amplification

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. In general, a fluorescent reporter is used to monitor the PCR reaction as it occurs. The fluorescence of the reporter molecule increases as products accumulate with each successive round of amplification. The point at which the fluorescence rises appreciably above background is called the threshold cycle. Taking advantage of the linear relationship between the log of the starting amount of template and the corresponding threshold cycle, a standard curve can be constructed. This standard curve can then be used to determine the starting amount for each unknown template, based on its threshold cycle.

The first demonstrated real-time PCR assay used the intercalation dye ethidium bromide (Higuchi et al. 1993). This intercalation dye as well as others demonstrate a significant increase in fluorescence when bound to double-stranded DNA. Currently, SYBR Green I is the intercalation dye of choice. While this method of detection is relatively simple and straightforward, it indiscriminately binds all double-stranded DNA products. Therefore, nonspecific amplification will result in an increased fluorescent signal.

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 two 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.

A molecular beacon (Tyagi and Kramer 1996) represents another example of a specific fluorescent probe. 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 have increased fluorescence, and those that do not bind are dark. With each successive cycle of amplification, the proportion of molecular beacons that bind to target and emit light during annealing increases, so that the increase in fluorescence directly corresponds to the accumulation of product.

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 four-color/four-gene/four-template multiplex assay in a real-time PCR. Using four specific fluorescent probes targeting four 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 iCycler iQ system.

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Methods

Multiplex

The following master mix was prepared to demonstrate multiplex capabilities:

> 1.5 ml 2x custom supermix (Life Technologies)*
> 12 µl 5 U/µl Platinum Taq polymerase (Life Technologies)
> 30 µl 100 mM dNTPs
> 6 µl 100 µM a-tubulin forward primer
> 6 µl 100 µM a-tubulin reverse primer
> 6 µl 100 µM a-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 (Cy5-labeled)
> 9 µl 100 µM b-actin forward primer
> 9 µl 100 µM b-actin reverse primer
> 6 µl 100 µM b-actin probe (Texas Red-labeled)
> 1.13 ml ddH2O

* 1.1x supermix, modified by manufacturer 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 eight different 2.0 ml skirted tubes.

A stock of each plasmid was quantitated using a PicoGreen dye assay measured with 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 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, four of the replicates were identified as standards and the other two 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 MgCl2
> 20 µl Platinum Taq polymerase (Life Technologies)
> 50 µl 100 mM dNTPs
> 15 µl 100 µM b-actin forward primer
> 15 µl 100 µM b-actin reverse primer
> 100 µl b-actin plasmid diluted to 104 copies/µl
> 3.95 ml dd H2O

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 MgCl2
> 45 µl 100 mM dNTPs
> 13.5 µl 100 µM b-actin forward primer
> 13.5 µl 100 µM b-actin reverse primer
> 18 µl 5 U/µl Platinum Taq polymerase (Life Technologies)
> 5.0 µl 180 µM TaqMan probe (PE Biosystems)
> 3.55 ml dd H2O

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

A stock of plasmid pEGFP-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
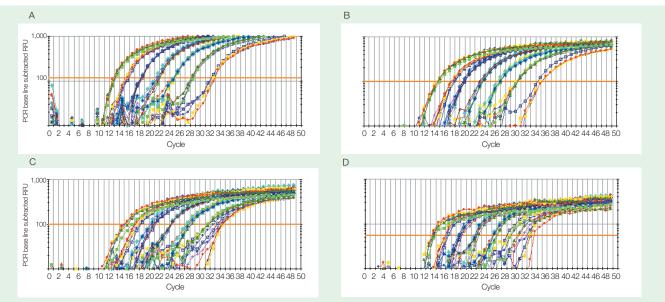

Fig. 1. iCycler iQ screen view showing results of the different dye layers from the multiplex four-target dilution series with four specific molecular beacons. The dynamic range covers 10^2 to 10^8 molecules of each of the four different targets. A, FAM dye layer, α-tubulin target. B, HEX dye layer,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

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 supermix (Life Technologies)
> 10.5 µl 100 µM b-actin forward primer
> 10.5 µl 100 µM b-actin reverse primer
> 4 µl 180 µM TaqMan probe
> 225 µl H2O

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

Human genomic DNA, 100 ng/µl (Clontech), was partially digested with *Bam*HI 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 eight 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, four wells of each replicate group within a column were defined as standards and the other four 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 iQ software. Viewing the amplification plot on a log scale is useful when assigning the threshold. The quality of the standard curves was 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 iQ 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 four 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 use: cycles 2–10 to establish the baseline, 10 times the mean standard deviation of the data collected over the baseline cycles to establish the threshold, and the last 10% of the data collected during the data collection step (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 had 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 (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 concentrations of genomic DNA ranged from 100 ng to 781 pg in 2-fold dilutions, or approximately 16,000 to 125 genomic equivalents, respectively, with eight 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 (Table). Theoretically, the threshold cycles of each replicate group should be separated by exactly one 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.
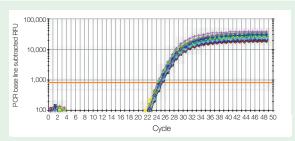
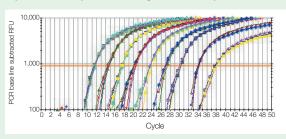**Fig. 2. iCycler iQ screen view showing uniformity of threshold cycle in 96-well plate containing identical samples.**

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 (pEGFP).
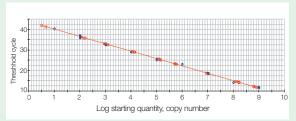

Fig. 4. Standard curve generated by the iCycler iQ software from the data in Figure 3. Circles, standards; squares, unknowns.
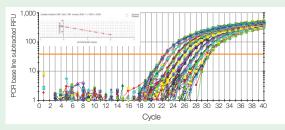

Fig. 5. Standard curve generated by the iCycler iQ software showing sensitivity to approximately 125 genomic equivalents. The inset shows the standard curve for the data, where half were defined as standards, and half as unknowns. The slope of the curve is –3.58.

Practice of the patented polymerase chain reaction (PCR) process requires a license. The iCycler iQ system includes a licensed thermal cycler and may be used with PCR licenses available from Applied Biosystems. Its use with authorized reagents also provides a limited PCR license in accordance with the label rights accompanying such reagents. Some applications may require licenses from other parties.

Table. Sensitivity of iCycler iQ system to target DNA concentration.

pg DNA	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

Discussion

This is the first single-tube amplification and simultaneous detection of four targets in a real-time PCR. Each well of the multiplex experiment contained four sets of primers, four plasmid-specific probes, and four individual plasmids; that is, four 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 iCycler iQ system is able to separate data collected from the four 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 iCycler 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 iCycler 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.

References

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Tyagi S and Kramer FR, Molecular beacons: probes that fluoresce upon hybridization, Nature Biotechnol 14, 303–308 (1996)

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