The iCycler iQ™ Detection System for Multiplex Real-Time PCR* Assays

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Introduction

The introduction of real-time PCR has made it possible to accurately quantitate starting amounts of nucleic acid during the PCR reaction without the need for post-PCR analyses. At the end of the amplification, the reaction mixture can be discarded without opening the tube, thus avoiding contamination of the laboratory with PCR product. In real-time PCR, a fluorescent reporter is used to monitor the PCR reaction as it occurs; the reporter can be of a nonspecific or specific nature. The fluorescence of the reporter molecule increases as products accumulate with each successive round of amplification. In the early cycles of amplification, the change in fluorescence of the reporter is usually undetectable, but at some point during amplification, the accumulation of product results in a measurable change in the fluorescence of the reaction mixture. The point at which the fluorescence rises appreciably above background has been called the threshold cycle. There is a linear relationship between the log of the starting amount of template and the corresponding threshold cycle during real-time PCR. Given known starting amounts of the target nucleic acid, a standard curve can be constructed by plotting the log of starting amount versus the threshold cycle. This standard curve can then be used to determine the starting amount for each unknown template, based on its threshold cycle.

The first demonstration of real-time PCR used the nonspecific reporter ethidium bromide (Higuchi et al. 1993). The fluorescence of ethidium bromide increases significantly as it binds and intercalates into double-stranded DNA during the extension step of the amplification cycle. SYBR® Green I can also be used as a fluorescent reporter in real-time PCR. Several suppliers sell PCR reaction mixtures containing optimized amounts of SYBR Green I. While this is a relatively simple technique useful for any PCR reaction, intercalating dyes bind to all double-stranded DNA products. Therefore, the increase in fluorescence does not necessarily accurately reflect the increase in desired product. When nonspecific binding is a problem, post-PCR corrections to the data may be necessary in order to obtain accurate quantitative information.

Fluorescent oligonucleotide probes enable real-time monitoring of the PCR assay, and ensure that increases in fluorescence result only from the accumulation of the desired product. One popular probe strategy is the TaqMan® assay (Applied Biosystems), which capitalizes on the 5' exonuclease activity of Taq polymerase to cleave a labeled hybridization probe during the extension phase of PCR (Holland et al. 1991). In a fluorescent TaqMan assay, the probe is labeled at the 5' end with a fluorescent reporter molecule such as fluorescein and at the 3' end with another fluorescent molecule, usually a tetramethylrhodamine derivative, which acts as a quencher for the reporter (Heid et al. 1996). When the 2 fluorophores are fixed at opposite ends of the 20–30 nt probe and the reporter fluorophore is excited by an outside light source, the normal fluorescence of the reporter is absorbed by the nearby quencher, and no reporter fluorescence is detected. When Taq polymerase encounters the bound probe during extension from one of the primers, it digests the probe, freeing the reporter from the quencher, and the reporter fluorescence can be detected and measured.

Molecular beacons (Tyagi and Kramer 1996), represent another example of specific fluorescent probes. The molecular beacon is a short oligonucleotide (25–40 nt) that forms a hairpin structure with a loop and stem. The loop is designed to hybridize to a 15–30 nt section of the target sequence. On either side of the loop are another 5 or 6 nt that are complementary to one another. A fluorescent reporter molecule is placed at the 5' end of the molecular beacon and a nonfluorescent quencher is placed at the 3' end. At room temperature, the molecular beacon assumes the hairpin formation, bringing the reporter and the quencher into intimate contact. In the hairpin structure, no fluorescence is detected from the reporter molecule. During the annealing step of the amplification cycle, thermodynamics favors the binding of the molecular beacon to its target rather than formation of the hairpin structure. Therefore, molecular beacons that bind to target during annealing are bright and those that do not are dark. With each successive cycle of amplification, the proportion of molecular beacons that bind target to and emit light during annealing increases, directly corresponding to the accumulation of product.

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Performance of a real-time PCR instrument is usually characterized by the sensitivity, uniformity, and dynamic range of linear response to a variety of input sample concentrations. The iCycler iQ system demonstrates excellent performance characteristics in these areas. In this article we illustrate the performance of the system when used with SYBR Green I dye, TaqMan, and molecular beacon chemistries. We also show the first 4-color/4-gene/4-template multiplex assay in a real-time PCR. Using 4 specific fluorescent probes targeting 4 genes — α-tubulin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclophilin, and β-actin — we have performed a real-time PCR showing specific amplification of each of these target genes in the same reaction tube on the iQ system.

Methods

Multiplex

The following master mix was prepared to demonstrate multiplex capabilities:

1.5 ml 2x custom Super mix (Life Technologies)**
12 µl Platinum Taq Polymerase (Life Technologies)
30 µl 100 mM dNTP
6 µl 100 µM α-tubulin forward primer
6 µl 100 µM α-tubulin reverse primer
6 µl 100 µM α-tubulin probe (FAM-labeled)
6 µl 100 µM GAPDH forward primer
6 µl 100 µM GAPDH reverse primer
6 µl 100 µM GAPDH probe (HEX-labeled)
9 µl 100 µM cyclophilin forward primer
9 µl 100 µM cyclophilin reverse primer
6 µl 100 µM cyclophilin probe (Cy™5-labeled)
9 µl 100 µM β-actin forward primer
9 µl 100 µM β-actin reverse primer
6 µl 100 µM β-actin probe (Texas Red®-labeled)
1.13 ml ddH_2O

** 1.1x Super mix, modified to contain 3 mM Mg^{2+} and 1.5 units of enzyme per reaction and adjusted to a 2x concentration

This master mix was then vortexed thoroughly and 322 µl was pipetted into 8 different 2.0 ml skirted tubes.

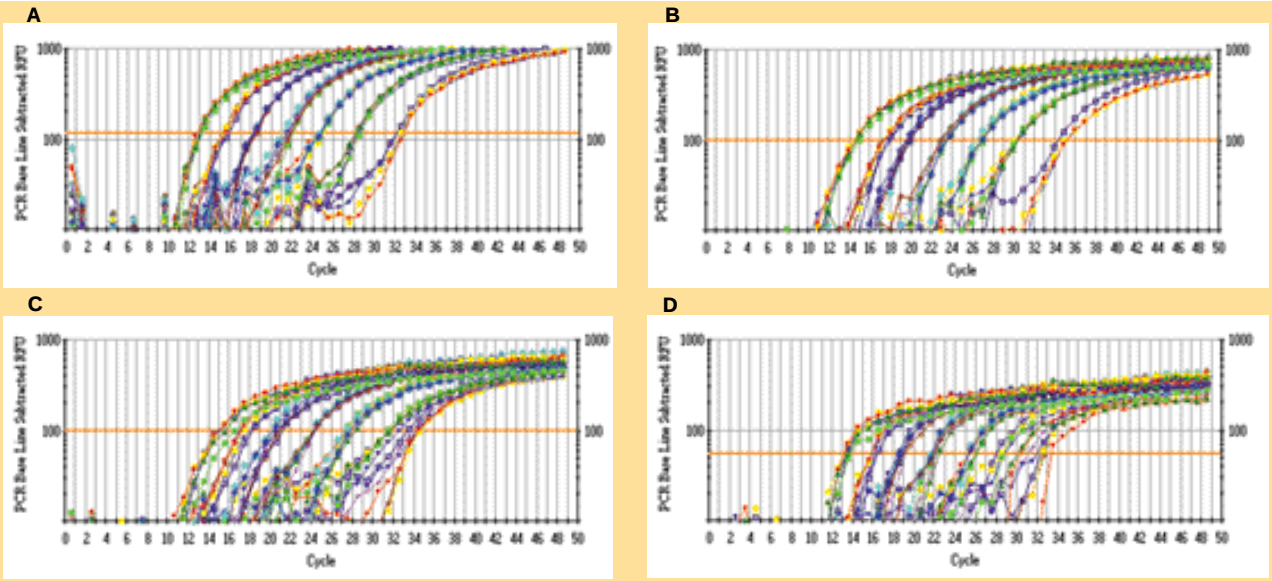

Fig. 1. iCycler iQ screen view showing results of the different dye layers from the multiplex 4-target dilution series with 4 specific molecular beacons. The dynamic range covers 10^2 to 10^8 molecules of each of the 4 different targets. A, FAM dye layer, α-tubulin target. B, HEX dye layer, GAPDH target. C, Texas Red dye layer, β-actin target. D, Cy5 dye layer, cyclophilin target.

A stock of each plasmid was quantitated using PicoGreen® dye binding measured by the VersaFluor™ fluorometer (Bio-Rad), then diluted to 10^8 copies/µl. The plasmids used were pEGFP-Actin (β-actin) and pEGFP-Tub, (α-tubulin; Clontech) and IMAGE Consortium clones 522094 (GAPDH) and 71154 (cyclophilin 40; ATCC). A 10-fold dilution series was prepared, from 10^8 to 10^2 copies/µl, and then 7 µl of each different plasmid at each dilution was added to each of the skirted tubes containing master mix. Finally, six 50-µl aliquots of each mix were pipetted into the rows of a 96-well thin-wall PCR plate. The plate was covered with a piece of optically clear sealing tape, spun briefly to bring all reagents to the bottom, and the plate was placed in the iCycler iQ detection system. PCR conditions were 3 min at 95°C followed by 50 cycles of 10 sec at 95°C and 60 sec at 55°C. Fluorescent data were collected during the 55°C step. In the plate setup, 4 of the replicates were identified as standards and the other 2 as unknowns

Uniformity

The following master mix was prepared to demonstrate system uniformity using SYBR Green I as the detection reagent:

500 µl 10x reaction buffer (SYBR Green PCR core reagent kit, PE Biosystems)
350 µl 50 mM $MgCl_2$
20 µl Platinum Taq polymerase (Life Technologies)
50 µl 100 mM dNTPs
15 µl 100 µM β-actin forward primer
15 µl 100 µM β-actin reverse primer
100 µl β-actin plasmid diluted to 10^4 copies/µl
3.95 ml dd H_2O

The master mix was thoroughly vortexed and 50 µl aliquots were pipetted into a 96-well thin-wall PCR plate. The plate was prepared for PCR as above. PCR conditions were 3 min at 95°C, followed by 40 cycles of 10 sec at 95°C and 30 sec at 60°C. Fluorescent data were specified for collection during the 60°C step. In the plate setup file, all wells were defined as unknowns.

Linearity

The following master mix was prepared to demonstrate linearity using a TaqMan probe as the detection reagent:

450 µl 10x Platinum PCR buffer (Life Technologies)
315 µl 50 mM $MgCl_2$
45 µl 100 mM dNTP
13.5 µl 100 µM β-actin forward primer
13.5 µl 100 µM β-actin reverse primer
18 µl Platinum Taq polymerase (Life Technologies)
5.0 µl 180 µM TaqMan probe (PE Biosystems)
3.55 ml dd H_2O

This master mix was vortexed thoroughly and then 441 µl of master mix was pipetted into 9 different 2.0 ml skirted tubes.

A stock of plasmid pEFGP-Actin was quantitated as above, then diluted to 10^9 copies/µl. A 10-fold dilution series was prepared from 10^9 to 10 copies/µl, and then 9 µl of each dilution was added to one of the skirted tubes containing master mix. Finally, eight 50 µl aliquots of each mix were pipetted into wells of a single column of a 96-well thin-wall PCR plate. The plate was prepared for PCR as above. PCR conditions were 3 min at 95°C followed by 50 cycles of 10 sec at 95°C and 30 sec at 60°C. Fluorescent data were collected during the 60°C step. In the plate setup file, half the replicates from each set in the series were identified as standards and the other half as unknowns.

Sensitivity

The following master mix was prepared to demonstrate sensitivity using a TaqMan probe as the detection reagent:

3.18 ml 1.1x Platinum PCR Super mix (Life Technologies)
10.5 µl 100 µM β-actin forward primer
10.5 µl 100 µM β-actin reverse primer
4 µl 180 µM TaqMan probe
225 µl H_2O

The master mix was thoroughly vortexed and then 416 µl aliquots were transferred into eight 2-ml skirted tubes.

Human genomic DNA, 100 µg/µl (Clontech) was partially digested by BamHI at 37°C for 2 hr, then heated to 100°C for 5 min before being plunged into an ice-water bath. A 2-fold dilution series was prepared from the genomic DNA, ranging from 100 ng/µl to 781 pg/µl. For each tube of the dilution series, 8.25 µl was pipetted into one of the 8 skirted tubes and vortexed. Finally, for each of the tubes, eight 50 µl samples were transferred into one column of a 96-well thin-wall PCR plate. The plate was prepared for PCR as above. PCR conditions were 3 min at 95°C followed by 40 cycles of 10 sec at 95°C and 30 sec at 60°C. Data were collected during the 60°C annealing/extension step. In the plate setup file, 4 wells of each replicate group within a column were defined as standards and the other 4 as unknowns.

Results

Multiplex

When the background-corrected data were brought down to the PCR baseline, the standard curve had a correlation coefficient (r^2) of 0.949 for the FAM dye layer, corresponding to the α-tubulin plasmid, 0.917 for the HEX (GAPDH plasmid) dye layer, 0.994 for the Texas Red (β-actin plasmid) dye layer, and 0.995 for the Cy5 (cyclophilin plasmid) dye layer across the dilution range tested (10^2–10^8 copies/reaction). The statistical evaluation of the data can be significantly improved by exploiting the data analysis options of the iCycler software. Viewing the amplification plot on a log scale is useful to assign the threshold. The quality of the standard curves could be improved by changing the threshold-relative fluorescence units (RFU) to between 50 and 105, depending on the dye layer. A unique feature of the iCycler software is that it allows use of any subset of the data that is collected through the Set Data Analysis option. By including the last 99% of the data collected in each cycle, the final standard curves had correlation coefficients from 0.996–0.998 over a range of 6 orders of magnitude (see Figure 1A–D). Each of the 4 standard curves had slopes close to the theoretical values (not shown), indicating that near maximum efficiency was attained.

Uniformity

After the data were brought to baseline by choosing PCR Base Line Subtraction from the onscreen Noise Reduction dialog box of the iCycler, the mean threshold cycle for the 96 replicates was 21.7 with a standard deviation of 0.91 cycles and a 4.2% coefficient of variation (CV). The default analysis conditions are to use cycles 2–10 to establish the baseline, to assign a threshold of 10 times the mean standard deviation of the data collected over the baseline cycles, and to use the last 10% of the data collected during the data collection cycle (the annealing/extension step in this experiment).

Extending the baseline cycles from cycle 4 to cycle 24 changes the mean threshold cycle for the 96 replicates to 23.7 with a standard deviation of 0.28 (1.2% CV). By using all data collected in this experiment over the 30 sec annealing/extension cycle (as many as 30 data points, depending on exposure time), combined with appropriate selections for baseline cycles and threshold, the final result for 96 wells (Figure 2) is a mean threshold cycle of 24.8 and a standard deviation of 0.185 (0.75% CV).

Linearity

When the background-corrected data were brought down to the PCR baseline, the standard curve has a correlation coefficient of 0.995, but the two most concentrated samples showed little separation. This occurred because the default baseline cycles are cycles 2–10, and the most concentrated samples had already come above baseline before cycle 10 (see Figure 3). The baseline cycles should be limited to cycles in which none of the samples have come above baseline, so the baseline cycles were changed to include cycles 2–5 only. The quality of the standard curve could be further improved by changing the threshold to 900 RFU and including the last 75% of the data collected in each cycle. The final standard curve shows a correlation coefficient of 0.998 over a range of 8 orders of magnitude (Figure 4).

Sensitivity

The starting concentration of genomic DNA ranged from 100 ng to 781 pg in 2-fold dilutions, or approximately from 16,000 to 125 genomic equivalents, respectively, with 8 replicates for each dilution. These data were optimized by extending the baseline cycles to include cycles 2–21, setting the threshold to 30 and using the last 75% of the data collected during the annealing/extension step. The resulting standard curve (Figure 5) showed a correlation coefficient of 0.999, and the threshold cycles of each successive replicate group were separated from its predecessor and successor by at least 3 standard deviations, down to approximately 250 genomic equivalents (see table). Theoretically, the threshold cycles of each replicate group should be separated by exactly 1 cycle; in this experiment, the difference between threshold cycles ranged from 1.01 to 1.19 cycles with a mean separation of 1.07 cycles.

Sensitivity of iCycler iQ to Target DNA Concentration

pg	Genomic Equivalents	Mean Threshold Cycle	SD	Δ Cycles
100,000	16,000	22.18	0.093	
50,000	8,000	23.19	0.046	1.01
25,000	4,000	24.22	0.114	1.03
12,500	2,000	25.24	0.146	1.02
6,250	1,000	26.29	0.117	1.05
3,125	500	27.43	0.131	1.14
1,562.5	250	28.62	0.146	1.19
781.25	125	29.65	0.166	1.03

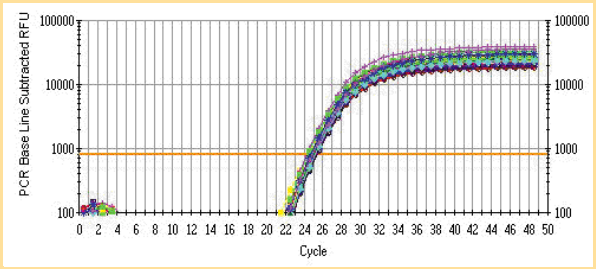

Fig. 2. iCycler screen view showing uniformity of threshold cycle in 96-well plate containing identical samples.

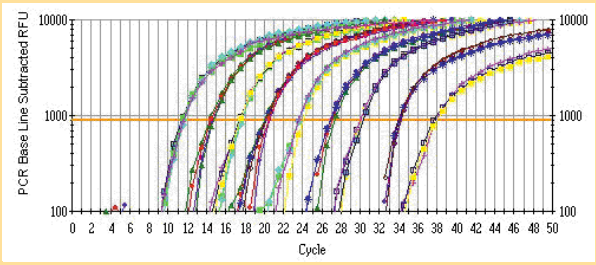

Fig. 3. iCycler iQ screen view showing results of a target DNA dilution series with a TaqMan probe. The dynamic range covers 10 to 10^9 molecules of the β-actin target (pEFGP).

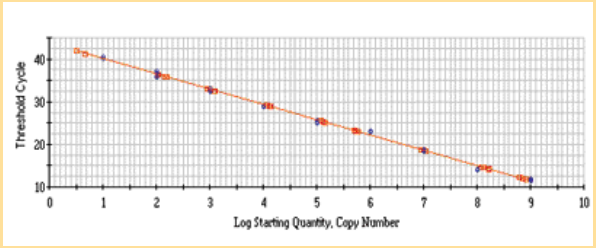

Fig. 4. Standard curve generated by the iCycler software from the data in Figure 3. Circles, standards; squares, unknowns.

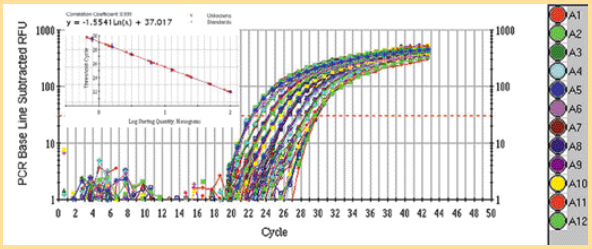

Fig. 5. Standard curve generated by the iCycler software showing sensitivity to approximately 125 genomic equivalents. The inset shows the standard curve for the data, where half were defined as standards, and half as unknowns. The slope of the curve is -3.58.

Discussion

This is the first single-tube amplification and simultaneous detection of 4 targets in a real-time PCR. Each well of the multiplex experiment contained 4 sets of primers, 4 plasmid-specific probes, and 4 individual plasmids; that is, 4 separate real-time reactions were combined in each well. Even with this level of complexity added to the chemistry of the reaction, correlation coefficients for the standard curves generated for each dye layer ranged from 0.996 to 0.998. The iQ system is able to separate data collected from the 4 different reactions occurring in a single well, and allows the maximum flexibility in a 96-well real-time format.

Using the intercalation dye SYBR Green I, the iQ system shows a uniformity for 96 wells with a standard deviation of 0.185 (0.75% CV). Using a TaqMan probe designed to detect β-actin as the target sequence, the iCycler iQ system shows linearity over a range of 8 orders of magnitude. In addition, we show the ability to distinguish between 125 and 250 genomic equivalents using the iCycler iQ system.

Baseline cycles, threshold levels, data analysis windows, and other parameters can be set by the user. Because the iQ system collects data over the entire cycle, the kinetic profiles of data sets can be evaluated for each detection chemistry. In addition to the multiplex ability, uniformity, sensitivity, and dynamic range of the instrument, we have shown the versatility of its data analysis.

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