

Advanced Cell Biology Lab 7.

Real-Time PCR (qPCR): the theory and interpretation of results

March 28th, 2011

Background

Conventional PCR does well to detect the presence of the DNA that the primer pair targets. Conventional PCR detects the amplified product (amplicon) by an end-point analysis—running the DNA on an agarose gel after the reactions are completed.

If the target DNA sequence is not there, no amplicon will appear on the agarose gel. If as little as a single DNA molecule that contains the target DNA sequence is in the sample. The amplification by 25–30 cycles is sufficient to generate detectable amplicons via electrophoresis. Thus, conventional PCR makes a highly sensitive assay for specific DNA sequence, which is useful for the diagnosis of diseases, especially viral types. It is also a rapid, highly sensitive and specific assay for microbes in environmental samples. It is, of course the assay of choice for genotyping human samples as you have seen in the PV92, Alu dimorphism analysis. Results of such assays are often used in paternity testing and in crime scene analysis.

Real-time PCR is based on the same principles as conventional PCR. The reaction requires both forward and reverse primers bracketing the target region (amplicon), nucleotides, and a DNA polymerase such as *Taq*. However, real-time PCR allows the accumulation of amplified product to be detected and measured as the reaction progresses—in “real time”. The difference is the addition of a fluorescence chemistry, which enables product amplification to be monitored throughout the entire real-time reaction using specialized thermal cyclers equipped with fluorescence detection modules. The measured fluorescence reflects the amount of amplified product in each cycle. Real-time PCR results can either be qualitative (presence or absence of a sequence) or quantitative (number of copies of DNA). Real-time PCR that is quantitative is also known as qPCR. The main advantage of using real-time PCR over conventional PCR is that real-time PCR allows you to determine the starting template copy number with accuracy and high sensitivity over a wide dynamic range. Conventional PCR can at best be semi-quantitative and the methods to obtain quantitative data can be quite complicated. One advantage of conventional PCR is better determination of the sizes of the amplified PCR products using conventional gel electrophoresis. Therefore, separating the real-time PCR products on a gel following amplification allows the visualization and determination of the size of the amplified products.

How Real-Time PCR Works

To best understand how real-time PCR works, think of what is happening in a PCR reaction. During the first cycles of a PCR reaction, the amount of amplicon doubles. The amount of amplicon after each cycle then multiplies exponentially in proportion to the starting amount of template in the sample. At some point, this doubling slows as the amount of substrate, nucleotides, and primers become used up. The DNA polymerase also becomes less active after the prolonged heating within the thermal cycler. The loss of doubling efficiency results in a plateau effect and the amount of amplicon produced with the later thermal cycles is no longer proportional to the amount of template DNA in the sample (Fig. 1). After enough cycles, all amplicons reach a certain maximum concentration, regardless of the initial concentration of template DNA.

The key to determining the quantity of original template DNA present in a sample during amplification is to examine the initial thermal cycles before reaching the plateau region of amplification. To

do this, the level of amplification is monitored continuously during the thermal cycling. Initially, fluorescence remains at background levels, and increases in fluorescence are not detectable (cycles 1–18 in Fig. 1) even though PCR product accumulates exponentially. Eventually, enough amplified product accumulates to yield a detectable fluorescent signal. The cycle number at which this occurs is called the threshold cycle, or CT. Since the CT value is measured in the exponential phase when reagents are not limited, real-time qPCR can be used to reliably and accurately calculate the initial amount of template present in the reaction.

The CT of a reaction is determined mainly by the amount of template present at the start of the amplification reaction. If a large amount of template is present at the start of the reaction, relatively few amplification cycles will be required to accumulate enough product to give a fluorescent signal above background. Thus, the reaction will have a low, or early, CT. In contrast, if a small amount of template is present at the start of the reaction, more amplification cycles will be required for the fluorescent signal to rise above background. The most commonly used chemistries for real-time PCR are the DNA-binding dye SYBR Green I and TaqMan hydrolysis probe. We provide an overview of SYBR Green I fluorescence chemistry below.

SYBR Green I is a DNA dye that binds non-discriminately to double-stranded DNA (dsDNA). SYBR Green I exhibits minimal fluorescence when it is free in solution, but its fluorescence increases dramatically (up to 1000-fold) upon binding to dsDNA (Fig. 2). As the PCR reaction progresses, the amplified product accumulates exponentially, more SYBR Green I binds, and fluorescence increases. The advantage of using SYBR Green I is its simplicity. This is similar to the action of ethidium bromide, but unlike ethidium bromide, SYBR Green I does not interfere with DNA polymerases, so it can be added directly to a PCR reaction mixture. SYBR Green I also has less background fluorescence than does ethidium bromide, is able to detect lower concentrations of double-stranded DNA, and is not hazardous.

Optimizing qPCR

Since real-time quantitation is based on the relationship between initial template amount and the CT value obtained during amplification, an optimal qPCR assay is absolutely essential for accurate and reproducible quantitation of your particular sample. The hallmarks of an optimized qPCR assay are:

- Linear standard curve
- Consistency across replicate reactions

A powerful way to determine whether your qPCR assay is optimized is to run a set of serial dilutions of template DNA and use the results to generate a standard curve. The template used for this purpose can be a target with known concentration (for example, nanograms of genomic DNA or copies of plasmid DNA) or a sample of unknown quantity (for example, total cell DNA—cDNA). A standard curve is constructed by plotting the log of the starting quantity of template (or the dilution factor, for unknown quantities) against the CT value obtained during amplification of each dilution. The equation of the linear regression line, along with Pearson's correlation coefficient (r) or the coefficient of determination (R^2), can then be used to evaluate whether your qPCR assay is optimized. Ideally, the dilution series will produce amplification curves that are evenly spaced, as shown in Fig. 3. If perfect doubling occurs with each amplification cycle, the spacing of the fluorescence curves will be determined by the equation $2^n = \text{dilution factor}$, where n is the number of cycles between curves at the fluorescence threshold (in other words, the difference between the CT values of the curves). For example, with a 10-fold serial dilution of DNA, $2^n = 10$. Therefore, $n = 3.32$, and the CT values should be separated by 3.32 cycles. Evenly spaced amplification curves will produce linear standard curves, as shown in Fig. 4. The equation and r values of the linear regression lines are shown above the plot.

Assignment

In today's lab we will analyze results of qPCR optimization experiment. This experiment utilized serial dilutions of the same template to illustrate the quantitative application of real-time. Each sample amplified the available template exponentially. The more starting template, the quicker the amplified product has been accumulated. Samples with more template reach the threshold for fluorescence detection sooner (at an earlier cycle) than samples with less template. We call this threshold cycle the CT value for the experiment.

We have three graphs (Figures 3, 4, and 5) and the table which reflects the results of qPCR. Please review them, discuss within a group and answer these questions:

Fig. 3.

- What is the average difference between CT for every group of dilution?
- Known dilutions have 10-fold differences. Why CTs differ only on several cycles?
- Why CTs for same dilutions are not exactly the same?

Fig. 4.

- This graph summarizes our dilution scheme and the corresponding CT. Why is the log value used?

Fig. 5.

A very important final check on your qPCR run is analysis of the endpoint product. Since SYBR green will show amplification of any double-stranded product, it is essential that you check to see that a single, expected product is produced. To check this, at the end of the run, the thermal cycler goes through continuous series of temperature changes (from the annealing temp up to about 95°C). The melt curve plots the change in fluorescence (negative first derivative of fluorescence vs. temperature). A sharp peak will form at the temperature at which fluorescence changes rapidly. This corresponds to the melting temperature (T_m) for an amplification product in the tube.

- As the temperature increases, what will happen to the PCR products?
- What is the melting temperature (T_m) in the experiment?

Table 1.

- Please estimate the unknown dilution. Explain how you did it.

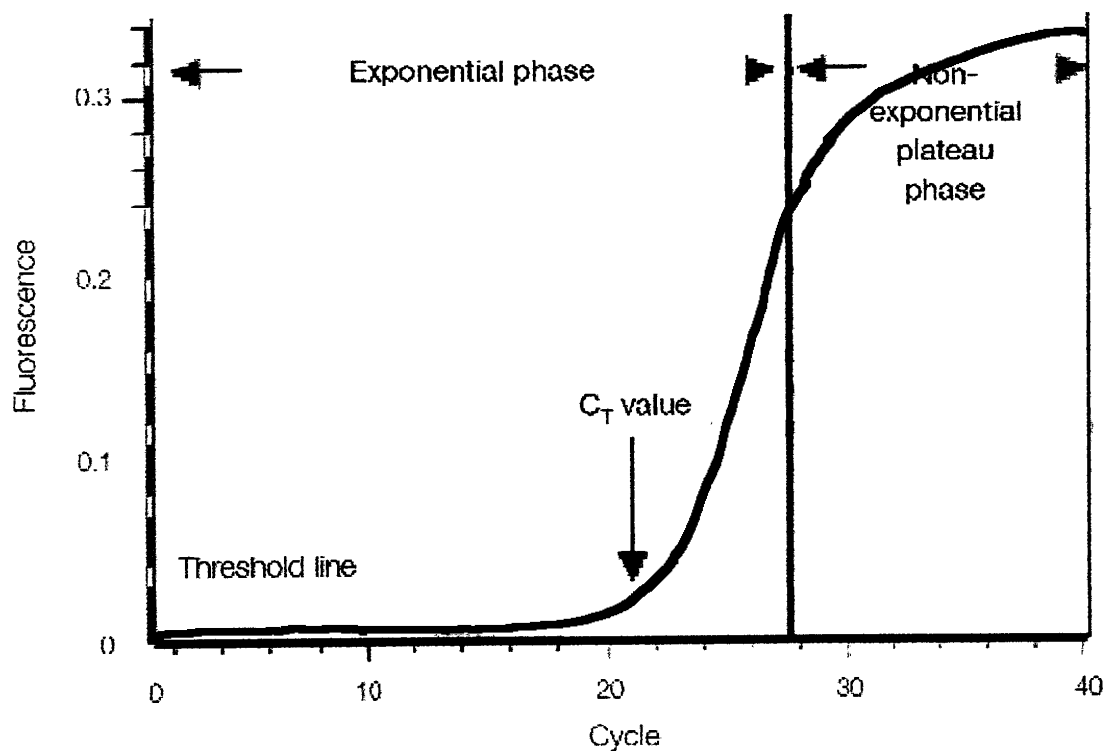


Fig. 1. Amplification plot. During the exponential phase, the amount of PCR product approximately doubles in each cycle. As the reaction proceeds and reaction components are consumed, the reaction slows and enters the plateau phase.

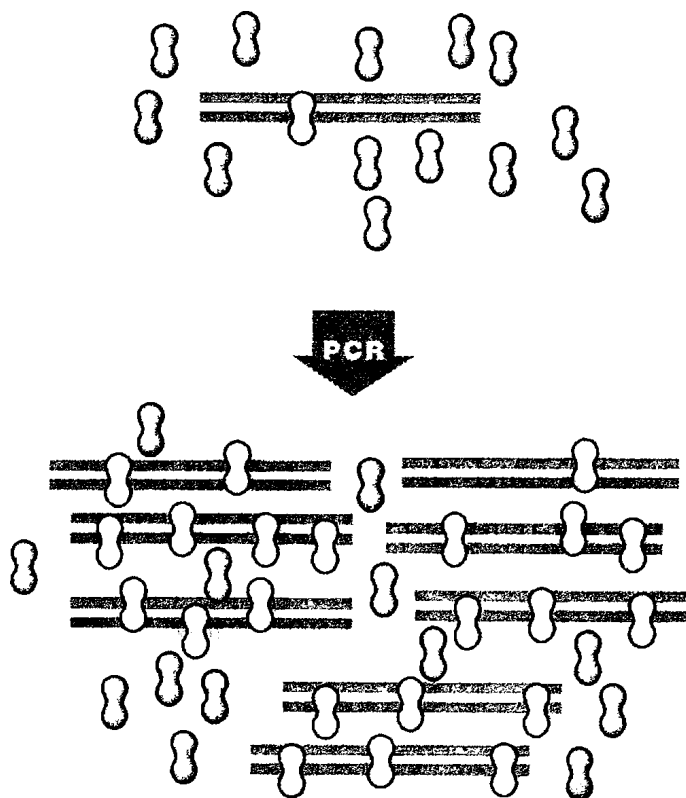


Fig. 2. SYBR Green I.

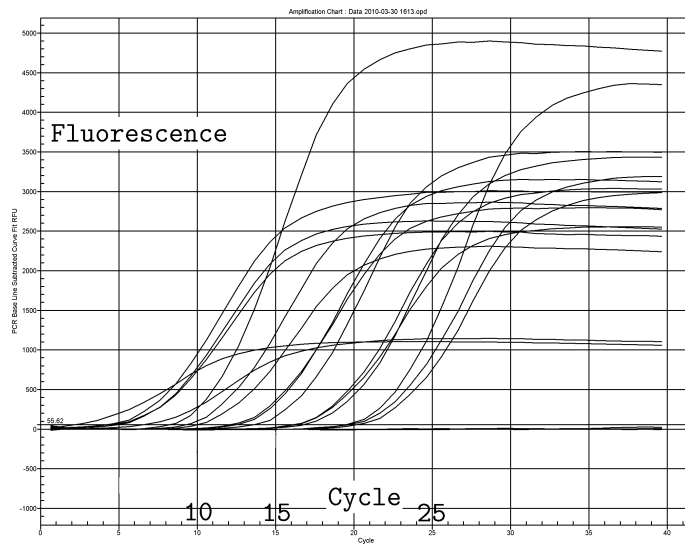


Fig. 3. Generating a standard curve to assess reaction optimization. A standard curve was generated using a 10-fold dilution of a template amplified on the real-time system. Each dilution was assayed in triplicate. Graphs are amplification curves of the dilution series.

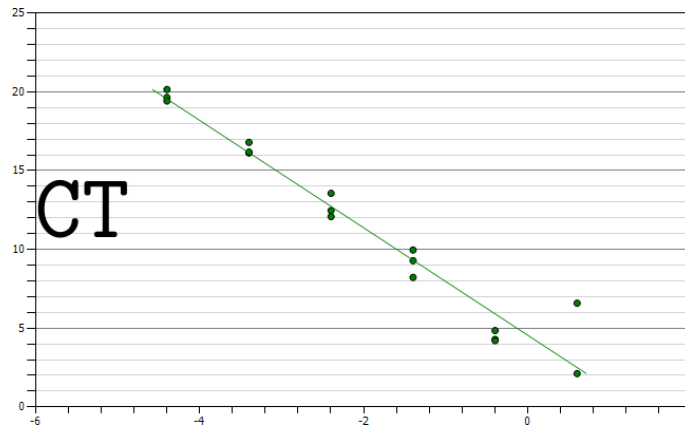


Fig. 4. Standard curve with the CT plotted against the log of the starting quantity of template for each dilution. The equation for the regression line and the r value are shown above the graph.

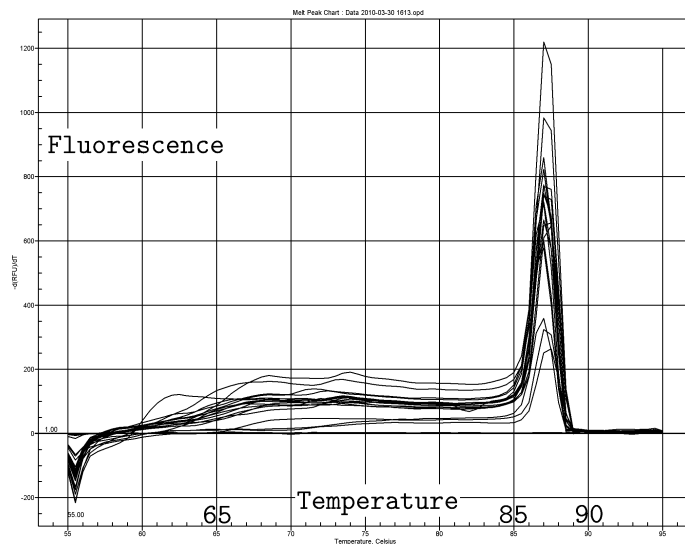


Fig. 5. Melt curve for the products made during the qPCR amplification.

Table 1. Numerical results of dilution experiment. One of dilutions is unknown.

Well	Fluor	Identifier	Threshold Cycle (CT)	CT Mean	CT Std. Dev
A01	SYBR1	undiluted	6.59	04.35	3.159
A02	SYBR1	undiluted	2.12	04.35	3.159
A03	SYBR1	undiluted	NA	00.00	NA
B01	SYBR1	1:10	4.29	04.45	0.349
B02	SYBR1	1:10	4.85	04.45	0.349
B03	SYBR1	1:10	4.21	04.45	0.349
C01	SYBR1	1:100	9.97	09.16	0.877
C02	SYBR1	1:100	9.29	09.16	0.877
C03	SYBR1	1:100	8.23	09.16	0.877
D01	SYBR1	1:1000	13.56	12.71	0.767
D02	SYBR1	1:1000	12.47	12.71	0.767
D03	SYBR1	1:1000	12.09	12.71	0.767
E01	SYBR1	1:10000	16.80	16.37	0.375
E02	SYBR1	1:10000	16.12	16.37	0.375
E03	SYBR1	1:10000	16.18	16.37	0.375
F01	SYBR1	?	20.16	19.74	0.374
F02	SYBR1	?	19.65	19.74	0.374
F03	SYBR1	?	19.43	19.74	0.374