Direct Quantitation of RNA Transcripts by Competitive Single-Tube RT-PCR and Capillary Electrophoresis


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ABSTRACT

Attempts are frequently made to semi-quantitate mRNA as a means of circumventing the laborious and time-consuming process of quantitation that is inherent in the use of competitor templates. However, semiquantitative approaches present the risk of generating non-reproducible data due to tube-to-tube variability and/or misinterpretation of quantities of product being generated during the plateau phase of PCR. Subsequently, it is difficult to compare semiquantitative data from separate experiments, and comparisons of levels of mRNA transcript from genes that amplify with different primer pairs cannot be made. Thus, reliable methods for mRNA quantitation continue to rely on the use of internal standards. In this report, we describe a strategy for dependable quantitation of low-abundance mRNA transcripts based on competitive competitive reverse transcription PCR (QC-RT-PCR) coupled to capillary electrophoresis (CE) for rapid separation and detection of products. Recommendations are included for the design of RNA competitors that can be paired with target RNA for cDNA synthesis primed with a gene-specific primer; these synthesized cDNAs are then co-amplified directly in the same tube using a single primer pair. We describe (i) a protocol for a single-tube RT-PCR that provides for cDNA synthesis and subsequent PCR amplification of target and competitor in identical reaction environments at each critical enzymatic step, (ii) a unique hot-start provision for optimizing precise and consistent PCR amplifications and (iii) a method for rapid PCR product separation, detection and quantitation by CE and laser-induced fluorescence.

INTRODUCTION

Quantitative-competitive, reverse transcription polymerase chain reaction (QC-RT-PCR) has been generally accepted as a useful tool for the quantitation of mRNA transcripts; however, numerous research efforts continue to rely on what have been referred to as semiquantitative RT-PCR techniques to attempt quantitation of gene expression. The latter methods are limited by the following: tube-to-tube variation (6), inability to identify arrival at the plateau phase of PCR (2) and the inability to compare mRNA transcripts that have been amplified with different primer pairs. Despite these limitations, there are salient reasons to simplify RT-PCR-based quantitation of mRNA transcripts. (i) The design, production and testing for efficacy of suitable competitors can be time-consuming. (ii) It can be difficult to achieve consistent RT-PCR results for low-abundance mRNAs, even with a good competitor, primarily due to the extreme sensitivity of PCR to initial-cycle kinetics and/or minute differences in delivery of the desired amounts of target and competitor templates. (iii) Final data acquisition requires separation of two PCR products, which is labor-intensive and potentially hazardous if radioisotopes are used.

Despite the technical difficulties involved in quantitative RT-PCR, the use of semiquantitative RT-PCR procedures is undermined by the risk of acquiring misrepresentative data, thus further necessitating development of a reliable method for quantitation of mRNA transcripts. To address this need, we have developed methods and procedures to quantitate relative amounts of mRNA transcripts in a reliable, workable manner. In this report, we define basic criteria for the design of internal RNA competitors that provide excellent reliability when used in single-tube RT-PCRs. Recommendations for the accurate delivery of templates are described for use with a novel single-tube RT-PCR protocol that incorporates a “hot start” for prevention of mis-priming in the critical first round of PCR cycling (9). To our knowledge, the protocols we provide are the first to provide a wax-tablet-based hot start to a single-tube RT-PCR. The use of capillary electrophoresis (CE) for rapid separation of PCR products and laser-induced fluorescence (LIF) for sensitive detection provides a safe and reliable approach for product analysis. Overall, three steps in a QC-RT-PCR have been optimized and combined: (i) gene-specific cDNA synthesis to limit the cDNA population, (ii) hot-started, single-tube RT-PCRs and (iii) CE analysis of products. This combination has resulted in a rapid and precise analytical system for quantitation of
mRNA in a competitive format. This system can be used to determine relative mRNA transcript levels that are altered following selected treatments either in vivo or in vitro. Here, we describe the application of these methods to the quantitation of perforin (a pore-forming, cytolytic protein) mRNA transcripts expressed by T lymphocytes infiltrating mouse-skin allografts.

MATERIALS AND METHODS

Target Primers

Two target primers, PA and PB, were selected to enable PCR amplification of a 198-bp product of 54% G/C content; these primers correspond to bases 665–684 and 844–863, respectively, of the mouse perforin gene (GenBank Accession No. M23182). The selected primer sites span an intron to prevent primer sites span an intron to prevent residual genomic DNA following total RNA extractions. These primers, and those described below, were synthesized by the Mayo Clinic Molecular Biology Core (Rochester, MN, USA) with retention of the 5′-protecting group (dimethoxytrityl) for subsequent purification with Oligonucleotide Purification Cartridges (OPC; PE Applied Biosystems, Foster City, CA, USA).

RNA Competitor Design and Production

A 158-bp RNA internal competitor of 53% G/C content that can be primed for cDNA synthesis with primer PB and amplified with target primers PA and PB was constructed by subcloning the PCR insert, an upstream primer consisting of a downstream primer consisting of (5′→3′) an XbaI site, primer sequence in a standard Prime, Boulder, CO, USA) and sequenced on a Model 373A Automated Sequencer (PE Applied Biosystems) to confirm insertion of the desired sequence (Mayo Molecular Biology Core).

Competitor Transcription Reactions

The competitive RNA was transcribed from 1 µg of BamHI-digested PET-Pcomp using T7 RNA polymerase and NTPs (Promega), following the suppliers recommended protocol. The newly synthesized RNA was subjected to a 5-min incubation at 75°C to inactivate T7 RNA polymerase, followed by a 30-min incubation with 20 U of RNase-free DNase (Boehringer Mannheim, Indianapolis, IN, USA) at 37°C to degrade the DNA template, and a 5-min incubation at 75°C to inactivate the DNase. The competitor RNA was separated from post-reaction components by passage through a Micro Scale Rapid Total RNA Extraction Kit (5 Prime → 3 Prime, Boulder, CO, USA) according to the manufacturer’s instructions (omitting the guanidium isothiocyanate step), aliquoted and stored at -70°C. The effective elimination of competitor DNA template was verified by RT-PCR amplification without reverse transcriptase. Before use in experiments, the RNA was quantitated by absorbance at 260 nm (A₂₆₀), estimated by the average of triplicate spectrophotometric readings and diluted in Molecular Biology Grade Ultrapure Water (5 Prime → 3 Prime) to appropriate concentrations.

Total Target RNA Extraction

Extractions of total RNA were made with a Micro Scale Rapid Total RNA Extraction Kit. The sources for RNA were mouse orthotopic tail skin grafts (2 mm × 5 mm) transplanted to histoincompatible recipients according to the previously described method (1). C57BL/6 recipients of primary grafts from BALB.B donors received second-set allografts from B10.129 (21 M) donors. Second-set grafts were harvested one day before expected rejection and RNA was extracted. RNA pellets were redissolved in RNase-free water, and aliquots were stored at -70°C. At the time of the experiment, triplicate A₂₆₀ readings were averaged, and the appropriate dilutions were made with Molecular Biology Grade Ultrapure Water.

RNA Storage and Handling

Standard procedures for handling and storing RNA were rigorously followed. All reagents prepared in the laboratory, and all RNA dilutions, were made with Molecular Biology Grade Ultrapure Water. Both total RNA samples and competitor RNA were stored at -70°C in aliquots, and a single aliquot of each was thawed, quantified and used at the time of the experiment. Remaining RNA was either discarded (competitor RNA) or reserved for other purposes (target RNA).

To promote complete and homogeneous solution of RNAs, RNA samples were first pipetted up and down 40–50 times and allowed to incubate at room temperature for 30 min before aliquoting for spectrophotometric readings.

RT-PCR Protocol

The first-strand cDNA synthesis for both the target and the competitor was primed with the gene-specific primer PB. A cDNA synthesis mixture for a final reaction volume of 20 µL/tube was prepared as a master mixture and aliquoted into a series (usually 5–9) of 0.5-mL PCR tubes (PE Applied Biosystems). The final reaction concentrations were 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂ and 0.25 mM each dNTP (Promega); 20 pmol of primer PB and 0.5 U of PRIME RNase INHIBITOR™ (5 Prime → 3 Prime) were also added. A constant amount of specimen-derived total RNA, followed by an incrementally increasing number of copies of RNA competitor, were then added to the tubes. Each template was added to its designated tube in the maximum allowable volume with TRIPLE CHECK® calibrated, aerosol-resistant pipet tips (Bio 101, La Jolla, CA, USA). As a final step, four units of Moloney murine leukemia virus Reverse Transcriptase (M-MLV RT; Life Technologies, Gaithersburg, MD, USA) were added to each tube to pro-
with CsOH. Hydroxyethyl cellulose (HEC; Aldrich Chemical, Milwaukee, WI, USA) was added to the buffer to 1% (wt/vol) as a sieving matrix. The buffer was then filtered through a 0.8-µm acetate filter (Nalgene®; Nalge Nunc International, Rochester, NY, USA) to avoid complexes that may foul the capillary. Immediately before use, 1 µM YO-PRO™-1 (Molecular Probes, Eugene, OR, USA) was added as a fluorescent intercalator. Sample injection involved (i) a 3-s × 3.5-kV electrokinetic pre-injection with water, (ii) a 20-s × 2-kV electrokinetic sample injection and (iii) a 3-s water post-injection at 0.5 psi (5.4 nL vol) (7). The separation was carried out at 7.5 kV (277 V/cm; 9 µA) for 10 min with the inlet functioning as the cathode and the outlet functioning as the anode (reversed polarity). The capillary temperature was thermostatically maintained at 20°C. The capillary was flushed with fresh (non-electrophoresed) separation buffer for 4 min (2 min pre- and 2 min post-electrophoresis) between electrophoresis of samples. Capillary regeneration was accomplished by flushing the capillary with methanol for 20 min and then equilibrating with separation buffer for an additional 20 min at the start of the day. A HaeIII digest of pBR322 (Boehringer Mannheim) was diluted 1:100 in water and used as a DNA standard to gauge the performance of the capillary at the start of each day’s experiments.

**Data Processing**

Following CE, peak areas were quantified by Gold System Data Acquisition Software (Version 8.1; Beckman Instruments). The ratio of the integrated peak areas was used to calculate the relative amounts of target and internal competitor products in each tube. To compensate for different levels of YO-PRO-1 intercalation that are dependent on the lengths of the PCR products, the competitor peak area was multiplied by the ratio of the number of target base pairs/number of competitor base pairs. The log of the ratio of the adjusted competitor peak area/target peak area was plotted on the ordinate axis, and the log of the competitor copy number was plotted on the abscissa. This produced a linear plot, and the point zero on the ordinate axis (the log value at the point where target and internal control were present in a 1:1 molar ratio) was interpolated to yield the approximate number of the copies of competitor and, thus, the approximate number of copies of target mRNA/ng total RNA (12).

**RESULTS AND DISCUSSION**

A reliable quantitative system for analysis of RNA transcripts is best accomplished by producing an RNA internal competitor that can be primed for cDNA synthesis and subsequently amplified with the same primers, and with a similar efficiency, as the target of interest. It has been observed by others that the primer sequences have the greatest effect on amplification efficiency, provided the sizes of the amplified sequences are under 1 kb (10,14). It has also been shown that the amplification efficiency of an RNA standard can be the same as that of its corresponding target even though the sequence between the shared primer binding sites is completely different (14). However, while it may not be necessary to do so, we select competitors and targets with similar G/C content ratios and distribution to help avoid differences in transcription efficiency that could occur as a result of differences in secondary structure and to avoid differences in denaturation efficiencies in the PCR (8). Intuitively, shorter target and competitor products have less opportunity for secondary structure differences than longer products, and it is well documented that decreasing the length of double-stranded (ds)DNA lowers its melting point (8). Thus, we prefer competitor and target products that are 200 bp or less, even though longer products (300–400 bp) have been successfully used for quantitative, competitive PCR (4). The sharpness and singularity of peaks observed on CE electropherograms serve as indicators that both products are being synthesized to completion at the PCR level, since incomplete products would be manifested either in variable slopes on the “left” side of the peaks or in additional peaks.

However, despite precautions taken in design of competitors, final confirmation of matched amplification kinet-
ics for target and competitor pairs must be determined experimentally. It is generally considered that any target/competitor pair will eventually reach a limiting value as template ratios drift from a 1:1 molar ratio, beyond which the kinetics of amplification are no longer similar enough to yield reliable data (8). Fortunately, for quantitative purposes, it is the 1:1 molar ratio of templates that needs to be ascertained, thus the points on either side of the 1:1 molar ratio (in a workable range, i.e., ca. 50-fold–100-fold) are the critical points that must be experimentally demonstrated to be reliable.

Identical micro-environments can be provided throughout the entire procedure by using a single tube for (i) the cDNA synthesis from both target and competitor templates with the same gene-specific primer and (ii) the subsequent PCR. The use of a single gene-specific primer to prime first-strand cDNA synthesis of both the target and the competitor in the same mixture vastly reduces background cDNA synthesis as the transcripts of interest are preferentially transcribed. The reduction of background cDNA synthesis enhances the ability to amplify low-abundance transcripts in the PCR stage. In addition, the use of total RNA as the target source in single-tube RT-PCR reduces the introduction of pre-amplification error by minimizing template processing, pre-reaction quantitation and transfer steps. To facilitate unbiased amplification of both target and competitor during the critical initial cycle of the PCR, we have incorporated the use of wax tablets to provide a hot start for single-tube RT-PCRs. Without a hot start, primers and Taq DNA polymerase have an opportunity to initiate synthesis of nonspecific templates while reactions are still at room temperature. These initial nonspecific products would be amplified throughout the PCR and confound the separation and analysis of final products by CE. The consistency and precision of the hot-started RT-PCRs presented here

![Electropherograms of perforin internal competitor and experimental target DNA.](image)

**Figure 1.** Electropherograms of perforin internal competitor and experimental target DNA. (A) Whole-length, 0–10.0 min, electropherogram of QC-RT-PCR competitor and target products. (B–E) Electropherogram interval, 7–9 min (the area of interest), of successive analyses with increasing competitor copy number (Panel C is 7–9 min of Panel A). Separation conditions as described in text. (RFU = relative fluorescence units.)
The RNA competitor for perforin described in this report was designed to meet the following basic requirements for an effective competitor: (i) incorporation of target primer sites on the 5′ and 3′ ends of vector sequence (target primer sites should be selected to span at least one intron, if possible); (ii) choice of lengths of target and competitor of 200 bp or less, and within 15–50 bp of one another, to promote unbiased amplification in the PCR while allowing for subsequent CE separation; and (iii) G/C content and distribution as similar as possible to those of the target. Internal competitors that are used in a single-tube, hot start RT-PCR protocol with target total RNA must also meet basic requirements for reproducibility and reliability; replicates of the same experiment should provide comparable estimations of copy number of target mRNA per ng of total RNA, and the target and competitor should amplify with similar kinetics. Replicate experiments provide an assessment of base-line error and attest to their efficacy.

The separation exhibited excellent base-pair resolution of the peaks, which were incrementally increased, focusing on the area of the electrophoretic separation (total analysis time of 10 min). Unincorporated DNA primers from the RT-PCRs are evident at approximately 5.25 min, while the competitor (158 bp) and target (198 bp) DNA products are evident at 7.75 and 8.4 min, respectively. The separation exhibited excellent baseline resolution of the peaks, which were then easily quantified by the data acquisition software. The total analysis time was approximately 12 min per sample, including 10 min for electrophoresis and 2 min to replace the separation matrix and regenerate the capillary. Sample analysis times could be shortened by decreasing the ionic strength of the separation buffer to 0.5× TBE and/or decreasing the concentration of HEC from 1% to 0.6% and separating the PCR products at a higher field strength (data not shown). However, a loss in electrophoretic separation could also result from differential migration between target and competitor DNA lengths difference of 40 bp.

Three replicate assays of QC-RT-PCR amplification of perforin transcripts were carried out to determine the variance in the estimated copy number generated by independent amplifications. The templates used in these experiments were the RNA competitor for perforin and target RNA extracted from a skin allograft that was in the process of rejection. The amount of target total RNA was held constant at 50 ng per reaction tube in each experiment, while the competitor for perforin was increased over a range from 10^4–10^5 copies in four reactions. Figure 1 shows electropherograms resulting from CE analysis for the first of these assays. Figure 1A shows the electropherogram for an entire electrophoretic separation (total analysis time of 10 min). Unincorporated DNA primers from the RT-PCRs are evident at approximately 5.25 min, while the competitor (158 bp) and target (198 bp) DNA products are evident at 7.75 and 8.4 min, respectively. The separation exhibited excellent baseline resolution of the peaks, which were then easily quantified by the data acquisition software. The total analysis time was approximately 12 min per sample, including 10 min for electrophoresis and 2 min to replace the separation matrix and regenerate the capillary. Sample analysis times could be shortened by decreasing the ionic strength of the separation buffer to 0.5× TBE and/or decreasing the concentration of HEC from 1% to 0.6% and separating the PCR products at a higher field strength (data not shown). However, a loss in electrophoretic separation could also result from differential migration between target and competitor DNA lengths difference of 40 bp.
ng of total RNA was calculated to be 1350, 1420 and 1200 with a mean ±SD of 1323 ± 92 (CV 7.0%) (Figure 2). The observed linearity was deemed acceptable with \( r^2 \) values of 0.997, 0.988 and 0.995, respectively, for each of the replicates. This SD and percent SD compare favorably to those previously reported for CE-based quantitation of PCR products (11). These previous experiments utilized homogeneous tissue culture cell lines as sources of RNA; it was shown that the percent standard errors in those experiments were inversely proportional to copy numbers per cell and ranged from 1.2%–12.5% (11). Note that the target RNA in the experiments reported in this communication is expressed by infiltrating T cells that comprise only a minority of the cells in skin allografts. Assuming that quantifications in this and the previous report (11) produced comparable results in terms of copy numbers per cell, the variability reported here is comparable to that previously reported.

A clear advantage in the use of CE-derived peak areas to estimate competitor/target ratios in these experiments is the quantitation of both products from a single reaction tube, where any differences in sampling volume will equally affect both competitor and target products. However, in semiquantitative methods, it is necessary to determine the total amount of a single PCR product, and such a determination is strongly affected by potential errors in handling independent samples. Also, an advantage associated with plotting a series of competitor/target ratios is the ability to determine whether proper amplification occurred within each reaction tube. An experimental error made in one of the reactions in a QC-RT-PCR series will manifest itself as a log ratio that deviates from the linear relationship calculated from all other data points. Such error could only be observed in semiquantitative procedures by means of very careful titration. Furthermore, the use of an internal competitor obviates the need to determine whether reactions have reached the plateau phase of PCR (3,11,15).

The observed discrepancy among replicate analyses is likely the result of slight differences in template dilutions and/or deliveries to the reaction mixture. When the experimental end point is a peak area ratio of target and competitor products following a dual template-based amplification, slight differences in quantitation and/or handling of these templates are exacerbated dramatically in the final result. It is, therefore, critical that steps be taken to minimize the discrepancies that can occur in template quantitation and handling. We have found that the use of an average of three spectrophotometric readings (A\(_{260}\)) for quantitation of both RNA templates is helpful, provided that sufficient template is available. It is also important that all spectrophotometric readings be taken just before RT-PCR since sample evaporation during storage will affect the final RNA concentration. Additionally, calibrated pipets should be used for preparation of template dilutions, and calibrated pipet tips should be used for transferring template volumes of 10 µL or less (see Materials and Methods). All template deliveries should be made in as large a volume as possible. The care necessary in template quantitation and delivery underscores the importance of using a one-tube approach for both the cDNA synthesis step and subsequent PCR, thereby minimizing loss associated with numerous template transfers.

Differences in amount of starting cDNA have been shown to have a significant impact on reproducibility in semiquantitative RT-PCR assays (13). Hence, as discussed above, it is imperative to experimentally demonstrate similar amplification kinetics when using varying ratios of members of a selected target/competitor pair. Three additional perforin QC-RT-PCR assays were performed varying the initial concentration of target RNA and/or ranges of competitor RNA. Separate perforin assays were performed with 60 and 30 ng of total target RNA from a second skin allograft while keeping the internal competitor range from 7.8 \times 10^3 to 2.5 \times 10^5 copies (Figure 3, A and B, respectively). A third assay (Figure 3C) was performed with 72 ng of target RNA and a range of competitor increasing from 1.56 \times 10^3 to 5 \times 10^5 copies. The approximate copy numbers of perforin mRNA transcripts per ng of total RNA calculated from this data (Figure

![Figure 3](image-url)

Figure 3. Linear graphs resulting from three QC-RT-PCR assays of perforin mRNA levels with varying target and competitor concentrations. Target total RNA extracted from a mouse-tail allograft was held constant at 60, 30 and 72 ng in experiments A, B and C, respectively, and varied competitor ranges were used. The respective calculated approximate copy numbers of perforin mRNA transcript/ng of total RNA are 1390, 1560 and 1420.
3, A, B and C) are 1390, 1560 and 1420, respectively, with a mean ±SD of 1457 ± 74 (CV 5.1%). Linearity of the results was acceptable as indicated by the respective r² values of 0.996, 0.997 and 0.989 for Panels A, B and C.

The appropriate range of competitor dilutions needed for a successful assay must be determined empirically. It is useful to perform an initial pilot over a wide range of internal standard copy numbers to first estimate the copy number of a particular transcript. Using this information, a more restricted range of competitor copy number with smaller increments can be defined, tested for reproducibility and implemented. We have observed that reproducible CE data can be obtained within a somewhat limited range by use of the integrated peak areas on either side of the point where target and competitor RNAs are present in a 1:1 molar ratio. This limitation of CE analysis is likely in addition to the target/template ratio limitations described above and has been noted by others (5,11). The reduction of number of samples in a given series that need to be amplified and quantitated is, of course, desirable. Tube reduction is particularly important when quantities of the sample RNA are limited, and it provides the added bonus of reducing experimental time and expense. The number of preliminary investigations required is generally not extensive, as one preliminary run normally suffices for the subsequent assay of many samples in the same or similar categories.

In the course of QC-RT-PCR experiments, a significant change in transcription levels may first be observed when one fails to bracket the 1:1 molar ratio of target and competitor. In this event, it is a simple matter to determine the adjustments that need to be made on the basis of the first experiment. One “walks” through transitions in transcription levels in a manner similar to chromosome walking, using the information gained from one experiment to advance to the next. Finally, while separation of products on an agarose gel following a QC-RT-PCR is not necessary if CE analysis is used, gel electrophoresis makes it possible to select only the samples on either side of the cross-over point for CE analysis.

In addition to perforin, we have designed and tested RNA competitors according to the guidelines discussed above for mouse tumor necrosis factor-α, interleukin-2 and Fas-ligand. These competitors have also proven to be reliable by meeting the amplification criteria described in this report for perforin. We routinely obtain r² values in the 0.995–1.00 range and consider that amplifications resulting in rounded values of less than 0.990 must be repeated. The analyses in which the initial concentrations of templates were varied without changing the estimated copy number are stringent and will only be successful when both templates (target and competitor) amplify with similar kinetics. While the predominant use for quantitation of mRNA transcripts is the acquisition of relative values, competitors that pass the kinetic tests described here will also allow the estimation of absolute values. Thus, reliable comparisons of transcript levels among different genes that amplify with different primers can be made.

Future research will explore applicability of the QC-RT-PCR system outlined here for multiplex quantitative PCR. This will enable the analysis of two or three genes of interest simultaneously and/or permit direct comparison of transcription of genes of interest to the transcription of a housekeeping gene. The ability of CE to separate products that differ by only 15 bp offers a distinct advantage over agarose gel electrophoresis for separation of multiple products in a multiplex analysis.

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