Quantitative PCR: How does it work?

Real Time quantitative PCR (qPCR) is very similar to traditional PCR. The major difference being that with qPCR the amount of PCR product is measured after each round of amplification while with traditional PCR, the amount of PCR product is measured only at the end point of amplification.

The concept of qPCR is simple: amplification products are measured as they are produced using a fluorescent label. During amplification, a fluorescent dye binds, either directly or indirectly via a labeled hybridizing probe, to the accumulating DNA molecules, and fluorescence values are recorded during each cycle of the amplification process. The fluorescence signal is directly proportional to DNA concentration over a broad range, and the linear correlation between PCR product and fluorescence intensity is used to calculate the amount of template present at the beginning of the reaction.

The point at which fluorescence is first detected as statistically significant above the baseline or background, is called the threshold cycle or Ct Value.

The Ct Value is the most important parameter for quantitative PCR. This threshold must be established to quantify the amount of DNA in the samples. It is inversely correlated to the logarithm of the initial copy number. The threshold should be set above the amplification baseline and within the exponential increase phase (which looks linear in the log phase). Most instruments automatically calculate the threshold level of fluorescence signal by determining the baseline (background) average signal and setting a threshold 10-fold higher than this average.

In theory, an equal number of molecules are present in all of the reactions at any given fluorescence level. Therefore, at the threshold level, it is assumed that all reactions contain an equal number of specific amplicons. The higher the initial amount of sample DNA, the sooner the accumulated product is detected in the fluorescence plot, and the lower the Ct value.

There are two ways to graph qPCR fluorescence data: a standard X-Y plot of fluorescence versus cycle number and a semi-log plot of log fluorescence versus cycle number (Fig. 1). Since PCR is a geometric amplification, ideally doubling every cycle, a linear plot of the data should show a classic exponential amplification as it does in the standard X-Y plot. A logarithmic plot of a successful geometric reaction will result in a straight line in the exponential region of the graph. The slope of this portion of the semi-log plot can be used to calculate the efficiency of the PCR.

Both plots can be broken into different regions showing the phases of PCR amplification. The different graphing techniques emphasize different reaction phases. During a typical qPCR experiment, the initial concentration of template is extremely low; therefore the resulting product-related fluorescence is too low to be detected. The background signal is shown as baseline in Figure 1. After the yield has reached the detection threshold, shown as the dotted line, the reaction course can be followed reliably through the exponential phase, which is best tracked in the semi-log plot. Once the reaction reaches significant product inhibition, or limiting reagent, the reaction reaches a linear phase, which is best tracked in the linear plot. After this point, the reaction is at the maximum yield, or the plateau phase.

There are two main methods used to perform quantitative PCR: dye-based, or non-specific detection, and probe-based, or specific detection. Both methods rely on calculating the initial (zero cycle) DNA concentration by extrapolating back from a reliable fluorescent signal.

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**Figure 1. PCR Amplification Plots**

**Standard X-Y Plot**

- **Baseline**
- **Exponential**
- **Linear**
- **Plateau**

**Semi-log Plot**

- **Baseline**
- **Exponential**
- **Linear**
- **Cycle threshold** $Ct \approx 15.5$
- **Plateau**

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