Four-color multiplex reverse transcription polymerase chain reaction—Overcoming its limitations

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Abstract

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) conducted in real time is a powerful tool for measuring messenger RNA (mRNA) levels in biological samples. Multiplex PCR is defined as the simultaneous amplification of two or more DNA (cDNA) targets in a single reaction vessel and may be carried out only using uniquely labeled probes for each target. Up to four genes can be detected in a multiplex 5′ nuclease assay when using the appropriate instrument and the right combination of fluorophores. One of the more important advantages of multiplexing is a reduced sample requirement, which is especially important when sample material is scarce. Additional benefits are saving time on reaction setup and lower cost compared to singleplex reactions. Although multiplexing has several advantages over singleplex qRT-PCR, limited work has been done to show its feasibility. Few publications on four-color multiplex qRT-PCR have been reported, and to our knowledge no work has been done to explore the assay’s limitations. In this paper, we report the first in-depth analysis of a four-gene multiplex qRT-PCR. To achieve a better understanding of the potential limitations of the qRT-PCR assay, we used in vitro transcribed RNA derived from four human genes. To emulate gene expression experiments, we developed a model system in which the in vitro transcripts were spiked with plant total RNA. This model allowed us to develop an artificial system closely resembling differential gene expression levels varying up to a million fold. We identified a single “universal” reaction condition that enabled optimal amplification in real time of up to four genes over a wide range of template concentrations. This study shows that multiplexing is a feasible approach applicable to most qRT-PCR assays performed with total RNA, independent of the expression levels of the genes under scrutiny.

Keywords: Real-time PCR; RT-PCR; PCR; Gene expression

Multiplex PCR is defined as the simultaneous amplification of two or more DNA or cDNA targets in one reaction well. Multiplexing in real time with the 5′ nuclease assay [1] of up to four genes is possible because fluorophores with different emission maxima and instruments that are able to detect and quantify the signal emitted by each different reporter dye have become commercially available. Once the assay conditions have been optimized, multiplex PCR is a strategy that produces considerable time savings. There is also a potential for cost savings that depends on the number of genes to be included in the multiplex PCR. More importantly, multiplexing saves sample material in situations in which sample availability is restricted. In a fourplex PCR, only one fourth of the target sample (cDNA or DNA) is used for amplification rather than an aliquot of the sample for each single reaction. For example, when analyzing tissue biopsies or samples prepared with a laser capture microdissection instrument, sample availability could be very limited. When sample size is small, analysis of multiple gene expression levels using single reactions with replicates may not be feasible unless the target RNA is previously amplified with an RNA amplification method.

Although the benefits and applications of four-color real-time PCR assays are numerous, only a few
scientific studies applying this technology have been published [2–4]. Further, no thorough studies on multiplex optimization and possible limitations of the technology have been reported. Accurate qRT\(^1\) PCR is dependent on the amplification efficiency of the templates tested in the reaction. Accurate quantification of differential gene expression levels requires the optimization of the reaction components such as Taq DNA polymerase, divalent ions (e.g., Mg\(^{2+}\)), dNTPs, and PCR primers and probes. In a non-optimized assay, a large difference in the expression levels between two genes can cause a reduced amplification efficiency of the gene(s) expressed at lower levels that eventually could lead to inaccurate results. We had previously reported a four-gene qRT-PCR multiplex assay (Kent Persson’s personal communication). The assay included \(\beta\)-actin and three genes from the polyamine biosynthesis pathway [antizyme inhibitor (AZI), ODC antizyme (AOZ), and ornithine decarboxylase (ODC)] [5]. In a later developmental stage we replaced \(\beta\)-actin with \(S\)-adenosylmethionine decarboxylase (AdoMetDC), another member in the polyamine biosynthesis pathway. While satisfactory multiplex results were achieved with both four-color reactions, the starting differential gene expression levels were never greater than beyond 32-fold. In an attempt to deepen our understanding of the multiplex assay and its limitations, we designed a model system to perform an in-depth analysis. Different amounts of in vitro transcripts generated from PCR templates for our genes of interest (AZI, OAZ, ODC, and AdoMetDC) were mixed and spiked with plant total RNA. The transcripts, with lengths longer than 700 bp, included a 5\(^\prime\) UTR and a poly(A) tail. We used the in vitro transcripts to generate 15 template sets spanning from identical target concentration up to a million fold difference in concentration. This outlined approach allowed us to develop an extremely flexible multiplex analysis.

While analyzing the data produced with the 15 sets, we discovered that our original multiplex qRT-PCR condition failed when amplifying targets whose starting concentrations differed by more than 32-fold, corresponding to a difference of five or more cycles in threshold cycle (Ct). Additional experiments to optimize our assay allowed us to identify a new reaction condition that was able to tolerate differences in template concentrations of at least a million fold. We believe that this newly developed condition is a “universal” one in which different levels of gene expression can be successfully multiplexed.

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### Materials and methods

**Materials**

iScript cDNA Synthesis Kit, dNTP Mix (10 mM each dATP, dCTP, dGTP, and dTTP), iTaq DNA Polymerase, 3% ReadyAgarose Gels, and the Aurum Total RNA Mini kit were obtained from Bio-Rad Laboratories (Hercules, CA). Human prostate total RNA, MEGAscript T7 in vitro transcription system, and The RNA Storage Solution were purchased from Ambion (Austin, TX). Primers for the in vitro synthesis constructs and primers and probes for the 5′-nuclease assay were purchased from Integrated DNA Technologies (Coralville, IA) and Trilink Biotechnologies, Inc. (San Diego, CA). The RNA 6000 Nano chips and reagents were purchased from Agilent Technologies (Palo Alto, CA). The QIAquick PCR Purification kit was from Qiagen (Valencia, CA). The Omiga 2.0 software was purchased from Accelrys (San Diego, CA). SYBR Green was purchased from Molecular Probes (Eugene, OR).

**Primer and probes**

All primers and hydrolysis probe sequences were designed with aid from the Omiga 2.0 software. Nucleotide sequences used for primers and probes design were retrieved from NCBI using the Omiga software.

All in vitro constructs were generated by PCR using primer pairs specific for ODC antizyme, antizyme inhibitor, ornithine decarboxylase, and \(S\)-adenosylmethionine decarboxylase transcripts. The forward primers (F) had the T7 minimal required promoter sequence incorporated 5′ to the gene-specific sequence. The reverse

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1. Abbreviations used: qRT, quantitative reverse transcription; AZI, antizyme inhibitor; ODC, ornithine decarboxylase; AOZ, ODC antizyme; AdoMetDC, \(S\)-adenosylmethionine decarboxylase; Ct, threshold cycle.

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**Table 1**

<table>
<thead>
<tr>
<th>Primer pairs used for in vitro transcript template generation</th>
<th>Primers used for in vitro construction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T7AIN F:</strong> 5′-TAATAACGACTCATATAGGGCTGAGATGAAAGG-3′</td>
<td><strong>T7AIN R:</strong> 5′-TTTTTTTTTTTTTTTTTTTACACACATCGAGCA CT-3′</td>
</tr>
<tr>
<td>Amplicon size: 684 bp in vitro transcript approximate length: 665 nt</td>
<td></td>
</tr>
<tr>
<td><strong>T7OAZ F:</strong> 5′-TAATAACGACTCATATAGGGCAGCAGCGATGA-3′</td>
<td><strong>T7OAZ R:</strong> 5′-TTTTTTTTTTTTTTTTTTTACACACATCGAGCACT-3′</td>
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<tr>
<td>Amplicon size: 688 bp in vitro transcript approximate length: 669 nt</td>
<td></td>
</tr>
<tr>
<td><strong>T7ODC F:</strong> 5′-TAATAACGACTCATATAGGGGTTCGCTCCCATG-3′</td>
<td><strong>T7ODC R:</strong> 5′-TTTTTTTTTTTTTTTTTTTACACACATCGAGCATC GTGCA-3′</td>
</tr>
<tr>
<td>Amplicon size: 689 bp in vitro transcript approximate length: 670 nt</td>
<td></td>
</tr>
<tr>
<td><strong>T7SAMDC F:</strong> 5′-TAATAACGACTCATATAGGGATGTCGCAATGT-3′</td>
<td><strong>T7SAMDC R:</strong> 5′-TTTTTTTTTTTTTTTTTTTGTAACTCATTGAACTAGTC TAGC-3′</td>
</tr>
<tr>
<td>Amplicon size: 858 bp in vitro transcript approximate length: 839 nt</td>
<td></td>
</tr>
</tbody>
</table>
Table 2  
Primers and probes used for 5′-nuclease real-time RT-PCR assay  
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′→3′)</th>
<th>Fluorophore quencher</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIN-F</td>
<td>GAAATATCCGTGACAGGGGA</td>
<td></td>
</tr>
<tr>
<td>AIN-R</td>
<td>CCAAGAGCAGTCCAAA</td>
<td></td>
</tr>
<tr>
<td>AIN-P</td>
<td>CGTGGAGAATCCTATTCCTGTGGTC</td>
<td>Cy5/BHQ2</td>
</tr>
<tr>
<td>OAZ-F</td>
<td>CGTTGAGAATCCTCTGTC</td>
<td></td>
</tr>
<tr>
<td>OAZ-R</td>
<td>CGTTGAGAATCCTCTGTC</td>
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<tr>
<td>OAZ-P</td>
<td>CGTTGAGAATCCTCTGTC</td>
<td></td>
</tr>
<tr>
<td>SAM</td>
<td>CGTTGAGAATCCTCTGTC</td>
<td></td>
</tr>
<tr>
<td>DC-F</td>
<td>CTAAGCCATCAGCTGTTAC</td>
<td></td>
</tr>
<tr>
<td>DC-R</td>
<td>GCGGTCAAGATCAGCTGTTAC</td>
<td></td>
</tr>
<tr>
<td>DC-P</td>
<td>TCGAGGCCATCAGCTGTTAC</td>
<td>HEX/BHQ1</td>
</tr>
<tr>
<td>SAM</td>
<td>GGAATTCGTGACAGGGGA</td>
<td></td>
</tr>
<tr>
<td>SAM</td>
<td>GTCCAATAAGTCCATCACC</td>
<td></td>
</tr>
<tr>
<td>DC-F</td>
<td>CATTGATCCACATGGTCCATCACC</td>
<td>FAM/BHQ1</td>
</tr>
<tr>
<td>DC-R</td>
<td>CATTGATCCACATGGTCCATCACC</td>
<td></td>
</tr>
<tr>
<td>DC-P</td>
<td>CATTGATCCACATGGTCCATCACC</td>
<td></td>
</tr>
</tbody>
</table>

Primers (R) included a 3′ poly(T) sequence to generate an equal-length poly(A) tail during the reverse transcription procedure; see Table 1.

Primers used for the real-time 5′ nuclease RT-PCR assays were originally generated for an earlier application. The primers were designed to be specific for the mRNA homologous sequence, i.e., cDNA, and not to amplify genomic-derived sequences including pseudo genes colinear to mRNA. Further, all primers and probe sets were designed for optimal PCR efficiency and to avoid secondary structure and primer dimers. All primers were designed to have a similar melting temperature ($T_m$), i.e., close to 59 °C, and were selected to generate PCR products of similar lengths, i.e., 80–162 bp. Hydrolysis probes were designed with a $T_m$ close to 68 °C. Fluorophores for probe labeling were chosen to minimize overlaps in their emission spectra. Selected fluorophores were FAM (6-carboxyfluorescein), HEX (hexachloro-carbonylfluorescein), Texas Red, and Cy5.

Appropriate Black Hole quenchers were matched for each fluorophore.2 See Table 2 for primer and probe sequences.

PCR and qRT-PCR assays were carried out using 1× PCR buffer (10× PCR buffer is 200 mM Tris–HCl, pH 8.4, 500 mM KCl). SYBR Green primer pair validation experiments were carried out in 50 µl using Taq (0.025 U/µl reaction volume), 5 µl of a 1/10,000 SYBR Green dilution, 300 nM each PCR primer, 1× Taq buffer, dNTPs (200 µM each dATP, dCTP, dGTP, and dTTP), and 3.5 mM MgCl₂. The performance of primers and probe sets included in our multiplex qRT-PCRs was evaluated using serial dilutions (1/5 dilutions) of human cDNA spanning three orders of magnitude.

### Total RNA cDNA synthesis

Template cDNAs for the synthesis of the T7 in vitro PCR constructs were synthesized with the iScript cDNA synthesis kit. cDNA synthesis reactions were performed in a total volume of 200 µl containing 500 ng of human total RNA from prostate tissue. Reactions were carried out according to the iScript cDNA synthesis kit protocol.

### Synthesis of the in vitro constructs

In vitro constructs for AZI, OAZ, ODC, and AdoMetDC, containing the T7 minimal promoter at the 5′ end and the poly(A) sequence at the 3′ end, were generated using the primer pairs shown in Table 1. The optimal annealing temperature ($T_a$) for each primer pair was determined using the iCycler iQ Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA) Gradient feature. In separate reactions each primer pair was incubated with cDNA (from human prostate total RNA) according to the manufacturer’s suggestions. PCR products were first analyzed on a 3% ReadyAgarose Gel and then purified with the QIAquick PCR Purification kit. Purified PCR products were quantified by measuring absorbance at 260 nm with a spectrophotometer, diluted in TE to the desired concentration, and stored at 4 °C until further processing.

### In vitro transcription

In vitro transcripts were generated with the MEGAscript kit; please refer to Synthesis of the in vitro constructs. Approximately 300 ng of purified PCR products were used as a template for in vitro transcription according to manufacturer’s instructions. Optimal in vitro transcription time was found to be 3 h for all the templates. After the reverse transcription step was completed, the samples were subjected to DNase I treatment as described by the kit instruction manual. In vitro transcripts were purified using the Aurum Total RNA Mini kit according to manufacturer’s instructions. Subsequently, the samples were subjected to a second DNase I treatment while the RNAs were still bound to the column. The integrity of the in vitro transcripts was analyzed on the Bioanalyzer 2100 using the RNA 6000 Nano chips followed by quantification using a spectrophotometer. The in vitro transcripts were diluted in The RNA Storage Solution to the desired concentration and then frozen at −70 °C.

### qRT-PCR analysis

The qRT-PCR assays were performed on the iCycler iQ Real-Time PCR Detection System using the primers–probe combinations described in Table 1. Fifteen in vitro
dGTP, and dTTP, and 6 mM MgCl2. Optimal multiplex reaction conditions were achieved when multiplex and singleplex assays. A successful qRT-PCR optimization by analyzing in parallel the performance of both singleplex and multiplex assays. A successful qRT-PCR multiplex reaction is achieved when multiplex and singleplex assays performed simultaneously on the same plate produce similar Ct values for the amplification of a single target. We measured the success of our multiplex optimization by analyzing in parallel the performance of both singleplex and multiplex assays. A successful qRT-PCR multiplex reaction is achieved when multiplex and singleplex assays performed simultaneously on the same plate produce similar Ct values for the amplification of a single target.
particular gene. In our laboratory we accept a maximum Ct difference of 0.5 cycles between single and multiplex data. In addition, successful multiplex assays can be achieved only when the exponential phase of the multiplex reaction can be superimposed over that of the singleplex reactions; i.e., both singleplex and multiplex reaction amplify with near identical efficiencies.

During the early stages of the optimization of our multiplex assay we used a reaction condition that was previously determined. This multiplex condition, which had been verified only for a Ct difference of five cycles in gene expression levels (Kent Persson’s personal communication), consisted of 300 nM each primer, 200 nM each probe, 200 μM each dATP, dCTP, dGTP, and dTTP, 5 mM MgCl₂, and 0.075 U/μl reaction volume of Taq DNA polymerase (condition A). In vitro assemblies 1–4 (Fig. 1) were selected to perform an initial screening using condition A. In vitro assembly 1 was successfully amplified with condition A; however, this condition failed when applied to assemblies 2–4. Results generated with assemblies 2–4 clearly demonstrated that increasing differences in template concentrations worsened the amplification efficiency of the less-concentrated target. It became apparent that the multiplex assay was not ready to sustain large differences in template concentrations. The amplification problem was most pronounced for in vitro assembly 4. While the three cDNAs with a Ct of ~25 (i.e., the highest concentration of in vitro transcripts in the sample) amplified well, the cDNA with a Ct of ~35 (i.e., the less-concentrated target) showed a severe inhibition of its amplification. To overcome the amplification problems found with assemblies 2–4, we decided to further optimize some reaction components. Keeping the primers and probes concentration unaltered, we changed the concentrations of dNTPs, Taq DNA polymerase, and Mg²⁺. These modifications were subsequently applied to the in vitro assemblies 1–4. Fig. 3 shows how modifying the concentration of both dNTPs and Taq DNA polymerase impacts the amplification of the less-concentrated template in assembly 4. Data from the analysis of assemblies 1–4 showed that when using 0.1 U/μl reaction volume of Taq DNA polymerase and 400 μM each dATP, dCTP, dGTP, and dTTP, the exponential phase of the multiplex reactions became practically identical to that of the singleplex reactions. Additional analysis showed that 6 mM MgCl₂ produced better replicates especially at lower Ct values (data not shown).

**Discovery of a universal four-color multiplex condition**

The new reaction condition, i.e., 400 μM each dATP, dCTP, dGTP, and dTTP, 0.1 U/μl reaction volume Taq DNA polymerase, and 6 mM MgCl₂ (condition B), was successfully applied for the amplification of in vitro assemblies 1–4. To investigate whether condition B could sustain even larger differences of target RNA concentrations, we subsequently applied this condition for the amplification of assemblies 5–15. Condition B successfully amplified assemblies 5–15 without any decline in PCR efficiency. Furthermore, condition B was able to act as a “universal” multiplex reaction condition allowing for optimal amplification of all in vitro assemblies 1–15. For the in vitro assemblies 1–15 shown in Fig. 1, PCR efficiencies for singleplex reactions were equivalent to their multiplex counterpart (Figs. 4–6).
Discussion

qRT-PCR is a powerful tool for measuring messenger RNA (mRNA) levels in biological samples [6,7]. The technique has found application in the study of gene function [6–8], molecular diagnostics [9,10], pharmacogenomics [11], and other fields. The introduction of technologies that combine RT-PCR with the use of fluorescent dyes [12] or fluorescently-labeled hybridization probes [13] was a major advance for the development of qRT-PCR. Because of the numerous advantages intrinsic to the use of qRT-PCR for mRNA quantification (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 [1] is one of the most widely used methodologies for mRNA quantitation in qRT-PCR. The assay takes advantage of the 5′→3′ endonuclease activity of Taq DNA polymerase to cleave fluorescently-labeled oligonucleotide probes that have hybridized to PCR products during the reaction annealing phase.

Multiplex PCR allows for the simultaneous amplification and detection of several different mRNA/cDNA targets in a single reaction vessel. Although multiplex qRT-PCR is a greater challenge than singleplex qRT-PCR, it offers several advantages over singleplex assays. One of the most important advantages of multiplexing is sample savings, which can be quite important when sample material is scarce. When performing a four-gene multiplex assay, a substantial amount of the sample material can be saved compared to the single-gene assay. The savings allow one to perform additional experiments or to carry out complementary analysis using supplementary techniques. Multiplexing also reduces time, labor, and cost compared to singleplex reactions. In addition, multiplex reactions can considerably increase throughput.
Despite the numerous advantages provided by the multiplex approach, up to date there has been only one report on four-color qRT-PCR [2] and none exploring the limitations of the multiplex assay or defining a set of universal conditions. It is possible to imagine multiplex users being satisfied with multiplex assays optimized for a limited difference in gene expression since the assay could be well suited for many experimental conditions.

However, differences in gene expression levels that are not supported by an underoptimized multiplex assay will lead to unreliable experimental results.

Primers and probes design, selection of fluorophores, the use of a hot-start Taq DNA polymerase, and assay optimization are important factors to consider when designing a multiplex qRT-PCR assay. Primers-probe sets for our four-color assays were optimized for both
specifity and efficiency. Probes for the primers pairs used in the multiplex assay were designed to avoid self-complementarity and hairpin formation and to hybridize at about 68 °C. Ideally, all the primer pairs in a multiplex reaction should amplify their respective targets with similar efficiencies. For our multiplex qRT-PCR assay, we used primers with nearly identical Tm (59 °C). In addition, primer design was aimed to reduce the formation of nonspecific amplification products by avoiding hairpin formation and primer–primer and primer–probe interactions. Inappropriate primer design can lead to the development of multiplex PCR assays with poor amplification efficiencies. Even when using the standard rules that satisfy good primer design [14,15], predicting the performance characteristics of a PCR primer pair in a multiplex reaction is not possible. Therefore, primer pairs in a multiplex setup are empirically tested and a trial-and-error approach is used.

The number of genes that can be detected in a 5′ nuclease multiplex qRT-PCR assay depends on the design of the instrument and on fluorophore choice. Ideally there is no overlap in the emission spectra of the fluorophores and when there is overlap the instrument must be able to separate out the contributions of each fluorophore for every measurement taken. The wide-ranging excitation source of the iCycler iQ and the filter-based optical design make it possible to select fluorophores with well-differentiated emission spectra, though it is not possible to select four with no overlap in emission from among the commercially available fluorophores. Overlapping spectra are deconvoluted into their individual contributions by the sophisticated data analysis algorithms of the instrument. The combination of four fluorophores selected for our multiplex qRT-PCR (FAM, HEX, Texas Red, and Cy5) generated highly specific results.

It is well known that the initial PCR cycles have significant effect on the overall sensitivity and specificity of the reaction. The use of multiple primer sets in multiplex PCR increases the chance of synthesizing both spurious PCR fragments due to unspecific priming and primer–primer and primer–probe interactions. In addition to a good primer and probe design strategy, the use of a hot-start enzyme is a straightforward solution to the problems affecting the first few amplification cycles. We used a Taq DNA polymerase that contains a monoclonal antibody against Taq polymerase, which binds to the enzyme until the temperature is increased. Antibody binding at low temperatures prevents the nonspecific amplification due to mispriming and primer–dimer formation that occurs during reaction setup.

We previously reported the development of a four-color qRT-PCR assay for the study of four genes from the polyamine pathway (see Introduction). Although the quality of our multiplex data when testing small differences in concentration of target cDNAs was good, we felt the need to achieve a better understanding of the limitations of the multiplex approach. For this reason, we decided to explore the limits of a successful four-color multiplex qRT-PCR using the 5′ nuclease assay.

For assay development and validation, we needed a flexible system for the precise analysis of large differences in gene expression. For the model system to be valid, it had to mimic in vivo gene expression for qRT-
PCR analysis. Based on the DNA sequences used for the polyamine gene multiplex assay, we designed four T7 RNA-polymerase-driven PCR constructs to generate in vitro transcribed RNAs. The purified transcripts were then combined in 15 different in vitro RNA assemblies spanning from small to vast differences in template concentrations. The assemblies were generated to contain template combinations that would result in differences of 0, 10, and 20 threshold cycles when amplified in real-time PCR (Fig. 1). The amounts of each in vitro transcript in the various assemblies were either the same or differed by 1000- to 1,000,000-fold. To mimic potentially “real” experimental situations using total RNA, we performed reverse transcription reactions after spiking Arabidopsis total RNA into each of the 15 assemblies.

Data generated using the original condition A with the first four in vitro assemblies clearly showed that the original multiplex condition allowed for only limited multiplexing. The analysis showed a more efficient amplification for the more-concentrated targets while the target present at the lowest concentration was amplified with a reduced efficiency, thus generating unreliable results. We then decided to further optimize our four-color reaction to make it able to efficiently amplify all the targets present in the reaction even in situations in which there was a wide range of template concentrations.

To clarify how we could compensate for the difference in target concentrations, we analyzed the impact of using higher concentrations of Taq DNA polymerase, dNTPs, and Mg2+ keeping the primers and probe concentrations unaltered.

We found that the use of a higher concentration of Taq DNA polymerase was key to generating a four-color qRT-PCR protocol that sustained reliable multiplex amplification over a wide dynamic range. The use of higher Taq DNA polymerase concentrations for multiplex purposes has been previously reported. Chamberlain et al. [16] reported a multiplex PCR assay that required four- to fivelfold higher amounts of Taq polymerase than the amount used for the single-gene assay. Shuber et al. [17] also described the use of a fivelfold Taq DNA polymerase concentration for multiplexing protocols. It was subsequently demonstrated that the presence of PCR products accumulated in later PCR cycles completely inhibited the activity of DNA polymerase [18]. The required concentration of Taq DNA polymerase is therefore related to the number of primer pairs included in the reaction and the expression levels of the genes tested in the assay.

We made an interesting observation most notably while analyzing assembly 15. As shown in Fig. 6, the amplification of the AZI, OAZ, and ODC targets (Ct ~15) reached the PCR plateau phase when the amplification of AdoMetDC (Ct ~35) was taking off. This result suggests that our reaction protocol would support multiplexing even in the presence of larger differences of template concentrations. Based on our results, we believe that we have found a “universal” multiplex condition applicable to any properly designed primers–probe sets able to produce high PCR efficiencies.

Although our assay requires 0.1 U/μl reaction volume of Taq DNA polymerase, this amount is identical to that required to carry out four singleplex PCRs. During our study, we also noticed that there was a direct proportionality between the number of primer pairs included in the multiplex assay and the units of Taq DNA polymerase needed to perform the amplification reaction. Thus, a twoplex reaction required twice the amount and a threeplex reaction three times the amount of Taq DNA polymerase required by a singleplex reaction. This finding is in agreement with the results reported by Keinz [18].

When sample material is scarce, carrying out additional multiplex experiments and alternative assays or even archiving the material can be a problem or in some cases even impossible. The suggested multiplex protocol described here enables large sample savings since only one fourth of sample material is used as compared to four singleplex reactions. Even when only a single gene is studied, multiplexing can be a useful approach. Recent publications on normalizations using reference genes for the analysis of in vivo material [19] suggest a minimum of three internal control genes for accurate normalization. Therefore, a single gene of interest could be multiplexed in the same tube using three valid reference genes. For difficult normalization situations, sets of internal control genes could be multiplexed in parallel to the gene or genes of interest. Nevertheless, any application using a multiplex qRT-PCR assay would benefit from the advantages described in this paper. Our novel assay was thus able to support multiplex real-time PCR amplification from four targets present either at equal concentrations or having up to a millionfold difference in target concentration.

References