Comparison of Fluorometric Detection Methods for Quantitative Polymerase Chain Reaction (PCR)

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Abstract:

In this study, we compared the sensitivity of two different detection methods for quantitative polymerase chain reaction (PCR). Various amounts of a 75 mer single-stranded deoxyribonucleic acid (DNA) fragment, which can be used as a DNA label for the immuno-PCR (iPCR) assays, were amplified by PCR. The amount of amplified DNA fragments was determined by the fluorescence (FL) of SYBR$^{(R)}$ Green dye that specifically interacts with double-stranded DNA fragments. In the first selected detection method, real-time PCR, FL measurements were carried out at each thermal cycle, as the DNA was being amplified by PCR. This was achieved using the Applied Biosystems (ABI) Prism 7000 Sequence Detection System and its standard protocol. In the second detection method, referred to as end-point detection, after the PCR amplification was completed, off-line FL measurements were subsequently carried out using a conventional plate reader. In order to achieve the lowest limit of detection (LOD) from the off-line measurement, we have optimized a wide variety of parameters. Our data have indicated the LOD of real-time PCR method was approximately three orders of magnitude lower than the end-point measurement method, with a linear range spanning six orders of magnitude; 10 fmol to 10 zmol of PCR template. The lower LOD of the real-time PCR method could be partly due to the ability to maximize the number of thermal cycles that could be carried out in PCR, without increasing the nonspecific amplification of any contaminating DNA. The results of this study can be applied to the development of ultra-sensitive iPCR assays for various disease markers.

Keywords: Polymerase chain reaction | Fluorometric detection | chemistry | biochemistry | immunochemistry | DNA

Article:

Introduction

Nucleic acids are the building blocks of the human body, which makes studying them so rewarding and fascinating. The invention of the polymerase chain reaction (PCR) by Mullis in the mid-80s caused a surge in nucleic acid research.1 This one idea, of in vitro deoxyribonucleic

acid (DNA) amplification, pioneered and improved many applications such as gene cloning and expression, DNA fingerprinting, disease diagnosis, and DNA sequencing.1 It was the PCR invention that assisted the completion of the human genome project in 2003 and has opened doors to even more scientific innovations.

With the advances in nucleic acid analysis, Cantor and colleagues at Boston University developed a novel detection method for immunoassay called immuno-PCR (iPCR), based on the enzyme linked immunosorbant assay (ELISA).2 By altering the signal amplification from enzymatic turnover to the amplification of PCR, Cantor pushed the limit of detection (LOD) of iPCR beyond those of conventional ELISAs.2 During the past decade, iPCR has been applied successfully to many other immunoassays that require high sensitivity; for example, the detection of various tumor markers, hormones, and pathogens.3–10 These applications have been successful, primarily because iPCR uses the specificity of antibody–antigen interactions in coordination with the sensitivity of PCR to produce a high performance assay for low-level analyte detection.

A very important aspect of the iPCR technique is not only the ability to detect trace amounts of analyte, but also in determining the absolute quantity of the analyte. Standard molecular biology techniques are usually employed to achieve quantitation of PCR products.2 In one such method, agarose gel electrophoresis is used to first separate the PCR products. This is followed by staining the agarose gel with ethidium bromide and analyzing the fluorescent image of the gel with densitometry.11 Using the earlier approach to quantitate PCR products, Cantor's iPCR assay could detect as little as 580 molecules of bovine serum albumin.2 The earlier detection method, often referred to as end-point PCR detection, performs PCR product quantitation at the plateau portion of the exponential PCR amplification. At this point of the PCR reaction, the amplification of DNA has virtually stopped, since most of the primers are consumed. The most important drawback of the end-point PCR detection method is the possibility to contaminate subsequent PCR amplifications with previous PCR products. This is due to the fact that the post-PCR procedure for detecting PCR products is physically carried out with a different experimental setup (gel electrophoresis) in the laboratory.

One solution to the problems of end-point PCR quantitation is the availability of fluorescent chemistries that can detect the accumulation of PCR products cycle-by-cycle or what is more commonly referred to as real-time PCR. In this method, the detection of PCR products can be performed during the early stages of PCR amplification, when the rate of amplification is exponential and the primers are still ample. Real-time PCR detection not only decreases the sample-to-sample variability, but also avoids any post-PCR sample handling. Even with this technique, certain laboratory precautions should be taken.12 First, samples must be prepared very carefully. Before any work is done, it is customary to irradiate the working surface, tubes, pipetters, and tips with ultraviolet (UV) light. The UV light will cause any DNA in the area to crosslink with each other, preventing amplification. If any residual DNA in these areas were

introduced into the reaction, the irradiated DNA would not be denatured in the PCR cycles and, therefore, could not be extended and contaminate the assay. Reagents for PCR should be separated into stock solution and working solution groups, so that if contamination were to occur in the working solutions, they could be discarded and the stock solutions would be contamination free. The PCR template to be amplified should be stored separately from the other reagents and kept that way, at all times. Gloves should be worn at all times and changed whenever anything outside the working area is touched. Finally, after sample preparation is completed, the working area should be cleaned with a 10% bleach solution. In every laboratory, there should be designated pre-, and post-PCR areas. Ideally, these areas should be separate laboratories; however, this cannot be achieved sometimes. This precaution is taken so that any PCR reaction tubes that have to be opened will not inadvertently, contaminate a working area, solution, pipette, or pipette tip that might be used in future experiments. These might seem like exhaustive steps; however, the problem with contamination is not easily overcome. In the viewpoint of analytical chemistry, the elimination of post-PCR sample handling can completely get rid of the possibility for subsequent PCR contamination.

The most common drawback of the real-time PCR method is the expense of the instrument that combines the thermocyclcer with an on-line fluorescence (FL) detector. With that in mind, other methods of post-PCR end-point PCR detection have been established, such as the Amplifluor™ qPCR system from Intergen (Norcross, GA), which incorporates fluorescein into the PCR products so that end-point FL signal is proportional to the initial amount of PCR template in the PCR amplification.13 An alternative method of end-point detection has been established by using a laser induced FL capillary electrophoresis system.14 Although these techniques may improve upon the limitations pertaining to end-point measurements, without the need for a more expensive real-time PCR instrument, there are also increased reagent cost and longer analysis time to take into account. Despite the cost of real-time PCR instrument, its capability to minimize PCR contamination, higher sample throughput, and ease of use make it well worth the investment.

Common chemistries used in real-time PCR detection vary from DNA intercalating dyes to specific fluorescently labeled nucleic acid probes. Intercalating dyes, such as SYBR$^{(R)}$ green, bind specifically to any double-stranded DNA.15 When bound, the FL of the dye increases proportionally with the amount of DNA in the sample. The chemistry is very simple and does not interfere with the PCR reaction. However, if more than one DNA sequence happens to be amplified in the same PCR, the SYBR$^{(R)}$ green method is incapable of distinguishing different PCR products. Either using more stringent PCR conditions or the use of sequence specific fluorescently labeled DNA probes can overcome this problem.

Labeled DNA probes can bind specifically to their complementary DNA sequences on the PCR products, and FL intensity increases in proportion to the number of probes bound. For example, molecular beacons16 are one type of DNA probe that uses an initial hairpin shape to quench the

detection fluorophors signal through the principle of fluorescent resonance energy transfer (FRET).17 When the Molecular Beacon binds to its complementary DNA sequence, the hairpin remains denatured and allows the fluorophor to fluoresce and be detected. A similar technique is implemented in Invitrogen's Light Upon Extension (LUX®) primers.18 19 The LUX PCR primer is labeled with two fluorophors that are quenched via its hairpin shape just as the Molecular Beacon through FRET. However, when the PCR reaction begins, the LUX primer is denatured and extended, compared to the Molecular Beacon, that is merely used as a detection probe. As the LUX primer is extended, the FL increases in proportion to the number of DNA molecules amplified. Although, these probe-based methods are normally more specific than the SYBR(R) green method, each probe is synthesized and labeled for only one particular sequence. With adequate precaution to avoid PCR cross contamination, the SYBR(R) green method is apparently a quick and low cost universal method for the detection and quantitation of PCR products.

In this article, we have directly compared two different methods for PCR quantitation, namely end-point and real-time measurements. Two simultaneous experiments were run in order to establish LOD and limit of quantitation for both methods. The real-time method was done using the Applied Biosystems (ABI) Prism 7000 Sequence Detection System and SYBR(R) green intercalating dye for detection, Whereas, for the end-point detection method, the FL measurements of SYBR green dye was only carried out following the completion of PCR amplification by using a ThermoLabsystems Fluoroskan Ascent FL plate reader.

Experimental

Real-Time PCR Quantitation Using ABI Prism 7000 Sequence Detection System

We determined the linear range of PCR quantitation using the following oligodeoxynucleotide PCR template and primers.

PCR template (Genelink, Hawthorne, NY):

5′-
CTAGCCTCCTCCTGGGTTTTCCAGCCACCCATAGCATGATTCGACCCTGCT
TTCTGTTTCAGGGGCAGACCGTAC-3′

Forward primer (Integrated DNA Technologies, Coralville, IA):

5′-GTA CGG TCT GCC CCT GAA ACA-3′

Reverse primer (Integrated DNA Technologies, Coralville, IA):

5′-CTA GCC TCC TCC TGG GTT TT-3′

Each 50 µL PCR reaction contained 25 µL SYBR(R) Green Master Mix (ABI, Foster City, CA), 3 µL 5 µM Forward Primer, 3 µL 5 µM Reverse Primer, 14 µL UV irradiated deionized water, and 5 µL of various amounts of PCR template. The following thermocycling settings were used: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 sec at 95°C, 30 sec at 61°C, and 1 min at 72°C. All PCR reactions were carried out in optical PCR tubes obtained from ABI. Data analysis was analyzed using the ABI Prism 7000 SDS software.

To construct a calibration graph, the amount of PCR template was plotted against the delta threshold cycle (Ct), which was equal to the total number of allotted cycles (40 cycles) minus the thermal cycle number, at which point changes in FL intensity had reached a pre-selected value.3

Use of SYBR(R) Green I Dye for DNA Quantitation

SYBR(R) Green I dye (ABI), which is usually used for gel staining, has been used as a FL probe for DNA quantitation in solution. SYBR(R) Green I dye is the same intercalating dye used in the real-time PCR master mix described previously. Since this dye does not interfere with any PCR reagents, such as Taq polymerase, we can assume it will behave the same as during the real-time experiments. To measure the FL from SYBR(R) green, the Fluoroskan Ascent FL plate reader (Thermo Labsystems, Woburn, MA) equipped with a 30 W quartz/halogen light source and 485 nm excitation and 520 nm emission filters were used. Readings were taken at 37.2°C with a 20 msec integration time and 10 msec blanks before and after each well, taken immediately after the plate moved into position with an acceleration of 0.5 m/sec^2. Calf thymus DNA (Sigma-Aldrich, St. Louis, MO) was used as a standard to construct a calibration curve. The linear calibration graph showed that this dye could be used for DNA quantitation in solution as well as gel staining (data not shown).

End-Point Quantitative PCR

We investigated the possibility of end-point quantitative PCR by combining the use of an Eppendorf Mastercycler gradient thermocycler and the ThermoLabsystems Fluoroskan Ascent FL Plate Reader. The same PCR template and primers were used as described earlier as well as the same intercalating dye and thermocycling conditions. After the thermocycling was completed, the entire PCR products were transferred via pipette to a Nunc Maxisorb 96 well plate (Nalge Nunc International, Roskilde, Denmark) in black color with flat bottom before being read on the plate reader with the same settings as described earlier. To construct a calibration graph, the amount of PCR template was plotted against the FL reading after the PCR reaction was completed.

Results and Discussion

The ability to use SYBR(R) green as a detection dye for DNA quantitation on the Fluoroskan Ascent FL has been previously established.20 However, in order to obtain the lowest LOD from this detection method for our end-point quantitative PCR measurements, we optimized a series of parameters such as PCR cycle number, different FL instrument settings, amount of SYBR(R) green dye, and DNA-SYBR(R) green complex stability.

First, we amplified different amounts of PCR template with 20, 30, or 40 thermal cycles and determined how many PCR thermal cycles would give the highest signal-to-noise ratios (S/N) and the lowest coefficients of variation (CV). We found that at 20 PCR cycles, the S/N was the highest (Table 1). From the plot of cycle number vs. change in FL (data not shown), the results indicated that the PCR reaction with 20 thermal cycles was still in its exponential growth phase. Hence, the reaction materials were still abundant and not only the S/N was the highest but the CVs were lowest as well (Table 1). With 30 thermal cycles, the signals began to drop with more PCR template. With 40 thermal cycles, both signal and data correlation were incoherent. For samples with more PCR template, at thermal cycle numbers above 20, it appeared that the rate of amplification began to slow down. The difference in sample conditions, such as low volume pipetting variations that can slightly change primer and deoxynucleotide triphosphate (dNTP) concentrations tube-to-tube, became more prevalent after the reaction has completed the exponential amplification. Also, using SYBR(R) green dye for detection does not allow for the use of an internal standard (IS), due to the inability of the dye to distinguish between different DNA sequences. Without using an IS, the CVs have significantly increased with 30 or 40 thermal cycles (Table 1), which according to our data were beyond the exponential growth phase of PCR amplification. In order to establish the LOD for our quantitative PCR assay using SYBR(R) green detection, not only was the highest S/N taken into consideration, but also the CV was equally important. Hence, we have chosen to amplify our selected template for only 20 cycles.

Table 1. Optimization of thermocycle number for end-point detection

Total cycle number	Moles of PCR template	Fl. (arb. unit)	S/N*	CV** (n = 3)
20	1.00E-12	10.84	4.36	5.26
	1.00E-15	8.49	3.42	8.25
	1.00E-18	3.87	1.56	5.17
	0.00	2.48	1.00	27.16
30	1.00E-12	9.49	3.17	7.49
	1.00E-15	6.07	2.02	15.65

	1.00E-18	4.36	1.46	16.05
	0.00	3.00	1.00	20.00
40	1.00E-12	7.15	1.54	1.67
	1.00E-15	7.08	1.53	28.32
	1.00E-18	4.43	0.96	12.22
	0.00	4.63	1.00	36.03

Note: All PCR template (75 mer) concentrations were determined in triplicate (n = 3), using an Eppendorf Master gradient thermocycler at 95°C for 10.00 min, then 40 cycles of 95°C for 15 sec, 61°C for 30 sec, and 72°C 1.00 min. Samples were then transferred into a NuncMaxisorb 96 well plate (Nalge Nunc International, Roskilde, Denmark) and the FL read on a ThermoLabsystems Fluoroskan Ascent FL at 485 nm excitation and 520 nm emission. Four different concentrations of sample were allowed to thermocycle to the noted PCR cycle then immediately had its FL read. The chart represents the differences in average FL, S/N, and CV (n = 3) for the samples stopped at the indicated cycle. *S/N, signal-to-noise ratio; **CV, coefficient of variation.

After studying the effects of PCR cycle numbers, the integration time for FL measurements in the plate reader was studied in order to further improve the detection sensitivity. The difference in sensitivity with 20 (default setting) and 200 msec integration times were insignificant (data not shown).

It is known that SYBR$^{(R)}$ green dye binds specifically to double-stranded DNA and its FL intensity is proportional to the amount of DNA. To ensure that an optimal amount of SYBR$^{(R)}$ green dye was present, we had added extra SYBR$^{(R)}$green dye into the PCR products after the amplification with the master mix was completed. The results showed that the extra SYBR$^{(R)}$ green had increased both signal and noise to the same extent, hence no effect on improving the sensitivity of our end-point detection method (data not shown).

For the purpose of using the end-point detection method to develop an ultra-sensitive iPCR assay, we had also studied the stability of the complex between double-stranded PCR products and SYBR$^{(R)}$ green dye. In this study, we chose to measure the FL signals immediately after 20 thermal cycles, and 30 min and 1 hr after 20 cycles were completed (Fig. 1). FL intensity of PCR production was stable when read approximately 5–10 min after the cycle had ended. The FL intensity then gradually decreased over 1 hr after the thermocycling was completed, whereas the R^2value was maintained at 0.99 for each of the measuring time points. One possible explanation for the decrease on the FL signals could be the effects of photobleaching the SYBR$^{(R)}$ green dye that has been complexed with the PCR products. To investigate this hypothesis, pure calf thymus DNA solutions with known concentrations were mixed with

SYBR(R) green master mix solution. The FL measurements of the mixtures were carried out sequentially at 0, 30, and 60 min after mixing the solutions, while the mixtures were incubated at constant room temperature under complete darkness. The control of this experiment included DNA-SYBR(R) green mixtures that had not been measured until the end of the 60 min incubation. In comparison to the control experiment, the FL signals that were obtained from the sample mixtures, which had been repeatedly irradiated by the excitation laser beam, were significantly lower. Hence, we concluded the major cause for the lowering of the FL signals from measuring the PCR products was in fact due to the adverse effects of photobleaching. With these results in mind, samples should be read immediately after the cycle has completed, in order to maintain the optimal S/N ratio and LODs.

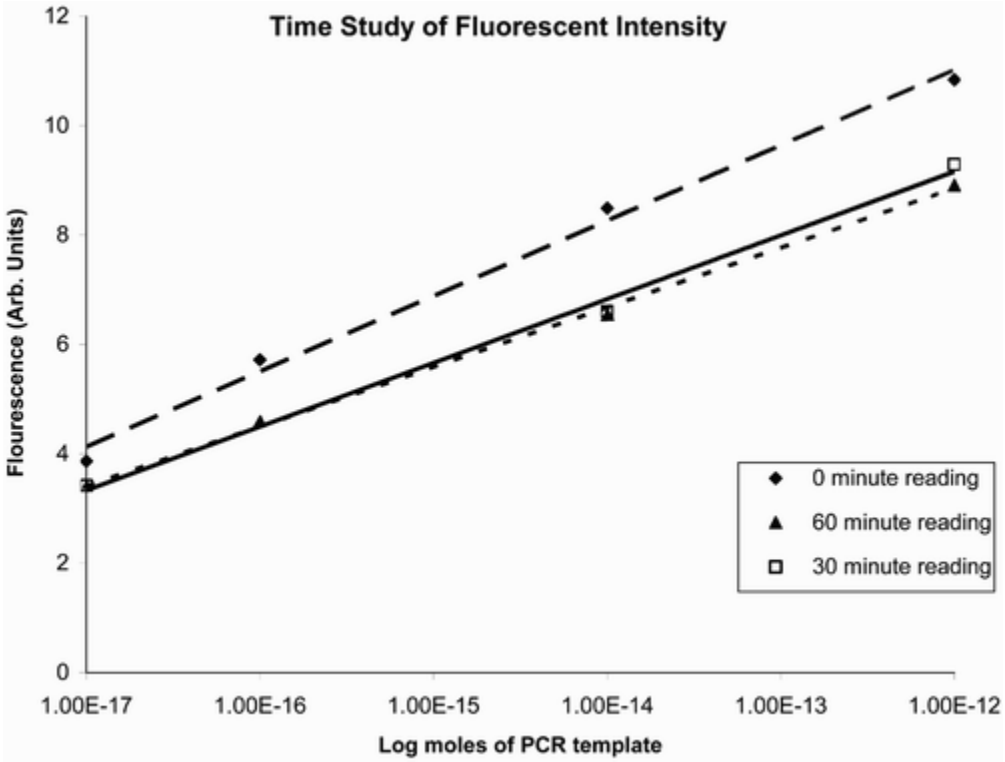

Figure 1. FL intensity time study of PCR products after 20 thermocycles. The product's FL was read immediately after the thermocyles finished, 30, and 60 min after the cycles finished. The range linearity remained constant with all three R^2 values at 0.99.

Before any comparative studies were performed, we chose to run a simple experiment to ensure the two thermocyclers, ABI and Eppendorf, had the same thermocycling efficiency. If this was so we could be sure we were making a true direct comparison of the two methods, since the PCR template, primers, intercalating dye, thermocycling times, and efficiency would all be indistinguishable. The only difference would be the technique of quantitation between the two methods. To check the efficiencies, we ran, simultaneously, in the two thermocyclers, two different quantitites of PCR template in triplicate. We then took the fluorescent readings of the

samples on the Fluoroskan Ascent FL as in the end-point method and compared the fluorescent intensity between the samples cycled in the Eppendorf from those cycled in the ABI Prism. No significant difference in FL was observed between the samples from the two methods, and we could, therefore, assume the two instruments performed identically.

After optimizing the off-line detection method, experiments were performed to establish the linear dynamic ranges for PCR quantitation. The range of PCR template quantities was expanded, in order to yield a sigmoidal standard curve and properly demonstrate the upper and lower LODs. With either 1 : 100 or 1 : 10 serial dilutions of PCR template, nine orders of magnitude of PCR template quantities (1 pmol to 1 zmol) were analyzed. From the results in Figure 2, the linear ranges of quantitation for both methods were 10 fmol to 10 zmol and 5 pmol to 5 amol of PCR template for the real-time and end-point methods, respectively. The error bars represent the standard deviations. In the real-time detection method, triplicate samples of each dilution of PCR template were examined. The end-point detection method also consisted of triplicate samples of each PCR template dilution, and the FL of each sample was measured five times. The real-time detection method did not appear to show an upper plateau of signal. This was because, the highest PCR template quantity (1 pmol) was undetected due to the change in FL being above the threshold value even before the PCR amplification. Owing to this reason, the second highest PCR template quantity (10 fmol) was considered as the upper LOD, whereas the end-point detection method showed high CVs in the FL signals from more than 5 pmol of template, which is considered as the upper LOD for the method. It should also be noted that samples above milli-molar concentrations could be quantitated using standard UV 260 nm absorbance where an optical density of 1 corresponds to 20–30 μg/mL in concentration.21

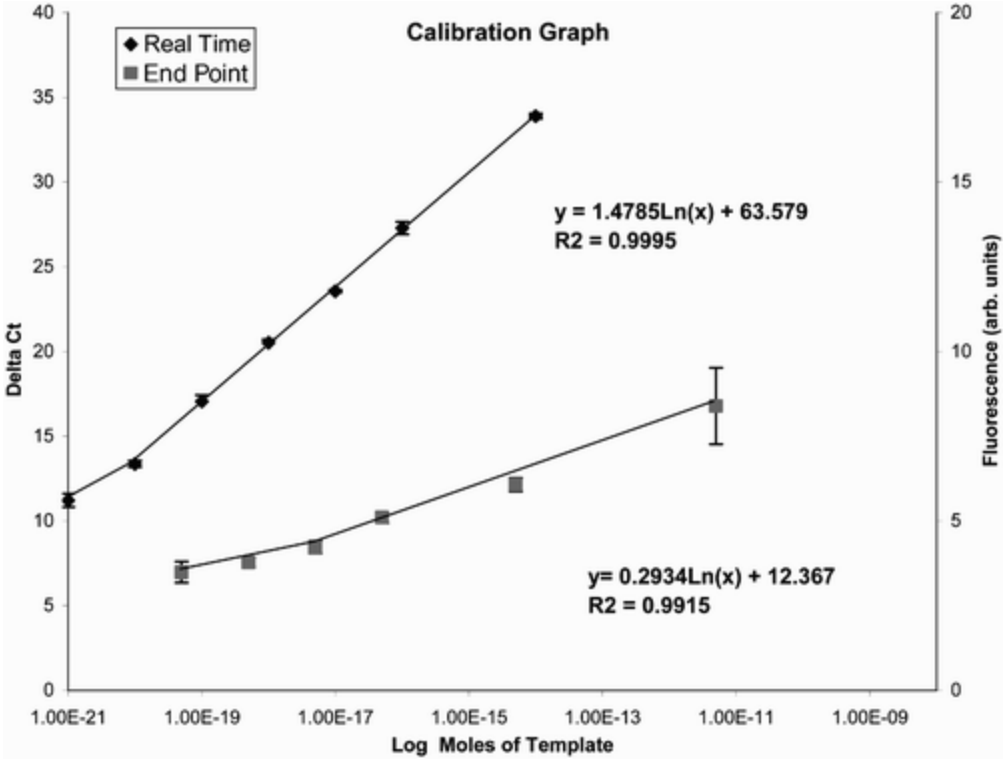

Figure 2. Calibration plot of both the real-time and end-point PCR quantitation methods using the same 75 mer PCR template, SYBR$^{(R)}$ green dye, and thermocycling conditions. The real-time method spans six orders of magnitude in its linear range from 10 fmol to 10 zmol of PCR template, while the end-point method spans six orders of magnitude from 5 pmol to 5 amol. Error bars indicate the standard deviations at each concentration in which $n = 3$ for the real-time method and $n = 15$ for the end-point method.

The PCR template used for this analysis was not 100% efficient for PCR amplification. As we have identified earlier, there are some concerns with contamination when using these techniques, and unfortunately the DNA sequence used in these assays has a high propensity toward nonspecific amplification.12 Keeping this in mind, it should be noted that the LOD and linear ranges established are not optimal for real-time or end-point analysis using SYBR$^{(R)}$ green detection. It has recently been shown by Morsczeck and colleagues that SYBR$^{(R)}$ green could be used to quantitate a 366 base pair gene segment down to ~15 copies.22 The numbers found in our study were optimal for only the PCR template analyzed, and it is expected that a more appropriate sequence for PCR, such as the one used in the Morsczeck study, would lower limits and increase efficiency. However, the LOD difference between the two methods studied here, three orders of magnitude, should remain constant no matter which PCR template is used. Overall, the real-time method is undoubtedly an easier and more sensitive technique for PCR quantitation, whether it is for iPCR or any other PCR application.

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