Convenient Multiplex PCR Assays With iQ™ Multiplex Powermix

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Multiplex Quantitative Studies of Gene Expression

Gene expression analysis is a fundamental element of research on disease and drug development. To study gene expression, the corresponding mRNA levels must be assessed either quantitatively or qualitatively. One reliable method of assessment is reverse transcription of the mRNA into copies of DNA (cDNA) using reverse transcriptase, followed by quantitation of the cDNA using quantitative PCR. Reverse transcription-quantitative PCR (RT-qPCR) has been used for validating expression results obtained from microarrays as well as for more detailed quantitation.

Absolute quantitation of gene expression results in limited information about the transcripts of a specific gene. More relevant information can be obtained from relative gene expression studies in which one looks at internal reference gene(s) within the sample to normalize the relative change in the gene of interest (Bustin 2000, Vandesompele et al. 2002). Determination of relative gene expression can be accomplished by setting up separate reactions to analyze the gene of interest and the internal reference gene (this is required when using SYBR Green I); alternatively, the target genes can be amplified concurrently in a single reaction tube (referred to as multiplexing). In addition to gene expression analysis, multiplex real-time PCR has been used to detect different bacteria in the same sample (Templeton et al. 2003, 2004, Khanna et al. 2005), to detect mutations in more than one PCR product (Ugozzoli et al. 2002), and to analyze multiple transcripts in the same sample (Persson et al. 2005). The advantage of performing multiplex qPCR assays is that one can assess up to five different PCR products in the same tube (using the iQ™5 multicolor real-time PCR detection system). Thus, it is possible to assess several internal reference genes and genes of interest in the same tube, using much less sample than when each reaction is set up individually.

To multiplex, each PCR product requires a gene-specific primer set and a fluorophore-labeled gene-specific probe along with standard PCR reagents. Until recently, qPCR multiplexing was accomplished by supplementing a real-time 2x supermix with additional dNTPs, $MgCl_2$, and *Taq* polymerase, so that limiting reagents would not influence any of the reactions. Another approach sometimes used in optimizing multiplex reactions is to determine the limiting primer concentration for the more highly expressed targets by testing a series of concentrations. This approach conserves reagents so that amplification of lower-abundance targets can occur unhindered. This practice not only is time consuming, but also uses more of the required reagents. A more practical approach is to use iQ multiplex powermix, which allows multiplexing of up to five unique targets with minimal need for optimizing reaction conditions, allowing considerable savings in cost and time.

This tech note describes several points that must be addressed for optimal multiplexing of reactions.

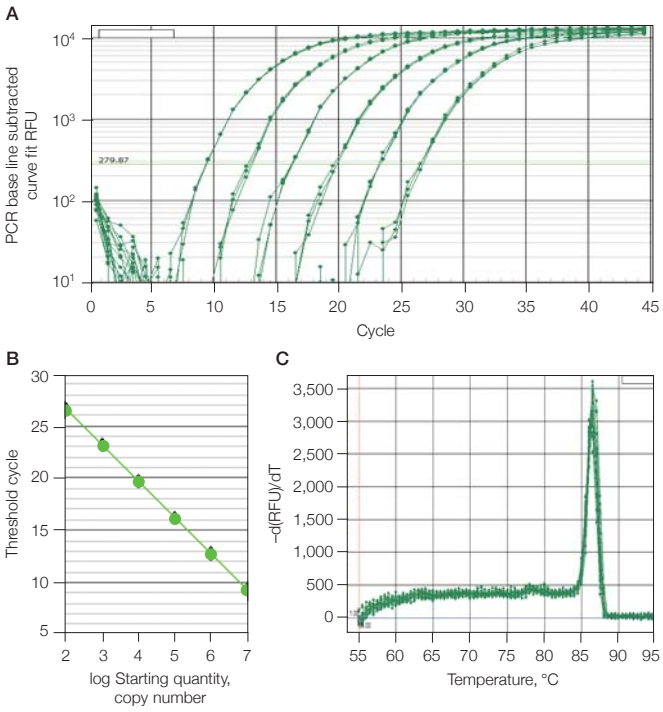

Fig. 1. SYBR Green I assay validation and melt curve for the β-actin primer set. A, amplification curve for 10-fold serial dilutions of β-actin plasmid (10^7–10^2 copies per reaction); **B**, resulting standard curve with efficiency = 94.6%, slope = –3.459, R^2 = 1.00; **C**, melt curve run immediately after amplification.

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Guidelines for Experimental Design

Primers, Probes, and Internal Reference Genes

Primers and their associated probes should be designed at the same time, but only the primers should be ordered for the initial validation experiments (see next section). It is often advisable to design several primer and probe sets per PCR product, and order and validate the primer sets first.

It is critically important in qPCR multiplex assays that there be no complementarity between any of the primers and probes that will be included in the reaction. During the experimental design stage, it is important to assess all the primer and probe sequences for complementarity. A convenient rule of thumb is to have no more than four complementary bases in a row, to avoid any possible cross-hybridization. Many tools are available to test these interactions and assist in the design of multiplex reactions, including oligonucleotide design web sites and more extensive software packages such as Beacon Designer. This software will perform all pairwise comparisons and will suggest only primers and probes that will not cross-hybridize with each other.

Another important experimental design consideration is the choice of appropriate internal reference genes for normalizing sample input across treatment conditions or samples (Bustin 2000). Several researchers have investigated the use of appropriate housekeeping genes in detail, suggesting that up to three internal reference genes be used to reduce the likelihood of incorporating large errors into the results (Vandesompele et al. 2002). These internal reference genes must be shown to be stably expressed across the proposed experimental conditions (Livak and Schmittgen 2001, Guo et al. 2002).

Validation of Primer and Probe Specificity and Efficiency

Each real-time reaction should be characterized individually using SYBR Green I before ordering the probe. By doing so, if primers need to be redesigned, one will not incur the added potential cost of probe redesign. Figure 1 shows an example of a reaction run with SYBR Green I to determine reaction efficiency, with melt-curve analysis to show specificity. The melt curve shows the absence of primer-dimers even at low template concentrations. The reaction efficiency is calculated from the slope of the standard curve (expected efficiency >90%). In the case shown, the efficiency was 94.6%.

Specificity (amplification of only one PCR product) is verified using melt-curve analysis as well as by running representative samples on a high-resolution gel. The optimal annealing temperature of the primers can be determined using the gradient feature included in iCycler iQ®, MyiQ™, iQ5, and Opticon Monitor™ software. Primer and $MgCl_2$ concentrations can also be titrated to improve reaction efficiencies. If none of these methods results in adequate reaction optimization, we recommend redesigning the primers.

When both the reaction efficiency and the specificity are acceptable, probes should be ordered and validated over the relevant template concentration range. These assays should display the same performance with the probe as with SYBR Green I (>90% efficiency for each reaction). Because the goal of a multiplex gene expression experiment is to compare expression levels of a gene of interest to a reference gene, it is important that the efficiencies of all reactions be similar to one another (generally within 5%).

Multiplex Reactions

Once all individual reactions have been tested, it is important to compare the individual reactions with the multiplex reaction. This comparison will expose any effects of one reaction on the others in the multiplex reaction. There should be no significant difference between the threshold cycle (C_T) value obtained for a reaction in the singleplex format and the value obtained in the multiplex format. If this is not the case, further investigation of primer interactions will be necessary.

Figures 2 and 3 display a validation experiment showing that the C_T of each singleplex reaction does not shift when the same assay is performed in a four-target multiplex reaction. Each of the singleplex reactions was previously validated following the strategies described above. Amplification was performed using iQ multiplex powermix and the same primer (300 nM) and probe (200 nM) concentrations for singleplex and multiplex reactions. We recommend running a two-step PCR protocol (combining the annealing and extension steps) and allowing adequate time (about 1 min) for the instrument to collect data through all four or five filter sets.

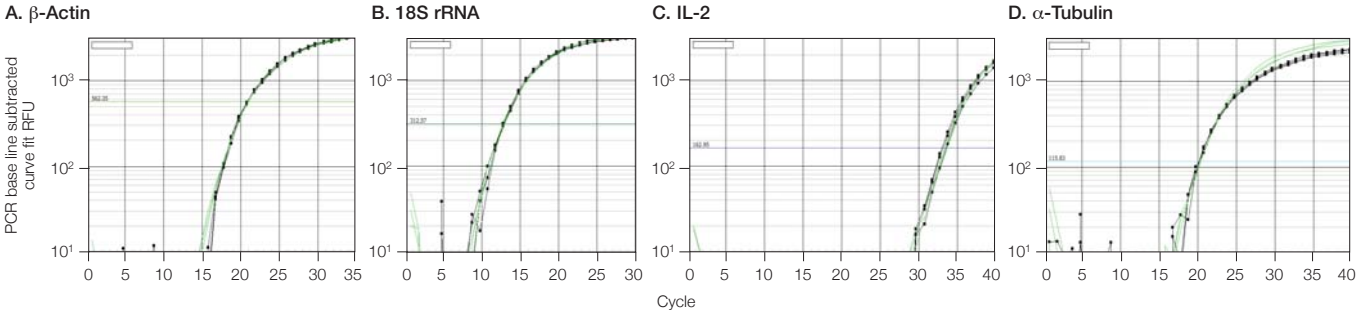

Fig. 2. Comparison of singleplex and multiplex results. Template was human spleen cDNA prepared from purchased RNA, using iScript™ reverse transcriptase. One-tenth of a 1 µg cDNA synthesis reaction was added to each 50 µl reaction. (▬), multiplex reactions; (—), singleplex reactions. **A**, β-actin primers and FAM-labeled probe; **B**, 18S rRNA primers with HEX-labeled probe; **C**, interleukin-2 (IL-2) primers and Texas Red-labeled probe; **D**, α-tubulin primers with Cy5-labeled probe.

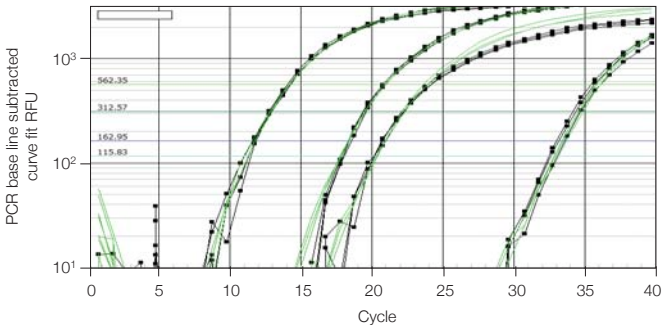

Fig. 3. Multiplex reactions showing all four fluorophore traces on the same graph. From left to right: 18S rRNA (HEX), β-actin (FAM), α-tubulin (Cy5), and IL-2 (Texas Red). Data set is the same as in Figure 2.

These data demonstrate that each of the singleplex reactions performs similarly whether run independently or in the four-target multiplex assay. Additionally, the difference in C_T values of 20.6 between the 18S rRNA (HEX) and IL-2 (Texas Red) traces (Figure 3) represents over 6 orders of magnitude difference in template concentration, yet the presence of more highly expressed targets had no effect on the amplification of IL-2. From these data, we have determined that multiplexing these reactions will not skew our data on the relative expression of one gene compared with the others. Furthermore, no additional optimization was required when using iQ multiplex powermix.

Table 1 shows the average C_T values obtained for four replicate amplifications using each primer/probe set in singleplex and corresponding multiplex reactions. The C_T values are essentially the same when including the SD; there is a slight shift of ~0.12 C_T for α-tubulin, which is acceptable.

Table 1. Comparison of C_T of each reaction in singleplex and multiplex. Values shown are averages ± SD.

Target	Singleplex	Multiplex
β-Actin	20.89 ± 0.07	20.82 ± 0.08
18S rRNA	12.85 ± 0.07	12.82 ± 0.08
IL-2	33.46 ± 0.18	33.21 ± 0.30
α-Tubulin	20.27 ± 0.04	20.09 ± 0.02

At this point, the validation of our model system has led to an optimized experimental design for gene expression studies. Modifications of the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001, Vandesompele et al. 2002) are most often used to present relative gene expression data. Figure 4 illustrates the type of relative and normalized gene expression graphs that can be displayed with iQ5 software. The data suggest that HeLa cells express about 8 times more α-tubulin than kidney, and spleen expresses about 8 times more IL-2 than kidney.

iQ multiplex powermix permits multiplexing of five PCR products in a single tube (Figure 5). Singleplex assays run alongside the five-color multiplex assay verified that there was no significant difference in C_T values between the two formats. In contrast, when another commercially available supermix designed for multiplex qPCR was used, there was a shift in C_T values between singleplex and multiplex reactions for targets expressed at low levels (Figure 6B). Therefore, the reaction would require further optimization. Analysis of this suboptimal multiplex data might cause one to conclude that α-tubulin is expressed over 100-fold lower relative to singleplex data, and normalized to β-actin and 18S rRNA expression levels.

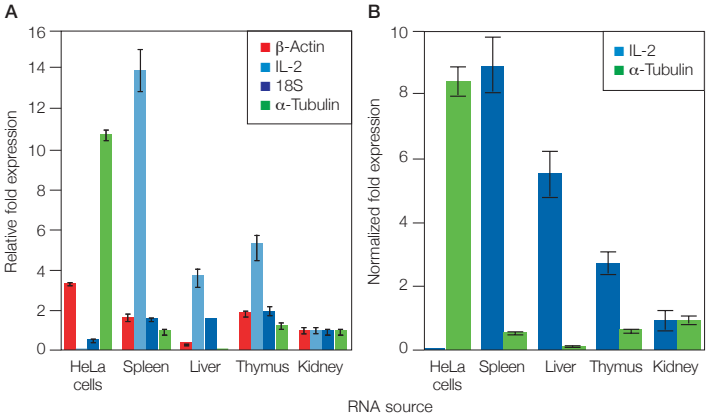

Fig. 4. Four-color multiplex reactions carried out using cDNAs prepared from different RNA sources. Reactions were carried out in triplicate with one-tenth of a 1 µg cDNA synthesis reaction using total human RNA in each 50 µl reaction. Genes analyzed were β-actin, 18S rRNA, IL-2, and α-tubulin. Kidney was set as the control sample. **A**, relative quantity (ΔC_T). **B**, normalized expression data ($\Delta\Delta C_T$) using β-actin and 18S as reference genes.

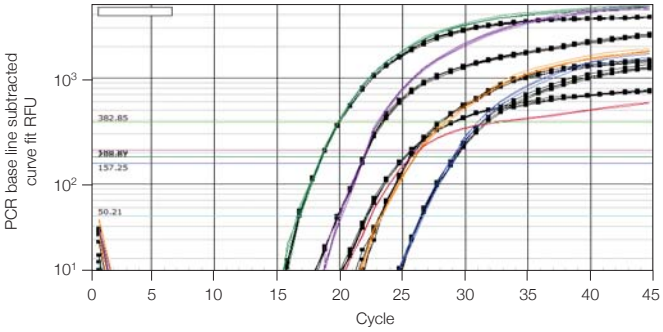

Fig. 5. Five-target multiplex reaction. One-tenth of a 1 µg cDNA synthesis reaction of human spleen total RNA was used in each 50 µl reaction. (■), multiplex; singleplex reactions: (—), FAM-labeled β-actin probe; (—), Cy5-labeled α-tubulin probe; (—), TET-labeled GAPDH probe; (—), TAMRA-labeled cyclophilin probe; (—), Texas Red-labeled IL-2 probe.

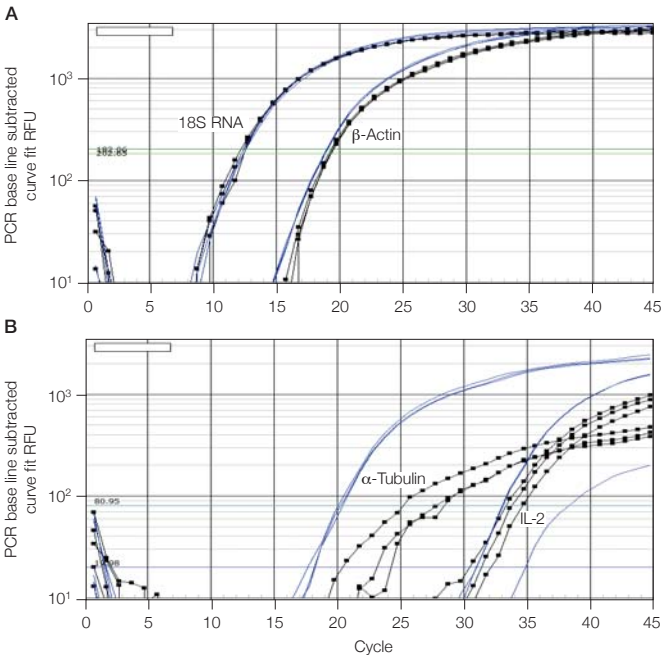

Fig. 6. Four-target multiplex reactions with another supplier's reagent are not as robust for low-expression targets. One-tenth of a 1 µg cDNA synthesis reaction was used in each 50 µl reaction. Genes analyzed were β-actin (FAM), 18S rRNA (HEX), α-tubulin (Cy5), and IL-2 (Texas Red), and the experiment was performed according to the supplier's protocol. (▪), multiplex; (—), singleplex reactions. The four traces are plotted in two panels for clarity: Higher-expressing targets are shown in **A**, lower-expressing targets in **B**.

Conclusions

Multiplexing requires some initial optimization of individual reactions. Once the assays have been validated, multiplexing is a powerful tool that can provide a large amount of data from small sample quantities. The versatility of iQ multiplex powermix reduces the need to optimize multiplex reaction conditions.

References

Bustin SA, Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays, J Mol Endocrinol 25, 169–193 (2000)

Guo D et al., The iCycler iQ detection system for evaluating reference gene expression in normal human tissue, Bio-Rad bulletin 2804 (2002)

Khanna M et al., The pneumoplex assays, a multiplex PCR-enzyme hybridization assay that allows simultaneous detection of five organisms, *Mycoplasma pneumoniae*, *Chlamydia* (*Chlamydophila*) *pneumoniae*, *Legionella pneumophila*, *Legionella micdadei*, and *Bordetella pertussis*, and its real-time counterpart, J Clin Microbiol 43, 565–571 (2005)

Livak KJ and Schmittgen TD, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method, Methods 25, 402–408 (2001)

Persson K et al., Four-color multiplex reverse transcription polymerase chain reaction — overcoming its limitations, Anal Biochem 344, 33–42 (2005)

Templeton KE et al., Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza A and influenza B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, 3, and 4, J Clin Microbiol 42, 1564–1569 (2004)

Templeton KE et al., Development and clinical evaluation of an internally controlled, single-tube multiplex real-time PCR assay for detection of *Legionella pneumophila* and other *Legionella* species, J Clin Microbiol 41, 4016–4021 (2003)

Ugozzoli LA et al., Fluorescent multicolor multiplex homogeneous assay for the simultaneous analysis of the two most common hemochromatosis mutations, Anal Biochem 307, 47–53 (2002)

Vandesompele J et al., Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol 3, research0034.1–0034.11 (2002)

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