

The iCycler iQ™ Detection System for TaqMan Assays

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Introduction

Specific fluorescent oligonucleotide probes allow for real-time monitoring of the polymerase chain reaction (PCR^{*}). One popular probe strategy is the TaqMan assay (PE 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 tetramethyl- rhodamine 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.

The fluorescence of the reporter molecule increases as products accumulate with each successive round of amplification. In early cycles of amplification, the change in the reporter's fluorescence is usually undetectable, but at some point during amplification, the accumulation of product results in a measurable change in the total fluorescence of the reaction mixture. This point, at which the fluorescence rises appreciably above the background, has been called the threshold cycle, and there is a linear relationship between the log of the starting amount of a template and its 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.

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 application note we illustrate the performance of the system when used with TaqMan probes.

Methods

Uniformity

The following master mix was prepared:

- 4.55 ml Platinum PCR supermix (Life Technologies)
- 15 μ l 100 μ M forward primer (5'-TGC GTGACATTAAGGAGAAG)
- 15 μ l 100 μ M reverse primer (5'-GCTCGTAGCTCTTCTCCA)
- 5.6 μ l 180 μ M probe (5'-FAM-CACGGCTGCTCCAGTCCTC-TAMRA)
- 100 μ l pEFGP-Actin (β -actin) plasmid, diluted to 10⁵ copies/ μ l (Clontech)
- 314 μ l dd H₂O

The master mix was thoroughly vortexed and then 50 μ l aliquots were transferred to the wells of a 96-well thin-wall PCR plate. The plate was covered with a piece of optically clear sealing film and spun briefly to bring all reagents to the bottom of the wells. PCR conditions were 3 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 30 sec at 60°C. Fluorescent data were collected at the 60°C step. In the plate setup file, all wells were defined as unknowns.

Linearity

The following master mix was prepared:

- 450 μ l 10x Platinum PCR buffer (Life Technologies)
- 315 μ l 50 mM MgCl₂
- 45 μ l 100 mM dNTP
- 13.5 μ l 100 μ M forward primer
- 13.5 μ l 100 μ M reverse primer
- 18 μ l Platinum *Taq* polymerase (Life Technologies)
- 5.0 μ l 180 μ M probe
- 3.55 ml dd H₂O

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

A stock of plasmid pEFGP-Actin encoding β -actin (Clontech) was quantitated using PicoGreen dye (Molecular Probes) binding with the VersaFluor™ fluorometer (Bio-Rad), then diluted to 10^9 copies/ μ l. A 10-fold dilution series was prepared from 10^9 to 10 copies/ μ l, and then 9 μ l of each dilution was added to one of the skirted tubes containing master mix. Finally, eight 50 μ l 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:

- 3.18 ml 1.1x Platinum PCR supermix
- 10.5 μ l 100 μ M forward primer
- 10.5 μ l 100 μ M reverse primer
- 4 μ l 180 μ M probe
- 225 μ l H₂O

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

Human genomic DNA, 100 μ g/ μ l (Clontech) was partially digested by *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/ μ l to 781 pg/ μ l. For each tube of the dilution series, 8.25 μ l was pipetted into one of the 8 skirted tubes and vortexed. Finally, for each of the tubes, eight 50 μ l 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

Uniformity

When the background-corrected data were brought down to the PCR baseline, the 96 wells had a mean threshold cycle of 23.4 with a standard deviation of 0.6 cycles and a coefficient of variation (CV) of 2.6%. However, these data could be optimized by first extending the baseline cycles to include cycles 2–24; this changes the mean threshold cycle to 25.5 with a standard deviation of 0.16 cycles (0.63% CV). Increasing the data window to include the last 75% of the data collected during the annealing/extension step, and changing the threshold to 32.5 relative fluorescence units (RFU) — or 15 times the mean standard deviation during the baseline cycles — further improved the data, so that the final mean threshold cycle was 25.9 with a standard deviation of 0.12 cycles with 0.48% CV (Figure 1).

Linearity

When the background-corrected data were brought down to the PCR baseline, the standard curve has a correlation coefficient (r^2) of 0.995, but the 2 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 2). 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 3).

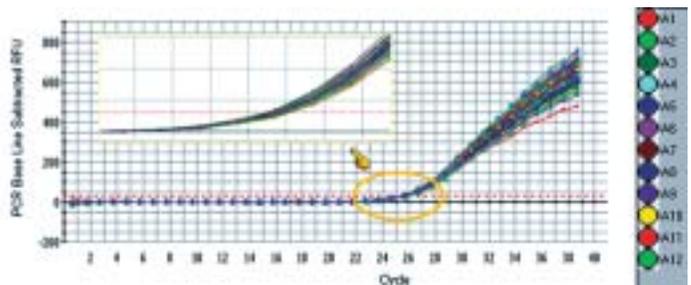


Fig. 1. iCycler iQ screen view showing threshold cycle uniformity in 96 replicate samples. Inset shows a zoom view of the data around the threshold cycle. The iCycler iQ software was used to optimize the data as described in the text.

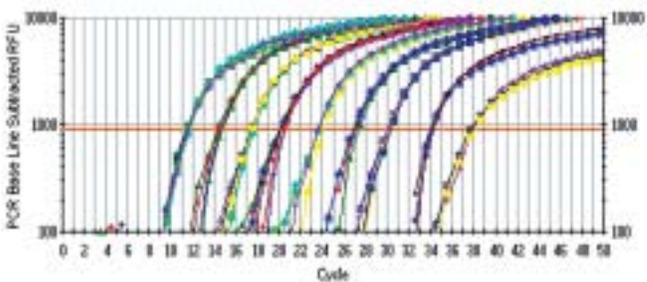


Fig. 2. iCycler iQ screen view showing results of a target DNA dilution series with a TaqMan probe. The dynamic range covers 10 to 10^9 molecules of the β -actin target (pEFGP-Actin).

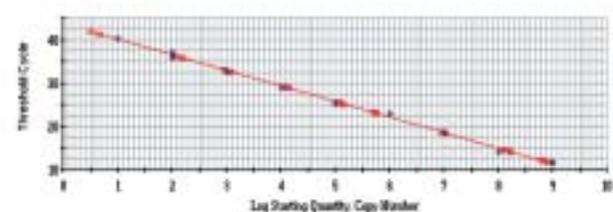


Fig. 3. Standard curve generated by the iCycler software from the data in Figure 2. Circles, standards; squares, unknowns.

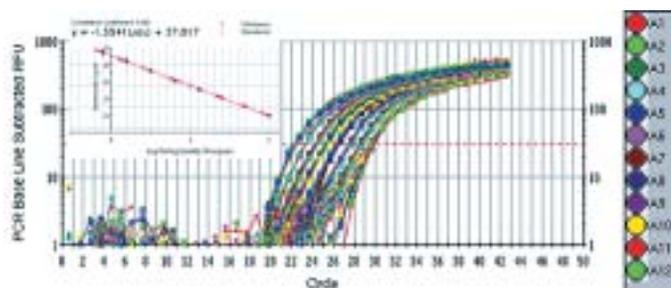


Fig. 4. Standard curve generated by the iCycler software showing sensitivity to approximately 125 genomic equivalents.

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 4) 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 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

Using a TaqMan probe designed to detect β -actin as the target sequence, the iCycler iQ system shows uniformity over a 96-well plate and 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. When obtaining threshold cycles for a 2-fold dilution series in a real-time PCR, these threshold cycles should be separated by exactly one cycle for each dilution. The data for threshold cycle separation obtained using the TaqMan probe for β -actin and human genomic DNA on the iCycler iQ system ranges from 1.01 to 1.19 cycles (mean = 1.07).

References

- Heid CA et al., Real time quantitative PCR, *Genome Res* 6, 986–994 (1996)
- Holland PM et al., Detection of specific polymerase chain reaction product by utilizing the 5' \rightarrow 3' nuclease activity of *Thermus aquaticus* DNA polymerase, *Proc Natl Acad Sci USA* 88, 7276–7280 (1991)

* 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.

TaqMan is a trademark of Roche Molecular Systems.



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