Guidelines for **Optimization of Quantitative Multiplex RT-PCR**

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Quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Freeman et al. 1999) is a powerful tool for measuring messenger RNA (mRNA) levels in biological samples. The technique has found application in the study of gene function (Giuletti et al. 2001), molecular diagnostics (Bernard and Wittwer 2002), pharmacogenomics (Pagliarulo et al. 2002), and other fields.

The introduction of technologies that combine RT-PCR with the use of fluorescent dyes (Higuchi et al. 1993) or fluorescently labeled hybridization probes (Lee et al. 1993) was a major advance for the development of qRT-PCR. Because of the numerous advantages intrinsic to the use of qRT-PCR for mRNA quantitation (wide dynamic range, no post-PCR steps required, shorter turnaround time, and reduced cost), research and molecular diagnostics laboratories have been implementing these methods over the past seven years.

The 5'-nuclease assay (Holland et al. 1991) is one of the most widely used methodologies for mRNA quantitation in real time. The assay takes advantage of the 5'→3' endonuclease activity of *Taq* DNA polymerase to cleave fluorescently labeled oligonucleotide probes that have hybridized with PCR products during the annealing phase. In a typical 5'-nuclease assay for mRNA quantitation, a target-specific dual-labeled probe is mixed with a primer set specific for the amplification of the target sequence. The probe, which can be complementary to either DNA strand of a PCR product, is labeled at the 5' end with a reporter dye (6-FAM, HEX, Texas Red, Cy5, etc.) and at the 3' end with a quencher (Black Hole Quencher 1, Black Hole Quencher 2, etc.).

After the denaturation step, the dual-labeled probe hybridizes to its complementary sequence prior to the hybridization of both primers. As the reaction reaches the annealing temperature, the primers hybridize to their complementary sequences, thus becoming available as a substrate for a thermostable DNA polymerase (most commonly, *Taq* DNA polymerase). During polymerization, *Taq* DNA polymerase synthesizes a new DNA strand; upon encountering the hybridized dual-labeled probe, *Taq* DNA polymerase cleaves and degrades the probe, resulting in the separation of the fluorophore from the quencher. As a consequence, the reporter fluorophore is no longer quenched and is free to fluoresce. The energy emitted is directly proportional to the number of PCR products present after each amplification cycle.

Multiplex PCR is defined as the simultaneous amplification of two or more DNA targets in one reaction. The number of genes that can be detected in a multiplex 5' nuclease assay depends on the design of the instrument and the choice of fluorophores. A wide selection of fluorophores that emit light at different wavelengths is now commercially available. Bio-Rad's iCycler iQ® detection system allows the development of multiplex assays for detection in real time of up to four target sequences in a single tube.

> "To achieve optimal performance, the development of a four-color multiplex PCR requires optimization of five aspects."

Multiplex PCR is likely to produce considerable time savings. Depending on the specific assay, there are also potential cost savings. However, the main advantage of the multiplex approach is that only a small aliquot of the target sample (RNA or DNA) is used for amplification, rather than an aliquot for each single reaction. This could be extremely important in situations in which the amount of material (for example, a tissue biopsy) available for sample preparation is limited. In addition, normalizing in the same well reduces the variability generated by pipetting inaccuracy and by PCR itself (due to the presence of inhibitors and other factors).

To achieve optimal performance, the development of a four-color multiplex PCR requires optimization of five aspects: sample preparation, the reverse transcription reaction, primer and probe design, primer and probe performance, and multiplex reaction conditions. In this article, we describe our experience with optimization of a four-color multiplex RT-PCR assay. We selected as an experimental model three genes from the polyamine biosynthetic pathway (Shantz and Pegg 1999) and β-actin.

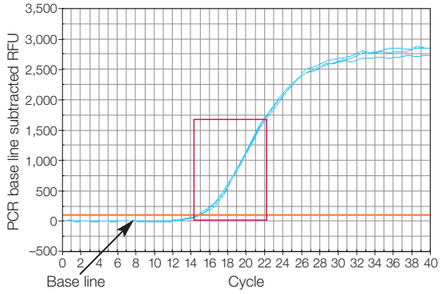

Fig. 1. Obtaining the threshold cycle (C_T) value. During the polymerization step of each PCR cycle, labeled probes are cleaved, resulting in an accumulation of fluorescence (blue line). C_T is defined as the cycle at which the fluorescence reaches a determined threshold level significantly higher than background (base line). The threshold value (indicated by the orange bar at 100 RFU) should be set within the exponential phase (red box) of the amplification plot. For each reaction, the cycle number at which the plot crosses the threshold is the C_T value. In this example the C_T values for triplicate reactions were 15.0, 14.9, and 14.8.

Terminology and iCycler iQ System Features

To better understand how a multiplex PCR is optimized, we first present the terminology used in real-time PCR and how these parameters are visualized on the iCycler iQ detection system.

Figure 1 shows a representative amplification plot and defines some of the terms used for quantitative analysis with real-time PCR. The plot shows relative fluorescent units (RFU) versus PCR cycle number. During the initial cycles, the increase in PCR product formation leads to little detectable change in fluorescent signal. The background fluorescence defines the base line for the amplification plot.

The threshold cycle (C_T), which is used to determine the abundance of a particular nucleic acid (for example, an RNA transcript) in a sample, is the number of amplification cycles required for the accumulated fluorescence to obtain a value significantly higher than the background. With the iCycler iQ, the threshold can be manually selected in the software anywhere along the exponential phase of the plotted fluorescence. Alternatively, the software can automatically select the threshold value. In an optimized real-time PCR, the C_T is inversely proportional to the initial target copy number and therefore can be used for accurate quantitation. Because endpoint analysis does not yield reliable quantitative data (Gibson et al. 1996), real-time PCR has become the method of choice to determine nucleic acid concentration. As the amplification reaction proceeds, some of the reagents (*Taq* DNA polymerase, deoxynucleotides, etc.) become limiting, and the rate of accumulation of PCR products decreases until a plateau is reached with little or no increase in the PCR product synthesis (Kainz 2000).

When the sample contains a low number of target molecules, the C_T values are more scattered due to the random chance of pipetting the same number of copies in each replicate. Note that an increase of one C_T corresponds to a decrease of the original target copy number by half, and conversely, a decrease of one C_T corresponds to an increase of the initial copy number by a factor of two.

The iCycler iQ offers choices for data visualization including a linear amplification plot and a logarithmic amplification plot. Figure 2 illustrates both plots obtained from a dilution series of a cDNA template. The cDNAs (four replicates per dilution) were amplified and detected in real time. The threshold was set at 50 in both cases. A standard curve is retrievable whenever a dilution series is amplified, and allows easy evaluation of primer and probe set performance.

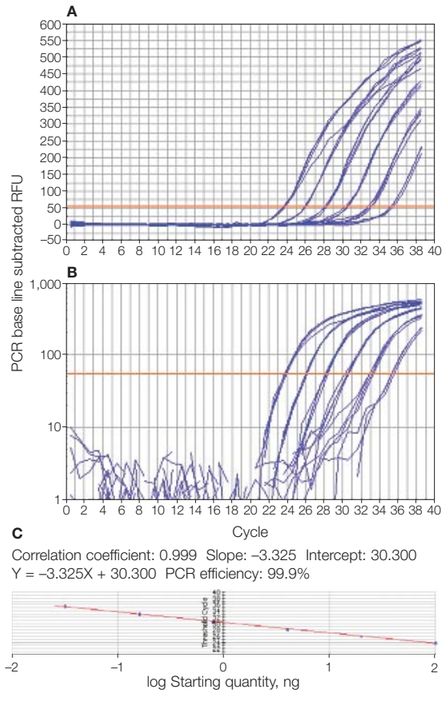

Fig. 2. iCycler iQ data presentation and analysis features. In this example, real-time amplification using a 5-fold dilution series of cDNA is shown in both linear (A) and log (B) plots. Data to evaluate the performance of a primers and probe set are derived from the standard curve (C) generated from the dilution series.

Sample Preparation

Although sample preparation is not directly involved in qRT-PCR optimization steps, the quality of the real-time RT-PCR results depends heavily on the quality of the template RNA.

To generate reliable real-time RT-PCR data, the sample material used for RNA preparation should be as homogeneous as possible. Even when a section of tissue is seemingly homogeneous, it may not have had a uniform growth environment or may be contaminated with nearby cells of other types. A heterogeneous sample may alter data due to differential expression of the target gene. In disease samples, inflammatory and normal cells surround the cells of interest (for example, tumor cells). Cell types of interest may constitute a small percentage of the size of a biopsy sample. To overcome this problem, researchers are using the laser capture microdissection (LCM) technique (Emmert-Buck et al. 1996). LCM provides a means of isolating a homogeneous population of cells from heterogeneous tissues such as clinical samples.

Most real-time RT-PCR applications use total RNA as a template for the preparation of cDNA. When using mRNA as template, accurate normalization becomes more complex because the choice of normalization method is more limited.

Several methods for preparing high-quality RNA have been developed (Boom et al. 1990, Chomczynski and Sacchi 1987). In our laboratory, we use both AquaPure™ total RNA and Aurum™ total RNA kits. To avoid sample contamination, RNA should be prepared in a location separate from both the PCR setup and post-PCR analysis areas. An important difficulty associated with RNA preparation is the presence of ribonuclease (RNase) activity. For current laboratory protocols on RNA work and procedures, see Sambrook and Russell (2001).

The quality of the real-time RT-PCR data depends not only on RNA quality but also on the accurate quantitation of the mass of nucleic acid included in the reaction. Measurement of absorbance at 260 nm, which is the most common way to determine RNA concentration, is a reliable method for RNA concentrations >100 ng/µl (Bustin 2002). Higher sensitivity and greater accuracy are obtained with RiboGreen (Molecular Probes), a nucleic acid binding dye that can be used in combination with a standard fluorometer, a fluorescence microplate reader, or a filter fluorometer; the limit of detection is as low as 1 ng/ml of RNA. Assessing the quality of the RNA samples is equally important since degraded RNA will reduce the number of target molecules analyzed. The quality of the RNA should be analyzed by agarose gel electrophoresis or with the 2100 bioanalyzer from Agilent Technologies. This instrument not only quantitates the RNA sample, but also performs a quality check.

Quantitation of RNA from a small number of cells or from a minute amount of tissue sample can present a practical problem. For these circumstances, relative quantitation against reference genes can be considered (see Normalization).

DNase treatment is highly desirable for reliable quantitation of gene expression because genomic DNA contamination may alter the observed gene copy number. Some samples, like LCM-captured material or certain tissue biopsies, may not be amenable to DNase treatment due to reduced sample availability and the possibility that additional manipulation may increase material loss. In these instances, the design of primer sets that avoid genomic DNA sequences is an alternative strategy; see Primer and Probe Design.

Reverse Transcription

The reverse transcription step is critical to attain accurate quantitation of the sample material. The amount of cDNA synthesized in the RT reaction should accurately represent the RNA input. This requires a reverse transcriptase with appropriate sensitivity, specificity, and dynamic range. Reverse transcriptases that retain activity at higher temperatures can overcome problems associated with mRNA secondary structure, while RNase H activity is necessary to efficiently amplify certain templates (Polumuri et al. 2002).

RT reactions can be performed with oligo(dT), random, or gene-specific primers. The choice of primers may influence the quantitation of the target gene in a specific assay; therefore, the primers used in the RT step should be carefully chosen. Gene-specific primers are specific and sensitive and result in less background priming (Lekanne Deprez et al. 2002, Pallisgaard et al. 1998), but also provide less flexibility of use than random or oligo(dT) primers. When oligo(dT) primers are used, it is a good practice to design the PCR primers close to the 3' end of the mRNA sequence. This avoids loss of sensitivity due to truncated messages when assaying long RNA transcripts. Naturally, oligo(dT) priming should be avoided when working with transcripts or species lacking or having short poly(A) tails (Lakey et al. 2002).

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It has been reported that cDNA primed using gene-specific primers or oligo(dT)primers is amplified with more complete coverage than randomly primed cDNA in SYBR Green-based qRT-PCR (Lekanne Deprez et al. 2002). When using the 5' nuclease assay, we found oligo(dT)-primed cDNA to be amplified satisfactorily. However, we also found that randomly primed cDNA was amplified at least as well as oligo(dT)-primed cDNA for most sequences, and for other sequences randomly primed cDNA clearly was amplified better than oligo(dT)-primed cDNA. Furthermore, in a limited experiment we found gene-specific primer performance equal to that of random primers. Bio-Rad's iScript™ cDNA synthesis kit is a modified RNase H^+ MMLV reverse transcriptase optimized for real-time applications. It uses a proprietary mix of random oligomers and oligo(dT) primers that demonstrated excellent performance in all our RT-PCR applications.

RT-PCR reactions can be divided into two approaches. The first consists of the use of a single tube in which both the RT step and the PCR are performed. In the second approach, the RT step and the PCR amplifications are performed in different tubes. The same-tube, directly linked reaction approach is referred to as one-step RT-PCR. This can be achieved either by utilizing a *Thermus thermophilus* (*Tth*) polymerase with inherent reverse transcription activity, or by combining a reverse transcriptase with a thermostable DNA polymerase. *Tth* DNA polymerases allow use of higher temperatures during the reverse transcription step. This can be beneficial for GC-rich target mRNA, which easily forms secondary structures. However,

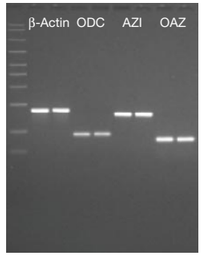


Fig. 3. Verification of specific amplification by gel electrophoresis. Primer pairs were used in four RT-PCR assays with human prostate cDNA as a template. Two aliquots of each PCR were loaded on a 3% ReadyAgarose™ gel. Each primer pair amplified only the single predicted fragment.

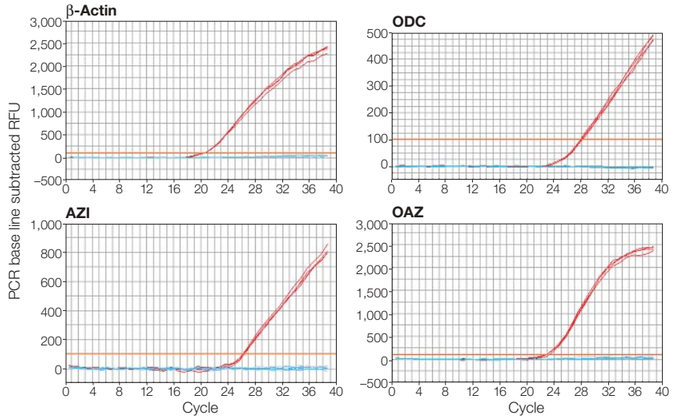
Tth-based RT-PCR is less sensitive than one-step RT-PCR using two enzymes (Cusi et al. 1994). While the one-step approach is very convenient, it is less sensitive than the two-step procedure with separate RT and PCR steps (Leutenegger et al. 1999). We corroborated these findings in our laboratory in comparisons of one- and two-step RT-PCR reactions. Furthermore, in multiplex applications one-step RT-PCR may cause mispriming because of the low temperature used in the RT reaction. Another advantage of the two-step RT-PCR approach is that it minimizes RNA work since the cDNA generated in a single reaction can be used for several PCR amplifications.

Normalization

Although accurate normalization is one of the unresolved cornerstones of real-time qRT-PCR, a few different approaches have been proposed. Normalization against a single housekeeping gene and rRNA is no longer considered acceptable other than perhaps for in vitro systems (Bustin 2002, Tricarico et al. 2002, Vandesompele et al. 2002). Vandesompele et al. recommend the use of multiple internal control genes, with a minimum of three per assay. Even though this approach works well, the drawback is the amount of labor and time involved in finding appropriate control genes for individual experiments. In addition, this approach is not feasible when sample material is a limiting factor.

Other means of normalization involve relating the sample to the number of cells, the mass of tissue, or the total RNA concentration (Bustin 2000, 2002). Normalization against total RNA performs acceptably; however, it requires accurate determination of the RNA concentration and quality (Tricarico et al. 2002). This can be achieved by the means described in the Sample Preparation section. Normalizing against total RNA concentration may currently be the most feasible and accurate method (Tricarico et al. 2002). However, the method has some limitations.

Fig. 4. Specificity of primer pairs for cDNA in singleplex assays. Reactions were negative controls (blue) or contained cDNA (red) or genomic DNA (gray, obscured by blue traces). Only reactions that contained template cDNA generated fluorescence.



Normalizing to total RNA is not possible when mRNA has been used as a template. In addition, the method does not take into account any differences in transcriptional activity of tissues or differences in cellular content of mRNA, and it may be difficult to apply when sample material is a limiting factor.

Primer and Probe Design

For most real-time RT-PCR applications, the primer pairs are designed to generate PCR fragments that are between 60 and 200 bp long. Short PCR products are preferred; however, the actual length of the amplified DNA fragments will depend on the gene of interest and the nature of the assay. To accurately quantitate an RNA target with real-time qRT-PCR, a primer set should exponentially amplify the template. For optimal performance, primers should be target-specific, hairpin and primer-dimer free, and compatible in multiplex PCR reactions. Moreover, a high priming efficiency is expected along with a lack of self-complementarity. The primer and probe sequences should not cross-hybridize to other sequences within the genome. Several software programs, both commercial and noncommercial, can be utilized for primer and probe design. We usually design PCR primers with a length between 15 and 22 nucleotides and an ideal melting temperature (T_m) between 55 and 60°C. For multiplex purposes, the optimal annealing temperature of individual primer sets should not differ by more than 2°C. Primers are usually used at a concentration of 50–300 nM.

We previously mentioned that when sample is limiting, researchers are reluctant to treat RNA preparations with DNase in order to minimize sample handling and avoid sample loss. In these situations, primers may be designed to avoid genomic DNA amplification. When the DNA sequence at the intron/exon boundaries is known, the design of a primer pair that will not amplify genomic DNA is ideal. This can be achieved by designing one primer to span the exon-exon junction of the mRNA. This primer will hybridize to cDNA synthesized from spliced mRNA, but not to the genomic intron-containing sequence.

The above procedure does not avoid the amplification of pseudogenes, which in most cases are colinear with the cDNA. When pseudogenes are a potential problem and their sequences are known, the primers can be strategically designed to avoid their amplification. For this purpose, primers can be designed by applying strategies used for allelic discrimination, such as allele-specific PCR (Ugozzoli and Wallace 1991) and allele-specific oligonucleotide (ASO) hybridization (Ugozzoli et al. 2002). Primers designed to avoid genomic amplification must fulfill the primer design criteria mentioned above. In some cases, strategies for avoiding amplification of genomic DNA and pseudogenes can be combined. We successfully combined them for the design of the primer sets in our four-color multiplex RT-PCR.

Primer performance can be evaluated in different ways. To determine the optimal annealing temperature for a primer set, we perform a temperature gradient experiment using the iCycler iQ gradient tool. Up to eight annealing, polymerization, and denaturation temperatures can be tested with the gradient tool in a single experiment. iQ™ SYBR® Green supermix along with the melt curve feature of the iCycler iQ are useful tools to perform gradient experiments since they allow us to verify the reaction conditions that generate only specific PCR products (shown by single peaks in the melt curve) and not primer-dimers. Primer-dimers, when present, can be detected in melt curves because they usually melt at lower temperatures than PCR products. However, the synthesis of the expected DNA fragment must be corroborated by agarose gel electrophoresis. The iQ and iTaq™ SYBR Green supermixes allow cost-effective evaluation in real time of primer pairs before the design of the dual-labeled probes required for 5'-nuclease assays, including multiplex assays.

The T_m for the probes used in 5'-nuclease assays should be slightly higher (7–10°C) than that of the primers, and they should be no longer than 30 nucleotides. This difference in T_m ensures that the probe will hybridize to its target sequence prior to primer hybridization, thus allowing strand displacement and subsequent hydrolysis by *Taq* DNA polymerase. Probes should hybridize to their target sequences close to the 3' end of a primer, ideally to the reverse primer. Probes must not have a G at their 5' end since this could result in quenching of the signal even after hydrolysis. Ideally, the probe should be complementary to the DNA strand that contains more Gs than Cs. In addition, the target sequence for the probe should be selected such that the GC content is 30–80%.

We developed a four-color multiplex RT-PCR assay for the detection of three human genes involved in the polyamine biosynthetic pathway (ornithine decarboxylase [ODC], ODC antizyme [OAZ], and antizyme inhibitor [AZI]) along with human β-actin. All the primers were designed to avoid priming on genomic DNA sequences. Analysis of PCR products by agarose gel electrophoresis showed the presence of a single band for each primer pair (Figure 3). Further, when primer-probe pairs were used in singleplex real-time analysis, only the cDNA template produced target-specific PCR products (Figure 4).

Verification of Primer and Probe Performance

When developing a four-color multiplex assay, it is crucial that all the primer pairs amplify their target sequences with similar efficiencies (within a few percent of each other). Once the primers and probes have been designed, the next step is to verify their performance. To achieve this goal, a serial dilution of the templates is prepared and these dilutions are PCR amplified (Figure 5). After amplification, iCycler iQ

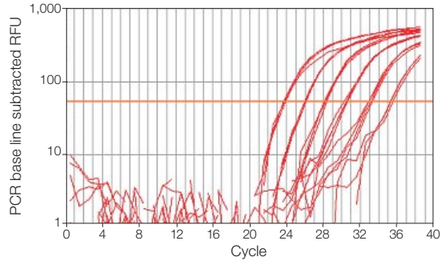


Fig. 5. PCR performance verification of AZI primer and probe set. Human prostate cDNA was used as a template in a dilution series spanning 3 orders of magnitude. Standard curve had slope –3.325, r = 0.999, and PCR efficiency of 99.9%. The primer and probe sets specific for the ODC, OAZ, and β-actin sequences produced nearly identical results (data not shown).

software calculates the PCR efficiencies for each primer-probe set. We routinely use cDNA prepared from total RNA to prepare dilutions covering at least 3 orders of magnitude of template concentration. The real-time data are then plotted as the log starting template concentration versus C_T, and a standard curve is generated. The slope of the curve shows the efficiency of amplification for the PCR primer-probe sets. For exponential amplification at 100% (ideal) efficiency, the corresponding theoretical slope is –3.322. Acceptable PCR efficiencies range from around 92% to 102%. Another parameter calculated from the standard curve is the correlation coefficient, r. This number indicates how accurately the data points fit the values assigned to the standards. Ideally, r should be close to 1.0; however, we consider values down to 0.990 valid.

Optimization of a Four-Color Multiplex Assay for Gene Expression

Once the primer-probe sets have been validated, the next step toward development of a four-color real-time PCR assay for gene expression is multiplex reaction optimization.

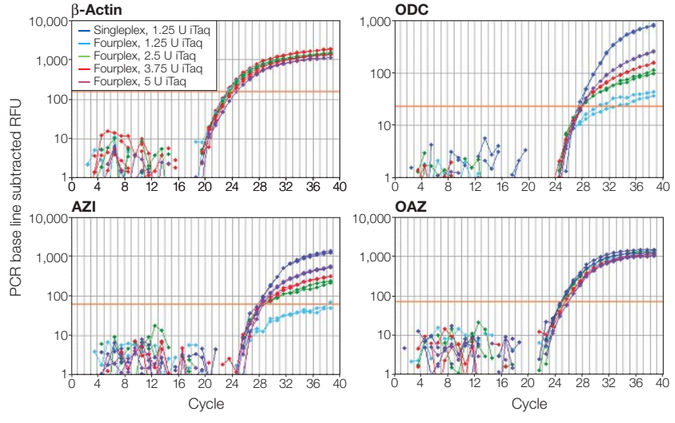


Fig. 6. Optimization of iTaq DNA polymerase concentration in fourplex reactions. $MgCl_2$ and dNTP concentrations were kept constant while the singleplex iTaq concentration (1x) was compared to multiplex conditions using increasing amounts of iTaq DNA polymerase (1–4x). The β-actin and OAZ genes (those with the highest expression in our example) were amplified with similar efficiency in singleplex and multiplex conditions at all iTaq concentrations tested. The less expressed AZI and ODC genes required additional iTaq polymerase to attain multiplex amplification comparable to the singleplex amplification. Subsequent optimization experiments continued using 3x iTaq per reaction (see Figure 7).

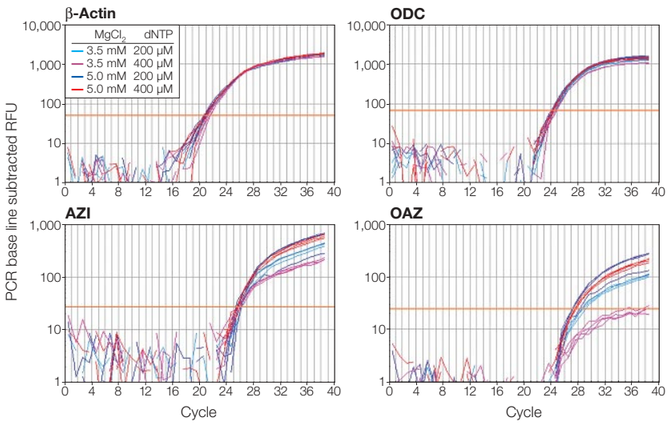
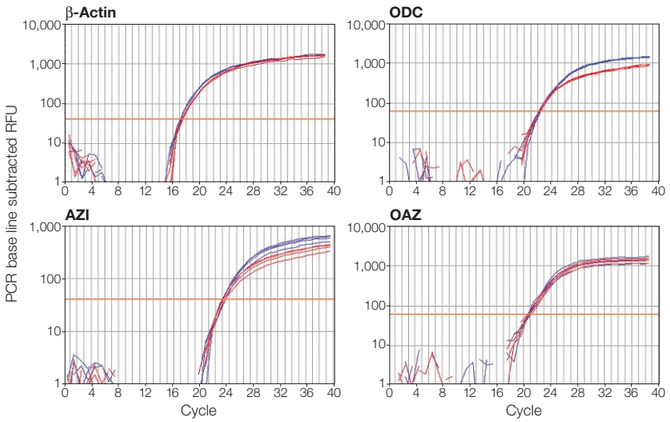


Fig. 7. Optimization of $MgCl_2$ and dNTP concentrations in fourplex reactions. Keeping the iTaq DNA polymerase concentration constant at 3.75 U per 50 µl reaction (3x iTaq), the effect of $MgCl_2$ and dNTP concentration was assessed. The combination of 5.0 mM $MgCl_2$, 200 µM dNTPs, and 3.75 U iTaq DNA polymerase gave the best amplification in multiplex reactions (no singleplex reactions are shown here).



	C_T Values			
	β-Actin	ODC	AZI	OAZ
Singleplex	17.0 ± 0.0	23.0 ± 0.2	22.2 ± 0.1	20.8 ± 0.1
Multiplex	17.3 ± 0.0	23.1 ± 0.2	22.2 ± 0.1	20.8 ± 0.2

Fig. 8. Verification of multiplex performance. Data from singleplex (blue) and fourplex (red) reactions (four replicates each using human prostate cDNA as template), were superimposed. The plots overlapped completely during the exponential phase. The table shows the average C_T values for the singleplex and multiplex reactions.

For optimal performance of a multiplex PCR assay, each participating primer pair must amplify at its peak performance after being mixed with the other primer sets. An increasing number of multiplexed primer pairs along with a large difference in target copy number for each template in the reaction make optimization challenging. When the reaction conditions are not fully optimized, the least expressed gene(s) cannot be amplified exponentially. Consequently, amplification results would be equivocal and therefore would not reflect the initial number of target molecules in the reaction. Reagents can be limiting (*Taq* DNA polymerase, dNTPs, and $MgCl_2$), resulting in a plateau effect (Kainz 2000). The addition of extra DNA polymerase to the amplification reaction requires an increase in the concentration of Mg^{2+}. The concentration of free Mg^{2+} in solution is related to the dNTP concentration; therefore, both components should be optimized at the same time. A potential problem could arise when the primers for different genes are not compatible in multiplex. In this case, the problem primer set(s) have to be identified and replaced.

We optimized our multiplex RT-PCR conditions by adjusting the iTaq DNA polymerase (Bio-Rad Laboratories), Mg^{2+}, and dNTP concentrations. The concentration of iTaq DNA polymerase for singleplex real-time RT-PCR was the same as that used for conventional RT-PCR, or 0.025 U/µl (1.25 U per 50 µl reaction). With Mg^{2+} and dNTPs at singleplex conditions (3 mM Mg^{2+} and 200 µM dNTPs), a 3-fold increase in iTaq DNA polymerase seemed to maintain optimal amplification for all the primer pairs in our multiplex PCR (Figure 6). The 5'-nuclease assay usually requires about 200 µM of dNTPs and 3 mM Mg^{2+} for proper probe hybridization. Once the iTaq polymerase concentration was set at 3.75 U/µl, our four-gene multiplex assay seemed to benefit from 5 mM Mg^{2+}, with no increase necessary in dNTPs (Figure 7).

To achieve optimal multiplex performance, each individual primer-probe set needs to amplify the target gene exponentially, as it does in a single reaction. For verification, we performed a side-by-side comparison of a single- and a multiplex reaction for each primer-probe set. The real-time graphs generated using singleplex and multiplex conditions should show the same C_T value and be superimposable during the exponential phase of the PCR amplification.

When we used both human prostate and small intestine total RNA samples, successful multiplex conditions were achieved by increasing the iTaq DNA polymerase concentration 3-fold and the concentration of Mg^{2+} to 5 mM (Figure 8). We did not find it necessary to titrate primer and probe concentrations in our four-gene multiplex reaction to generate consistent results. However, when the genes being multiplexed have large differences in expression, it may be necessary to lower the primer and probe concentrations for the highest expressed gene. Although it may not always be the case, our four-color multiplex assay consumed fewer reagents than used for amplification of the four genes in separate reactions.

Conclusions

Achieving a successful multiplex real time RT-PCR assay requires multiple optimization steps. Once optimized, the multiplex approach saves sample, time, and in some cases, costs. The outlined procedures should help your laboratory through the optimization steps to acquire a functional multiplex assay.

For more information on tools for real-time PCR and literature highlighting applications, visit our web site at **www.bio-rad.com/amplification/**

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Introducing Two Sensitive and Reliable Kits for One-Step Quantitative RT-PCR

Real-time RT-PCR has revolutionized the study of gene expression. It is now theoretically possible to detect the RNA transcripts of any gene, regardless of the scarcity of the starting material or relative abundance of the specific mRNA. When reverse transcription is coupled with real-time PCR, the combination results in a powerful methodology to study gene expression. Benefits of real-time RT-PCR include accurate and specific quantitation, high sample throughput with less input sample, and gel-free data analysis.

It is the number of discrete enzymatic steps that distinguishes the two methods used to perform RT-PCR, namely one-step or two-step RT-PCR. Bio-Rad now offers products for both methods. Bio-Rad's newest offerings, the one-step quantitative RT-PCR kits, were developed to offer convenient choices for a variety of applications.

Advantages of iScript™ One-Step RT-PCR Kits

- **The broadest linear dynamic range for quantitative RT-PCR** — Reliable results over the widest range of input, down to 100 fg of total HeLa RNA
- **Proprietary antifoaming agent** — Enables smoother pipetting and more sensitive detection at low input concentrations
- **Compatibility with all real-time detection chemistries** — The iScript one-step RT-PCR kit with SYBR® Green is optimized for single-color detection, while the iScript one-step RT-PCR kit for probes is optimized for probe hybridization and hydrolysis in singleplex or multiplex RT-PCR

Features of iScript One-Step RT-PCR Kits

Bio-Rad's one-step quantitative RT-PCR kits allow highly sensitive detection on a wide range of thermal cyclers. cDNA synthesis and PCR amplification are conveniently carried out in the same tube, using the powerful combination of iScript RNase H^+ reverse transcriptase and iTaq™ hot-start DNA polymerase.

The choice of reverse transcriptase can play a major role in the efficiency of the first-strand cDNA synthesis reaction. Several attributes of the iScript reverse transcriptase allow it to effectively transcribe high yields of cDNA for PCR. One of the key attributes of the iScript reverse transcriptase is that it is RNase H^+. RNase H^+ reverse transcriptases degrade RNA molecules present in RNA-DNA hybrids, ensuring a clean pool of cDNA for PCR. These reverse transcriptases increase the sensitivity of quantitative RT-PCR detection and analysis. Thus, the sensitivity of both iScript one-step RT-PCR kits exceeds that of other kits on the market, generating linear results down to 100 fg of input, as shown in Figures 1A and 2.

The iScript one-step RT-PCR kit with SYBR Green is one of the few kits that effectively minimizes the incidence of primer-dimers, a common pitfall of the one-step RT-PCR procedure. Primer-dimers can compete with the RT-PCR reaction being studied, and can lead to inaccurate results. This kit contains a balanced combination of cations to prevent primer-dimer formation in a SYBR Green background, as shown in Figure 1B.

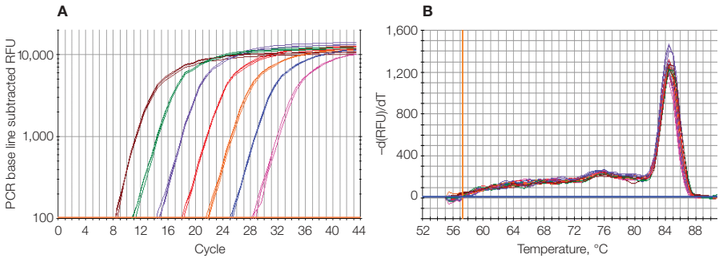

Fig. 1. The iScript one-step RT-PCR kit with SYBR Green provides high reproducibility and sensitivity across a broad range of concentrations. Panel A, amplification plot. Panel B, melt-curve analysis showing absence of nonspecific PCR artifacts. Reactions were performed in triplicate, along with no-template controls, using GAPDH primers and 100 ng to 100 fg of total HeLa RNA. Reactions were carried out on the iCycler iQ® real-time detection system. Standard curve had r = 1.000, slope = –3.466, efficiency = 95%. The melt-curve protocol was performed after RT-PCR, revealing a single clean melting peak for all samples tested.

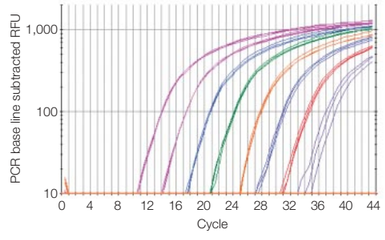

Fig. 2. The iScript one-step RT-PCR kit for probes delivers unparalleled results over an extremely wide dynamic range. RNA (1 µg to 100 fg) was isolated from HeLa cells using the Aurum™ total RNA kit. Primers and a FAM-labeled β-actin probe were used for RT-PCR. Each reaction was performed in triplicate on the iCycler iQ system. Standard curve had r = 1.000, slope = –3.39, efficiency = 97.2%.

The iScript one-step RT-PCR kit for probes has been tested in a multiplex setting using two targets, with results shown in Figure 3. This is the only kit that has demonstrated duplex reaction results in a one-step quantitative RT-PCR format. The ability to duplex successfully is largely dependent on input template concentrations; both templates should be present in roughly equal amounts.

The iScript one-step RT-PCR kits are linear over the broadest dynamic range and are capable of detecting even trace amounts of input RNA. In just one easy step, these kits provide a convenient and sensitive means of performing quantitative RT-PCR.

For more information, visit us on the Web at **www.bio-rad.com/iscript/**

iScript One-Step RT-PCR Kit for Probes

iScript One-Step RT-PCR Kit With SYBR Green

Ordering Information

Catalog #	Description
iScript One-Step Quantitative RT-PCR Kits	
170-8892	iScript One-Step RT-PCR Kit With SYBR Green, 50 reactions
170-8893	iScript One-Step RT-PCR Kit With SYBR Green, 200 reactions
170-8894	iScript One-Step RT-PCR Kit for Probes, 50 reactions
170-8895	iScript One-Step RT-PCR Kit for Probes, 200 reactions
Related Products	
170-8890	iScript cDNA Synthesis Kit, 25 x 20 µl reactions
170-8891	iScript cDNA Synthesis Kit, 100 x 20 µl reactions
170-8860	iQ™ Supermix, 100 x 50 µl reactions
170-8862	iQ Supermix, 500 x 50 µl reactions
170-8864	iQ Supermix, 1,000 x 50 µl reactions
170-8880	iQ SYBR Green Supermix, 100 x 50 µl reactions
170-8882	iQ SYBR Green Supermix, 500 x 50 µl reactions
170-8884	iQ SYBR Green Supermix, 1,000 x 50 µl reactions
170-8885	iQ SYBR Green Supermix, 2,000 x 50 µl reactions, 50 ml bottle
170-8850	iTaq SYBR Green Supermix With ROX, 200 x 50 µl reactions
170-8851	iTaq SYBR Green Supermix With ROX, 500 x 50 µl reactions
170-8852	iTaq SYBR Green Supermix With ROX, 1,000 x 50 µl reactions
170-8853	iTaq SYBR Green Supermix With ROX, 2,000 x 50 µl reactions, 50 ml bottle
170-8870	iTaq DNA Polymerase, 5 U/µl, includes 250 U polymerase, 1.25 ml 10x PCR buffer, 1.25 ml 50 mM $MgCl_2$ solution
170-8875	iTaq DNA Polymerase, 5 U/µl, includes 5,000 U polymerase, 25 ml 10x PCR buffer, 25 ml 50 mM $MgCl_2$ solution
170-8874	dNTP Mix, 200 µl, 10 mM each dNTP

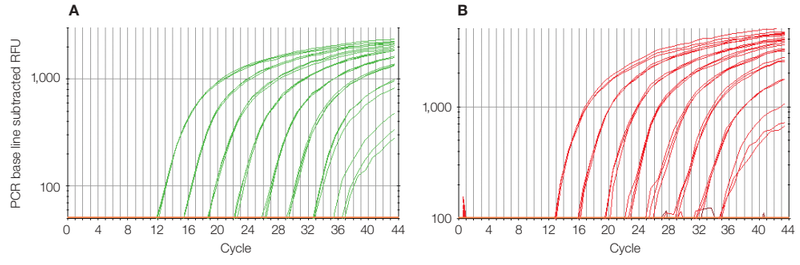

Fig. 3. The iScript one-step RT-PCR kit for probes yields compelling results in a duplex real-time reaction. Tubulin and β-actin gene targets from HeLa total RNA were reverse transcribed and amplified in duplex over a 10-fold serial dilution. Each reaction was performed in triplicate, and RT-PCR was carried out on the iCycler iQ system. A, a dual-labeled FAM probe was used to detect the β-actin gene in the first dye layer. Standard curve had r = 1.000, slope = –3.53, efficiency = 92%. B, in the second dye layer, a dual-labeled Cy5 probe was used to detect the tubulin gene. Standard curve had r = 1.000, slope = –3.27, efficiency = 102%.