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RT-qPCR Made Simple: A Comprehensive Guide on the Methods, Advantages, Disadvantages, and Everything in Between

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Abstract
Introduction: In the world of science, many technologies and methodologies exist to contribute to the process of research. Polymerase Chain Reaction (PCR) is a technology that aids in amplifying specific DNA sequences. PCR can be used to determine the presence of a certain DNA gene. In contrast, reverse transcriptase quantitative PCR (RT-qPCR) converts ribonucleic acids (RNA) into complementary DNA (cDNA) which can then be amplified to give a C_t (threshold cycle) value, a representation of how much of the original RNA transcript was present in the sample.
Utility: RT-qPCR is a technique that can be used in many areas of research, including forensic pathology to identify individuals through polymorphic repetitive regions of the DNA called short tandem repeats. This method can also be used in diagnosing various viral diseases such as the recent COVID-19 virus. RT-qPCR is also used in numerous laboratory procedures, such as determining cell growth, cell survival, genetic persistence, and more.
Challenges: This method does come with many challenges, such as determining the normalization technique to be used in order to effectively compare the C_t value of the sample with the C_t value of the control gene, as there are numerous ways to perform this comparison. This challenge can be mitigated by establishing a common technique within each lab. Determining which housekeeping gene should be used in the normalization process is also a persistent challenge. This can be addressed by researching the different genes and determining which housekeeping gene will best be established as an accurate control. Ensuring the purification of RNA and gathering knowledge of a few base pairs to design primers are other challenges that must be considered as well but can be resolved fairly easily.
Limitations: Limitations such as the difficulty in replication can hinder the reliability of the method. The 'Monte Carlo' effect and the lack of an established method for normalization further contribute to the difficulty in comparing studies with differing RT-qPCR protocols used. These limitations can be addressed by publishing data with the exact conditions and methods used in the RT-qPCR reaction.

Keywords: RT-qPCR; polymerase chain reaction; reverse transcriptase; amplification; research method; cDNA; normalization

Introduction

With the ever-changing scientific innovations and the need for cures for diseases and disorders, it was about time a technique arose that accurately determined the presence of various sequences in a fast and efficient manner. Polymerase chain reaction (PCR) is a technique in which specific sections of DNA are amplified, mimicking the DNA replication machinery in cells just by adding deoxynucleotide-triphosphates, a DNA polymerase, a buffer, primers, and a template with the gene of interest.

Although PCR helps produce DNA products, identifying the expression of specific genes or proteins is also necessary for research. This has resulted in the innovation of Reverse Transcriptase quantitative PCR (RT-qPCR). Reverse transcriptase is an enzyme found primarily in viruses [1] that can transcribe RNA into new double-stranded DNA, known as complementary DNA (cDNA). Thus, mRNA found within a cell can easily be transcribed into this double-stranded DNA by reverse transcriptase which can then undergo a normal PCR. RT-qPCR also allows for real-time detection of the number of cycles and amplification through the use of fluorescent probes and dyes.

This RT-qPCR reaction is advantageous to other methods of DNA amplification, such as Southern Blotting and RNA Microarray Analysis. Southern blotting is more time intensive and requires high DNA amounts in comparison to RT-qPCR [2]. Furthermore, RT-qPCR is preferred over RNA microarray analysis as it is more sensitive for smaller changes in genetic expression [3].

To run this reaction, specific primers must be designed in that they only anneal and thus direct the amplification of

the sequence of interest. These primers are sequence-specific oligonucleotides that are prepared beforehand - this step requires some knowledge of the genome pertaining to the sequence of interest.

Once the PCR tubes have the necessary components - the sample, deoxynucleotide-triphosphates (dNTPs), primers, a buffer, and the essential enzymes - the PCR can occur. There are two types of RT-qPCRs; the one-tube reaction involves the conversion of the mRNA to cDNA, and then continuing to amplify the cDNA, all in a single reaction mixture tube. The two-tube reaction separates the reverse transcriptase step from the PCR step; the RNA is converted into cDNA in one tube which is then, after selective filtration, subjected to another tube containing the DNA polymerase for the amplification step [4]. The advantage of the one-tube reaction involves the low chance of contamination and product loss, whereas the two-tube allows for a stable cDNA pool that can be stored for later use [4]. The general steps involved are simple. After adding the essential components, and separating them appropriately if conducting a two-step reaction, the reaction mixture will be subjected to a series of temperatures and steps during the DNA amplification part. The first step is the denaturation of the sample nucleic acids step, which is often done at 95 degrees Celsius [5]. Then, the annealing step is when the primers bind to the sequence of interest. The temperature at this stage is usually variable, depending on the primer length, primer content, etc. [5]. Finally, the elongation step is the step that actually allows the DNA polymerase to copy and amplify the target sequence. This is again tailored to the enzyme used [5]. The timings and cycle numbers for these phases also vary, as these factors depend on the efficiency of the enzymes and the machinery output. Figure 1 displays a quick visual of the differences of PCRs and RT-qPCRs.

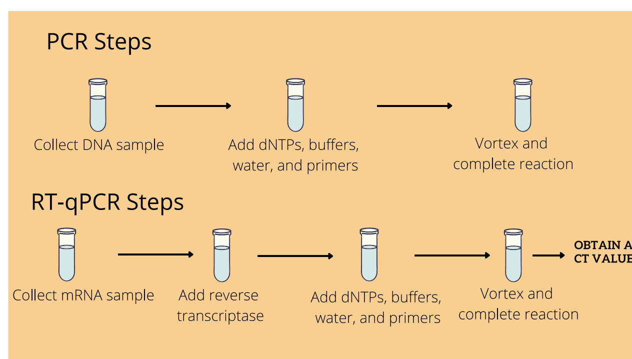

Figure 1. Steps in PCRs and RT-qPCRs. (created using www.canva.com).

The use of fluorescent dyes helps the observation of the PCR in real-time. When a fluorescent marker is attached to a probe (usually a sequence-specific probe), a quencher is also attached. The quencher stops the marker's fluorescence when it is in proximity to the marker. However, when the probe is able to bind to the sequence of interest during the amplification reaction, the quencher changes position, resulting in the fluorescence of the marker, providing a visually detectable signal [4] which can be graphed.

After the reaction, a C_t value (threshold cycle value) is obtained - this is the cycle number for when the fluorescence signal of the fragment of interest became significantly higher than the background signals. This value is compared against the C_t of another control gene, usually a housekeeping gene [4]. A housekeeping gene is expressed at a constant amount throughout almost all cells at any point of the cycle; this consistency allows RT-qPCR to be done at any time and establishes a baseline detection [6]. These genes can act as feasible controls as the researchers are aware of the gene's presence and the sequences for the primers are well-known. The most used reference genes for the normalization of qPCR data are GAPDH and ACTB [7]. It is established that the housekeeping gene will amplify; if the housekeeping gene does not show sufficient amplification, the data generated for the sequence of interest cannot be credible as there may be something wrong with the experimental conditions. The normalization of comparing the C_t value of the sequence of interest to that of a housekeeping gene can be done in many ways but introduces a few limitations and challenges which are discussed in further detail below. The C_t value, in general, is used to determine the amount of cDNA present in the sample which can ultimately be used to estimate the amount of mRNA in the sample [8]. This can then be used for a variety of applications, such as diagnosis of diseases, presence of proteins, and more. An important note is that the C_t value is inversely proportional to the initial cDNA in the reaction mixture. Thus, as the template amount increases, the number of cycles needed for significant amplification decreases [4]. Hence, a lower C_t value indicates higher levels of gene expression compared to a higher C_t.

Thus, RT-qPCR is an efficient and productive technology with many applications in the research laboratory as well as practical applications in other fields. Numerous challenges and limitations do exist; however, it is possible to mitigate the challenges and limitations with careful consideration and thoughtful processing.

Utility

RT-qPCR is utilized in many different applications due to its accuracy, high sensitivity, and high specificity [9]. For example, a practical application of RT-qPCR is within the use of forensic pathology [10]. Amplification of human DNA left on crime scenes or other areas in which suspects need to be identified can all be done in a single PCR analysis, by amplifying specific regions known as short tandem repeats (STR), which are common biomarkers for individuals [11]. STRs are highly polymorphic, so they can

produce highly accurate results for the identification of individuals. Most recently, RT-qPCR has aided in the COVID-19 pandemic. In this diagnosis, the RT-qPCR experiment amplifies a few known genes of the virus, such as the *RdRP* gene, the *E* gene (the envelope gene of the virus), and the *N* gene (the gene for the nucleocapsid of the virus) [12]. Significant amplification of these genes is indicative of the presence of the virus and is thus used to classify a test as "positive". RT-qPCR dominated as the primary diagnostic tool because of the virus's high spread rate, meaning a quick and efficient way of diagnosing this respiratory illness was needed. However, many arguments were made about the quality and consistency of this method – some studies concluded that the genomic load of the virus is independent of the severity of the disease [13], making the test unreliable for COVID-19 [12]. This could be due to the differing use of reagents, protocols, and various analysis methods by each hospital laboratory, as a major limitation of RT-qPCR is that quantifications made with different protocols and materials cannot be compared [15]. Nonetheless, it is still the most widely accepted tool for detecting COVID-19 [12].

RT-qPCR can also be utilized in research settings, such as measuring the gene expression associated with cell growth, cell survival, and detecting genetic cancer markers. It is also the method of choice in the determination of the presence of microorganisms in food items [9].

Therefore, since this method has an amazing and unique feature – the ability to detect amplification in real time – many practical applications can utilize this research tool for numerous applications.

Challenges

Several challenges following this technique need to be addressed before data from the RT-qPCR data is analyzed. The most significant challenge is the fact that normalization is required but choosing which method to use is the challenging aspect. Normalization refers to the addition of another known sample (usually a housekeeping gene) and its amplification along with the sample, serving as a control and a baseline. This allows for a significant C_t value as well as confirming that the sample is properly amplified. Numerous methods of normalization have been proposed and used, but a great deal of them still provide inaccurate data or additional problems. For example, using a similar sample size of the sample and the housekeeping gene may allow for the required C_t comparisons between the two. However, if human or animal tissues are the sample of interest, there is a necessity for the different samples to have similar tissue volume to weight ratio, or similar cells per volume ratios [4]. This is difficult to obtain as different cells and tissues often consist of varying densities. Furthermore, it has been pointed out that the various traditional genes used for normalization are variable within different tissues, species, etc. [16]. Moreover, other solutions have been suggested, such as the normalization of genomic DNA by an artificial molecule, or by RNA concentration quantification [4]. However, all the aforementioned ideas have their own concerns. Genomic DNA amplification may not be the best choice since RNA extraction procedures are used on the sample before the RT-qPCR, and these procedures often yield low DNA amounts, varying the extraction rates between the normalization sample and the sample of interest. Additionally, artificial molecules are costly to generate and would require previous validation [4]. Thus, the disadvantages associated with these normalization techniques make it more difficult to pick the best possible option; the current solution is to use one of these methods consistently within a lab.

Another challenge associated with RT-qPCR is that the RNA used needs to be free of contaminating DNA [17]. This can be achieved by the addition of special enzymes called DNases, which digest any DNA encountered to avoid unnecessary DNA amplification. On top of that, the RNA also needs to be free of inhibitors, which is made easier by the availability of RNAse-free reagents [17]. RNA extraction procedures have been well optimized from previous projects, but there needs to be a well-known concentration of inhibitors at which they do not affect the RT-qPCR. In order to mitigate this challenge, this concentration of inhibitors needs to be determined empirically to confirm that the RNA is ready to be amplified [18].

Limitations

Despite all efforts, limitations will often persist within various scientific research methods. In RT-qPCR, one of the major limitations is the difficulty in replication. Different laboratories have their own normalization methods, PCR setup protocols, and enzymes; all of these can contribute to the C_t value of a reaction. Thus, it can become difficult to compare the C_t values of the same sample between different studies as different experimental conditions within each study may alter the C_t value in unmeasurable and unnoticeable ways. This limitation can be mitigated by noting the techniques and conditions used by the study being analyzed, as well as by ensuring that publications consist of the exact conditions used, so that the reader can be aware of the potential variations in C_t values.

Another limitation involves a phenomenon termed the 'Monte Carlo' effect. This results from the limited ability of amplification of small, complex templates from a large cDNA pool. To elaborate, when the mRNA is converted into cDNA, there is a varying chance for every template of the target to either be lost or amplified [20]. This will conclude in varying amplification quantities and decreased yields, which can be challenging to deal with if the goal is to amplify a certain gene and to measure it quantitatively. This may be mitigated by adding more replicates within the same reaction. Another limitation that persists with this methodology is that there is no general published guideline

on how to interpret controls without templates [20]. There has to be an arbitrary cut-off and baseline point that the personal researcher has to establish in order to determine that the C_t value obtained is significant. Again, by stating the cut-off and baseline point in publications of the data, as well as by using consistent cut-offs, can alleviate this limitation. Moreover, deciding between one-step and two-step reactions may be difficult if looking to maximize specificity. This is due to the split between studies that show one-step methods having a higher specificity while others concluded that two-step methods are better in terms of specificity [21]. This limitation requires an in-depth analysis of both the gene of interest as well as the stated reaction conditions while interpreting the data.

In addition, a limitation that was introduced in the previous discussion on challenges with RT-qPCR includes the various methods of normalization. Although there are multiple ways with sufficient advantages with each protocol as clarified in the previous section, there is no single universally established method. This may result in difficulties in comparing studies from two different authors who have used differing normalization techniques. Thus, without a unified opinion on a normalization approach, the generalizability of RT-qPCR and its data becomes limited. Again, it is important to take into account the normalization techniques used when analyzing data and confirm that published data consists of the exact conditions of the reaction.

Another limitation that often limits the use of RT-qPCR in quantifying a specific gene is the prerequisite knowledge of at least part of the sequence of interest, as the primers that initiate the amplification process need to be long and specific enough to attach to the sequence. If a sequence is completely unknown in which no primers can be designed, random sequences could substitute as primers, however, this may cause amplification of a diverse set of sequences. Even if part of the sequence is known, if the known sequences are not long enough for the following primers to anneal specifically to the target sequence, there is a chance for other nucleotide sequences to be amplified, altering the data [19]. To overcome this limitation, various sequencing techniques may need to be used on the isolated main sequence.

Conclusions

All in all, despite the challenges and limitations, RT-qPCR is still an excellent way to generate quantitative data from small concentrations of mRNA in human cells, animals, plants, and more. RT-qPCR has an advantage over traditional PCR as it can use mRNA presence to determine the quantity of a sequence of interest, providing a tool to evaluate gene expression.

An RT-qPCR setup has numerous steps and components, and there are many ways in which one can carry out this reaction. Furthermore, one must normalize the data generated by the sequence of interest to a housekeeping gene, however, this may pose several challenges as there are various methods to do so, each with its own advantages and disadvantages. Other challenges include the requirement of similar densities of the samples if the comparison between two or more samples is being made, the RNA used needs to be free of contaminating DNA and inhibitors, and a sufficient part of the target sequence needs to be known for the adequate design of sequence-specific primers in order to reduce unintentional annealing and incorrect amplifications. These challenges, although daunting, need to be addressed and solved or at the very least, minimized, before using data generated from RT-qPCR. In a larger sense, there are still some limitations of this method that may prevent it from being utilized in various circumstances. These include the 'Monte Carlo' effect, which describes the difficulty of the accurate quantitative amplification of small sequences from large cDNA pools, the lack of a generalized consensus on how to interpret controls/normalization techniques, as well as the complication of trying to compare two different studies that used different normalization methods. Ways to reduce the effects of these limitations are available, although these limitations may persist in some aspect.

RT-qPCR is an efficient and revolutionary research laboratory method. However, there are still challenges and limitations associated with this technique that must be addressed and refined to better this method for the purpose of furthering scientific research.

List of Abbreviations Used

DNA: deoxyribonucleic acid
RT-qPCR: reverse transcriptase quantitative polymerase chain reaction
STR: short tandem repeat
PCR: polymerase chain reaction
cDNA: complementary DNA
dNTP: deoxynucleotide-triphosphates

Conflicts of Interest

The author (Nidhi Mehta) declare that they have no conflict of interests.

Ethics Approval and/or Participant Consent

This study did not require any human participants or had ethical concerns as this is a comprehensive analysis of the research method RT-qPCR and is a review article of different articles of this method.

Authors' Contributions

NM: made contributions to the initial research, collected data, drafted the manuscript, and gave final approval of the version to be published.

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