Quantification of mRNA Using Competitive RT-PCR with Standard-Curve Methodology


ABSTRACT

The use of reverse transcription polymerase chain reaction (RT-PCR) with internal RNA competitive standards (competitors) provides a means for measuring absolute amounts of mRNA transcripts in small numbers of cells. Most quantitative competitive (QC)-RT-PCR methods require analysis of multiple reactions to determine the equimolar point of the products produced from mRNA vs. competitor RNA. Herein, we present a method to produce one standard curve for each assay with all unknown samples compared directly to this standard curve. The standard curve is produced with differing amounts of standard RNA (native) amplified with one constant amount of competitor RNA. The number of transcripts in an unknown sample mRNA can be directly determined by RT-PCR of the sample with the same amount of competitor RNA and comparison of the ratio of products to the standard curve. This method has been used to quantify expression of multiple gene products from cultured cells or limited amounts of tissues and was found to be straightforward, sensitive, repeatable and quantitative. A complete protocol for producing standard and competitor RNA, subsequent QC-RT-PCR steps, and the evaluation of accuracy, sensitivity and precision for this assay are described using bovine prostaglandin F_2a receptor mRNA as an example.

INTRODUCTION

Many different techniques have been utilized to evaluate concentrations of specific mRNA; however, most of these procedures yield results that are either nonquantitative or semiquantitative. Recently, quantitative competitive reverse transcription polymerase chain reaction (QC-RT-PCR) has been used to quantify absolute amounts of mRNA (4,12,17). Most QC-RT-PCR applications use serial dilutions of competitor with a constant amount of unknown mRNA in the RT-PCR (12). This procedure requires 3–5 reactions for each sample in order to plot the ratio of products against the amount of added competitor to determine the equimolar amount for each unknown sample. Obviously, the procedure can be fairly labor-intensive, particularly if multiple samples and gene products are being analyzed. Recently, a procedure has been introduced that allows a quantitative single-tube RT-PCR procedure (5); however, this procedure still relies on comparison of results to a quantified competitor. Comparison to a competitor that differs in any way from the native mRNA provides the possibility that any amplification differences between native and competitor will produce nonquantitative results (10). The present manuscript reports a procedure that uses a competitor RNA to account for variation between PCR assay tubes but with final quantitative results obtained by comparison to a standard curve that utilizes a native RNA with sequence identity to the mRNA of interest.

A variety of different detection methods have been used to quantify RT-PCR products (1,5,9,11,12,14). Solid-phase assays have used immobilized probe or PCR products with subsequent use of fluorescent-, biotin- or digoxigenin-labeled PCR products or probes for detection (1,7,9,12). The denaturation of immobilized DNA, prehybridization and/or hybridization followed by multiple washing steps and subsequent color development or fluorescence excitation are not only labor-intensive but also time-consuming. In addition, to distinguish the products of native and competitor, two kinds of labeling probes or primers are necessary. Anion-exchange HPLC and UV absorbance have also been used successfully to separate and quantitatively examine QC-RT-PCR products (5,12). Separation of products by gel electrophoresis followed by quantification of fluorescence-labeled nucleic acids with an automated DNA sequencer has also shown considerable promise (11,12). Unfortunately, the expense of this technology has limited utilization.

Herein, we report a standard-curve method of QC-RT-PCR that allows us to differentiate PCR products on an acrylamide gel and directly quantify each sample by comparison to a standard curve. The procedure minimizes the amounts of mRNA needed because multiple reactions at different concentrations of competitor RNA are not needed for each unknown sample. We have used this technique to quantify 3 different mRNA transcripts (prostaglandin G/H synthase-2 [PGHS-2], prostaglandin F_2a receptor [FP receptor] and prostaglandin E_2 receptor EP_3 subtype [EP_3 receptor]) in mRNA samples isolated from less than 25 000 bovine luteal cells or cultured granulosa cells (15). Here we report the standard-curve QC-RT-PCR method using bovine FP receptor as an example.

MATERIALS AND METHODS

Synthesis of Standard (Native) and Calibrate (Competitive) RNA

Bovine corpus luteum mRNA was reverse-transcribed using oligo(dT)_{12-18} (Promega, Madison, WI, USA) and SuperScript™ M-MLV RNase H- reverse transcriptase (Life Technologies, Gaithersburg, MD, USA) by standard methods (6). One-tenth of RT product was PCR-amplified using bovine FP specific primers (A0034 and A0035), which were designed according to the published complementary DNA sequence (13). A 288-bp PCR fragment was obtained and cloned into BlueScript® SK (Stratagene, La Jolla, CA, USA) and named bPGF1. A 576-bp fragment from this plasmid (containing 288-bp FP sequence) was digested with HinfI (New England Biolabs, Beverly, MA, USA) and re-ligated with T4 DNA ligase (Promega). The ligation of two fragments retaining consensus primer sequences resulted in a 55-bp deletion of bPGF1 and was subcloned into Bluescript SK and termed bPGF1Δ55. This fragment was then sequenced using PRISM™ Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer/Applied Biosystems Division, Foster City, CA, USA) and was found to be 100% identical to the published cDNA sequence (13). Plasmids bPGF1 and bPGF1Δ55 were linearized by BamHI (Promega) and transcribed in vitro using T7 RNA Polymerase (Promega) to yield a 399-bp RNA with 288-bp FP receptor sequence (native) and a 344-bp RNA with 233-bp FP receptor se-
quence (competitor), respectively. Both native and competitor RNA were treated with RNase-free DNase I (Boehringer Mannheim, Indianapolis, IN, USA) and precipitated twice with 1/10 vol of 2 M sodium acetate (pH 4.0) and 2 vol of 100% ethanol. RNA concentration was determined by spectrophotometry. All RNA was tested to be free of DNA contamination by RT-PCR without reverse transcriptase and was then aliquoted and stored at -80°C until used.

Isolation of mRNA Directly from Tissues or Cultured Granulosa Cells

Whole tissue or cultured cells were solubilized in homogenization buffer, homogenized, centrifuged at 16 000 × g for 2 min at 4°C to remove cellular debris and had mRNA isolated using oligo(dT)₂₅ magnetic beads (Dynal, Lake Success, NY, USA) according to the manufacturer’s protocol (3). Isolated mRNA was aliquoted and stored in a -80°C freezer until used.

QC-RTPCR

A constant amount of competitor RNA was added into 1× RT master mix (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂, pH 8.3, 10 mM dithiothreitol [DTT], 100 pmol random primer, 4 mM dNTPs and 50 U SuperScript RNase H⁻ reverse transcriptase). This mixture was then dispensed into 0.2-mL thin-wall PCR tubes (USA Scientific Plastics, Ocala, FL, USA), and known amounts of native RNA in 2 µL of diethylpyrocarbonate (DEPC)-treated water or 2 µL of unknown mRNA samples were added individually to each tube. The final volume of RT mixture was 20 µL, and reverse transcription was performed at 42°C for 60 min followed by heating to 95°C for 10 min and quick-chilled to 4°C in a programmable thermal cycler (PTC-100™, MJ Research, Watertown, MA, USA). Ten microliters of RT products were mixed with 20 µL of 1× PCR buffer (10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, 0.1 mg/mL gelatin, pH 8.3, 0.2 mM dNTPs, 0.5 U Taq DNA Polymerase [Boehringer Mannheim] and 0.4 µM of primers) and subjected to 25, 28, 31 or 34 cycles of amplification (30 s denaturation at 95°C, 30 s annealing at 57°C and 30 s elongation at 72°C) followed by final elongation at 72°C for 5 min. Ten microliters of PCR products were directly separated on 5% acrylamide gel with 1× TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) at 110 V for 40 min using Mini-PROTEAN® II Electrophoresis System (Bio-Rad, Hercules, CA, USA). The gel was then stained with ethidium bromide and placed on an ultraviolet (UV) illuminator equipped with a camera connected to a Macintosh® computer (Apple
RESULTS AND DISCUSSION

Figure 1A schematically shows the bovine FP receptor cDNA (native) partial sequence and internally deleted DNA (competitor). The sequence of competitor, bPGF1Δ55, is identical to the sequence of native, bPGF1, except for the removal of a 55-bp HinfI fragment. Both native and competitor had consensus primer sequences (indicated as hatched boxes) and can be amplified by a single primer pair (Figure 1B). In this study, we chose to produce our competitor by restriction enzyme digestion and re-ligation of the products. Other PCR-based methods for the production of competitors have been described (6). The PCR products were directly quantified after separation on 5% acrylamide gel. The logarithmic ratio of native to competitor was plotted against the logarithmic initial amounts of native to produce the standard curve. The coefficient of variation (CV) can be calculated within an assay and between assays as a measure of the precision of the assay. In our assay with FP receptor, the intra- and inter-assay CVs were 8.7% and 9.9% (mean of high, medium and low mRNA samples evaluated in triplicate on 4 occasions). As expected, the lowest intra- and inter-assay CVs were found near the point of equimolar ratio of mRNA to competitor with larger CVs found when either mRNA or competitor were in excess. Thus, precision of standard-curve QC-RT-PCR assays can and should be evaluated by providing quality-control CVs for each assay.

There are numerous amplification-based procedures for relative or absolute quantification of mRNA. Excellent precision (CVs ≤10%) has been obtained with some of these assays including: single-tube QC-RT-PCR with HPLC (5), NASBA with electrochemiluminescence (16) and equimolar QC-RT-PCR (12). Many of these procedures have provided detailed evaluations of precision and sensitivities of assays; however, most studies lack a critical analysis of absolute accuracy or comparison of amplification efficiency between competitor and native mRNA. The nucleic acid-based sequence amplification (NASBA) procedure for human immunodeficiency virus type 1 (HIV-1) has been found to be absolutely quantitative in comparison with a “gold standard” of HIV particles quantified by electron microscopy (16).

A prerequisite for equimolar QC-RT-PCR requires similar reverse transcription and PCR efficiencies between samples and competitor RNA. Amplification efficiency differences of greater than 10-fold have been found between competitors of different length (10).
Use of competitors with different amplification efficiencies would obviously result in substantial variation in the calculated equivalence points, unless corrections are made (10). The present assay using standard-curve methodology did not require that the amplification efficiency of native and competitor RNA be equivalent. This was because the absolute amount of competitor RNA was not used in any of the calculated values. The slope of the standard curve will be related to the amplification efficiency of the competitor vs. native (S.-J. Tsai and M.C. Wiltbank, unpublished). Obviously, the amount of competitor must be constant between all wells, and this is assured by adding the competitor to the RT master mixture. In addition, accurate quantification requires that the amplification efficiency of the native RNA used in the standard curve and the unknown mRNA must be similar. In this study, no direct comparison of amplification efficiency was made; however, both amplified products are of identical size and nucleotide sequence, making it likely that amplification efficiency is similar (10). Addition of known amounts of standard RNA to a background of mRNA (0.1 and 1.0 ng liver mRNA) did not change the slope or intercept of the standard curve (data not shown), providing evidence that nonspecific mRNA does not alter amplification efficiency. It cannot be ruled out that differences in size of native mRNA vs. standard RNA could produce differences in RT efficiency and thus alter the absolute, but not relative, accuracy of final results. As with an ELISA or RIA, the similarity of standard and unknown samples can be evaluated by comparing the parallelism of standard curves as a measure of assay accuracy. Figure 2 shows that parallel lines are produced using either the standard preparation of RNA from the bPGF1 plasmid or using mRNA isolated from bovine corpus luteum (50% serial dilutions of mRNA or bPGF1 RNA). It is apparent that a standard, quantified pool of mRNA could be used for the standard curve in place of the bPGF1 RNA.

Figures 2 and 3 also show that increased sensitivity can be obtained by increasing the number of PCR cycles. Amounts of competitor and standard RNA are decreased to maintain quantifiable band intensities. Acceptable standard curves were obtained between 25 and 34 cycles of PCR (Figure 3). It is important that amounts of standard RNA, competitor RNA, unknown mRNA and PCR cycle number are carefully selected in a particular assay to give bands with an intensity that can be accurately quantified by the imaging equipment. It is difficult to provide a precise measure of sensitivity for these assays; however, if the ratio at -1 log unit is arbitrarily selected as a measure of sensitivity, then the sensitivity increases from about 1 million molecules at 25 cycles to 3360 molecules at 34 cycles. This sensitivity is similar to what has been reported with NASBA (16). The present study was not designed to determine the limit of sensitivity for this assay, but theoretically this limit could be only a few molecules with sufficient cycles of PCR amplification.

Heteroduplex formation can occur at higher numbers of PCR cycles (14), and this can seriously confound results with the equimolar QC-RT-PCR method (10). In our assays, we have attempted to eliminate overamplification by minimizing the PCR cycle number or the amount of starting RNA, and this

Figure 3. Sensitivity of QC-RT-PCR can be increased by using more PCR cycles. More than 4 orders of magnitude of bPGF1 (256–0.004 amol) in 50% serial dilutions were quantified using 25 amol (25 cycles, open circle), 4 amol (28 cycles, filled circle), 0.5 amol (31 cycles, open square) and 0.05 amol (34 cycles, filled square) of bPGF1Δ55. Note that Y-axis values were justified with a constant, 0.809 (233/288), to reflect actual band intensity, since longer DNA tends to intercalate more ethidium bromide. This is required in calculating results from equimolar QC-RT-PCR procedure but is not necessary in standard-curve QC-RT-PCR, since constant is canceled out when comparing the unknown to the standard curve.
We have calculated the amounts of FP receptor mRNA in 5 different cell preparations using standard-curve QC-RT-PCR and equimolar QC-RT-PCR. The calculated values for these samples were similar (statistically) with a correlation of 94% between the 2 methods. Equimolar QC-RT-PCR required five times more mRNA sample, more lanes of gel space if more than 2 samples were being analyzed and greater analysis time because the equivalence point was determined for each sample. In addition, the discrepancy of amplification efficiencies of native vs. competitor products could influence the outcome in equimolar QC-RT-PCR (10). Thus, standard-curve QC-RT-PCR should yield similar or more accurate results as equimolar QC-RT-PCR with need for less mRNA sample, fewer gels and less analysis time.

In conclusion, we have reported a simplified method for QC-RT-PCR using a standard-curve methodology. The whole procedure from reverse transcription, PCR and image analysis is straightforward and can be done in a normal working day. Multiple samples can be analyzed by this procedure without a great deal of extra effort. The procedure is extremely sensitive and can be used to evaluate gene expression from limited amounts of experimental material. The sensitivity can be increased by simply increasing PCR cycles, thus eliminating the need for radioisotopes. Moreover, the results can be expressed in a biologically meaningful way as specific transcripts per cell. The procedure was found to be highly repeatable and validity of results could be tested by procedures that are used with ELISAs or RIAs. Most standard laboratories should be able to adopt this technology, since the only specialized equipment that is required is a densitometer equipped with image-analysis software.

REFERENCES


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