

Reproducibility in the Quantification of mRNA Levels by RT-PCR-ELISA and RT Competitive-PCR-ELISA

BioTechniques 24:652-658 (April 1998)

L.L. Hall, G.R. Bicknell, L. Primrose, J.H. Pringle, J.A. Shaw and P.N. Furness
University of Leicester,
Leicester, England, UK

ABSTRACT

The use of reverse transcription (RT) PCR for relative quantitation of gene transcripts relies on the reproducibility of the individual RT, PCR and product measurement steps. Semi-competitive RT-PCR (RT-cPCR) uses an internal competitor template in the PCR step to improve quantitation. We have surveyed the reproducibility of RT, PCR, RT-cPCR and measurement, amplifying the glyceraldehyde-3-phosphate dehydrogenase "housekeeping" gene from isolated renal glomeruli. We used an enzyme-linked immunosorbent assay (ELISA) to quantify PCR products. We also report our PCR-based method for constructing a competitor DNA identifiable independently of the native product. Our results show that the entire RT-PCR and ELISA process had a standard deviation (SD) of less than 10% (n = 10). This compared to an SD of less than 13% (n = 10) in PCR and ELISA. The SD for ELISA alone was less than 11% (n = 10). RT-cPCR quantitation gave an SD of approximately 15% (n = 10). These results support the use of standard RT-PCR for the relative quantitation of mRNA. RT-cPCR is also suited to relative quantitation, but it is also independent of the amplification saturation curve and permits the identification of differences in cellularity between samples.

INTRODUCTION

Quantification of levels of mRNA is important in the study of gene expression. Reverse transcription polymerase chain reaction (RT-PCR) is widely used in the investigation of low-copy-number mRNAs and all mRNAs from small samples (3,5,7,9). The sensitivity of RT-PCR has enabled the analysis of microdissected human tissue samples, providing some degree of localization. What remains to be investigated is the reproducibility of the RT-PCR assay and the sources of variation.

RT-PCR quantitation has been reported as being difficult to validate, first, due to the questionable reproducibility of the RT step (8) and second, because of the exponential increase in PCR product with each cycle, which would be expected to magnify any minute differences resulting from changes in RT efficiency (6). The use of competitive RT-PCR (cRT-PCR) can overcome these problems (4). This involves the addition of a competitive RNA (cRNA) template to the RT reaction that is subsequently amplified alongside the wild-type cDNA in the PCR. However, the production of the cRNA template can be laborious and must be repeated for each gene of interest. In addition, the competitor RNA needs to be added to the RNA sample before the RT reaction, limiting the analysis to the study of a single gene from a single RNA sample. This may be partially resolved by using a multi-gene competitor RNA (10), but since it may need to be added to the RNA at more than one concentration, it may not

be appropriate where there is a limited amount of mRNA from microdissected tissue samples.

RT-competitive PCR (RT-cPCR) requires the addition of an internal competitive DNA standard to the PCR after completion of the RT step (8). This method permits the PCR amplification and analysis of multiple genes from a single RT reaction, and it overcomes any problems with the stability of an RNA competitor. However, it assumes constant efficiency of the RT reaction. Although absolute quantitation is not achieved, relative quantitation is improved, as the technique is independent of the saturation curve characteristic of PCR. Moreover, if applied to a "housekeeping" gene, it permits the estimation of sample "size" or cellularity.

To select the most appropriate technique for mRNA measurement in any given situation, one must have information about the variation introduced by each of the steps involved. The reproducibility of RT-PCR has been widely assumed but not adequately tested. We have previously developed an RT-PCR/enzyme-linked immunosorbent assay (ELISA) strategy, based on the use of oligo(dT)-linked paramagnetic beads, to extract and quantify mRNA from extremely small tissue samples, such as a single human renal glomerulus (3). We have used this approach to investigate the reproducibility and sources of variation in the entire RT-PCR-ELISA process and separately for the PCR step and the product assay step (ELISA). We have used the expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping

Table 1. GAPDH Primer and Probe Sequences

Primer Sequences	
F	AGA ACA TCA TCC CTG CCT C
R	GCC AAA TTC GTT GTC ATA CC
F'	<u>CTC TAG AGT CGA CCT GCA GGC ATG CAC CTT TGA CG</u>
R'	<u>GCA TGC CTG CAG GTC GAC TCT AGA GTG TAG CCC AG</u>
ELISA Probe (native)	GTT GAA GTC AGA GGA GAC C
ELISA Probe (competitor)	TGC CTG CAG GTC GAC TCT AGA

Underlined sequences denote F' and R' complementarity.

gene in isolated rat glomeruli as a model system, because of the availability of rat tissue. Although there is a potential problem of amplifying GAPDH pseudogenes, the use of Dynabeads® to isolate the mRNA ensures that contamination by genomic DNA is minimal.

The introduction of a competitive DNA template (RT-cPCR) creates potential for further variation. We therefore used the same rat model to measure the reproducibility of this cPCR approach. We report a simple and quick

PCR-based method for constructing the necessary competitor DNA template.

MATERIALS AND METHODS

Isolation of Glomeruli and Extraction of mRNA

Single human glomeruli were plucked from fresh human renal biopsies by hand using ultrafine forceps under direct vision using a dissection microscope. Glomeruli were obtained in

less than 1 min after the biopsy had been taken, after the adequacy of the biopsy for diagnostic purposes had been confirmed. Glomeruli were immediately dropped into 100 µL of lysis/binding buffer (100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, pH 8.0, 1% wt/vol sodium dodecyl sulfate, 5 mM dithiothreitol).

Renal cortex from the kidneys of a freshly killed rat was diced and pressed sequentially through 50-, 100- and 200-gauge stainless steel meshes, flushing with ice-cold phosphate-buffered saline (Hanks' PBS). The rat glomeruli were trapped on the 200-gauge mesh and were flushed into a centrifuge tube and allowed to settle at 4°C. Rat glomeruli were then resuspended with 10–20 glomeruli in 100 µL of lysis/binding buffer. Human and rat mRNA was extracted and processed using oligo(dT)-linked Dynabeads (DynaL, Bromborough, England, UK), as described previously (3). Briefly, glomeruli in lysis/binding buffer were incubated with 50 µg/mL proteinase K (Boehringer Mannheim, Lewes, England, UK) for 1 h at 37°C. The lysate was centrifuged for 30 s at 10000× g, and the supernatant was mixed with oligo(dT)-linked Dynabeads. The mRNA was allowed to anneal to the Dynabeads for 10 min at room temperature. mRNA-linked Dynabeads were washed twice in a buffer containing LiDS (10 mM Tris-HCl, pH 8.0, 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS; Dynal) and three times in the same buffer but without LiDS. Dynabeads were finally resuspended in diethyl pyrocarbonate (DEPC)-treated water.

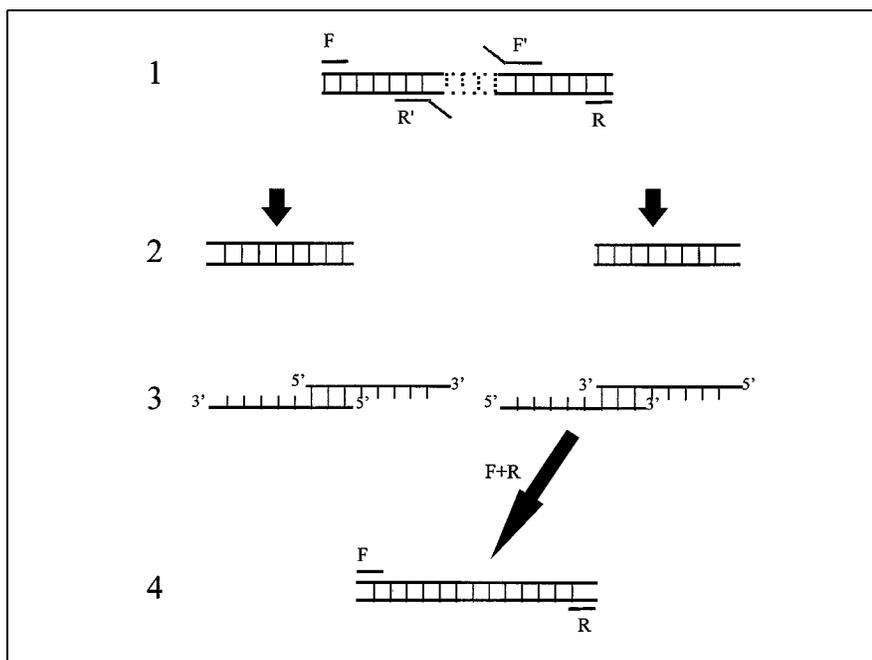


Figure 1. Schematic representation of the construction of the competitive template. 1) Native GAPDH PCR product. Dotted lines identify the region detected by the GAPDH ELISA probe. Also shown are the annealing site for the native primers F and R and the internal primers F' and R'. 2) The products of the two intermediate PCRs (F and R', F' and R) are shown. 3) When denatured, re-annealing at the sites of complementarity (identified by the gray strands) occurs, and one pairing can subsequently be extended. This is then amplified by the native GAPDH primers. 4) Competitor GAPDH PCR product. The gray region identifies the site recognized by the competitor DNA-specific ELISA probe.

Research Reports

RT-PCR

Dynabeads-linked mRNA was resuspended in reverse transcriptase buffer (Promega, Southampton, England, UK) containing 10 mM DEPC-treated dNTPs (Pharmacia Biotech, Uppsala, Sweden), 25 U RNasin® and 5 U AMV Reverse Transcriptase (both from Promega). Priming was by the oligo(dT) Dynabeads, and incubation was for 1 h at 42°C. As a control for genomic DNA contamination, a similar reaction was set up without the reverse transcriptase. Only glomerular mRNA without genomic DNA contamination was used in the variability studies.

cDNA-linked beads were washed once in Tris-EDTA and resuspended in PCR buffer [45 mM Tris, pH 8.8, 11 mM (NH₄)₂SO₄, 4.5 mM MgCl₂, 200 μM dNTPs, 110 μg/mL ultrapure BSA (Advanced Protein Products Ltd., Brierley Hill, England, UK), 6.7 mM β-mercaptoethanol, 4.4 μM EDTA, pH 8.0], containing 10 pmol forward primer and 10 pmol reverse primer. "Hot-start" PCR was performed as follows: one cycle of denaturation at 98°C for 5 min, holding at 59°C pending addition of 1 U *Taq* DNA Polymerase (Life Technologies Ltd., Paisley, Scotland, UK), and then primer extension at 72°C for 30 s; 4 cycles of denaturation at 95°C for 1 min, annealing at 59°C for 30 s and primer extension at 72°C for 30 s; 30 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 30 s and primer extension at 72°C for 30 s. We have previously verified that this number of cycles maintains the reaction in the exponential phase of amplification (data not shown). All PCR products were screened by agarose gel electrophoresis to confirm that the efficiency of each reaction was not compromised by the formation of primer-dimers.

GAPDH primers were designed to amplify both human and rat sequences. All primers and probes were designed in-house and synthesized by Life Technologies (Table 1). Forward primers were synthesized 5'-biotinylated to enable capture of forward-strand cDNA during ELISA. The primers and probe were purified to at least 98% purity by HPLC.

Construction of the Competitor DNA

A competitor GAPDH DNA template (cGAPDH), 26 bp smaller than the native GAPDH product, was produced from human glomerular GAPDH RT-PCR product using overlapping PCR primers. This entailed the deletion of 51 bp of native GAPDH product and the insertion of 25-bp novel sequence to be used as a target region for a specific ELISA probe enabling differential detection and measurement of the cGAPDH and native GAPDH products. Briefly, native RT-PCR GAPDH product was excised from a 3% agarose gel (Agarose MP™; Boehringer Mannheim), and melted in 3 volumes PCR-grade water. Two intermediate PCR templates were then amplified separately by PCR using 10 pmol primers, F' and R, and primers, F and R' (Figure 1, Panels 1 and 2 and Table 1). The intermediate products were isolated from an agarose gel, as above, and combined in approximately equal concentrations in a new PCR. The primers F' and R' had complementary 5' ends, such that a single annealed product is amplifiable using the original F and R primers to generate the competitor DNA (Figure 1, Panels 3 and 4). The PCR product was then run on a 2% Sepharide™ GEL

MATRIX Gel (Life Technologies Ltd.), excised and purified using the NUCLEON Easiclone Kit™ (Scotlab, Coatbridge, England, UK). The concentration of cDNA was then determined by spectrophotometry.

Measurement of PCR Products

An ELISA system was used to detect and quantify the RT-PCR products. CovaLink™ Plates (Life Technologies Ltd.) were biotinylated by incubation overnight with n-hydroxy-succinyl biotin (20 μg/mL in PBS) at room temperature. Plates were then washed three times with Buffer 1 (2 M NaCl, 40 mM MgSO₄, 0.05% vol/vol Tween® 20 in PBS) and treated with avidin (50 μg/mL in Buffer 1) for 30 min at room temperature, with agitation. Following three washes in Buffer 2 (0.02% Tween 20 in PBS), plates were treated with 3% PBS/BSA for 15 min at room temperature, with agitation. PCR products were dissolved 1:100 in PBS/BSA and allowed to bind to the avidin-coated plates for 1 h at room temperature, with agitation. Non-biotinylated (reverse strand) PCR products were denatured from the biotinylated (forward strand)

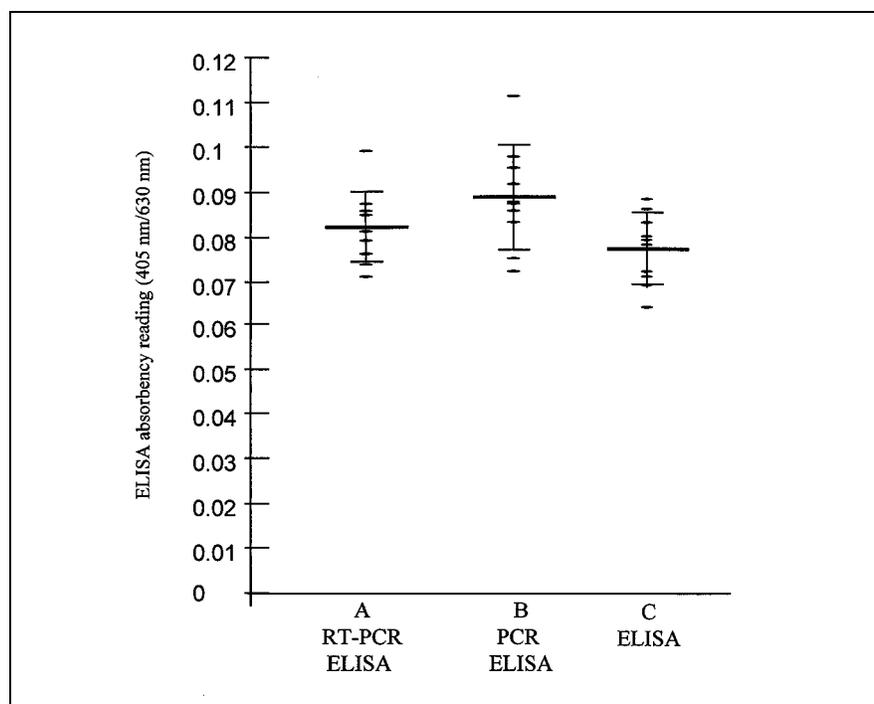


Figure 2. Reproducibility of RT-PCR-ELISA, PCR-ELISA and ELISA alone. Average ELISA data for each sample are plotted, along with the mean and SD.

by addition of an equal volume of 0.25 M NaOH for 10 min at room temperature. Plates were then incubated for 1.5 h at 42°C with oligonucleotide probes (Table 1; previously labeled with a 1:5 ratio digoxigenin-11-dUTP:dATP by terminal deoxynucleotidyl transferase [Promega] in the supplied buffer and diluted to 0.2 pmol/100 µL rapid hybridization buffer [Amersham International, Little Chalfont, Bucks, England, UK]). Plates were then washed three times in Buffer 2, before incubation with alkaline phosphatase-conjugated, anti-digoxigenin diluted 1:500 in PBS/BSA, for 30 min at room temperature, with agitation. Plates were washed three times in Buffer 2 followed by incubation in 1 mg/mL para-nitrophenyl phosphate in 1M diethanolamine, pH 9.8, for 2.5 h at 37°C. Samples were then read at 405 nm with a differential of 630 nm on a WellScan Multiwell Plate Reader (Denley Instruments Ltd., Billingshurst, England, UK).

All ELISA measurements were performed in duplicate with controls for nonspecific probe binding and plate quality.

Measurement of Variation

To determine the reproducibility of the entire RT-PCR and ELISA process, separate RT-PCRs were performed on ten 10-µL aliquots of pooled rat glomerular mRNA and subsequently measured using ELISA.

To assess the reproducibility of the PCR and measurement steps, excluding the RT step, we took ten 1-µL aliquots of a single cDNA, obtained from one of the RT reactions on pooled rat mRNA. These aliquots were amplified separately and measured using ELISA.

To test the ELISA measurement system, ten aliquots of a single GAPDH PCR product were measured.

To assess the reproducibility of RT cPCR, the procedure was performed using ten aliquots of a single RT reaction, with the addition of competitor DNA before amplification. In each reaction, 29 fg of competitor were added, as detailed in Results.

RESULTS

Measurement of Variation

To assess the reproducibility of the entire analysis in our model system, RT-PCR-ELISA was performed on ten equal aliquots of rat glomerular mRNA (Figure 2). Optical density readings ranged from 0.071 to 0.100, with a mean of 0.082 (Figure 2A). The standard deviation (SD) was 0.008 (10% of the mean).

When PCR ELISA was performed on 10 separate samples taken from a single rat RT reaction, the optical density readings ranged from 0.072 to 0.110, with a mean of 0.089 (Figure 2B). The SD was 0.011 (13% of the mean).

To determine the reproducibility of the ELISA measurement system, 10 equal aliquots of a single GAPDH RT-PCR product were measured. The variation in these readings ranged from 0.065 to 0.089 (Figure 2C). The mean was 0.078 with an SD of 0.008 (10% of the mean).

No significant difference was identified between the mean optical density readings of these three studies (Figure 2).

Construction and Determination of Concentration of the Competitor Template

The competitor DNA was constructed for use in studies of human renal biopsies, but is applicable to the current study because of sequence homology. We constructed the competitor DNA template (cGAPDH), from human GAPDH RT-PCR product, which when amplified with primers F and R, gave a product 26 bp smaller than the native product (Figure 3A). Competitor DNA template was titrated against a fixed amount of native human cDNA. The amount of competitor DNA required to give a 1:1 product ratio by visual inspection with the native cDNA was approximately 27 fg (Figure 3A). This was reproducible on 9 separate glomerular mRNA samples (data not shown). The quantification of results by ELISA allowed a standard curve to be constructed (Figure 3B). The ratio showed a linear relationship with the amount of competitor used over the range required to quantify GAPDH mRNA in single glomeruli.

Research Reports

Variation in cPCR

To determine the reproducibility of quantitative data achieved using RT-cPCR, 29 fg of competitor DNA were added to 10 separate PCRs containing rat cDNA. The amount of rat cDNA used was approximately equivalent to 1/25 of a single human glomerulus. Native and competitor PCR products were detected and quantified independently by ELISA. Figure 4 shows the ratio of native cDNA to competitor DNA, converted to absolute amounts (as detailed in the figure legend). The mean was approximately 11 fg (50 zmol) of cDNA per PCR. The SD was 1.65 fg (15% of the mean).

DISCUSSION

This study attempts to provide information that is of relevance to the design of RT-PCR-based strategies for the measurement of small amounts of mRNA. Accurate measurement of mRNA by RT-PCR relies on the efficiency of cDNA production, PCR amplification and product measurement. Our results show that the RT-PCR and ELISA measurement is highly reproducible (Figure 2A). Exponential amplification during PCR has the potential to magnify any slight errors. However, even with tiny samples (<50 zmol of cDNA), the PCR step shows minimal variation (Figure 2B).

The method of detecting and measuring the products of RT-PCR is important. Many studies have used, and continue to use, densitometry. The disadvantages of densitometry include the use of radioisotopes and increased variability due to differences in gel thickness and staining from gel to gel (8). The use of a phosphor imager can remove variability due to gel differences. However, this is specialized equipment, which is not present in many laboratories. In contrast, ELISA measurement requires relatively inexpensive equipment, is highly sensitive (1) and, as shown by this study, is very reproducible (Figure 2C).

The analysis of these data for RT-PCR and ELISA, PCR and ELISA and ELISA alone shows no one step to be more prone to error than another (Fig-

ure 2). The variation seen is likely a result of pipetting error.

The good reproducibility of RT-PCR and ELISA demonstrated by this study confirms that this is an effective method for the relative quantitative analysis of mRNA. The principal limi-

tation is that the results are expressed in arbitrary units, which cannot be converted to molar amounts. This may be adequate when all that is required is a simple relative comparison within or between samples, but if molar quantitation is needed, a competitive method is

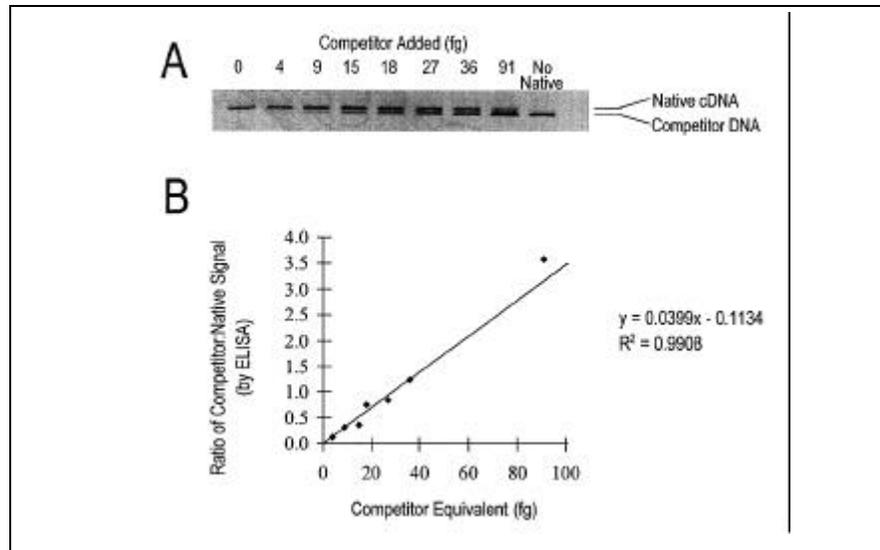


Figure 3. Quantitation of native GAPDH DNA by cPCR. Nine PCRs were performed, titrating known amounts of competitor DNA against 1/25 of the cDNA from a single human glomerulus (A). PCR products were measured using ELISA, and a standard curve was produced (B).

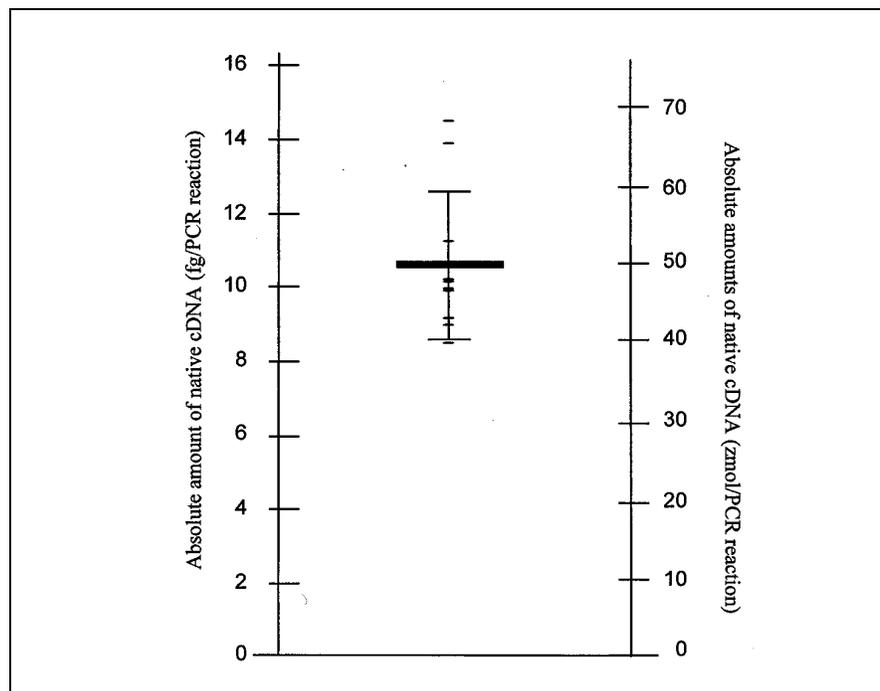


Figure 4. Reproducibility of RT-cPCR-ELISA, following conversion of data to absolute amounts of cDNA. Average absorbance readings were converted to absolute amounts (native cDNA absorbance/competitor DNA absorbance \times 29 fg). Absolute amounts of cDNA are plotted, along with the mean and standard deviation.

Research Reports

essential. There are two key types of competitor, a competitor RNA (cRT-PCR) and a competitor DNA (RT-cPCR). Competitive RT-PCR does not permit the accurate analysis of multiple genes from a single RT reaction, primarily since the competitor RNA can only be added to the RT reaction at a fixed concentration. However, it eliminates possible problems associated with variance in the RT step, even though our results suggest that this variance is minimal. For situations where the analysis of multiple transcripts is desired, several cRT-PCRs must be set up, using a dilution series of competitor to cover a wide range of native mRNA amounts. This is not practical when there is a limited amount of starting material.

In situations where absolute quantitation of starting material is not required, RT-cPCR can be used instead of cRT-PCR. RT-cPCR does not provide absolute quantitation of mRNA but it does give a measure of the amount of cDNA seeded into the PCR. It retains the advantages of cRT-PCR, in that the ratio of the native to competitor PCR product is independent of the PCR saturation curve, and any differences in

cellularity between samples are identified as shifts in this ratio. We have shown that the relationship between the ratio and the amount of competitor is linear over the range required to quantify GAPDH mRNA in single glomeruli.

Our method of constructing the competitor DNA template is simple and rapid (Figure 1). It is similar to other PCR-based approaches for synthesis of an exogenous competitor (e.g., Reference 1) but eliminates the need for cloning. Our approach requires only the synthesis of two "internal" primers for each gene of interest and then four separate PCR steps to generate a competitor DNA that can be amplified by the native primer pair F and R (Figure 1). The "internal" primers we have designed introduce a 51-bp deletion and a 25-bp insertion of unique sequence. The deletion and insertion sequences allow highly selective detection of the native and competitor PCR products by the use of specific oligonucleotide probes and ELISA measurement. No cross-hybridization between native probe and competitor DNA or competitor probe and native cDNA was detected. Our competitor template is 26 bp smaller than the native cDNA, and amplifies with the same primer pair, ensuring that the kinetics of amplification between competitor and native templates is similar. We have shown that the reproducibility of amplification is not significantly altered by the addition of competitor DNA (Figure 4). The method reported here can be adapted easily to other cPCR situations. The "insertion" sequence we have designed can be used for different competitor templates, eliminating the need to design a new probe for each gene of interest.

In summary, we have shown that RT-PCR and ELISA are ideally suited to relative quantitation of genes and can be applied when absolute quantitation by cRT-PCR is not required. RT-cPCR and ELISA measurement is independent of the saturation curve and permits the determination of differences in cellularity between samples when applied to a housekeeping gene. This enables more accurate quantitation of other genes of interest between samples.

ACKNOWLEDGMENTS

We are grateful for financial support from the U.K. Medical Research Council (Grant No. 9429414) and the University of Leicester.

REFERENCES

1. Alard, P., O. Lantz, M. Sebah, C.F. Calvo, D. Weill, G. Chavanel, A. Senik and B. Charpentier. 1993. A versatile ELISA-PCR assay for mRNA quantitation from a few cells. *BioTechniques* 15:730-737.
2. Becker-André, M. and K. Hahlbrock. 1989. Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach to a PCR aided titration assay (PATTY). *Nucleic Acids Res.* 17:9437-9446.
3. Bicknell, G.R., J.A. Shaw, J.H. Pringle and P.N. Furness. 1996. Amplification of specific mRNA from a single human renal glomerulus, with an approach to the separation of epithelial cell mRNA. *J. Pathol.* 180:188-193.
4. Bouaboula, M., P. Legoux, B. Pességué, B. Delpech, X. Dumont, M. Piechaczyk, P. Casellas and D. Shire. 1992. Standardization of mRNA titration using a polymerase chain reaction method involving co-amplification with a multispecific internal control. *J. Biochem.* 267:21830-21838.
5. Chelly, J., J.P. Concordet, J.-C. Kaplan and A. Kahn. 1989. Illegitimate transcription: transcription of any gene in any cell type. *Proc. Natl. Acad. Sci. USA* 86:2617-2621.
6. Gilliland, G., S. Perrin, K. Blanchard and H.F. Bunn. 1990. Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* 87:2725-2729.
7. Rappolee, D.A., A. Wang, D. Mark and Z. Werb. 1989. Novel method for studying mRNA phenotypes in single or small numbers of cells. *J. Cell. Biochem.* 39:1-11.
8. Riedy, M.C., E.A. Timm, Jr. and C.C. Stewart. 1995. Quantitative RT-PCR for measuring gene expression. *BioTechniques* 18:70-76.
9. Sarkar, G. and S.S. Sommer. 1989. Access to a messenger RNA sequence or its protein product is not limited by tissue or species specificity. *Science* 244:331-334.
10. Wang, A.M., M.V. Doyle and D.F. Mark. 1989. Quantitation of mRNA by the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* 86:9717-9721.

Received 20 August 1997; accepted 4 November 1997.

Address correspondence to:

Dr. Jacqueline A. Shaw
University of Leicester
Department of Pathology
Robert Kilpatrick Clinical Sciences Building
PO Box 65
Leicester Royal Infirmary
Leicester LE2 7LX, England, UK
Internet: js39@le.ac.uk