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REAL-TIME PCR APPLICATION AND GENE ANALYZING

Overview

PCR technology is widely used to aid in quantifying DNA. In an optimized reaction, the target quantity will approximately double during each amplification cycle (Fig. 1). Thus, the quantity of amplified product is linked to that of initial template DNA in quantitative PCR.

The point at which the quantity of amplified product is measured in order to calculate the initial template quantity can either be at the end of the reaction (endpoint semi-quantitative PCR) or while the amplification is still progressing (quantitative real-time PCR). Since the latter method has several advantages over the former one, quantitative real-time PCR is adopted in this course. In quantitative real-time PCR, a fluorescent reporter molecule (such as a double-stranded DNA binding dye, or a dye-labeled probe) is used to monitor the progress of the amplification reaction. With each amplification cycle, the fluorescence intensity increases proportional to the increase in amplicon concentration, with the PCR instrument system collecting data for each sample during each PCR cycle.

SYBR Green I is a common choice for quantification of amplified DNA. When free in solution, SYBR Green I displays relatively low fluorescence, but when bound to double-stranded DNA its fluorescence increases by over 1000-fold (Fig. 2). The more double-stranded DNA that is present, the more binding sites there are for the dye, so fluorescence increases proportionately to DNA concentration. This property of the dye provides the mechanism that allows it to be used to track the accumulation of PCR product. As the target is amplified, the increasing

concentration of double-stranded DNA in the solution can be directly measured by the increase in fluorescence signal.

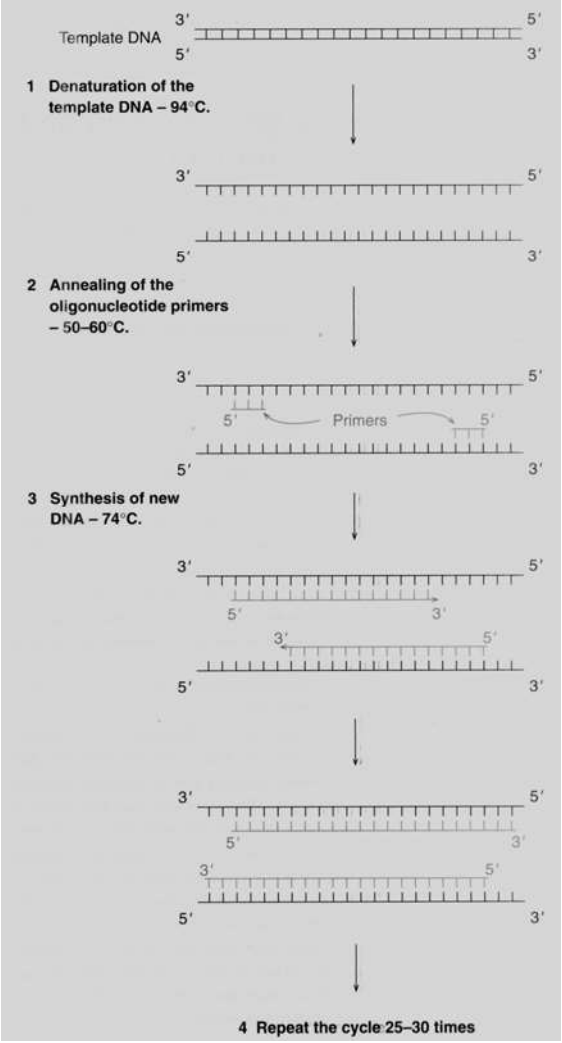


Figure 1. The basic steps in the polymerase chain reaction. Cited from Brown T. A. "Gene Cloning and DNA Analysis. 4th edition". Blackwell Science, 2001.

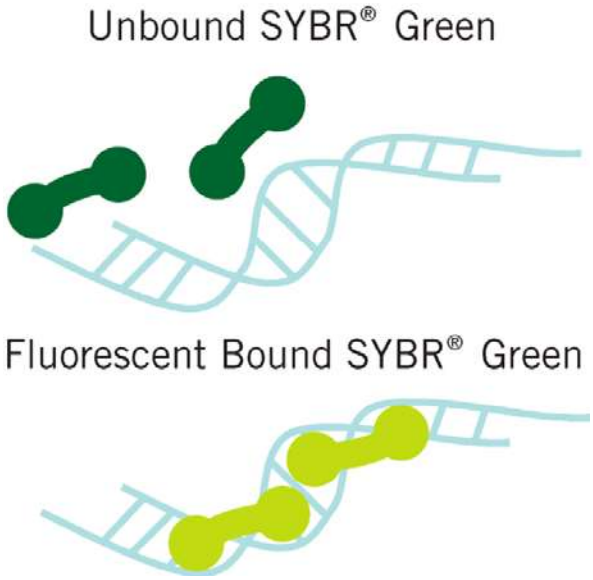


Figure 2. SYBR Green I detection mechanism. Cited from "Introduction to quantitative RT-PCR; methods and applications guide." Stratagene, 2007.

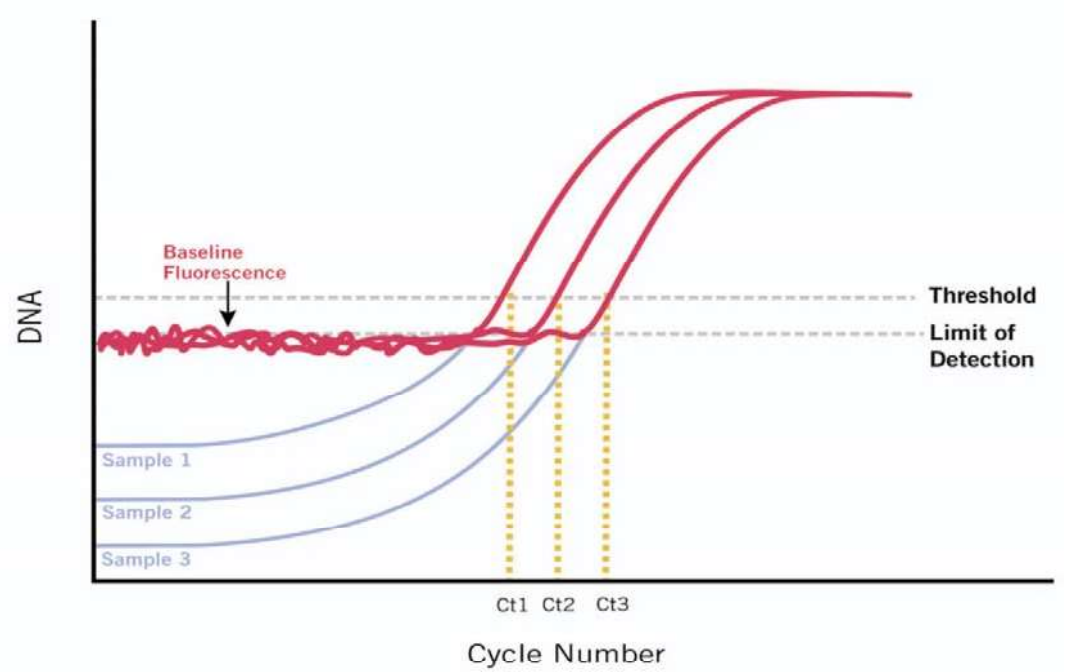


Figure 3. Principles of target concentration measurements using threshold cycle (Ct). The Ct is inversely proportional to the initial copy number. Only when the DNA concentration has reached the fluorescence detection threshold can the concentration be reliably inferred from the fluorescence intensity. A higher initial copy number will correlate to a lower threshold cycle. Cited from "Introduction to quantitative RT-PCR; methods and applications guide." Stratagene, 2007.

The resulting plots of fluorescence vs. cycle number for all the samples are then set with their background fluorescence at a common starting point (a process known as baseline correction). Then, a threshold level of fluorescence is set above the background but still within the linear phase of amplification for all the plots. The cycle number at which an amplification plot crosses this threshold fluorescence level is called the "Ct" or threshold cycle (Fig. 3). This Ct value can be directly correlated to the starting target concentration of the sample. The greater the amount of initial DNA template in the sample, the earlier the Ct value for that sample.

Comparative quantification is commonly used in real-time PCR analysis. This method allows us to calculate relative quantities of targets. Results are calculated as "relative quantity to the internal control gene", where the amount of the

internal control gene is assigned an arbitrary quantity of "1". Normally, some housekeeping genes such as β-actin gene and elongation factor-1α gene are used as the internal control because the relative copy number of these genes is considered to be constant.

Preprotocol considerations

1. RNA isolation

RNA isolation should be performed under RNase-free conditions. Wear gloves and use sterile tubes, pipet tips, and RNase-free water. Do not use DEPC-treated water, which can inhibit PCR. The RNase block that is included in the reaction mixture prior to RNA template addition provides additional protection against RNase contamination.

Contaminating DNA should be removed from the RNA preparation using an RNase-free DNase. Additionally, PCR primers may be designed to span adjacent exons in order to prevent amplification of the intron-containing genomic DNA.

2. Reference dye

A passive reference dye is included in this kit and may be added to compensate for non-PCR related variations in fluorescence. Addition of the reference dye is optional. Fluorescence from the passive reference dye does not change during the course of the PCR reaction, but provides a stable baseline to which samples are normalized. In this way, the reference dye compensates for changes in fluorescence between wells caused by slight volume differences in reaction tubes. The excitation and emission wavelengths of the reference dye are 584 nm and 612 nm, respectively.

Recommended control reactions

Performing no-RT control reactions is recommended for each experimental sample. The no-RT control is expected to generate no signal if there is no

amplification of genomic DNA. No signal indicates that the RNA preparation is free of contaminating genomic DNA or that the primers are specific for the cDNA.

It is also recommended to perform no-template control (NTC) reactions for each experimental sample to screen for contamination of reagents or false amplification. Slight, high cycle amplification indicates primer-only amplification (primer dimer, shown with a red line in Fig. 4). It is acceptable to observe a small amount of primer-dimer formation in the NTC wells, but if there is a corresponding peak in the sample amplification plots or dissociation analysis (Fig. 5, see 2-4) the Cts from these wells cannot be trusted as accurate.

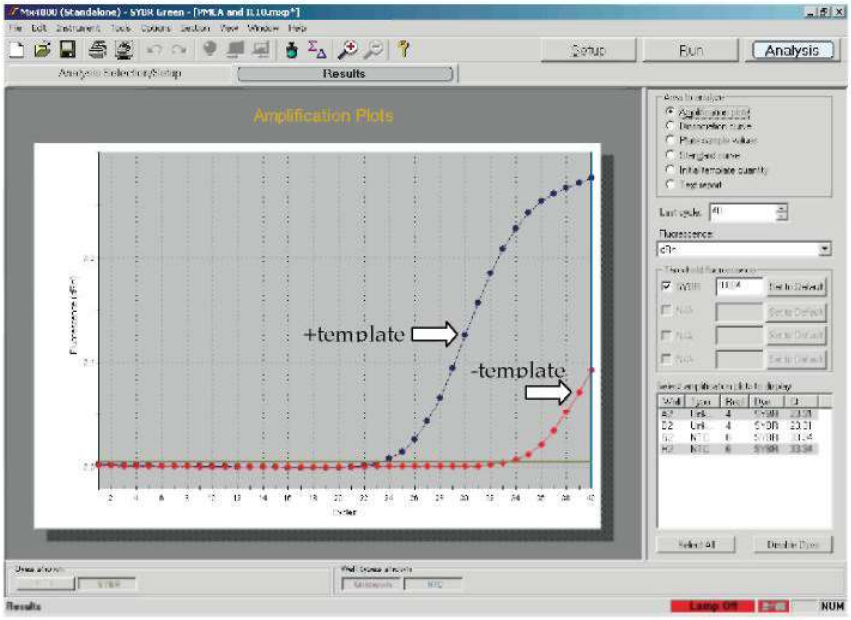


Figure 1. Typical amplification plot of quantitative real-time PCR. Blue and red lines indicate the results with and without DNA template, respectively. After "Brilliant SYBR green QRT-PCR master mix kit, 1-step instruction manual", Stratagene, 2007.

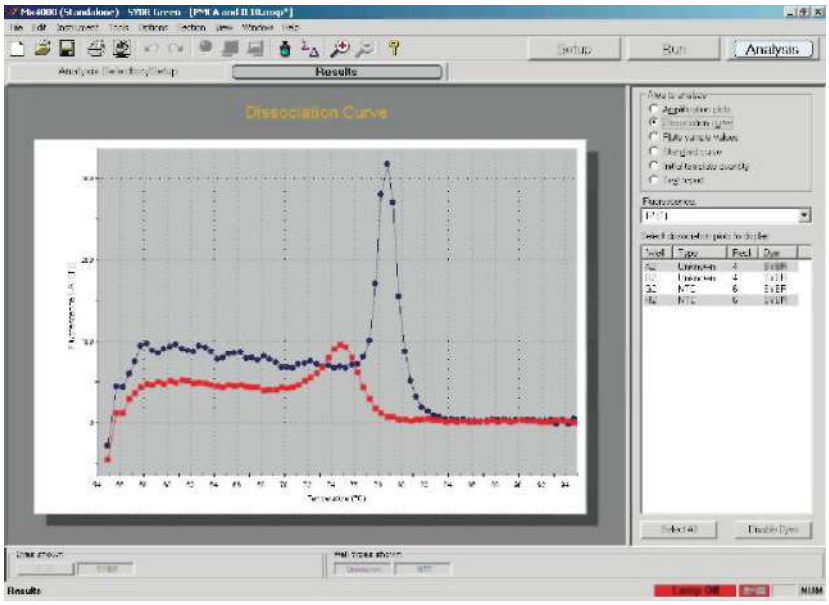


Figure 2. Typical result of dissociation analysis in quantitative real-time PCR. Blue and red lines indicate the results with and without DNA template, respectively. After "Brilliant SYBR green QRT-PCR master mix kit, 1-step instruction manual", Stratagene, 2007.

Dissociation (melting curve) analysis

To verify the proper amplification, a dissociation profile is generated. During a dissociation analysis, all PCR products are melted at 95°C, then annealed at 55°C and subjected to gradual increases in temperature. During the temperature increases, fluorescence data are collected until the reaction reaches 95°C. Figure 5 shows the melt data as the negative first derivative of raw fluorescence, R´(T), vs. temperature. Primer dimers can be detected by the analysis. Alternative splice forms, insertions, or deletions could also create alternatively melting PCR products, from specific priming sites.

Amplification efficiency of primers

In quantitative real-time PCR analysis, amplification efficiency of primers for the target gene and the internal control gene should be less than 0.1. Amplification efficiency is calculated as follows.

A three-fold to ten-fold dilution series over several orders of magnitude should be generated in triplicate. For example, for gene expression experiments, a typical serial dilution would consist of five points of a five-fold serial dilution, starting with 100 ng of total RNA per reaction (or the cDNA equivalent amount). Next, perform real-time PCR analysis using the primers, and make an amplification plot as shown in Fig. 6. The slope of the line indicates the amplification efficiency of the primer pair. Be aware that not all points of a standard curve will conform to high data quality metrics. Often, the high and low concentration points may not be in range, and elimination of these aberrant concentrations from analysis may result in a high quality assay, across a slightly lower linear quantitative range.

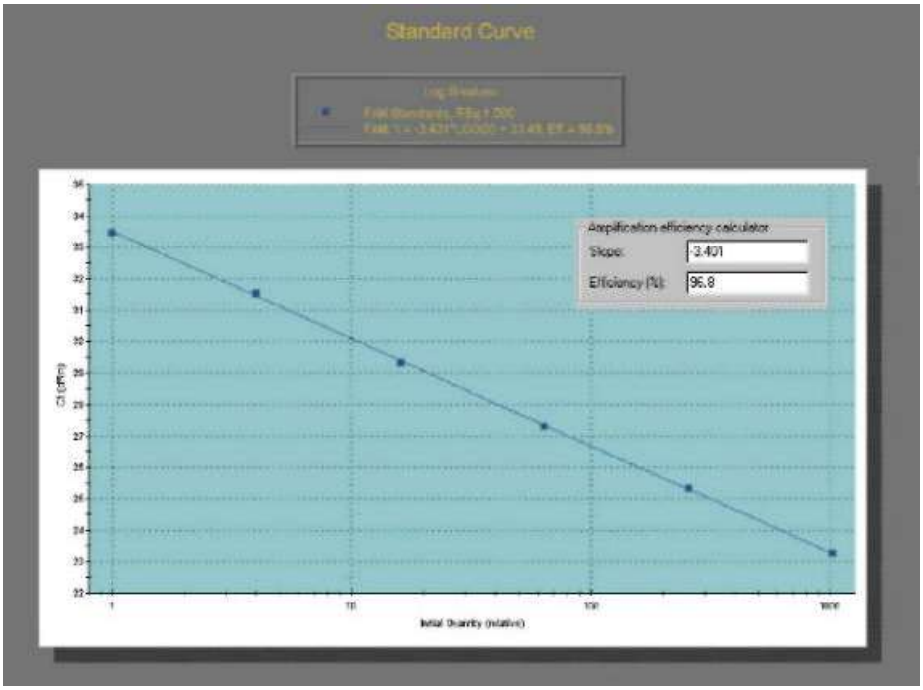


Figure 3. Calculation of amplification efficiency. X and Y axis indicate logarithmic plot of cDNA dilution factor and Ct values, respectively. Cited from "Brilliant SYBR green QRT-PCR master mix kit, 1-step instruction manual", Stratagene, 2007.

Protocols

1. RNA isolation

RNA is isolated by staff in this experiment.

Real-time PCR

1. If the reference dye is included in the reaction (optional), dilute the provided dye solution 1:500 (for the Mx3000P, Mx3005P, and Mx4000 instruments) or 1:50 (for the ABI PRISM 7700 instrument) using nuclease-free PCR-grade H_2O. Keep all solutions containing the reference dye protected from light.
2. Thaw the 2 x SYBR Green QRT-PCR master mix and store on ice. Mix the solution well by gentle inversion prior to pipetting.
3. Prepare the experimental reactions by combining the following components *in order.* Stratagene recommends preparing a single reagent mixture for duplicate experimental reactions and duplicate no template-controls using multiples of each component listed below.

Reagent Mixture

Nuclease-free PCR-grade H_2O to adjust the final volume to 25 µl (including experimental RNA)

12.5 µl of 2 x SYBR QRT-PCR master mix
x µl of upstream primer (50 - 100 nM final concentration)
x µl of downstream primer (50 - 100 nM final concentration)
0.375 µl of the diluted reference dye (optional)
0.0625 µl of StrataScript RT/RNase block enzyme mixture

1. Gently mix the reactions **without creating bubbles (do not vortex)**, then distribute the mixture to the experimental reaction tubes.
2. Add *x* µl of experimental RNA to each reaction. The quantity of RNA

depends on the RNA purity and the specific mRNA abundance. As a guideline, use 1 pg-1 µg of total RNA or 0.1 pg-1 ng of mRNA.

3. Gently mix the reactions without creating bubbles (do not vortex).

4. Centrifuge the reactions briefly.

5. Place the reactions in the instrument and run the appropriate RT-PCR program using the guidelines below. These amplification protocols are recommended initially, but optimization may be necessary for some primer/template systems.

Cycles	Duration of cycle	Temperature
1	30 min	50°C
1	10 min[a]	95°C
40	30 sec	95°C
	1 min[b]	50 - 60°C[c]
	30 sec	72°C

[a]Initial 10 minute incubation is required to fully activate the DNA polymerase.
[b]Set the temperature cycler to detect and report fluorescence during the annealing/extension step of each cycle.
[c]Choose an appropriate annealing temperature for the primer set used.

6. Run dissociation program according to the guidelines of PCR instrument.

Data analysis

Results of the real-time PCR analysis are shown as follows.

Sample	Detector	Task	Ct
Sample A: target gene	SYBR	Unknown	30.0
Sample B: target gene	SYBR	Unknown	28.0
Sample A: internal control gene	SYBR	Unknown	22.0
Sample B: internal control gene	SYBR	Unknown	22.0

Relative target gene quantity of sample B to sample A is $2^{-(\Delta\Delta Ct)}$
where $\Delta\Delta Ct = (Ct_{(target)} - Ct_{(control)})_{sampleB} - (Ct_{(target)} - Ct_{(control)})_{sampleA}$
In the above experiment, $\Delta\Delta Ct = (28.0 - 22.0) - (30.0 - 22.0) = -2.0$
Thus, relative target gene quantity of sample B to sample A is 22.0 = 4.0

References

1. Introduction to quantitative RT-PCR; methods and applications guide. Stratagene, 2007.
2. Brilliant SYBR green QRT-PCR master mix kit, 1-step instruction manual. Stratagene, 2007.
3. Brown T. A. Gene cloning and DNA analysis. Blackwell Science, 2001.