ct}$ method. These tools also provide data visualization, including amplification plots, standard curve graphs, and melt curve analysis. Using the software coupled with the qPCR machine is desirable for efficient and accurate data analysis.

Nonspecific amplification in RT-qPCR produces multiple T_m peaks during melting curve analysis. To overcome this issue, it is essential to use fresh reagents and thoroughly clean the workspace to eliminate potential contamination of PCR components. In addition, redesigning probes or primers is necessary because poorly designed probes or primers can lead to nonspecific amplification (Jalali et al., 2017). Optimizing PCR conditions, including primer concentration, enzyme levels, Mg^{2+} concentration, and cycling parameters, can further improve the specificity of the amplification (Bustin and Nolan, 2004).

CONCLUDING REMARKS

Here, we provide a brief overview of RT-qPCR assays for RNA quantification. Our article will serve as a concise and practical resource for biologists with minimum expertise in RT-qPCR assays, helping them effectively perform RNA quantification. Detailed protocols need to be modified depending on the specific RNA types that are analyzed. In addition, for more comprehensive research, users need to refer to additional publications, particularly primer design for different RNA types that are not described in this article.

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AUTHOR CONTRIBUTIONS

D.B., J.S., and S-J.V.L. wrote the article.

DECLARATION OF COMPETING INTERESTS

The authors have no potential conflicts of interest to disclose.

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