Optical Design of CFX96™ Real-Time PCR Detection System Eliminates the Requirement of a Passive Reference Dye

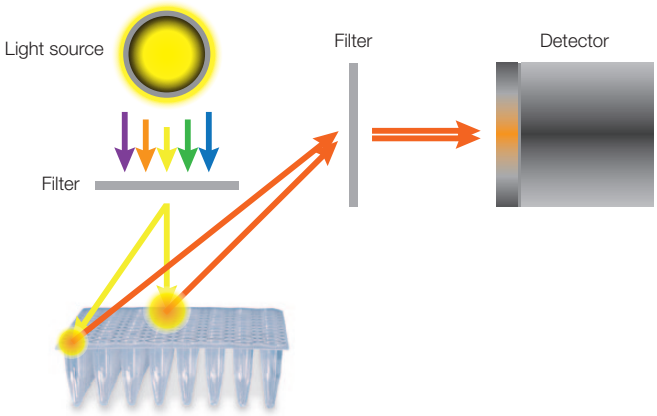
Liz Jordan and Richard Kurtz, Gene Expression Division,
Bio-Rad Laboratories, Inc., 2000 Alfred Nobel Drive, Hercules, CA 94537

Introduction

The optical design of most real-time PCR systems that use a lamp or other stationary single-light source creates illumination and detection light paths that vary for each well of the thermal cycler block. The variation in light path lengths produces different absolute fluorescence measurements for wells that contain the same concentration of a reporter fluorophore. A well with a shorter light path, typically located in the middle of the block, will have a higher fluorescence reading compared to a well with a longer light path, typically located on the perimeter of the block (Figure 1). The differences in absolute fluorescence levels do not impact real-time PCR results because results are determined by measuring the change in fluorescence of a reporter measured over the course of the reaction. For real-time PCR data processing, the sample fluorescence for each well is first baseline subtracted and then the quantification cycle (Cq) value is determined as a cycle number at which fluorescence has increased above background (Figure 2).

Some lamp-based real-time PCR systems utilize an internal reference dye to normalize well-to-well fluorescence signal differences resulting from optical path length variations. Normalizing the fluorescence intensity is accomplished in real-time PCR software by dividing the emission intensity of the reporter dye by the emission intensity of a reference dye. For example, the Bio-Rad MyiQ™2 real-time PCR detection system utilizes well factors collected at the beginning of the run to normalize fluorescence. Other manufacturers recommend the use of ROX fluorescent dye as a passive reference that is detected in a separate channel relative to the reporter dye. Dividing the emission intensity of the reporter dye by the emission intensity of ROX at each cycle yields the ratio defined as Rn (Figure 1B). Next, the Rn values are baseline subtracted on a per well basis to yield the ΔRn for a given reaction well. For these real-time PCR systems, the Cq value of a reaction is identified as the cycle number at which ΔRn has increased above background.

A



B

	Fluorescence Intensity, RFU		
Well	**Reporter**	**Passive Reference**	**Normalized Rn**
Center	1,500	750	2
Perimeter	1,000	500	2

Fig. 1. Light path differences between wells of a lamp-based real-time PCR system. A, the well in the center of the block has a shorter light path and, subsequently, emits a higher fluorescence signal compared to the well on the perimeter, which has a longer light path; **B**, fluorescence variation is corrected by normalizing the fluorescent reporter signal to the fluorescent signal of a passive reference dye. RFU, relative fluorescence units.

In contrast to lamp-based real-time PCR systems, the CFX96 and CFX384™ real-time PCR detection systems utilize solid-state optical technology with long-lasting light-emitting diodes (LEDs) for fluorescence excitation and photodiodes for detection. Scanning just above the sample plate, the CFX96 and CFX384 systems optics shuttle individually illuminates and detects fluorescence from each well with high sensitivity and no cross talk (Figure 3). At every position and with every scan, the optics shuttle is reproducibly centered above each well, so the light path is always identical, with the same intensity for all wells, eliminating the need to sacrifice data collection in one of the channels to normalize to a passive reference. For the CFX96 and CFX384 systems, Cq determination requires only baseline subtraction of individual well fluorescence readings, minimizing the amount of data processing performed by the real-time PCR software.

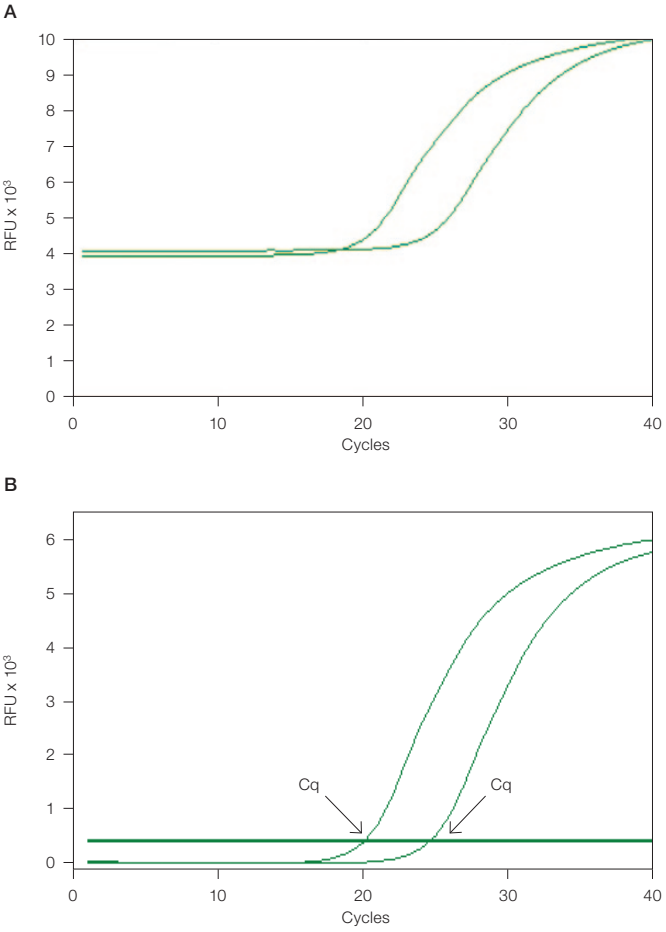


Fig. 2. Real-time PCR fluorescence data processing. Fluorescence readings were measured from two wells before (**A**) and after (**B**) baseline subtraction. The Cq value for each sample is indicated by the arrow. RFU, relative fluorescence units.

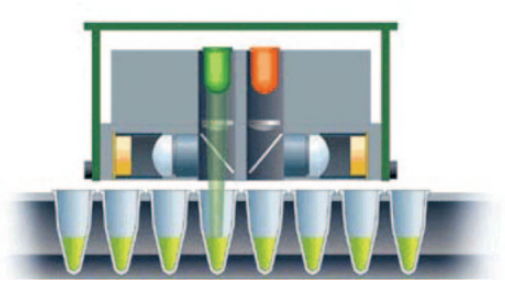


Fig. 3. Optics shuttle of the CFX96 real-time PCR detection system. As the CFX96 system optics shuttle travels across the plate, light is focused directly into the center of each sample well. Side view of the optics shuttle shows the green LED firing over a well.

It has been proposed that, in addition to normalizing fluorescence levels due to well-to-well optical path variation, ROX normalization can also control non–PCR-related fluctuations, such as reaction volume variation or input template variation resulting from pipetting error. In this study, we confirm that total reaction volume, including the amount of ROX dye added to a reaction, does not impact real-time PCR results. Instead, the amount of starting nucleic acid template determines the Cq value for a real-time PCR sample. Our results demonstrate that ROX normalization compensates only for fluorescence level differences due to optical path variation and not pipetting variations.

Methods

HeLa or kidney RNA (Ambion) was reverse transcribed with the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Inc.) in 100 µl reactions. The standard 20 µl iScript reverse transcription reaction (including up to 1 µg total RNA) was scaled up to 100 µl with 5 µg total RNA. cDNA was diluted in TE buffer to 10 ng/µl (based upon input total RNA). Primers and probe (Integrated DNA Technologies, Inc.) were diluted to 10 µM in TE buffer and 50x ROX passive reference dye was diluted to 5x in TE buffer. The CFX96 system was tested with the Hard-Shell® thin-wall plate (catalog #HSP-9601) and Microseal® 'B' adhesive seal (all from Bio-Rad). The ABI 7500 real-time PCR system was tested using the MicroAmp fast optical 96-well reaction plate (0.1 ml) with MicroAmp optical adhesive film (all from Life Technologies Corporation).

The same cycling conditions were used on both instruments: one cycle for 3 min at 95°C, then 40 cycles for 10 sec at 95°C and 30 sec at 60°C.

In the first experiment, reaction volumes ranging from 12 to 17 µl/well (in 1 µl increments) were tested. For each volume, a master mix was prepared such that the identical mix was pipetted into each of eight replicate wells per plate for each instrument tested. The amount of input template was held constant at 25 ng HeLa cell cDNA in all samples. iQ™ supermix was used for all qPCR reactions (at 1x final concentration) with the addition of the following (final concentrations shown): 300 nM each primer, 200 nM FAM-labeled β-actin probe, 0.075x ROX (as specified by the instrument manufacturer), and water to final reaction volume. ROX was not added to the reactions run on the CFX96 system.

In a second experiment, a master mix (based on 15 µl/well reaction volume) that included 25 ng kidney cDNA per reaction was prepared for all wells tested. Twelve to 17 µl (in 1 µl increments) of the master mix were dispensed into eight replicate wells (for each instrument), resulting in 1.667 ng increments in the amount of input template used in the reactions, starting with 20 ng template. iQ supermix was used for all qPCR reactions (at 1x final concentration) with the addition of the following (final concentrations shown): 300 nM each primer, 200 nM FAM-labeled β-actin probe, 0.075x ROX (as specified by the instrument manufacturer), and water to final reaction volume. ROX was not added to the reactions run on the CFX96 system.

Results and Discussion

In experiment 1, the amount of input template (25 ng cDNA) was held constant in the reactions but the total reaction volumes varied from 12 to 17 µl to mimic the variability potentially introduced by pipetting errors. Additional reaction component concentrations were identical in all reactions to ensure consistent amplification and ROX normalization effects. This experiment tested whether differences in total reaction volume, mimicking pipetting inaccuracies, impact real-time PCR results.

For experiment 1, analysis of the Cq values for the different replicate sets of reactions with different volumes indicated that they were equivalent on the ABI 7500 system. Applying ROX normalization to the data decreased the standard deviation for each replicate set, as shown in Figure 4. It should also be noted that processing the data with the additional step of ROX normalization decreased the average Cq value for each replicate by as much as 1.6 cycles for a 15 µl reaction.

For experiment 1, the Cq values for the eight replicate reactions at different volumes were statistically equivalent on the CFX96 system (Figure 5). Cq values on the CFX96 system were in a similar range (17–17.5) compared to the ABI 7500 system before ROX normalization, but results yielded smaller average Cqs across the replicates. The uniformity of the Cq results from experiment 1 on both instruments demonstrates that reaction volume does not significantly impact real-time PCR results — similar results can be obtained despite having as much as a 40% difference in the volumes of reaction components and ROX (12 µl reaction volume versus 17 µl reaction volume).

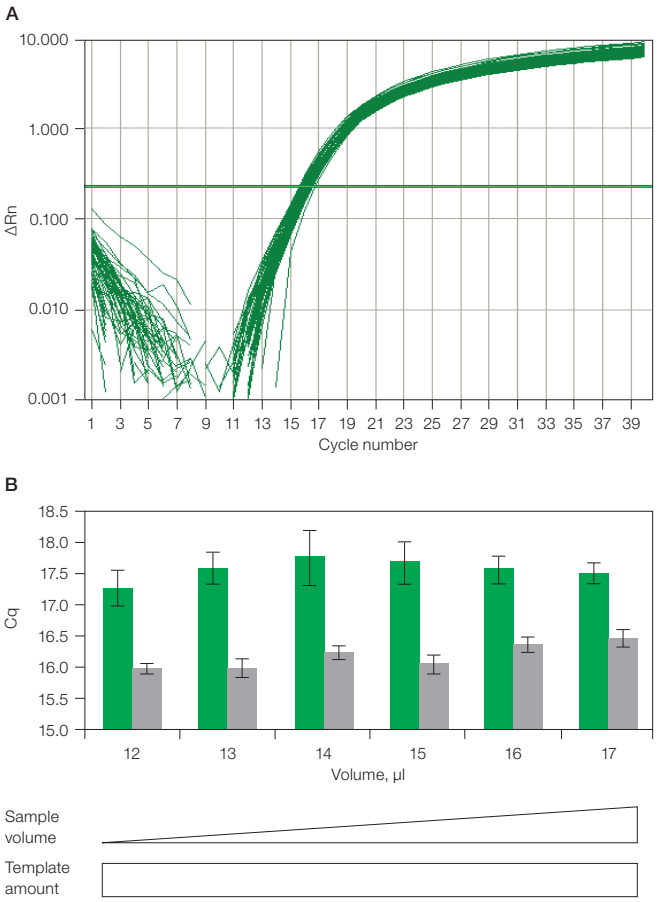


Fig. 4. Results on the ABI 7500 system for reactions with the same amount of input template and increasing final reaction volumes (experiment 1). A, real-time PCR amplification curves were analyzed with ROX normalization; **B**, average Cq values were measured for eight replicate reactions with identical input template amount (25 ng cDNA) and variable reaction volumes (12–17 µl). ROX normalization (■) lowered the standard deviations of Cq values for each replicate reaction set compared to Cq values for the identical reaction sets without ROX normalization (■). Cq, quantification cycle.

In experiment 2, the total reaction volumes varied from 12 to 17 µl, as in experiment 1. However, these reactions used increasing amounts of input template to mimic variability due to pipetting errors. All other reaction component concentrations were identical in all reactions to ensure consistent amplification and ROX normalization effects. This experiment tested how small differences in the input template amount impact real-time PCR results.

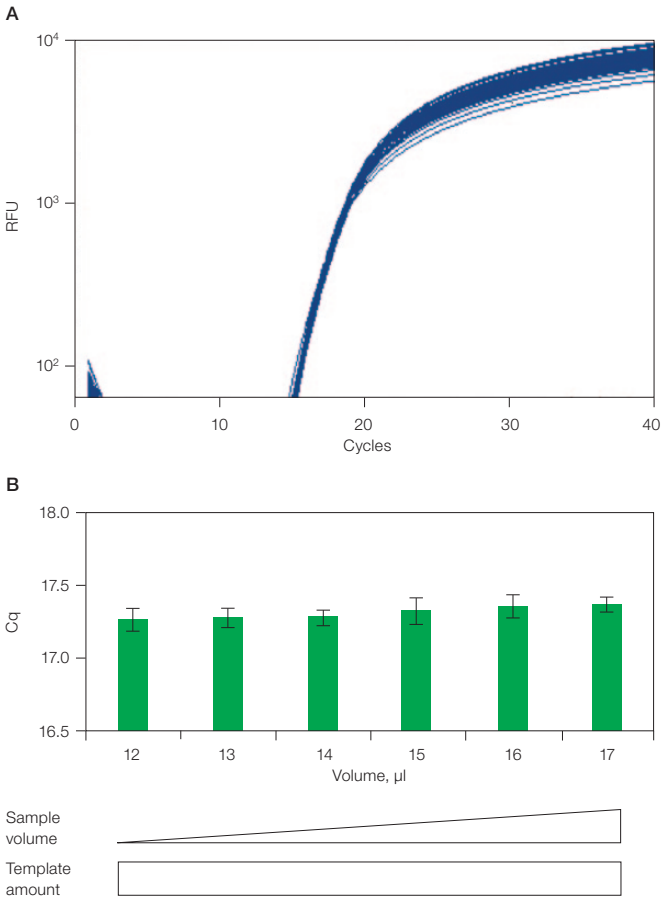


Fig. 5. Results on the CFX96 system for reactions with the same amount of input template and increasing final reaction volumes (experiment 1). **A**, real-time PCR amplification curves analyzed on a CFX96 system demonstrated tighter uniformity versus the ABI 7500 system (Figure 4B); **B**, average Cq values were measured for eight replicate reactions with identical input template amount (25 ng cDNA) and variable reaction volumes (12–17 µl). Cq, quantification cycle; RFU, relative fluorescence units.

In experiment 2, a slight decrease in Cq value paralleling the increasing amounts of sample template was expected. On the ABI 7500 system, a limited downward trend in Cq values was observed, with a more apparent decrease in Cq values when comparing data from the 14 to 17 µl reaction volumes (Figure 6). The data shown in Figure 6 have been normalized; an equivalent trend was observed in non-ROX normalized data (not shown). On the CFX96 system, a trend of decreasing Cq values can be observed with increasing template concentrations from 12 to 17 µl, suggesting that the CFX96 system provides extremely sensitive discrimination of input sample template (Figure 7).

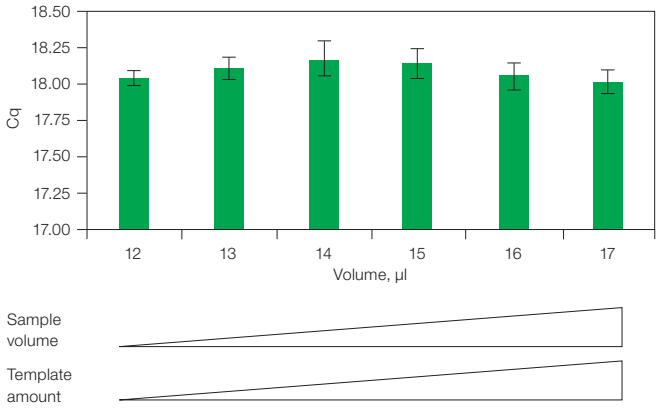


Fig. 6. Results on the ABI 7500 system for reactions with increasing input template and increasing final reaction volumes (experiment 2). Average Cq values were measured for eight replicate reactions with increasing input template amount (20–30 ng cDNA) and variable reaction volumes (12–17 µl). Cq, quantification cycle.

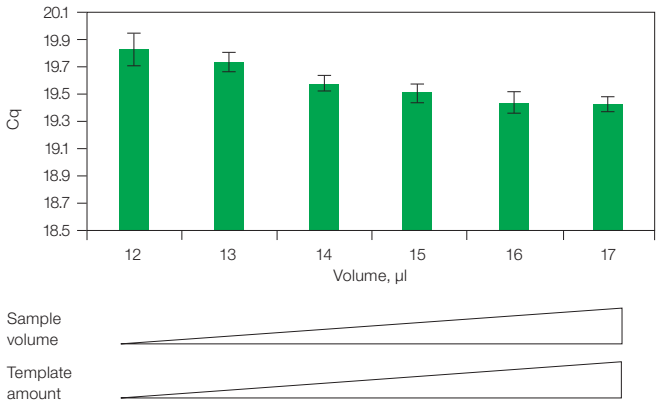


Fig. 7. Results on the CFX96 system for reactions with increasing input template and increasing final reaction volumes (experiment 2). Average Cq values were measured for eight replicate reactions with increasing input template amount (20–30 ng cDNA) and variable reaction volumes (12–17 µl). Cq, quantification cycle.

ROX reference dye does not participate in PCR and, thus, it is defined as a passive reference. The use of ROX normalization on the ABI 7500 system, a lamp-based real-time PCR system, did improve the standard deviation of Cq values for replicate wells compared to Cq values for replicate wells when ROX normalization was not applied. However, the additional data processing step of applying ROX normalization also lowered the Cq values. This decrease in Cq values resulting from data normalization should not be confused with an increase in instrument sensitivity relative to non-ROX normalization PCR systems. The starting quantity of template is determined by comparing Cq values to one another or to a standard curve.

For the CFX96 system, an identical optical path and light intensity reached all samples, providing sensitive and reproducible real-time PCR results across all wells without the need to normalize fluorescence signals in the software. The advantage of the fixed optical path is also supported by the lower standard deviations for the replicate reactions performed on the CFX96 system.

Conclusions

It has been proposed that ROX normalization performed after real-time PCR data collection can account for pipetting inaccuracies of reaction components or template amount and, subsequently, can correct for technical variations observed in real-time PCR experiments. Reaction components and template are dispensed independently of one another and no distribution correlation exists between ROX dye, real-time PCR fluorescent reporter, or template when pipetting reaction components into a sample well. Essentially, one must consider the pipetting of these components as independent events, with the randomness for each event associated with the total number of molecules being pipetted. This holds true regardless of components being pipetted one at a time or as a reaction master mix.

In this study, we have presented evidence that the total reaction volume and difference in amounts of reaction components other than template that can be caused by pipetting inaccuracies have limited to no impact on results. Our study also suggests the amount of SYBR® Green or fluorescence chemistry added to the reactions is in vast excess in these reactions and small differences in volume of one of these reaction components do not significantly impact results among replicate samples. The input template amount alone is the primary factor that determines when the fluorescence will rise above the background, yielding the Cq value. To control for differences in the input template amount distributed in replicate wells, we recommend performing real-time PCR reactions in replicates.

Information in this tech note was current as of the date of writing (2010) and not necessarily the date this version (rev A, 2010) was published.

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