Development and Assessment of a Quantitative Reverse Transcription-PCR Assay for Simultaneous Measurement of Four Amplicons

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Background: High-throughput and forward-deployable biological dosimetry capabilities are required for tactical and medical decisions after radiologic events. We previously reported a quantitative reverse transcription (QRT)-PCR assay for human radiation-responsive gene targets using a whole-blood ex vivo irradiation model, but we needed a multitarget assay on a smaller, less costly, real-time PCR detection system.

Methods: We developed a quadruplex QRT-PCR assay in a 96-well, closed-plate format suitable for use with RNA extracted from whole blood. Four cDNA targets were simultaneously amplified in a sealed tube by hybridization to exonuclease probes, each conjugated to distinct fluorogenic reporters. A novel primer-limited 18S rRNA reference target was validated from serial dilutions of human total RNA. To test assay precision, we incorporated a positive-control cDNA mimic into duplex and quadruplex PCR reactions. The master mixture was supplemented with more enzyme, MgCl2, and deoxyribonucleotides. Simultaneous detection of four targets was evaluated in comparison with respective duplex QRT-PCR assays.

Results: The simultaneous detection of three radiation-responsive genes by quadruplex QRT-PCR was quantitative, with gene expression changes similar to those observed with optimized duplex and triplex QRT-PCR assays. The 18S rRNA and GADD45 calibration curves (threshold cycle vs log10 cDNA) were linear and reproducible and showed optimal PCR efficiencies as indicated by slopes statistically equivalent to the theoretical value of −3.322.

Conclusions: This is the first study of a quadruplex QRT-PCR assay. Our approach has diagnostic utility in the detection of biomarkers, biological and toxicologic agents, and genes of inherited diseases and cancer. © 2003 American Association for Clinical Chemistry

With the advent of real-time PCR technology (1) and new options for affordable instrumentation, real-time quantitative reverse transcription (QRT)-PCR assays are becoming widespread methodologies for diagnostic purposes (2–9). The use of probes with modified chemistry, such as high-affinity DNA minor-groove binding probes (10), and dark quenchers (11) has further improved quantitative PCR (QPCR) sensitivity by increasing both probe hybridization efficiency and signal-to-noise ratios. Many new instruments are cost-effective, offer four-color detection, allow for multiple chemistries, permit shortened PCR cycling times, and are suitable for use in routine diagnostic laboratories (12, 13).

Even with these developments in real-time PCR technology, QRT-PCR and QPCR assays have been slow to find their way into diagnostic laboratories. The clinical concerns include the need for standardization of the methodology and interlaboratory validation of the technologies (12). Feasible clinical assays need to be economical, sensitive, and simple, and they need to offer high-throughput capabilities through automation (13).

QRT-PCR assays fulfill many requirements for clinical applications, although they should be multiplexed for analysis of multiple targets (14). Furthermore, for clinical applications, they should be multiplexed with an endogenous control to verify both the quality and quantity of RNA in the test samples (13–16). However, quantification
of multiple targets in one amplification reaction is problematic, and no single approach is easily adaptable to different PCR bioassays.

Novel multiplex detection assays, with simultaneous detection of four (17) or seven (18) targets, have been achieved by altering the operational design of the ABI Prism 7700 Sequence Detection System™ or using a fluorescent microplate reader with end-point analysis. Both of these multiplex assays demonstrated sophisticated technologic proof of principle for extending the multicolor, multiplex PCR system.

Several small multiplex units are designed specifically for measuring multiple excitations and emission wavelengths (12, 13). The use of hydrolysis probes with dark quenchers increased the usable spectra of possible fluorochrome reporters and significantly improved signal-to-noise ratios (11, 16–18). Transitioning from a duplex PCR assay to higher-order multiplex PCR assays offers improved assay flexibility, cost-effectiveness, and high-quality quantitative data generated with minimal amounts of cDNA template in multiplex assays. This transitioning could be extremely valuable for conservation of rare or not easily obtainable human RNA samples.

An example of a four-color detection and cost-efficient unit is the iCycler IQ™ (Bio-Rad Inc.). It provides a 96-well format with optical caps that can be set up with predeveloped reagent and assay validation plates of practical use to diagnostic applications. A broad-spectrum tungsten light source and multiple filters permit a wide range of excitation and emissions of 400–700 nm as well as the multiplexing of up to four different reporter fluorophores. Online display allows visual confirmation of the PCR amplification in progress. The output provides a useful advantage of instantaneous visual representation of the amplification process for each sample. This feature is particularly helpful in a military field laboratory (19) or a point-of-care medical facility, where QPCR and QRT-PCR assays are most challenging. Troubleshooting can be immediate by examining the real-time data for signal detection of endogenous and exogenous control targets.

To achieve high-throughput and portable biodosimetry capability for quantification of candidate gene biomarkers, we transitioned from our previously accepted “gold standard” ABI 7700 Sequence Detection System to the smaller spectrofluorometric iCycler IQ thermal cycler to develop cost-effective, simple, and predictive real-time PCR assays for sentinel biomarkers (20).

For radiation biodosimetry purposes, a whole-blood sample easily can provide prospective molecular biomarkers (19, 21). For example, genes and pathways involved in the induction and repair of radiation-induced DNA damage, apoptosis, and reduction-oxidation pathways are likely candidate biomarkers. Sentinel gene expression biomarkers should demonstrate diagnostic radiation dose-dependent changes that can be measured by sensitive QRT-PCR assays (19–21).

The expected increases in quantitative gene expression by real-time PCR assay for targets such as GADD45 and DDB-2, as shown, were closely related to previous microarray data (20, 21). However, these potential biomarkers need further elucidation as a function of tissue type, exposure conditions, and radiation type. Validation of gene expression changes observed for several genes in microarray analysis by QRT-PCR assay facilitates the development of dual-use assays for both diagnostic and research applications.

We developed and optimized a high-throughput multiplex QRT-PCR assay that can demonstrate radiation dose responses for three different low-copy genes with a reference target in one sealed tube, using our ex vivo whole-blood radiation model. Three up-regulated and one down-regulated radiation-responsive gene targets were chosen to demonstrate development of a multitarget, multicolor bioassay for biodosimetry applications. To our knowledge, this is the first report of simultaneous and quantitative detection of gene expression dose responses for three different target genes and an endogenous reference gene in a single tube with use of total RNA isolated from human whole blood in a real-time QRT-PCR assay.

Materials and Methods

Blood Samples

Whole blood was collected from one healthy human male donor by venipuncture, irradiated ex vivo at radiation doses 0, 1, 2, and 3 Gy in 15-mL conical tubes (1.5–3 mL; n = 20), and cultured at 37 °C for 24- and 48-h intervals (20). The total RNA of peripheral blood samples was extracted with the QiAamp RNA Blood Mini Kit (Qiagen), and 1 μg of the extracted RNA was reverse-transcribed into cDNA with random hexamer primers (20). Samples of cDNA were stored at −20 °C until tested.

Probes, Primers, and PCR Consumables

Gene sequences were obtained from GenBank®. Primers and probes were chosen using Primer Express (Applied Biosystems Inc.). Basic Local Alignment Search Tool (BLAST®) searches were conducted to confirm gene specificity of the primers and probes and the absence of genomic DNA amplification. Oligo 6 (Molecular Biology Insights, Inc.) was used to calculate the secondary structure and possible cross-reactivity of oligonucleotides based on the nearest-neighbor thermodynamic values.

Fluorochrome choices for the hydrolysis probes and their corresponding dark quencher (Black Quencher™; Biosource International Laboratories Inc.) for use on the iCycler IQ (Bio-Rad Inc.) were based on relative brightness and a range of fluorochrome excitations and emissions. Fluorochrome reporters used were 6-carboxyfluorescein (FAM; excitation, 488 nm; emission, 518 nm), hexachloro-6-carboxyfluorescein (HEX; excitation, 488 nm; emission, 556 nm), Texas Red (excitation, 595 nm; emission, 615 nm), and Cy 5 (excitation, 649 nm; emission, 670 nm).

The modifications and DNA sequences for the fluoro-
Taq amplification of two targets on the iCycler iQ, using FastStart multiplex amplification reactions was formulated for amplification mixture (20 ng of human cDNA/50 ribosomal subunit primers and probe. A standard PCR mixture with the BR18S polymerase (5 U/50 L), PCR-grade deoxynucleotides, MgCl₂, and MgCl₂, and buffer purchased from Roche Applied Sciences. All duplex PCR reactions on the iCycler iQ were amplified using a standard PCR mixture with the BR18S ribosomal subunit primers and probe. A standard PCR reaction mixture (20 ng of human cDNA/50 μL) for multiplex amplification reactions was formulated for amplification of two targets on the iCycler iQ, using FastStart Taq polymerase (1.25 U/50 μL), deoxynucleotide triphosphates (dNTPs; 800 mM), and MgCl₂ (3 mM).

A separate, fortified, extrastrength mixture, named 4Mix, prepared in our laboratory contained different concentrations of the same reagents [FastStart Taq polymerase (3.5 U/50 μL), dNTPs (1800 mM), and MgCl₂ (5 mM)] for multiplex amplification of four targets on the iCycler iQ.

Primer-limiting system. Accrual of the 18S amplicon after fluorescent detection was regulated by limiting both 18S primers to a final concentration of 50 nM in all multiplex assays, using 200 nM of the specific probe. Targets (GADD45, DDB-2, BAX, and MnSOD) were amplified with use of standardized concentrations of 400 nM for each primer set and 200 nM for the specific probes.

18S rRNA system. New primers (BR18Sr and BR18Sp) and an original 18S rRNA probe were synthesized with Texas Red reporter and Black Quencher (BR18SP) for higher order multiplexing on the iCycler iQ. A calibration curve was constructed by plotting the initial number of 18S rRNA target molecules in the dilution series of human cDNA amounts (log₁₀) against the threshold cycle (Cₜ). Serial 10-fold dilutions of total human cDNA were used as templates for fluorogenic 5'-nuclease PCR assays to verify the new ribosomal subunit primers and probe for BR18S. Serial dilutions of cDNA from human whole blood were prepared at final concentrations of 10, 1, 0.1, 0.01, and 0.001 ng in 50-μL reactions.

Probe performance for the BR18S PCR product was validated against the 18S reference sequence that amplified with the TaqMan 18S rRNA probe (VIC®) and primers (Applied Biosystems Inc.) in standard-strength PCR mixtures on the iCycler iQ. A universal two-step PCR assay was used throughout these studies, with the activation of hot-start Taq polymerase for 15 min at 94 °C, followed by a two-step PCR protocol for 40 cycles (15 s of denaturation at 94 °C and 1 min at 60 °C with fluorescent data acquisition at 60 °C during the combined annealing-extension step).

DATA ANALYSIS
Each target was identified by distinct emissions of the respective reporter dyes as recorded with use of spectral filters and computer software. Data from fluorogenic 5'-nuclease PCR studies were collected and analyzed by the iCycler iQ software (Bio-Rad), which calculates the relative fluorescent units (RFU). The baseline and fluorescent.

Table 1. Oligonucleotide primer and probe sequences used in this study.

<table>
<thead>
<tr>
<th>Target</th>
<th>Name*</th>
<th>Sequence, 5'→3'</th>
<th>Tₘ°, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>BR18Sr</td>
<td>5'-AGG AAT TCC CAG TAA GTG CG-3'</td>
<td>59.0</td>
</tr>
<tr>
<td></td>
<td>BR18Sp</td>
<td>5'-GCC TCA CTA AAC CAT CCA A-3'</td>
<td>55.7</td>
</tr>
<tr>
<td>DDB-2</td>
<td>DDB2f</td>
<td>5'-CAT GAT CTT CGC ATG CAG AGT-3'</td>
<td>62.8</td>
</tr>
<tr>
<td></td>
<td>DDB2r</td>
<td>5'-GGG ACT CCT GCT CTT GTT-3'</td>
<td>61.9</td>
</tr>
<tr>
<td>BAX</td>
<td>BAXf</td>
<td>5'-CAT GGA GCT GCA GAG GAT-3'</td>
<td>62.5</td>
</tr>
<tr>
<td></td>
<td>BAXr</td>
<td>5'-TTG CCG TCA GAA AAC ATG TCA-3'</td>
<td>62.5</td>
</tr>
<tr>
<td>GADD45</td>
<td>GADD45f</td>
<td>5'-TGC TCA GGA GCC CCG TGA TGT-3'</td>
<td>63.4</td>
</tr>
<tr>
<td></td>
<td>GADD45r</td>
<td>5'-GCA GGC ACA ACA CCA CTC TA-3'</td>
<td>63.0</td>
</tr>
<tr>
<td>MnSOD</td>
<td>MnSODf</td>
<td>5'-CAC ATC AAC GCG CAG ATC AT-3'</td>
<td>63.1</td>
</tr>
<tr>
<td></td>
<td>MnSODr</td>
<td>5'-CAG TGC AGG CTC AGG AGC TCT-3'</td>
<td>62.3</td>
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<tr>
<td></td>
<td>MnSODP</td>
<td>5'-HEX-CGT CTC AAC AGC AGC CAC CA-3'</td>
<td>73.3</td>
</tr>
</tbody>
</table>

* f, forward primer; r, reverse primer; P, hydrolysis probe.

Reporters: Cy5, FAM, HEX, Texas Red (TxRd), and Black Hole Quencher 1 or 2 (BHQ1 or 2).

° Melting temperature (Tₘ°), calculations used to adjust for salt at 50 mM (Na⁺).
cience threshold settings were adjusted to provide continuity of analysis between the ABI Prism 7700 Sequence Detection System and the iCycler iQ. These values were kept constant for the gene targets throughout the remainder of these experiments.

The cycle number at which the fluorescent signal of a given reaction well crossed the threshold value was denoted as the Ct. Ct data for multiplex targets (e.g., GADD45, DDB-2, BAX, and MnsOD) in each well were normalized to the internal standard, 18S ribosomal subunit BR18S Ct, by use of the formula: ΔCt = target Ct - internal standard Ct. Radiation effects on gene expression were analyzed further by comparison to a reference sample (i.e., blood calibrator or nonirradiated sham-treated samples) (20).

Microsoft Excel-fitted lines were obtained by use of standard regression analysis programs within Excel XP (Microsoft Corp.) and Sigma Plot 5.0 (SPSS Inc.). Standard errors of Ct were determined for each value. Samples were run in triplicate PCR assays in each experiment. PCR efficiencies were determined from the slope of the straight-line fit (12, 20, 22) of real-time PCR data, based on serial dilutions of human cDNA or GADD45 cDNA mimic.

RNA MIMIC SYNTHESIS
The RNA or cDNA mimics were generated by PCR and in vitro transcription, as described previously (20).

CONSTRUCTION OF CALIBRATION CURVES
In a singleplex PCR assay, the protocol was described for 18S rRNA data (18S rRNA system) used in the construction of calibration curves (data not shown). For a duplex PCR assay, 10-fold serial dilutions of human GADD45 cDNA calibrator, starting with 100 ng of cDNA, were added in 10-ng aliquots of xenogenic murine cDNA in QRT-PCR assays to measure the change in target Ct relative to 18S rRNA (20). Murine liver total RNA was purchased commercially (BD Biosciences). In a quadruplex PCR assay, the same dilution series of human GADD45 cDNA calibrator was added to human total cDNA (derived from a 3-Gy isolate) to coamplify all human targets.

MEASUREMENT OF GENE AMPLIFICATION VALIDATION STRATEGY
In these studies, all cDNAs from the ex vivo radiation model used were well characterized in duplex PCR assays initially run on the ABI Prism 7700 Sequence Detection System (data not shown). These assays were repeated on the iCycler iQ format with modifications (BR18S probe and primers, gene target probe modifications, and reaction mixture formulation). All amplification assays for triplex and quadruplex reactions were performed in triplicate. Three RNA isolations per dose were subjected to the reverse transcription reaction in triplicate (n = 9) per dose, giving 36 assays for each PCR run.

VALIDATION OF THE 18S rRNA PRIMERS AND PROBE SYSTEM
The duplex QRT-PCR protocol, reported previously (20), led to the development of a quadruplex QRT-PCR assay on the iCycler iQ platform. This transition required the development of a novel and robust sequence-specific 18S rRNA primer and probe system for quantification of 18S rRNA, compatible with the iCycler iQ platform.

An original 18S rRNA probe (BR18Sp) and primers (BR18Sf, BR18Sr) were developed for duplex PCR and higher order multiplexing assays (more than two targets). The dynamic range and PCR efficiency were compared with those of the ABI 18S rRNA primers and probe by creating PCR-based calibration curves for both primer and probe sets (described in Materials and Methods). Respective slopes detecting the 18S rRNA target, which were derived from serial dilutions of human cDNA, were compared with the theoretical value of −3.322 by use of the two-sided t-test (each with three degrees of freedom; data not shown). Neither test showed a statistical difference (at 5%) from the theoretical value (P = 0.073 and 0.184, respectively). Because the slopes did not differ statistically from −3.322, it follows that the PCR efficiencies did not statistically differ from 100%.

DUPLEX PCR ASSAY ON THE iCycler iQ FORMAT
There were major differences between the real-time PCR assay and instrumentation using the iCycler iQ and the Prism 7700 Sequence Detection System. For example, the halogen lamp excitation of the iCycler is less intense than the excitation of the Prism 7700 laser at its maximum wavelength. Major changes in assay reagents and fluorochromes also were required to change to the new format.

GADD45 cDNA MIMIC
A new GADD45 external calibration curve was produced to measure assay precision and sensitivity in a duplex PCR assay with the new 18S rRNA as the internal standard. The human GADD45 cDNA mimic was divided in aliquots with fixed amounts of murine-derived cDNA, as described previously (20). Murine cDNA was compatible with our validated 18S rRNA primers and probe because the 18S rRNA sequence crosses species, although the endogenous GADD45 target sequence is human species-specific and did not amplify murine GADD45 mRNA.

Three independent assays run in triplicate showed linear correlation (r ≥0.99) for the GADD45 Ct (Fig. 1). PCR efficiency for detection of 8 log10 units of GADD45 molecules was η = 92.6% (SE, 3.59%). The equation and coefficients for a straight-line fit of ΔCt data to the added number of GADD45 molecules (data not shown) were: ΔCt = −3.513 (0.10) log10(g) + 35.936 (0.827); r = −0.9975, where g equals the number of GADD45 mimic molecules. The slope (−3.513) from these data was not statistically different (P = 0.11) from −3.322. When the data were fitted with a fixed slope equal to −3.322 or −log2(10), the fitted line was: ΔCt = −log2(g) + 34.42 (0.273).
relative gene expression for three targets (of repair from one ex vivo experiment caused increased for four gene expression targets from one RNA isolation

MnSOD

and multiplexing

the duplex QRT-PCR assay on the ABI Prism 7700 Se-

dmurine cDNA. Data are derived from three independent studies. Real-time PCR

internal reference. See

ture, we attempted to detect two low-copy specific gene

In our initial experiments, using the standard PCR mix-

PCR assays of radiation-responsive gene expression

Functional PCR assays

Physiologically relevant (typically up to fourfold) duplex PCR assays of radiation-responsive gene expression changes were performed with standard PCR mixture for each of several targets (GADD45, BAX, DDB-2, and MnSOD) with the validated 18S rRNA as the internal standard. Representative examples of duplex PCR data for four gene expression targets from one RNA isolation experiment can be seen in the “Duplex” column in Table 2. Exposure to ionizing radiation (1–3 Gy) after 48 h of repair from one ex vivo experiment caused increased relative gene expression for three targets (GADD45, DDB-2, and BAX) and down-regulation of relative gene expression for MnSOD. These data were used for comparison and ultimate validation of higher order multiplexing.

$^{4}$Mix PCR reagent mixture with higher multiplexing

In our initial experiments, using the standard PCR mixture, we attempted to detect two low-copy specific gene expression targets simultaneously with the 18S rRNA reference target (triplex QRT-PCR assay). Quantitative function of the triplex PCR assay was diminished (Table 3) compared with the duplex assay, with data showing a higher degree of intraassay and interassay variability. We evaluated the use of a supplemented PCR reagent mixture with more $^{4}$Mix to overcome these limitations. The GADD45 gene target data from a PCR mixture optimization study (Table 3) illustrates improvements with the $^{4}$Mix in triplex PCR assays. Improvements with the $^{4}$Mix were clearly demonstrated by the relative gene expression data (column RQ in Table 3). We observed a statistical difference between mixtures for the 2-Gy radiation dose ($P = 0.04$), but not at 0, 1, and 3 Gy (Table 3). However, the variances for the RQ values with $^{4}$Mix were consistently smaller ($P < 0.05$, F-test) compared with the standard PCR mixture (Table 3).

In this experiment, data also indicated no statistical difference in $C_T$ values (t-test) for 18S rRNA (data not shown) at different doses in triplex PCR assays for both PCR mixes ($P \geq 0.05$). The mean (SE) value of the 18S rRNA $C_T$ was 11.9 (0.04) for the standard PCR mixture and 11.8 (0.05) for $^{4}$Mix. Therefore, competition for the available resources in the standard PCR mixture was the apparent limitation overcome by the $^{4}$Mix, which contained threefold more Taq enzyme and approximately twofold more dNTPs. To correlate previous results with higher order multiplexing measurements, we compared the standard PCR mixture with $^{4}$Mix, using a duplex PCR followed by triplex PCR assays. We used three RNA isolations and measured real-time GADD45 target data for each radiation dose. Three replicates for each RNA sample per dose for three RNA isolations were run on three different days with different batches of $^{4}$Mix. Similar slopes were obtained for the GADD45 target (Fig. 2, ■) from a duplex (GADD45 and 18S) QRT-PCR assay in $^{4}$Mix compared with a representative duplex QRT-PCR assay with standard mixture (Fig. 2, ○), showing no inhibition or advantage to using $^{4}$Mix in duplex QRT-PCR assays (Fig. 2). The introduction of primers and probe to detect a third gene expression target, MnSOD, appeared to produce GADD45 values (Fig. 2, ▲) with a dose-response slope 26% (SE, 9.2%) higher ($P = 0.012$).

To investigate whether the duplex and triplex PCR assays revealed the same quantitative relationship as the quadruplex PCR assay, we performed 12 variations of QRT-PCR assays with a different RNA isolate from the same donor (Table 2). In the triplex and quadruplex PCR assays, targets were coamplified with use of combinations of specific primer and probes (Table 1). These results for specific targets showed gene expression profiles similar to those in the duplex QRT-PCR assay (using standard PCR mixture) for two or three coamplified gene targets and 18S rRNA. ANOVA was used to compare the QRT-PCR assays for each gene target and radiation dose. Only 3-Gy
data from the GADD45 dose response showed a statistically significant P value of 0.034. These data were consistent with earlier findings of differences in gene expression changes for the GADD45 target compared with the results using duplex and triplex QRT-PCR assays (Fig. 2). None of the data from the other radiation doses for this target or any of the other gene targets had P values showing statistical significance for evidence of mean differences. Therefore, no noteworthy difference in the ability to quantify gene expression changes was found among the duplex, triplex, and quadruplex QRT-PCR assays for the targets shown.

**QUADRUPLE PCR ASSAY DETECTION LIMITS**

Finally, our abilities to maintain linearity, precision, and reproducibility of the quadruplex PCR assay were tested for potential depletion of key PCR mixture components in the 4xMix. Addition experiments were performed in which we added GADD45 cDNA mimic to the cDNA template from a 3-Gy-irradiated human blood sample. We evaluated the quantitative function of the assay for each coamplified target: GADD45, DDB-2, BAX, MnSOD, and S/18S rRNA. The number in front of the code is the number of PCR assays run for each combination of targets.

Table 3. Comparisons of relative quantification of GADD45 expression in triplex PCR assays between standard PCR mixture and 4xMix. a

<table>
<thead>
<tr>
<th>Dose, Gy</th>
<th>Standard PCR mixtureb</th>
<th>4xMixc</th>
<th>Targetb</th>
<th>RQd</th>
<th>SE</th>
<th>RQd</th>
<th>SE</th>
<th>Pd</th>
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<tr>
<td>0</td>
<td>1.10</td>
<td>0.15</td>
<td>1.01</td>
<td>0.05</td>
<td>0.56</td>
<td>0.05</td>
<td>0.56</td>
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<tr>
<td>1</td>
<td>2.12</td>
<td>0.37</td>
<td>2.15</td>
<td>0.16</td>
<td>0.94</td>
<td>0.16</td>
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</tr>
<tr>
<td>2</td>
<td>5.96</td>
<td>0.56</td>
<td>4.57</td>
<td>0.24</td>
<td>0.04</td>
<td>0.24</td>
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</tr>
<tr>
<td>3</td>
<td>5.56</td>
<td>2.37</td>
<td>5.35</td>
<td>0.37</td>
<td>0.93</td>
<td>0.37</td>
<td>0.93</td>
<td></td>
</tr>
</tbody>
</table>

The experimental procedure was the same as described in Table 2 except that the sample used was another RNA isolation from the same donor. The target code is 1 GMS. b Standard PCR mixture: Taq polymerase (1.25 U/50 μL), dNTPs (800 mM), and MgCl2 (3 mM). c 4xMix formulation: Taq polymerase (3.50 U/50 μL), dNTPs (1800 mM), and MgCl2 (5 mM). d RQ, relative quantification. e P values were derived from the Student t-test. P ≥ 0.05 indicates that results are the same.
Fig. 2. Dose responses for GADD45 gene target in a duplex and triplex RT-PCR assay with standard PCR mixture and 4× Mix. 

Dose-response gene expression changes are illustrated by an ex vivo whole-blood model at 48 h recovery time for detection of GADD45. The x axis depicts the reported nominal doses 0, 1, 2, and 3 Gy at a dose rate of 0.1 Gy/min. Detection of the GADD45 target was evaluated for reproducibility when the standard PCR mixture and 4× Mix were used. For the standard PCR mixture (○ and dashed line), a single GADD45 dose-response experiment of a typical duplex PCR assay with three replicates per dose is shown. For the 4× Mix PCR (solid lines), GADD45 gene expression changes for three independent qRT-PCR assays from the same RNA isolation in a duplex PCR assay (targets, GADD45 and 18S rRNA) are shown. Symbols represent the means, and error bars represent the SE.

Fig. 3. Four-color multiplex RT-PCR assay with the 4× Mix PCR reagents and RNA from 3-Gy-irradiated whole-blood sample: effect of added amounts of GADD45 mimic. 

The GADD45 cDNA calibrator was diluted from a stock solution of 100 ng/10 μL. Serial 10-fold dilutions of cDNA calibrator from 10 ng (1.85 × 10^11 molecules) to 1 fg (1.85 × 10^6 molecules) were added to the cDNA derived from 3-Gy ex vivo-irradiated sample before qPCR assay. The iCycler IQ instrument, GADD45 (A), BAX (C), and DDB2 (D) gene expression targets and the 18S rRNA (E) internal standard were concurrently detected in a real-time PCR assay with the 4× Mix PCR reagent. Curves 1–7 reflect decade dilutions of the authentic cDNA mimic; curve G indicates background expression of GADD45 at a 3-Gy dose.

data from curves 1, 2, and 7 (Fig. 3A). The measured GADD45 Cₚ values at the highest concentrations (Fig. 3A, curve 7) of the added GADD45 mimic (1.85 × 10^11 molecules) study were similar to the 18S Cₚ values at approximately cycle 11. The results demonstrated a slight nonlinearity compared with lower GADD45 mimic samples and hence were eliminated from subsequent analysis. Endogenous GADD45 Cₚ values from the 3-Gy sample represented ~3.2 × 10^6 GADD45 molecules (Fig. 3A, curve G), as calculated from the GADD45 calibration curve (20). Hence, this number of GADD45 molecules was similar to values obtained for the lower two dilutions of the GADD45 calibrator [concentration for curve 7, 1.85 × 10^9 molecules; concentration for curve 2, 1.85 × 10^6 molecules per reaction; Fig. 3A, curve 1 (as indicated by the arrow) and curve 2]. Consequently, the two lower GADD45 dilutions were eliminated from our analysis because their Cₚ values were nonlinear.

A least-squares fit using a straight-line model was determined from a dynamic range of the remaining four 10-fold dilutions (Fig. 3A, curves 3–6) of the GADD45 calibrator (data not shown). The slope of −3.270 (SE, 0.059) represented the mean of three repeated assays with a derived PCR efficiency of 102% (3.89%). Statistically, the slope of −3.270 was not different from the value −log₂(10) = −3.322. The fit of these data, therefore, was made with the slope fixed at −3.32, producing the fitted line of ΔCₚ = −log₂(g) + 35.15 (0.062). GADD45 detection was quantitative over a broad dynamic range in the quadruplex QRT-PCR assay, without loss of linearity for the GADD45 target.

Discussion

Transferring to the smaller iCycler iQ platform achieved both portable biodosimetry capability and the ability to perform cost-effective, real-time detection of several gene targets in a single well of a 96-well plate format. This study demonstrated quantitative and simultaneous amplification of three targets of low abundance under primer-limiting conditions of the 18S rRNA reference target.

Vastly different concentrations of target mRNAs, or the cDNA, can cause quantification problems for qPCR detection (23). However, coamplification of several gene products with extensive differences in starting copy number can retain the initial relative proportions of these
targets in a multiplex QRT-PCR assay, provided several caveats are observed. Violations of the principle for equivalent PCR efficiencies of two sequences can produce a systematic bias in the PCR assay, giving misestimates of the initial copy numbers (13, 24). During multiplex PCR conditions, PCR products from the more abundant target will accumulate until in sufficient molar excess to inhibit the enzyme and hence interfere with amplification of lower copy number targets (24). Beyond certain ratios of starting concentrations of cDNA for two amplicons, stochastic amplification can occur, leading to either a substantial imbalance of two amplicons or no detectable amplification of the low-copy target (24).

The first substantial increase in the amount of PCR product that correlates to the initial amount of target template occurs during the exponential phase of amplification. Quantifying the PCR reaction at this time provides a significant advantage over endpoint detection, where PCR plateau-phase inhibition artifacts can be a confounder (25). A compelling example is illustrated in the quadruplicate PCR addition experiment (Fig. 3). Amplification products of higher molar abundance for both GADD45 cDNA (Fig. 3A, curves 4–7) and 18S rRNA targets (Fig. 3B) had no inhibitory effect on measured Ct values for targets with later exponential phases, i.e., BAX (Fig. 3C) and DDB-2 (Fig. 3D). Therefore, in this example, the reaction efficiencies are unaffected during the exponential phase of amplification, as indicated by no differences observed in the Ct values.

Our central caveat to higher order multiplexing is that amplification efficiency highly influences PCR reproducibility. Factors affecting amplification efficiency in the QPCR process include the efficiency and activity of the enzyme, pH, annealing temperature, cycle numbers, Mg2+/dNTP/primer concentrations, primer specificities, and cDNA input. Observed evidence also suggests that Ct values obtained from short amplicons, 70–100 bp, show greater reproducibility than those obtained from longer ones (26). If equal PCR efficiencies are maintained by use of short amplicons, disparities between high- and low-abundance targets can be resolved by supplementing the PCR mixture, as we did with the 45Mix, so that all components remain in excess.

Primer limiting of the high-abundance reference target (18S rRNA) provides a convenient, cost-effective way to ensure adequate resources during PCR amplification of low-copy gene targets for achieving optimum PCR efficiencies (20). High primer concentrations are advisable for quantitative real-time detection of less abundantly expressed gene targets but should be limited to 100–500 nM for each primer. This limiting will offset mutual interference of multiple sets of PCR primers, which can reduce the dynamic range of detection and make quantification unreliable (13, 20, 26). Primer limiting worked well in both the ABI Prism 7700 Sequence Detection System and iCycler iQ formats because both use standard cycling volumes rather than reduced volumes (<10 μL) (20).

We found no statistically significant intraassay and interassay variability for the Ct's of the 18S ribosomal subunit in optimized singleplex, duplex, triplex, or quadruplicate PCR assays (data not shown). Others accomplished the same goal by use of primer sequences with different melting temperatures and alterations in PCR annealing and extension cycling temperatures at the plateau phase to restrict primers more efficiently for the high-copy target by the alternative rapid-methods approach (13).

The results in Fig. 3 also illustrate an inherent contradiction in the conditions for primer limiting of the high-abundance target. Indeed, GADD45 target curves 6 and 7 vs BAX or DDB-2 targets exemplify a more than 15-cycle difference in Ct (a 32 000-fold difference in expression) when quantitatively amplified in the same tube. Here, 18S rRNA was primer limited but GADD45 mimic cDNA was not. These data lend credence to a contention that primer limiting is not always necessary to obtain quantitative multiplex QPCR data.

Quantification is a crucial step in QRT-PCR analysis and can mean the difference between unreliable, inconclusive results or dependable data. Quantifying nucleic acids in the presence of possible inhibitors is a challenging and common problem facing those seeking to further develop diagnostic QRT-PCR and QPCR assays. Clinical samples for QRT-PCR assays are often minute in quantity, and the RNA could become degraded or contain inhibitors. Because RNA has special handling and storage requirements, additional RNA or DNA control templates in multiplex QRT-PCR assays would be practical for applications in a military portable field laboratory (19) or a point-of-care clinical laboratory. However, simultaneous quantification of a single target with an endogenous reference target and an exogenous control necessitates a minimum requirement for a triplicate QRT-PCR assay (13). With both types of controls, reliable comparisons of RNA can be made from different isolations, at different times, from different tissues, or under various suboptimal storage conditions.

The quality of PCR reagents is as important as the quality of the RNA template to maintain analytical performance of real-time assays. Different preparations of routine PCR mixtures, formulations of reagents by different vendors, and even lots of reagents from the same vendor may influence the analytical performance of real-time PCR assays (26, 27). It is important to test reagents before use in quantitative assays when using different vendor sources or lots.

Two criteria were used as quality-control measures for PCR performance: the dynamic range and the intraassay and interassay variability of the Ct value. The 45Mix was formulated using both empirical judgment and limited matrix testing in a 96-well plate by varying the concentrations of Taq enzyme and/or nucleotides and magnesium (data not shown). We plan first to test and optimize new targets with duplex QRT-PCR assays before perform-
ing quadruplex assays in 96-well plates. Thus, reagents can be prepared, lyophilized, and stored on the shelf as quality-controlled predeveloped PCR reagent plates.

Finally, a compelling reason for higher order multiplexing is savings in cost, RNA resources, and labor time. The costs of multiplexing with real-time PCR are high, but the quadruplex assay actually was cost-effective. Reagents for total RNA isolation from whole blood and cDNA synthesis cost US $19.10 for three cDNA samples (20 ng/each), compared with US $7.36 for one cDNA sample (20 ng) for a quadruplex assay. PCR reagents and consumables cost US $6.36 for three different duplex QPCR assays on the ABI 7700. In contrast, a quadruplex QPCR assay on the iCycler iQ costs US $3.66. This cost difference reflects a 57.5% savings for both cDNA synthesis and QPCR assay. Another persuasive motive is the preservation of limited RNA samples in addition to cost savings.

In conclusion, the ability to detect multiple target sequences simultaneously in a predeveloped real-time quantitative PCR assay lends itself to development of diagnostic systems designed to measure changes in gene expression within a particular application, such as biological diagnostics for assessment from radiologic, biological, and chemical agent exposures.

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