Real-Time PCR* using the iCycler iQ Detection System and Intercalation Dyes

Faye Boeckman, Keith Hamby, and Larissa Tan, Bio-Rad Laboratories,
2000 Alfred Nobel Drive, Hercules, CA 94547 USA

Intercalation Dyes

The introduction of real-time PCR has made it possible to accurately quantify 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 nature or of a 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 (Molecular Probes, Eugene OR) 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.

Methods

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 forward primer (5'-TGCGTGACATTAAGGAGAAG)
15 µl 100 µM reverse primer (5'-GCTCGTAGCTCTTCTCCA)
100 µl pEFGP-Actin (β-actin) plasmid (Clontech) 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 covered with a piece of optical-quality sealing film and briefly spun to bring all reagents to the bottom of the wells, then the plate was placed in the iCycler iQ detection system (Bio-Rad Laboratories). The 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 unknown.

A second experiment was conducted to show dynamic range using SYBR Green I detection. A master mix was prepared by combining:

350 µl 10x reaction buffer (SYBR Green PCR core reagent kit)
245 µl 50 mM $MgCl_2$
14 µl Platinum *Taq* polymerase
35 µl 100 mM dNTPs
10.5 µl 100 µM forward primer
10.5 µl 100 µM reverse primer
2.77 ml dd H_2O

This master mix was thoroughly vortexed and then 441 µl was pipetted into seven different tubes. Next a 10-fold dilution series of pEFGP-Actin (β-actin) was prepared starting from a stock of 10^9 plasmids/µl, and 9 µl of each DNA stock in the serieswas added to one of the seven tubes containing master mix. Finally the DNA and master mixes were vortexed and eight50 µl aliquots from each tube were placed in separate rows of a 96-well thin-wall PCR plate. The plate was prepared for PCR and the cycling conditions and data collection were the same as those described above. In the plate setup file, four wells from each replicate group were defined as standards and the other four were defined as unknowns.

*Practice of the patented polymerase chain reaction (PCR) process requires a license. The iCycler 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.

Results

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).

The statistical evaluation of the data can be significantly increased by exploiting the data analysis options of the iCycler software. 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).

Viewing the amplification plot on a log scale can be useful for assigning the threshold. In this experiment, the default threshold is about 810 relative fluorescence units (RFU), but the data were improved when the threshold was lowered to 800. This result was a mean threshold cycle of 24.9 with a standard deviation of 0.189 cycles (0.76% CV).

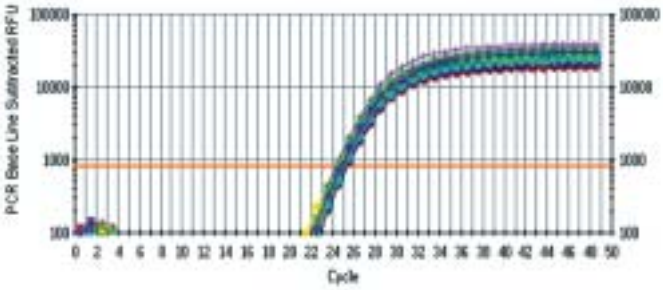

Fig. 1. iCycler screen view showing uniformity of threshold cycle in 96-well plate containing identical samples.

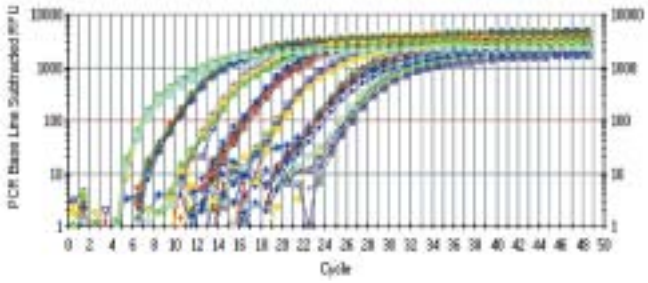

Fig. 2. iCycler screen view showing results of a target DNA dilution series. The dynamic range covers 10^3 to 10^9 molecules of the β-actin target (pEFGP-Actin).

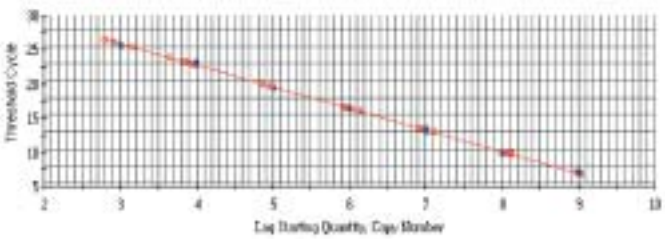

Fig. 3. Standard curve generated by the iCycler software from the data in Figure 2.

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 using all data collected over the 30 sec annealing/extension cycle (as many as 30 data points, depending on exposure time), combined with the selections made for baseline cycles and threshold, the final result for 96 wells (Figure 1) is a mean threshold cycle of 24.8 and a standard deviation of 0.185 (0.75% CV).

Dynamic Range

This experiment covered 6 logs of the starting plasmid concentration, from 10^3 to 10^9 plasmids/reaction. Because the concentrated samples came above threshold by cycle 7, cycles 2–5 were chosen for the baseline (Figure 2). All the data collected during the 30 sec annealing/extension cycle were used in the analysis and the threshold was raised from the default of 44 to 100. These manipulations result in a standard curve with a correlation coefficient of 0.999 (Figure 3).

For this experiment, the "unknowns" are identified below.

Actual Titer	Mean Calculated Titer
10^9	1.0 x 10^9
10^8	1.2 x 10^8
10^7	0.97 x 10^7
10^6	1.1 x 10^6
10^5	0.91 x 10^5
10^4	0.70 x 10^4
10^3	0.88 x 10^3

Discussion

Using the intercalation dye SYBR Green I, the Bio-Rad iCycler iQ accurately detects and quantitates PCR products over a wide range of starting concentrations, with a linear response over 6 orders of magnitude ($r^2 = 0.999$). In addition to the uniformity and dynamic range of the instrument, we have shown the versatility of its data analysis, which is not restricted to arbitrary constraints. 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, and any subset of the data can be selected.

Reference

Higuchi R et al., Kinetic PCR Analysis: Real-time monitoring of DNA amplification reactions, Biotechnology, 11, 1026–1030 (1993)

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