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Real-Time Multiplex PCR* Using

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Introduction

When researchers need to measure the amount of RNA or DNA in a preparation, they typically employ a traditional biochemical approach. High concentrations of nucleic acid can be estimated by staining agarose or acrylamide gels after electrophoresis. With less material, or for more exact determination, the concentration can be estimated by spectroscopy or fluorometry. Occasionally, the amount of nucleic acid present is so small that none of the traditional measurement methods are appropriate. In these cases, researchers have turned to the polymerase chain reaction (PCR) to make more copies of the desired nucleic acid. After amplification they measure the amount of DNA produced in the reaction and finally calculate the starting amount of nucleic acid based on post-PCR measurements. For a number of technical reasons, accurate calculation of the starting amount of nucleic acid is not possible, and even relative comparisons between two amplified samples are generally not valid because of differences in efficiency and specificity of the two amplification reactions. In gene expression studies, when the starting amount of nucleic acid is diminishingly small, or for the most demanding sensitivity, such as monitoring the number of viral particles present in a patient's blood sample, real-time quantitative PCR is the only reasonable approach. In real-time quantitative PCR, a fluorescent reporter molecule is added to the chemical reaction. This reporter may be specific for the desired amplification product, or it can be nonspecific, used only to report the accumulation of double-stranded product. In practice, quantitative PCR is carried out in an instrument that can monitor the reaction mixture and measure the fluorescence of the reporter system during each round of amplification, not just at the end point. During the early rounds of amplification, the change in fluorescence is negligible and beyond the sensitivity of the detector, but at some point, a change will register with the detection system. This point, called the threshold cycle (C_T), is proportional to the log of the starting amount of nucleic acid (Heid et al. 1996). When samples of known starting amount are amplified simultaneously under the same conditions as the unknowns, it is possible to construct a standard curve, and from this standard curve determine the starting amount of DNA in each of the unknowns.

In some instances it is desirable to simultaneously quantitate two or more genes; for example, in gene expression studies, one of the genes might be a housekeeping gene used as an internal standard. Simultaneous amplification and quantitation can also be more cost efficient and lead to higher throughput. However, optimization of experiments designed to accurately quantitate multiple target genes in one tube is not a trivial undertaking. In this article we discuss the optimization of a multiplex reaction system. We present data showing the simultaneous amplification of 4 targets from genomic DNA in a single tube and also showing the simultaneous amplification of 2 targets when one is present in great excess of the other.

Methods

Genomic DNA Multiplex

Prior to performing PCR, human genomic DNA (250 ng/µl; Promega) was partially digested with *Bam*HI at 37˚C for 2 hr, and then heated to 100˚C for 10 min before immediately cooling on ice. This DNA was then stored at 4˚C until use. To show that any gene amplified alone had an identical C_T when amplified with other genes in multiplex (on the same plate), single-gene reactions with 100 ng human genomic DNA were compared to the 100 ng multiplex reaction. Reaction conditions were identical for single and multiplex reactions as described below. To demonstrate a range of multiplex capabilities, 10-fold serial dilutions, from 500 ng to 50 pg, were assayed for α-tubulin, GAPDH, IL-1 β, and factor VIII DNA. The following 50 µl reactions were prepared to demonstrate multiplex capabilities:

25 µl 2x Life Technologies custom PLATINUM SuperMix (40 mM Tris pH 8.4, 100 mM KCl, 1.6 mM dNTPs, 6 mM $MgCl_2$, 50 U/ml PLATINUM *Taq* polymerase)
2 µl 50 mM $MgCl_2$ (Life Technologies)
0.4 µl 5 U/µl Life Technologies PLATINUM *Taq* polymerase
0.5 µl 100 mM CLONTECH Advantage ultrapure dNTPs
0.2 µl 100 µM α-tubulin forward primer (5'-CCAAGCTGGAGTTCTCTA-3', Operon)
0.2 µl 100 µM α-tubulin reverse primer (5'-CAGAGTGCTCCAGG-3', Operon)
0.15 µl 100 µM α-tubulin probe (5'-FAM-CCCAGGTTTCCACAGCTGTAGTTGAC-CTGGG-DABCYL-3', Operon)
0.15 µl 100 µM GAPDH forward primer (5'-CATGTTCCAATATGATTCCAC-3', Operon)
0.15 µl 100 µM GAPDH reverse primer (5'-CCTGGAAGATGGTGATG-3', Operon)
0.1 µl 100 µM GAPDH probe (5'-HEX-CAAGGCTGAGAACGGGAAGCTTGTCCA-GCCTTG-DABCYL-3', Operon)
0.075 µl 100 µM IL-1 β forward primer (5'-TGCTCCTTCCAGGACCT-3', Operon)
0.075 µl 100 µM IL-1 β reverse primer (5'-GTGGTGGTCGGAGATTC-3', Operon)
0.075 µl 100 µM IL-1 β probe (5'-Texas Red-CTCTGCCCTCTGGATGGCGGCAG-AG-DABCYL-3', Operon)
0.2 µl 100 µM factor VIII forward primer (5'-GACAGTGGAAATGTTACC-3', Operon)
0.2 µl 100 µM factor VIII reverse primer (5'-CATCCCAGCATGTAGATG-3', Operon)
0.15 µl 100 µM factor VIII probe (5'-Cy5-AGCTGGAATTTGGCGGGTGGAATGTCC-AGCT-BH2-3', Integrated DNA Technologies)
10.375 µl PCR-grade ddH_20
10 µl diluted human genomic DNA (Promega)

PCR conditions were 3 min at 95˚C, followed by 50 cycles of 10 sec at 95˚C, 60 sec at 55˚C.

the iCycler iQ™ Detection System

7-Order Concentration Difference

Ten-fold serial dilutions of GAPDH DNA, from 10^9 to 10^2 copies per 50 µl reaction, were prepared and amplified either alone or with 10^9 copies of α-tubulin. The following reaction conditions were used:

25 µl 2x Life Technologies custom PLATINUM SuperMix
2 µl 50 mM $MgCl_2$ (Life Technologies)
0.5 µl 5 U/µl Life Technologies PLATINUM *Taq* polymerase
0.5 µl 100 mM CLONTECHAdvantage ultrapure dNTPs
0.15 µl 100 µM α-tubulin forward primer (5'-GCAAGCTGGCTGAC-3', Operon)
0.15 µl 100 µM α-tubulin reverse primer (5'-CATAATCAACTGAGAGACG-3', Operon)
0.1 µl 100 µM α-tubulin probe (5'-HEX-CACCGGTCTTCAGGGCTTCTTGCCGGTG-BH1-3', Biosource International)
0.15 µl 100 µM GAPDH forward primer (5'-CAACTACATGGTCTACATGTTC 3', Operon)
0.15 µl 100 µM GAPDH reverse primer (5'-CTCGCTCCTGGAAGATG-3', Operon)
0.1 µl 100 µM GAPDH probe (5'-FAM-CGGCACAGTCAAGGCCGAGAATGGTGCCG-DABCYL-3', Operon)
1.0 µl plasmid DNA dilution
20.2 µl PCR-grade ddH_20

PCR conditions were 3 min at 95°C, followed by 50 cycles of 10 sec at 95°C, 45 sec at 55°C.

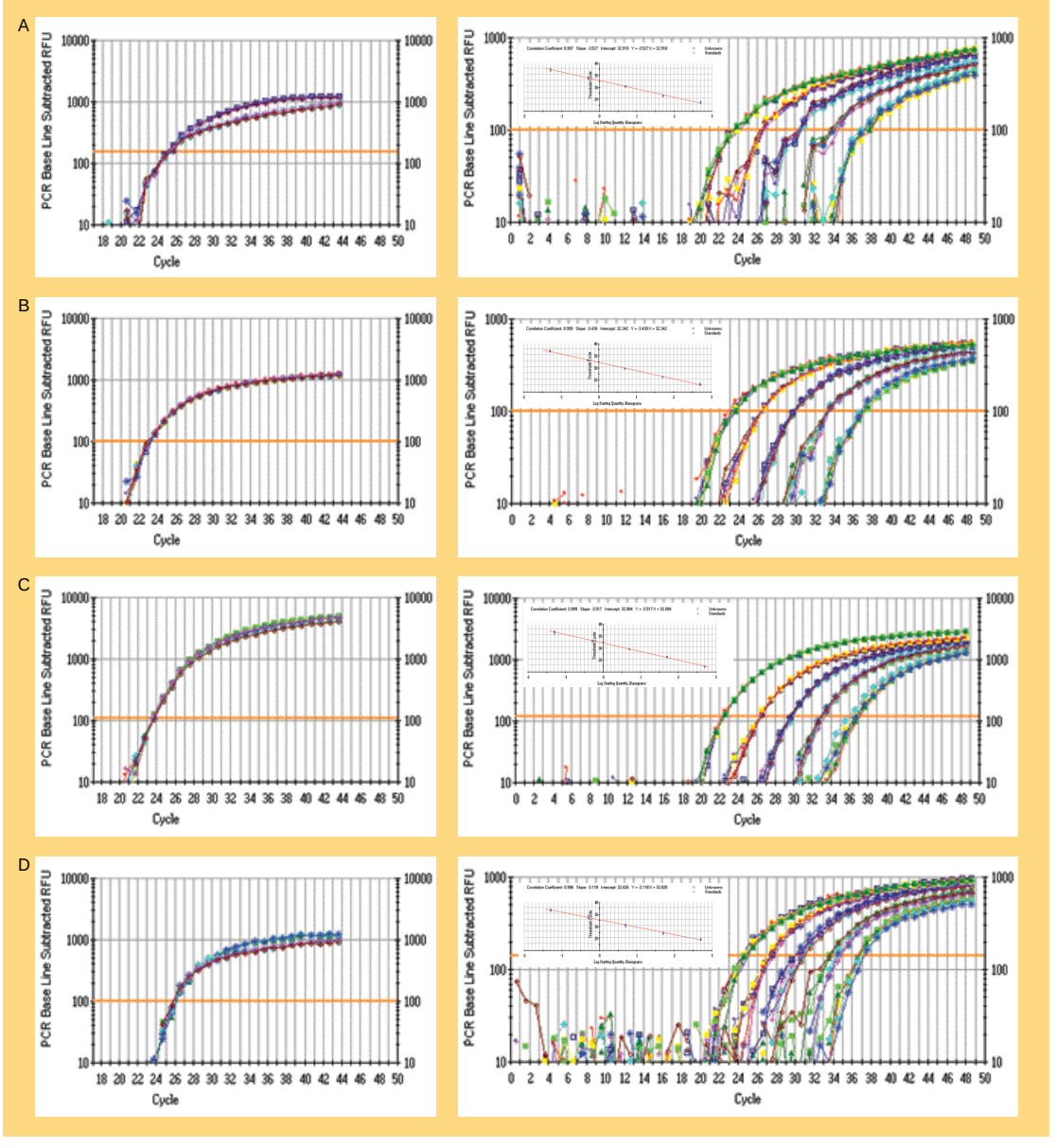


Fig. 1. Genomic DNA multiplex data. Left, Comparisons of single vs. multiplex reactions for 4 human genomic DNA targets. DNA (100 ng) was partially digested as described above. All reactions were prepared under multiplex conditions with primers and probes for either one gene target or for all 4 targets. Single and multiplex reactions had indistinguishable C_T values. Panel A, α-tubulin (C_T ± SD), single reaction = 24.13 ± 0.08, multiplex = 24.24 ± 0.08; B, GAPDH, single reaction = 23.11 ± 0.11, multiplex = 22.98 ± 0.14; C, IL-1 β, single reaction = 23.56 ± 0.07, multiplex = 23.62 ± 0.11; and D, factor VIII, single reaction = 26.15 ± 0.09, multiplex = 25.96 ± 0.10. Threshold values were not significantly different (P>0.09) by an unpaired Student's t test. Right, detection of 10-fold serial dilutions of human genomic DNA, from 500 ng to 50 pg, in real-time multiplex PCR. Panel A, detection of α-tubulin; B, GAPDH; C, IL-1 β; and D, factor VIII. Insets show standard curves.

Interpretation of Results

Gene expression studies of multiple gene targets require both precise and accurate quantitation of the same samples at the same time. The iCycler iQ system displays powerful multiplexing capabilities that we demonstrate here on genomic and plasmid targets. Furthermore, we demonstrate the need to optimize PCR efficiency when working with varying starting template concentrations.

In order to conduct successful multiplexing you must (1) maximize and equalize the efficiency of each reaction and (2) minimize any cross-reactivity between individual reactions. Maximizing the efficiency of a reaction allows accurate quantitation over a wider range of starting template concentrations and improves the reproducibility of replicate samples. It is equally important that the individual reactions have similar efficiencies because any differences in individual efficiencies will be amplified when the components of the two reactions are combined. If you know that the multiplex templates differ in concentration by less than 1,000-fold, then you can accept efficiency differences of about 10%. For example, one reaction may be 96% and the other 87% efficient. If you do not know the template concentration difference, or if it is greater than 1,000-fold, then the reaction efficiencies should not differ by more than 5% and the individual reaction efficiencies should be greater than 90%. The most powerful tool for adjusting reaction efficiency is to examine the secondary structures of the PCR product, primers, and probe. It is critical to eliminate all secondary structures that might interfere with DNA:DNA interactions, but the secondary structure must be evaluated at the appropriate temperature. Secondary structures that melt below the annealing temperature are not important. It is often necessary to raise the annealing temperature or relocate one or both primers or the probe in order to avoid secondary structure problems.

Cross-reactivity between primers and targets in the separate reactions can occur due to specific gene interaction effects, such as primer recognition, as well as to competition for substrates. It is straightforward to test for cross-reactivity of the individual reaction components by comparing a set of wells containing one reaction alone to a set containing all reaction mixtures. If there is no cross-reactivity, the C_T of template A when amplified alone will be identical to the C_T of template A in the presence of the other components. A labeled probe is not required to test for cross-reactivity.

Addition of extra polymerase, dNTPs, and $MgCl_2$ improves results for 2 or more individual reactions in multiplex. For a typical 50 µl reaction, we use 3.25–3.75 U *Taq* polymerase, 450 µM each dNTP, and 5 mM $MgCl_2$ (compared to 1.25 U polymerase, 200 µM each dNTP, and 3 mM $MgCl_2$ for a single reaction). If cross-reactivity is minimal, but efficiencies of multiplex reactions are lower than the individual reactions, minor adjustments to the primer concentrations may be necessary.

Genomic DNA Multiplex Discussion

Prior to multiplexing 2 or more PCR targets, it is important to show that amplification of each target is unaltered in a multiplex reaction containing additional PCR targets. We show that amplification of the PCR target is identical whether it is amplified alone or in multiplex. C_T values for a single gene target were not significantly different when amplifying the target alone or with 3 other genes under optimized conditions. This was true for plasmid or genomic DNA targets. Figure 1 (left-hand panels) shows the results for each of the 4 human genomic DNA targets, α-tubulin, IL-1 β, GAPDH, and factor VIII. Amplification of the gene alone or in multiplex resulted in identical C_T values and identical slopes (which indicate reaction efficiency), showing exponential amplification. This confirms that multiplex conditions do not alter any of the targets' amplification under these conditions. The reaction conditions were critical to this observation. Because the PCR efficiency is slightly different for each PCR product in this multiplex under identical reaction conditions (not shown), primer concentrations were adjusted to ensure similar amplification of each target. The right side of the figure shows 4 orders of magnitude amplification for each gene target in the multiplex reaction. The inset shows the standard curve for each reaction. The slopes and corresponding efficiencies of the reactions are –3.12 (~100%), -3.43 (96%), -3.52 (92%) and –3.53 (92%) for factor VIII, GAPDH, IL-1 β, and α-tubulin respectively.

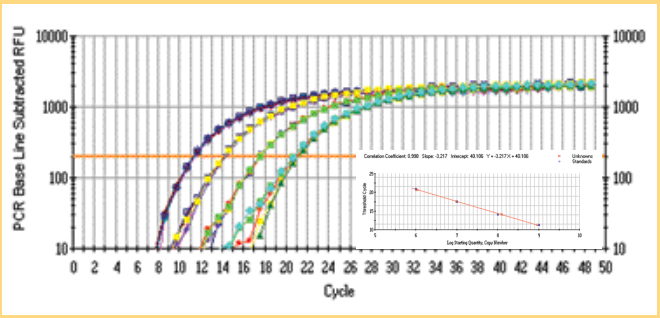
7-Order Concentration Difference

A wide difference between the starting concentrations of 2 gene products targeted for amplification is a common scenario when screening samples with a standard housekeeping gene and a rare message of interest. Here we show optimized conditions for up to a 10 million-fold difference in starting template concentrations between 2 PCR targets. Plasmids containing the human cDNAs for GAPDH and α-tubulin were multiplexed over varying concentrations. The α-tubulin plasmid was held constant at 10^9 copies per well while varying the concentration of GAPDH from 10^2 to 10^9 copies per well. Figure 2 shows the results of multiplexing. There is no significant difference between amplification of GAPDH alone or in multiplex with 10^9 copies of α-tubulin as shown by the C_T values (see table inset in Figure 2). Similar results were obtained by holding GAPDH constant at 10^9 copies and multiplexing with the same serial dilution of α-tubulin (data not shown). To avoid problems when multiplexing with extreme differences in target gene concentration (e.g., 10^9 copies α−tubulin with 10^2 copies of GAPDH), optimization of reaction components is required. We found that the effect of increasing the amount of enzyme and free nucleotides was additive (not shown). The $MgCl_2$ concentration was increased to compensate for the higher concentration of nucleotides, which chelate magnesium ions. In addition, we found that accurate quantitation of small amounts of one target in a reaction with large amounts of a second target depended heavily on the amplification efficiencies of both targets. Large differences

required high- (>90%) and equal-efficiency PCR amplifications of both genes. Amplification efficiencies of the GAPDH and α-tubulin plasmids were determined prior to multiplexing and found to be nearly equal and nearly 100% (data not shown).

Gene expression studies of multiple gene targets, as well as other applications, require both precise and accurate quantitation of the same samples at the same time. The optimization of PCR efficiency is essential in performing this accurate quantitation. We have demonstrated that the iCycler iQ system is a powerful tool to achieve this optimization and to perform multiplex real-time PCR with plasmid and genomic targets.

A



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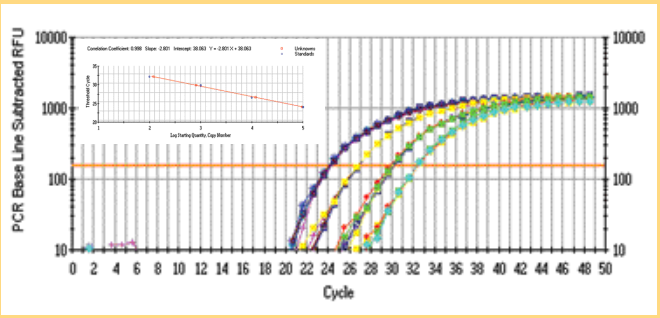

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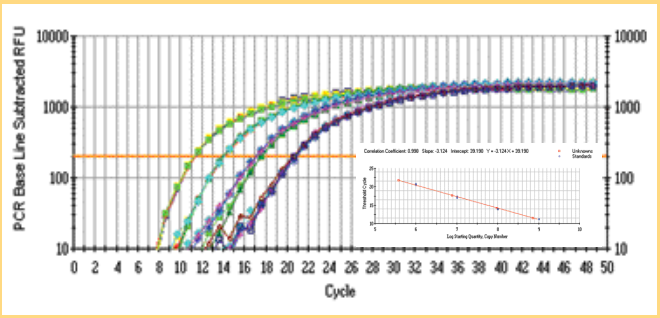

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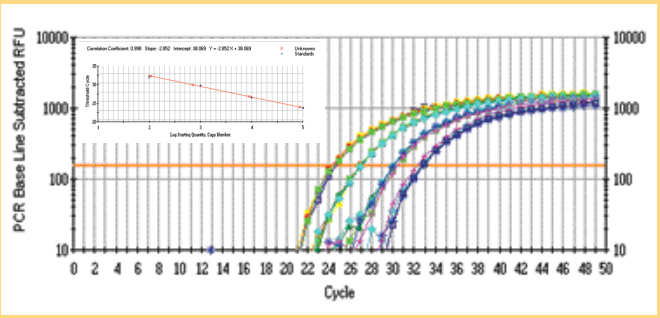

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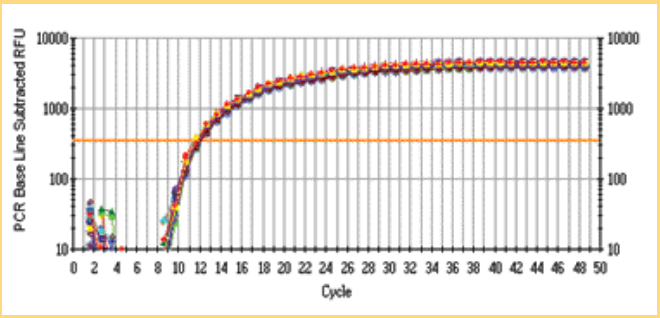

GAPDH Standard Curve in Single-Target and Multiplex Reactions

Starting Quantity	Single-Target Mean C_T	Multiplex Mean C_T
1.0×10^9	11.32 ± 0.096	11.32 ± 0.14
1.0×10^8	14.51 ± 0.063	14.41 ± 0.14
1.0×10^7	17.89 ± 0.110	17.70 ± 0.18
1.0×10^6	21.03 ± 0.061	20.99 ± 0.18
1.0×10^5	24.21 ± 0.095	24.25 ± 0.19
1.0×10^4	27.65 ± 0.240	27.54 ± 0.11
1.0×10^3	30.62 ± 0.094	30.46 ± 0.15
1.0×10^2	32.99 ± 0.140	32.91 ± 0.23

Fig. 2. Accurate quantitation over a 7-order-of-magnitude difference in DNA concentration for 2-gene multiplex real-time PCR. Panels A and B, GAPDH plasmid DNA only; C and D, GAPDH plasmid multiplexed with 10^9 copies/well of α-tubulin plasmid DNA. Detection of 10-fold serial dilutions of GAPDH plasmid, from 10^9 to 10^6 (A and C) and from 10^5 to 10^2 (B and D) copies/well. Insets for each panel show the standard curve for each dilution series. The accompanying table presents the calculated threshold cycles for each standard concentration of GAPDH, either alone or multiplexed with α-tubulin. Threshold values between single and multiplex reactions were determined not to be significantly different (P>0.08) by either an unpaired Student's t test or an unpaired t test with Welch correction.

Reference

Heid CA et al., Real time quantitative PCR, Genome Res 6, 986–994 (1996)