# Quantitative PCR by Continuous Fluorescence Monitoring of a Double Strand DNA Specific Binding Dye

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**Key Words**
*LightCycler™, PCR, SYBR® Green I Dye, quantification*

**Abstract**
A simple method for quantitative PCR using the LightCycler™ and the dsDNA binding dye SYBR® Green I Dye is demonstrated. The method has a dynamic range of at least six orders of magnitude and a sensitivity of a single copy per reaction. Two methods for removing the confounding signal from non-specific amplification products are described. The method is suitable for both DNA and RNA quantification.

## Introduction

While there are many methods available for quantification of nucleic acids, quantitative PCR is becoming the method of choice (1). The high sensitivity and the wide dynamic range of the technique are advantages that outweigh many of the difficulties of the procedure. While extremely useful, quantitative PCR can be laborious to perform. Most of the difficulties arise because only a very small number of the cycles in a PCR reaction contain useful information. The early cycles have undetectable amounts of the DNA product, late cycles (the so-called plateau phase) are almost as uninformative. The quantitative information in a PCR reaction comes from those few cycles where the amount of DNA grows logarithmically from barely above background to the plateau. Often only 4 or 5 cycles out of 40 will fall in this "log-linear" portion of the curve.

The position of these precious few cycles contains most of the quantitative information. The log-linear phase appears at higher cycle numbers as the number of template copies in the reaction decreases. How are these cycles identified? In traditional quantitative PCR, the sample is divided into aliquots and placed in multiple reaction tubes. One tube is removed for analysis after each cycle. The reactions are run out on a gel and the amount of DNA at each cycle is quantified, sometimes by staining with a double strand specific DNA binding dye such as ethidium bromide or SYBR® Green I Dye. The brightness of the fluorescent band is measured and the fluorescence is plotted against cycle number. The cycle number where the fluorescence of the sample is first detected (the "threshold") is compared to the threshold of samples of known concentration (2).

Quantification with the LightCycler™ greatly simplifies the process (3). The samples are continuously monitored during the PCR, so a single reaction takes the place of many reactions. The log-linear region is easily identified as the fluorescence data appears on the computer screen. The simplest way to monitor the progress of the reaction in the LightCycler is to include a dsDNA binding dye in the reaction (4). Ethidium bromide is the most common example of this kind of dye, but newer dyes like SYBR Green I Dye give stronger signals. This dye is thought to bind in the minor groove of dsDNA. In its unbound state SYBR Green I Dye has relatively low fluorescence, but when bound to DNA it fluoresces brightly. As the amount of DNA in the PCR increases, the amount of fluorescence from the dye increases proportionally.

SYBR Green I Dye has the virtue of being easy to use. Because it has no sequence specificity it can be used to detect any PCR product. All you need is a primer pair and a template and you can do quantitative PCR.However, this virtue has a drawback. SYBR Green I Dye binds to any dsDNA molecule, whether it is the intended product or a non-specific product like a primer dimer. This non-specific binding of SYBR Green I Dye will particularly make the quantification of low copy numbers more difficult.

To overcome this specificity problem, we have taken advantage of the LightCycler's continuous monitoring capability (5). The products of a PCR reaction can be melted by increasing the temperature of the sample. At the $T_m$ of the product, a sharp reduction is seen in the level of fluorescence. Non-specific products tend to melt at a much lower temperature than the longer specific products. This allows the amplification curves to be corrected for the contribution of the low melting non-specific products, extending the sensitivity of the technique down to a single copy per reaction (6).

## Materials and Methods

### β-*globin PCR conditions*

DNA amplifications were done using the LightCycler (Idaho Technology, Idaho Falls, ID) in a standard PCR reaction containing 0.5 µM of each primer and SYBR Green I Dye (Molecular Probes) and bovine serum albumin (BSA). β-globin primers have been previously described (7). Purified PCR product was used as a template and was obtained by phenol/chloroform extraction and ethanol precipitation (8) followed by multiple washing in a Centricon® 30 microconcentrator (Amicon). Template concentration was determined by absorbance at 260 nm.

The reactions were cycled 45 times with a 94°C denaturation for 0 s and a 60°C combined annealing/extension for 20 s. Fluorescence was acquired at the end of the annealing/extension phase. The melting curves were obtained at the end of amplification by cooling the sample at 20°C/s to 60°C and then increasing the temperature to 95°C at 0.1°C/s. Fluorescence was acquired every 0.1°C.

### *mRNA quantification conditions*

Fibroblast cultures were grown, and RNA was purified as previously described (9). cDNA was made from 5 µg of purified RNA using mMLV reverse transcriptase (in 50 mM Tris pH 8.3 (25°C), 75 mM KCl, 3 mM $MgCl_2$) at 37°C for 60 min. The cDNA was treated with RNase I for 5 min at 37°C and was purified by phenol/chloroform extraction and ethanol precipitation (8).

Amplification was done as above except that primers were at 5 µM of each and the *Taq* polymerase was pre-incubated with anti-*Taq* antibody. The template was either purified amplicon at the indicated copy number, or 20 ng of cDNA. The primer sequences have been previously reported (6).

The reactions were cycled 50 times with a 95°C denaturation for 0 s, a 60°C annealing for 0 s, and 72°C extension for 5 s, with slopes of 20°C/s, 20°C/s and 5°C/s respectively. Fluorescence was acquired after heating at 5°C/s to a temperature 2°C below the product melting temperature ($T_m$) and holding for 5 s.

### *Data analysis*

Quantification data was analyzed using the LightCycler analysis software. Background fluorescence is removed by setting a noise band. The log-linear portion of the standard's amplification curve is identified and the crossing point is the intersection of the best fit line through the log-linear region and the noise band. The standard curve is the plot of crossing point versus the log of copy number.

Correction of the amplification curves was done by taking a melting curve at the end of the amplification and then calculating the area under the specific product peak and the total area under the melting curve using the LightCycler melting curve analysis software. The SYBR Green I Dye signal for each curve was reduced in proportion to the relative peak areas and the zero template control fluorescence values were subtracted from all curves.*

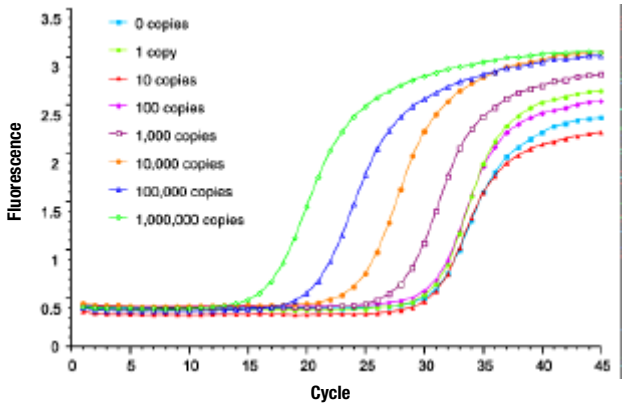


**Figure 1** **Amplification from zero to one million copies of a fragment of the human β-globin gene.** All samples contained SYBR Green I Dye. The reactions from one million to one thousand copies are easily distinguished. Below 1000 copies, the reactions begin to overlap due to the predominance of primer dimers and non-specific PCR products at these lower copy numbers.

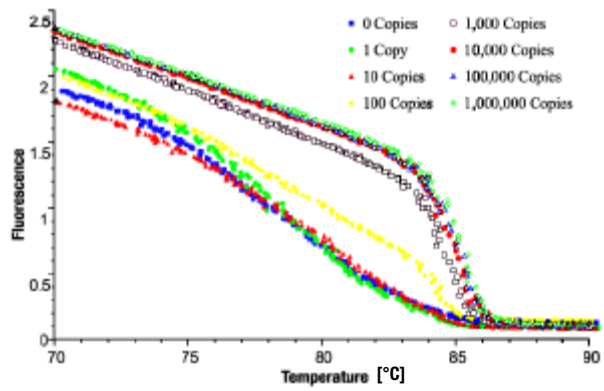


**Figure 2** **Fluorescence versus temperature "melting curves" for the products of DNA amplification reactions with from zero to one million starting copies of a 536 bp fragment of the human β-globin gene.** For the samples with a high initial copy number, the SYBR Green I Dye fluorescence declines linearly with increasing temperature, followed by a steep decline in fluorescence as the specific product melts (around 85°C). The primer dimers and non-specific products produced in the low copy number samples melt at much lower temperatures. The 100 copy reaction is clearly biphasic, with both low and high melting components.

## Results and Discussion

### *Quantification of DNA using SYBR Green I Dye and melting peak correction*

Monitoring the amplification of a 10-fold serial dilution of a fragment of the human β-globin gene with SYBR Green I Dye gives a series of amplification curves (Figure 1). The curves are shifted to increasingly higher cycle numbers with decreasing copy number down to 1,000

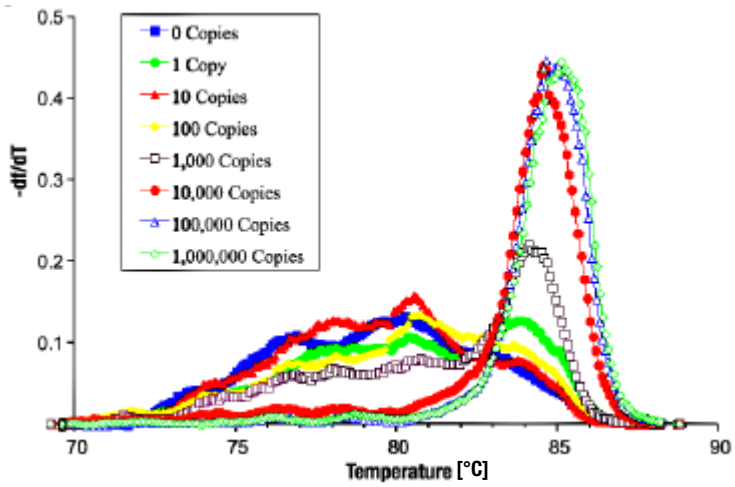


**Figure 3** **Taking the negative derivative of fluorescence with respect to temperature converts melting curves into "melting peaks."** The melting of the DNA product is easily identified as a sharp peak centered at the $T_m$ of the product. Non-specific amplification products tend to melt at much lower temperatures and over a broader range. The area under the overall melting peaks is related to the total amount of amplification products. The relative area under the melting peak at approximately 85°C, is related to the amount of specific PCR product.

copies. The amplification curves for reactions containing 100, 10, 1, and zero copies, however, all overlap.

After the PCR, these samples were heated from 70°C to 90°C. The result of this melt is shown in Figure 2. Initially, as the temperature increases, the one million copy sample shows a slow decrease in fluorescence. This is caused by the quenching effect of higher temperatures on SYBR Green I Dye. When the temperature reaches the $T_m$ of the β-globin fragment (about 85°C), there is a steep decrease in fluorescence as the product denatures to single stands that no longer bind SYBR Green I Dye. The 100,000 and 10,000 copy samples look similar to this, indicating mostly specific PCR product. The zero copy sample shows a lower temperature, broader melt, indicating that most of the fluorescence comes from a mix of short, non-specific products. The 100 and 1000 copy samples show both the broad, low temperature melt and a steep, high temperature melt suggesting that they contain both non-specific and specific products.

The melting transitions can be seen more easily on a plot of the negative derivative of fluorescence with respect to temperature(-dF/dT) versus temperature (Figure 3). This data transformation produces "melting peaks" in temperature regions of steep fluorescence decrease. The center of the melting peak is the $T_m$ of the DNA product melted. The one million copy sample shows only a single melting peak centered at 85°C. The zero copy sample shows only a broad, low temperature peak. The 1,000 and 100 copy samples show both peaks.

The area under these melting peaks is related to the amount of product melting at that temperature. Therefore the amplification curves shown in Figure 1 can be corrected to reflect the proportion of the signal coming from specific products. Figure 4 shows the result of this correction. While the high copy samples are mostly unaffected by the correction, the 1000 copy, 100 copy, and 10 copy samples are now easily distinguished. One copy is now different from zero copies. This correction has extended the dynamic range of SYBR Green I Dye quantification by 3 orders of magnitude.

### *Quantification of IL-1β message using SYBR Green I Dye*

Another method for removing the non-specific product signal from an amplification curve is to acquire the fluorescent signal at a temperature above the melting temperature of the primer dimers.

Figure 3 shows that most of the primer dimers are melted at 82°C but very little specific product has melted at this temperature. The contribution of primer dimers to a fluorescent signal acquired at 82°C would be minimal.

This technique was used to quantify messenger RNA for rat interleukin 1β. cDNA was made from cultured rat synovial fibroblasts that were treated with interleukin-1β (IL-1β) or tumor necrosis factor (TNF-α); the level of message was compared to untreated control fibroblasts. Figure 5 shows a standard curve of IL-1β

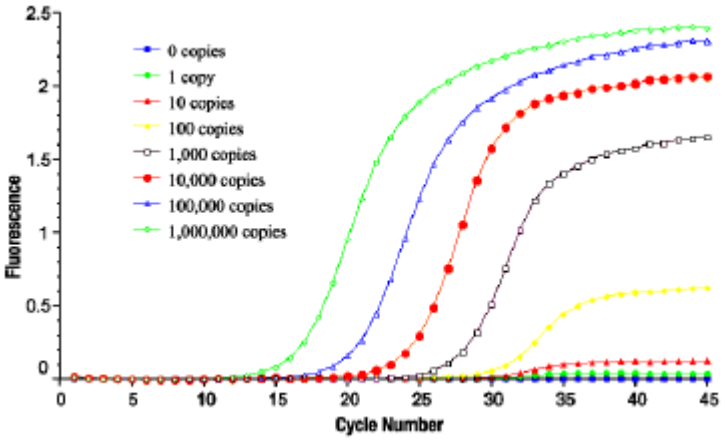


**Figure 4** **The dynamic range of quantification can be expanded several orders of magnitude by using melting curve correction.** Amplification profile data are multiplied by the fraction of the total area under the melting peaks that comes from the specific product. After correction it is possible to distinguish between 100, 10, and one initial copies.

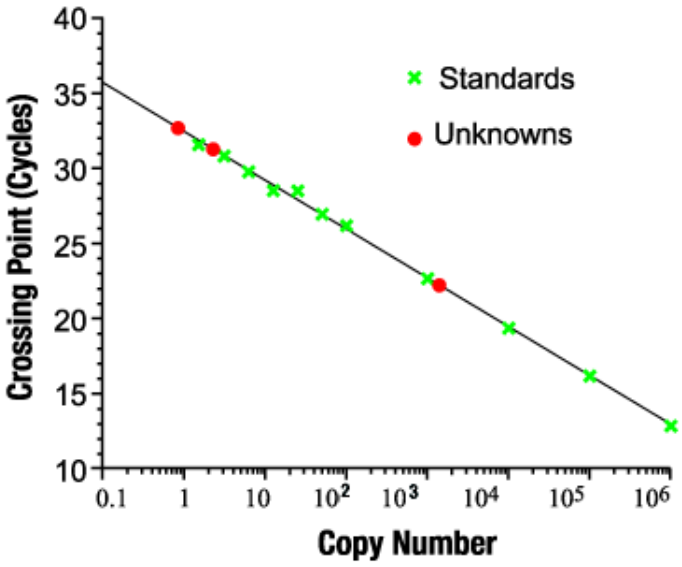


**Figure 5** **SYBR Green I Dye standard curve with three unknown samples.** The crossing point is calculated using the LightCycler software. A line is fit to the log linear portion of each amplification curve. The crossing point is the intersection of this line with the background noise band. All the data points represent a single reaction. **Note: The conclusions from Figure 5 are summarized in Table 1.**

| Treatment | Crossing point | IL-1β copies/ reaction |
|---|---|---|
| No Treatment | 31.3 Cycles | 2.3 |
| IL-1ß (10 U/ml) | 22.2 Cycles | 1390 |
| TNF-α (10 U/ml) | 32.7 Cycles | 0.8 |

**Table 1. The effect of treatment on IL-1β message.** Levels of IL-1β message are increased over 600 fold by treatment of the cells with IL-1β. Treatment with TNF-α however, has little effect on transcription. Coefficients of variation for mRNA quantification in this system ranged from approximately 50% at the low end of the curve to about 6% at the high end of the standard curve; a single copy of template could be reliably detected.

purified PCR product from one to one million copies per reaction. Note the log-linearity across all six orders of magnitude. Even at low copy number the log-linearity is maintained, suggesting that the confounding signal from primer dimers has been successfully removed.

## Conclusion

This paper has demonstrated a simple and rapid technique for quantification of nucleic acids. The use of the dsDNA binding dye SYBR Green I Dye makes this a generic technique that should be easily adaptable to any system.

Note: All data shown have been generated with the LC24 machine and have been kindly provided by Idaho Technology.

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*The described data correction is currently not part of the LightCycler software from Idaho Technology and was done by separate experimental software.