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Real-time PCR: Concept and Application in Livestock

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Abstract

Real-time PCR, also called quantitative PCR or RT-qPCR, is a technique which provide a simple and elegant method for determining the amount of a target sequence or gene that is present in a sample. It is a gold standard method for accurate and sensitive quantification of nucleic acid sequences. It completely revolutionizes the way one approaches PCR-based quantitation of DNA and RNA. Traditional PCR had the drawbacks like poor precision, low sensitivity, low resolution, Post-PCR processing etc., which are being eliminated by RT-qPCR. RT-qPCR requires dedicated instruments that are able to quantify amplification products in real-time during each cycle. The simplest detection technique for newly synthesized PCR products uses SYBR Green I fluorescence dye that binds specifically to the minor groove ds-DNA. The quantification method of choice depends on the target sequence, the expected range of the nucleic acid amount present in the tissue, the degree of accuracy required and whether quantification needs to be relative or absolute. Furthermore, a normalisation of the target gene with an endogenous standard is recommended. However, the reproducibility of RT-qPCR result varies greatly between tissues, isolation methodology and the reagents used. Therefore, MIQE provides a checklist for preparing a report of the RT-qPCR study. It allows reviewers to evaluate the work and other investigators to reproduce it.

1. Introduction

Real-time PCR, also called quantitative PCR (qPCR) is one of the most powerful and sensitive gene analysis techniques (Derveaux et al., 2010). Given its large dynamic range of linear quantification, high speed, sensitivity (low template input required) and resolution (small differences can be measured), this method is perfectly suited for validation of microarray expression screening results on an independent and larger sample panel, and for studies of a selected number of candidate genes or pathway constituents in an experimental setup (biopsies, treated cell cultures or any other sample collection). Frequently, real-time polymerase chain reaction is combined with reverse transcription to quantify messenger RNA (mRNA) and microRNA (miRNA) in cells or tissues.

As the name suggests, real-time PCR measures PCR amplification as it occurs (Bustin and Mueller, 2005). Data is collected throughout the PCR process rather than at the end of the PCR process. This completely revolutionizes the way one approaches PCR-based quantitation of DNA and RNA. Two different methods of analyzing data from real-time quantitative PCR experiments exist: absolute quantification (Bustin, 2000) and relative quantification (Applied Biosystems, 1997; Livak and Schmittgen, 2001). Absolute quantification determines the input copy number of the transcript of interest, usually by relating the PCR signal to a standard curve. Relative quantification describes the change in expression of the target gene relative to some reference group such as an untreated control or a sample at time zero in a time-course study.

2. Goal of RT-qPCR

As the name suggests, RT-qPCR monitors the progress of the PCR as it occurs i.e., in RT-qPCR, reactions are characterized by the point in time during cycling when amplification of a target is first detected rather than the amount of the target accumulated after a fixed number of cycle. In traditional PCR, results are collected after the reaction is complete, making it impossible to determine the starting concentration of nucleic acid.

3. Chemistry of RT-qPCR

Two types of chemistries can usually be used to detect PCR products in RT-qPCR namely Probe-based fluorogenic 5′

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nuclease chemistry (Livak et al., 1995) and SYBR Green I dye chemistry (Morrison et al., 1998). Both types of chemistries can be used for quantification assay types including:

a. one-step RT-PCR for RNA quantification
b. Two-step RT-PCR for RNA quantification
c. DNA quantification

Further, Probe-based fluorogenic 5′ nuclease chemistry can also be used for allelic discrimination. Small molecules that bind to dsDNA can be divided into two classes: intercalators e.g., ethidium bromide and minor groove-binders (MGBs) e.g., Hoechst33258 (Higuchi et al., 1992; 1993). Regardless of binding method, there are at least two requirements for a DNA binding dye for real-time detection of PCR products i.e. Increased fluorescence when bound to dsDNA and No inhibition of PCR.

Thus, every real-time PCR reaction contains a fluorescent reporter molecule—a TaqMan Probe or SYBR Green dye to monitor the accumulation of PCR product. As the quantity of target amplicon increases, so does the amount of fluorescence emitted from the fluorophore.

3.1. Probe-based fluorogenic 5′ nuclease chemistry

AmpliTaq Gold DNA polymerase has 5′ exo-nuclease activity. The 5′ exo-nuclease activity of AmpliTaq Polymerase and FRET (Fluorescent Resonant Energy Transfer) makes it possible to detect PCR amplification in Real-Time (Applied Biosystems, 1997). FRET is utilized in the 5′ nuclease assay based on the principle that when a high energy dye is in close proximity to a low-energy dye, there will be a transfer of energy from high to low. In the 5′nuclease assay, an oligonucleotide called a TaqMan Probe is added to the PCR reagent master mix. The probe is designed to a specific sequence of template between the forward and reverse primers. The probe sits in the path of the enzyme as it starts to copy DNA or cDNA. When the enzyme reaches the annealed probe the 5′ exo-nuclease activity of the enzyme cleaves the probe. The Probe is designed with a high energy dye termed a Reporter at 5′ end and a low energy molecule termed a Quencher at 3′ end. When the probe is intact and excited by a light source, the Reporter dye's emission is suppressed by the Quencher dye as a result of the close proximity of the dyes. When the probe is cleaved by the 5′ nuclease activity of the enzyme, the distance between the reporter and the Quencher increases causing the transfer of energy to stop. The fluorescent emissions of the reporter increase and the quencher decrease. The amount of reporter signal increase is proportional to the amount of product being produced for a given sample.

3.2. Amplification Plot

When the fluorescent signal of reporter increases to a detectable level it is captured by the Sequence Detection instrument and displayed as an Amplification Plot. The amplification plot contains valuable information for the quantitative measurement of DNA or RNA. The threshold line is the level of detection or the point at which a reaction reaches a fluorescent intensity above background. The threshold line is set in the exponential phase of the amplification for the most accurate reading. The cycle at which the sample reaches this level is called the Cycle threshold (Ct). These two values are very important for data analysis using the 5′ nuclease assay. Chemical modifications such as minor groove binder (MGB) moiety (Afonina et al., 2002), peptide nucleic acid (PNA) backbones or locked nucleic acid (LNA) analogs increases probe stability and enables efficient PCR probes to be generated.

3.3. SYBR green I dye chemistry

It provides an alternate method to perform RT-qPCR analysis. SYBR Green dye binds the minor groove of double stranded DNA (Morrison et al., 1998). When SYBR Green dye binds double stranded DNA, the intensity of the fluorescent emissions increases. As more double stranded amplicons are produced, SYBR Green dye signal will increase. SYBR Green dye will bind to any double stranded DNA molecule, while the 5′ Nuclease assay is specific to a pre-determined target.

4. Primer Designing

In case of *in vitro* amplification reactions, like PCR or reverse transcription (RT), primers are short oligonucleotides composed of DNA, which serve as starting points for DNA replication. By choosing specific primer sequences the desired regions of a DNA fragment can be amplified.

For most PCR reactions, the most important factors for determining the success of the whole reaction are the sequence and the quality of the primers. The typical primer length is 18-30 bases. Very short or very long primers result in reduced specificity and efficiency and hence lower PCR efficiency. Repetitive sequences should be avoided. The GC content should range between 40-60%. Complementarity within primers that exceeds 3 bases and homologies between primers especially in 3′-end should be avoided. A, G- or C- nucleotide at 3′-end will increase primer binding strength. However, more than 3 G/C bases will diminish the specificity of the reaction (Dieffenbach et al., 2011).

For special applications, e.g. gene expression analysis, mRNA-specific primer pairs are recommended to avoid the genomic DNA amplification. Two methods can be chosen. First, the primers can be designed to bind in two different exons due to which PCR fragments derived from genomic DNA and mRNA can be easily distinguished (Genomic DNA derived PCR fragments would be much larger than those of mRNA derived). Depending upon the intron length, the polymerization times can be shortened so that the long fragments won't be synthesized.

Another recommended method is to design primers that bind over exon-exon junctions on the mRNA. This also prevents the amplification of genomic DNA.

For RT-qPCR applications, the following recommendations should be followed:

- The amplicon length should be less than 150 bp
- For TaqMan assays, a G-residue on the 5′-end of the probe should be avoided to prevent conjugation of Fluorescein-based reporter dyes to a G-residue.
- The strand which gives the probe more C's than G's should be selected.
- For optimal quenching, the maximum TaqMan probe length is 30 bases.
- The Tm of the probe should be between 68 and 70 °C.
- The primers should be designed after the probe.
- They should be designed close to the probe without overlapping it. A distance of 3 bases from the probe would be good.
- The Tm of the primers should be at least 60 °C and should be 8-10 °C lower than the probe.

Only highly purified primers (e.g. HPLC-purified) should be used if possible. Non-purified primer samples would generally contain shorter fragments generated during their synthesis. These side products will favor the formation of primer dimers, consequently decrease the PCR sensitivity and yield.

Primers are usually supplied as lyophilisates and can be stored at 2-8 °C. Stock solutions of Primers should always be aliquoted and stored at -20 °C. Repeated freeze-thaw cycles should be avoided.

5. Quantification Strategies in RT-qPCR

In RT-qPCR, two quantification types are possible generally, (i) A relative quantification which is based on relative expression of a target gene opposed to a reference gene. This is adequate to investigate the physiological changes in gene expression. (ii) An absolute quantification which is based either on an internal or an external calibration curve.

5.1. $\Delta\Delta C_q$ method

When reporting expression of target genes relative to those of reference genes, PCR efficiency is particularly important. The $\Delta\Delta C_q$ or $\Delta\Delta C_t$ method is one of the most popular means of determining differences in concentrations between samples and is based on normalisation with a single reference gene. The difference in C_q values (ΔC) between the target gene and the reference gene is calculated and the ΔC_qs of the different samples are compared directly. The two genes must be amplified with comparable efficiencies for this comparison to be accurate. C_q values > 40 are suspect because of the low efficiency and generally should not be reported. However, more generalized quantitative models have been developed to correct the differences in amplification efficiency and allow the use of multiple reference genes.

5.2. Calibration curve

Calibration curve provides a more accurate means to establish PCR amplification efficiency. It provides a simple, rapid and reproducible indication of the mean PCR efficiency, the analytical sensitivity and the robustness of the assay. The slope of the log-linear portion of the calibration curve is usually used to determine the amplification efficiency. When the logarithm of the initial template concentration (the independent variable) and C_q (the dependent variable) are plotted on the x-axis and y-axis respectively, the PCR efficiency = $10^{-1/slope} - 1$. The theoretical maximum of 1.00 (or 100%) indicates that the amount of product doubles with each cycle. Differences in PCR efficiency will produce calibration curves with different slopes. Hence, the differences between the C_q values of the targets and the references will not remain constant and would yield misleading results due to inaccuracy in calculations of relative concentrations.

5.3. Normalisation

Normalisation is an essential component of a reliable RT-qPCR assay (Huggett et al., 2005). This process controls the variations in extraction yield, reverse-transcription yield and amplification efficiency and thus enables the comparison of mRNA concentrations across different samples. Normalisation of the target gene with an endogenous standard is the most appropriate strategy. However, their utility must be experimentally validated for particular tissues or cell types and specific experimental designs.

5.4. Housekeeping genes

As an endogenous standard, mainly non-regulated reference genes or housekeeping genes (Thellin et al., 1999) like glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), albumin, actins, tubulins, cyclophilin, hypoxanthine phosphoribosyltransferase (*HPRT1*), 18S rRNA or 28S rRNA etc., are applicable. They are present in all nucleated cell types since they are necessary for basis cell survival.

Reference gene mRNAs should be stably expressed and their abundances should show strong correlation with the total amounts of mRNA present in the samples. Normalisation against a single reference gene is not acceptable unless the investigators present clear evidence for the reviewers that confirms its invariant expression under the experimental conditions described. Hence, the optimal number and choice of reference genes must be experimentally determined.

6. MIQE guidelines

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (*MIQE*) is a set of guidelines

that describes the minimum information necessary for evaluating RT-qPCR experiments (Bustin et al., 2009). Included is a checklist to accompany the initial submission of a manuscript to the publisher. Full disclosure of all reagents, sequences and analysis methods is necessary to enable other investigators to reproduce results.

The purpose of these guidelines is as follows:

- To enable authors to design and report RT-qPCR experiments those have greater inherent value.
- To allow reviewers and editors to measure the technique quality of submitted manuscripts against an established yardstick.
- To facilitate easier replication of experiments described in published studies that follow these guidelines.

7. Advantages of RT-qPCR

- Traditional PCR is measured at End-Point (plateau), while RT-qPCR collects data in the exponential growth phase
- An increase in Reporter fluorescent signal is directly proportional to the number of amplicons generated
- The cleaved probe provides a permanent record amplification of an amplicon
- Increase dynamic range of detection
- No-post PCR processing
- Detection is capable down to a 2-fold change

8. Applications of RT-PCR

RT-PCR can be applied to traditional PCR applications as well as new applications that would have been less effective with traditional PCR. RT-qPCR is used for a broad range of applications including quantitative gene expression analysis, genotyping, SNP analysis, pathogen detection, drug target validation and for measuring RNA interference.

9. Conclusion

Introduction of real-time PCR technology into the field of molecular biology has simplified the quantification of nucleic acids that generates innumerable data within a short period of time. Although most of the real-time PCR assays at high precision and reproducibility, however the accuracy of the obtained data is largely depended on several other factors such as sample preparation, quality of the standard and choice of housekeeping gene. Reproducibility of RT-qPCR result varies greatly between tissues, isolation methodology and the reagents used for this MIQE provides a checklist for preparing a report of the study. The accuracy of the produced data has to be checked during the establishment of the assay by comparison with other established assays.

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