Real-Time PCR (also known as quantitative PCR or qPCR)

Adapted from Current Protocols Essential Laboratory Techniques
(DOI: 10.1002/9780470089941.et1003s00)

Overview

During PCR amplification, short DNA sequences are copied at each cycle. Theoretically, the amount of DNA in the reaction should double at each cycle, resulting in an exponential amplification of the initial target DNA. This is potentially true during the early cycles when the PCR components are in vast excess compared to the target sequence. However, as product accumulates, the substrates are depleted, resulting in the inhibition of the reaction. By examining how efficiently the amplicon is being produced, a PCR reaction can be broken into three distinct phases: exponential, linear, and plateau (Figure1).

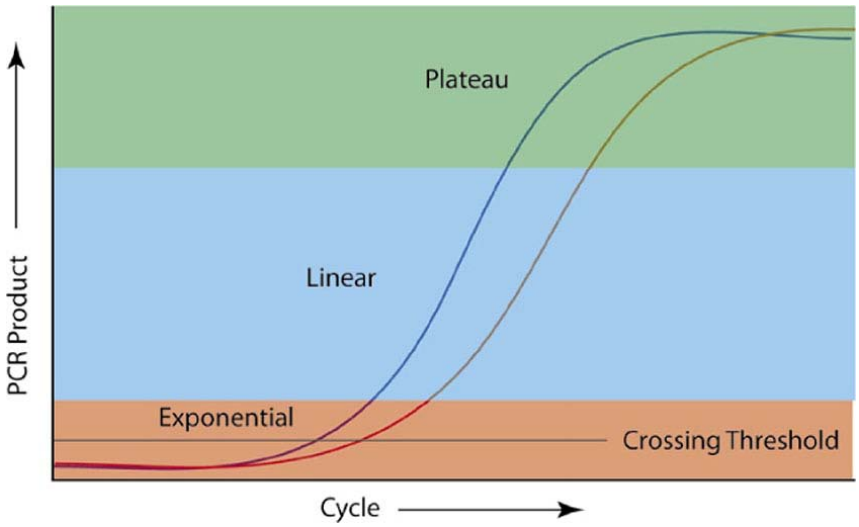

Figure 1. Phases of PCR**.** Initially, when the amount of product is small and enzyme and reagents are not limiting, product generation is exponential and the reaction is closest to 100% efficiency. During the linear phase products continue to accumulate, but the reaction efficiency begins to fall and reagents become limiting. Finally, in the plateau phase of the reaction, accumulation of product ceases as the reaction is exhausted for a number of reasons. Adapted from http://www.biotechniques.com/BiotechniquesJournal/specialissues/2008/April/Twenty-five-years-of-quantitative-PCR-for-gene-expression-analysis/biotechniques-45217.html?pageNum=2

Semi-quantitative Reverse Transcription PCR (RT-PCR) analysis generally relies on measuring the end point of a PCR reaction by ethidium bromide visualization of the DNA product separated by agarose gel electrophoresis (Figure 2). This approach has limitations in accurately and reliably quantifying samples, due to the low sensitivity of the ethidium bromide staining procedure and the variable kinetics of the PCR reaction.

Real-time PCR (or qPCR) allows precise quantification of specific PCR products even if the starting amount of material is at a very low concentration. This is achieved by quantifying amplicon production at the exponential phase of the PCR reaction in contrast to measuring the amount of product at the end-point of the reaction. The amplicon is monitored in “real-time,” or as it is being produced, by labeling and detecting the accumulating product with a fluorescently

tagged substrate during the amplification procedure. How quickly the amplified target reaches a threshold detection level correlates with the amount of starting material present. This method has many advantages over conventional PCR including increased speed due to reduced cycle number, lack of post-PCR gel electrophoresis detection of products, and higher sensitivity of the fluorescent dyes used for the detection of the amplicon.

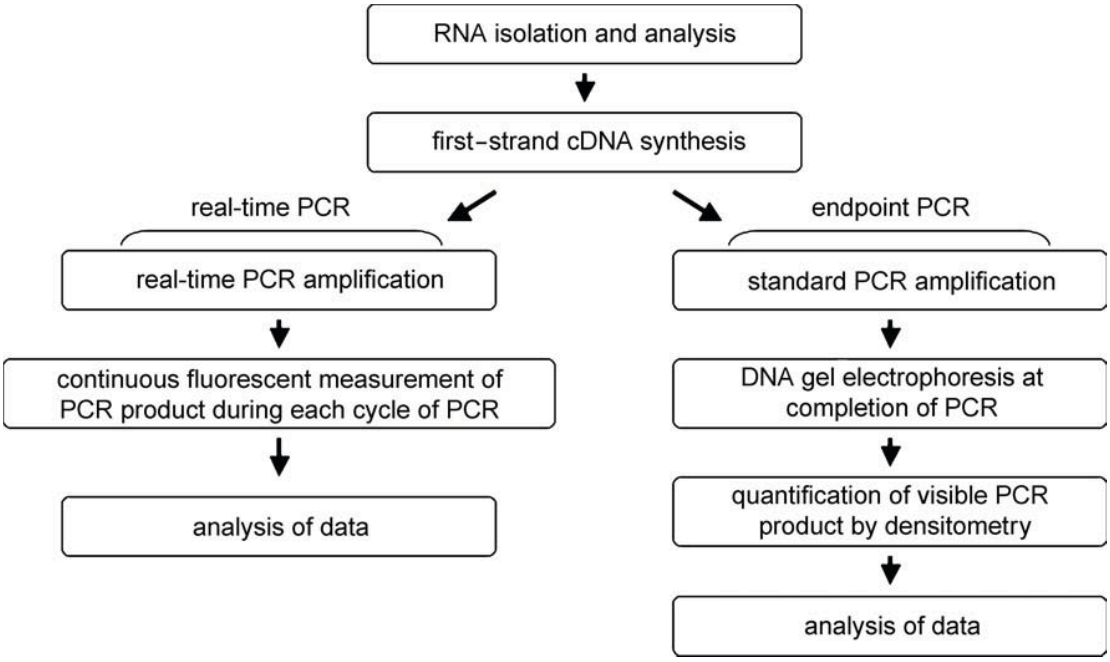

Figure 2. Comparison of endpoint RT-PCR and real-time RT-PCR. Adapted from Current Protocols Essential Laboratory Techniques (DOI: 10.1002/9780470089941.et1003s00).

A common strategy to fluorescently label DNA in qPCR is to use SYBR Green dye. The principle behind the SYBR Green family of dyes is that they undergo a 20- to 100-fold increase their fluorescence upon binding dsDNA that is detected by the real-time PCR machine's detector. Thus, as the amount of dsDNA increases in the reaction mix, there will be a corresponding increase in the fluorescent signal. However, this simplicity means that they do not distinguish between different dsDNA products and it is important that PCR reactions be optimized so that only the target amplicon is present or that other methods be employed to distinguish between different products (e.g., melting point analysis).

A. Primer design

Successful qPCR depends upon the ability to amplify a short product (<300 bp, ideally 100 to 200 bp) that is specific to the mRNA. Typically, this means that one or both primers straddle an intron by annealing to the exons at the 5' end and the 3' end of the intron (Figure 3). It is also possible to design effective primers that flank a large intron and are thus much more efficient at amplifying cDNA than any genomic DNA contamination. Effective design thus requires sequence information on the genomic DNA of a gene and direct alignment with the cDNA coding sequence. Sequence information can be downloaded from a number of publicly available websites, including the NCBI website (http://www.ncbi.nlm.nih.gov/).

To assist in the design of primers, a number of computer programs are available that can identify primers for the most efficient amplification of target sequence. These programs take into account several factors to optimize primer design, including: (1) elimination of primer dimers due to complementary sequence between primers, (2) matched annealing temperatures between the primers, and (3) suitable difference between the calculated annealing temperature of the primers and the annealing temperature of the PCR product. Two widely used programs are Primer3 and NCBI Primer-BLAST.

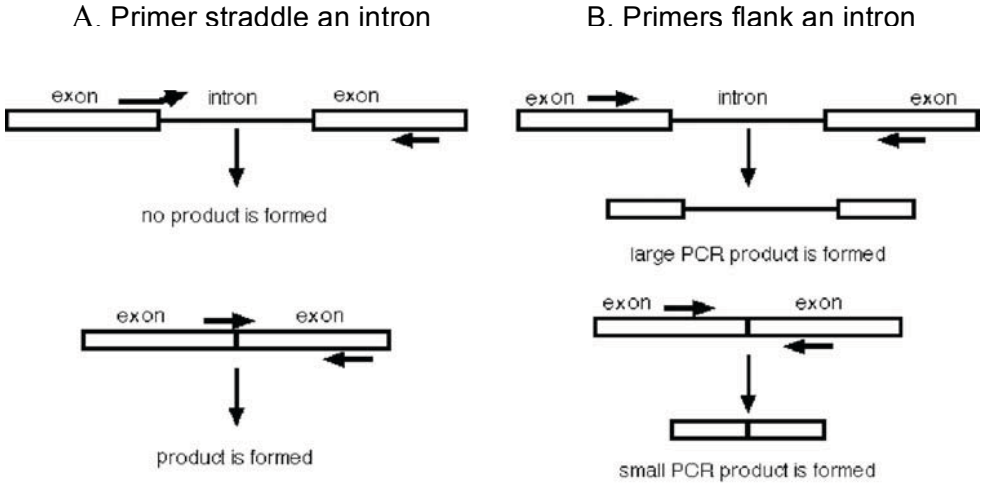

Figure 3. Designing primers for real-time PCR. Adapted from Current Protocols Essential Laboratory Techniques (DOI: 10.1002/9780470089941.et1003s00).

B. Optimizing the PCR Reaction for Real-Time Analysis

Important considerations:

Optimal annealing temperature

After primers have been synthesized, their annealing temperatures should be optimized. This can be done using a temperature gradient analysis to determine the optimal annealing temperature. A temperature gradient analysis takes advantage of a feature found in most thermal cyclers that allows one to simultaneously conduct several PCR reactions at different temperatures. The different reactions can then be compared and a decision made about which annealing temperature will give the best product.

Number of PCR products

Detecting the PCR product in real-time involves the use of a fluorescent dye. Nonspecific fluorescent dyes from the SYBR Green family are most commonly used, because they are more economical compared to strand-specific probes, and easier to optimize. When using this method of detection, a single, specific DNA fragment has to be obtained during PCR amplification, because any additional nonspecific DNA fragment accumulation will contribute to the fluorescence measured.

Amplification efficiency

In order to make valid comparisons between different samples it is important to determine the amplification efficiency of your primers. Ideally, amplification efficiencies for each primer set

should be roughly equal.

C. Experimental design

Prior to setting up the actual experiments it is important to consider your experimental design and several quality control steps, including negative and positive controls. These will ensure that the real-time PCR data being generated is of high quality. In addition, for quality control reasons, each sample is run in triplicate.

Real-time PCR experiments should routinely include the following controls:

- Positive control (when available)
- Negative controls:
  - No template
  - No reverse transcriptase control

The no-template control confirms that there is no contamination in the PCR reagents. If a band is produced in the no-template control, this might indicate that one or more of the reagents is contaminated with template, or, more likely, previously amplified PCR product. The specific reagent that is contaminated will have to be determined by troubleshooting. Using barrier micropipet tips with all PCR reagents can prevent most contamination problems.

Running a no-reverse-transcriptase control helps detect the presence of contaminating DNA in the RNA. If the no-template control is negative and the no-reverse-transcriptase control produces a band, most likely there is contaminating DNA that is being recognized by the primers. Treatment of the RNA template with RNase-free DNase should alleviate the problem. In some cases, the primers may have to be redesigned so as to avoid amplifying potential DNA targets. Typically this may require straddling a different intron or adjusting the 3' end of the primer slightly to have less potential to basepair at its 3' end with a genomic DNA sequence.

Lastly, when conducting real-time RT-PCR for the first time, it is helpful to also include a positive control. This is especially important if the absence of gene expression is a likely result.

D. Real-Time RT-PCR Analysis and Quantification

<u>Setting the threshold for detection of the amplicon</u>

Setting the threshold determines the level of fluorescence signal that is sufficiently above background to be considered a reliable signal. The cycle at which the threshold is met or exceeded is called the "cycle threshold" (CT), and is used for making comparisons between samples. Setting the threshold should take into consideration the detection limits of the equipment and background fluorescence due to the fluorescent chemistry used. Setting the threshold too low may result in unreliable data collection due to random fluctuations in the sample tube that result in premature 'detection' of product. Setting it too high may result in the detection of product after it has left the exponential phase and has entered the linear phase, again resulting in inaccurate data collection.

It is important that the threshold be set to allow detection of product while it is still in the exponential phase. The point at which the product's detected fluorescence crosses the threshold is called the CT value. The significance of threshold values is that they provide a useful measure for comparison between samples. In a 100% efficient PCR, the amplified

product will double at each cycle. This means that differences in CT between different samples correspond to differences in starting amount of the target sequence. For example, a CT difference of 1 corresponds to a 2-fold difference in starting material and the sample with the lower CT value has more starting material. Each difference in CT value corresponds to differences in starting material of 2 raised to the power of the CT difference.

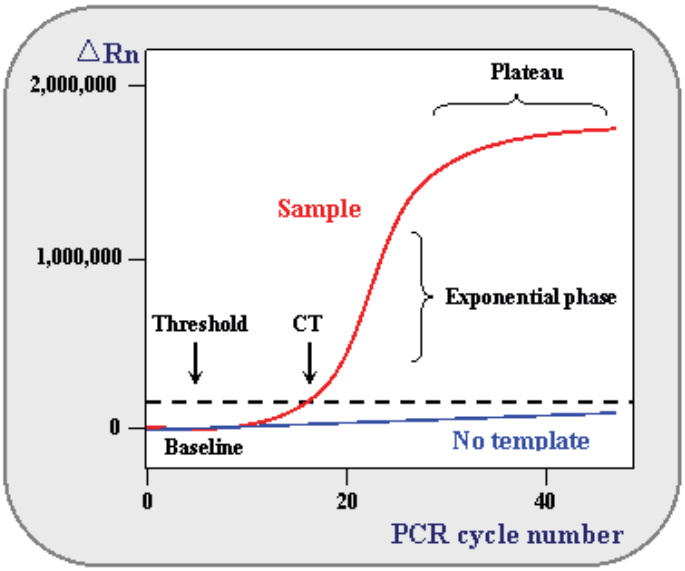

Figure 4. Model of real-time quantitative PCR plot. **Baseline** is defined as PCR cycles in which a reporter fluorescent signal is accumulating but is beneath the limits of detection of the instrument. **Threshold** is an arbitrary level of fluorescence chosen on the basis of the baseline variability. A signal that is detected above the threshold is considered a real signal that can be used to define the threshold cycle (CT) for a sample. Threshold can be adjusted for each experiment so that it is in the region of exponential amplification across all plots. **CT** is defined as the fractional PCR cycle number at which the reporter fluorescence is greater than the threshold. The CT is a basic principle of real time PCR and is an essential component in producing accurate and reproducible data.
Adapted from http://www.ncbi.nlm.nih.gov/genome/probe/doc/TechQPCR.shtml

Relative quantification

The data obtained from real-time RT-PCR analysis are CT values that are a measure of the amount of starting target material. The CT values need to be converted using different procedures to make valid comparisons. Ultimately what is obtained is a measure of differences in gene expression between two samples (e. g., treated and control).

When comparing samples using relative quantification, the CT values from different treatments will need to be compared after correction for amplification efficiency and normalization with the housekeeping gene. The normalized fold induction differences that are determined can be used for comparison purposes between treated and untreated samples. It is important to remember that these relative changes in gene expression are not a measure of how much actual message is present and is simply a relative measure of how much that gene has increased (or decreased) in gene expression in relative terms. However, for many experimental purposes this is sufficient.

Protocol

- Use a 96-well plate template to determine the plate set up. Run at least 3 technical replicates for each reaction in the same plate.

- Remember to wear gloves!

- To set up the PCR make 2 master mixes for each primer pair you want to test:
    - cdna + syber green =6 μl total
        - 5 μl Sybr Green
        - 1 μl cDNA

    - primers + dH_2O = 4 μl total
        - 3.4 μl dHH_2O
        - 0.3 μl 10 μM F primer
        - 0.3 μl 10 μM R primer

- Aliquot the primer mix first, then the cDNA so you don't contaminate the wells with mixed cDNA for each primer pair.

- Tap samples down gently.

- Centrifuge samples briefly before loading onto machine.

- Load into machine, making sure to place tubes in correct orientation. Run the adequate program.

- Analyze the data using the "Real-time PCR analysis" Excel template and the Nature Protocols paper "Analyzing real-time PCR data by the comparative CT method" (Schmittgen and Livak, 2008) as a reference.

References

Fraga, D., Meulia, T. and Fenster, S. (2014). Unit 10.3 Real-Time PCR. Current Protocols Essential Laboratory Techniques, DOI: 10.1002/9780470089941.et1003s08.

Real-Time qRT-PCR. http://www.ncbi.nlm.nih.gov/genome/probe/doc/TechQPCR.shtml

Schmittgen, T.D. and Livak, K.J. (2008). Analyzing real-time PCR data by the comparative CT method. Nature Protocols, doi:10.1038/nprot.2008.73

7.15 Experimental Molecular Genetics

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