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Limitations Imposed by Heteroduplex Formation on Quantitative RT–PCR

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RT–PCR, a rapidly emerging technique for the detection of RNA, is being used by many investigators to quantify small amounts of RNA. Accurate quantification of RNA content has been facilitated by the use of competitive amplicons as internal controls. We demonstrate that losses in sensitivity and accuracy are associated with an internal standard having sequence similarity to the primary amplicon. Analysis of PCR products under non-denaturing and denaturing conditions provided evidence that these losses were associated with heteroduplex formation. Subsequent analysis of factors associated with heteroduplex formation provides insights for future development of competitive assays. Assay considerations that can minimize limitations associated with competitive PCR protocols are discussed.

Polymerase chain reaction of reverse transcribed mRNA is rapidly gaining acceptance as a quantitative method (1). Our experience with RT–PCR indicated that substantial losses of sensitivity and accuracy can occur when RT–PCR products are quantified by non-denaturing electrophoresis unless heteroduplex formation is eliminated.

Competitive RT–PCR typically employs an internal standard with similarity to the primary amplicon to minimize differences in amplification efficiency (2,3). However, the denaturation/annealing process allows the formation of both homoduplexed and heteroduplexed DNA products. Electrophoresis under nondenaturing conditions separates the homo- and heteroduplexed products, the latter decreasing the amount of homoduplexed DNA in the bands being quantified. As a result, heteroduplex formation promotes decreased sensitivity and inaccurate evaluation of amplified PCR products. In spite of this, many laboratories use non-denaturing electrophoresis in conjunction with competitive RT–PCR. The use of gels stained with ethidium bromide (4), a common approach with low sensitivity, requires a greater amount of amplified product for detection. This requirement for more DNA product further enhances the probability of heteroduplex formation. The formation of heteroduplexes is an inherent limitation of the competitive RT–PCR method that must be assessed during the development of these assays.

MATERIALS AND METHODS

Plasmids used for RT–PCR. pG4TH, a pGEM vector containing a 1.7 kbp fragment of mouse TPH cDNA served as a template to synthesize synthetic TPH mRNA (pG4TH RNA) (5). pG4TH was cut with *Ngo*MI (within the PCR amplified region) and a DNA fragment of

5′CCGGCTCGCGACTCGATTCAACGAACATCTGGTACGTGTACGTATGCGATGGTAC3′

3′GAGCGCTGAGCTAAGTTGCTTGTAGACCATGCACATGCATACGCTACCATGGGCC5′

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Abbreviations: rTth, recombinant thermostable reverse transcriptase/DNA polymerase; bp, base pairs.

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was ligated into this site to form pG4TH+I. RNA was synthesized from the SP6 promoter of *Sal*I cut pG4TH or pG4TH+I using the RiboMAX kit (Promega, Madison, WI, USA) to yield 1795 base pG4TH and 1850 base pG4TH+I RNAs, respectively.

Cell line. A mouse mastocytoma cell line (P815) that contains TPH mRNA was used as a source of RNA (6).

RT–PCR. Recombinant thermostable reverse transcriptase/DNA polymerase, rTth, was used in conjunction with a two-buffer, single-tube system (Perkin Elmer Cetus, Norwalk, CT, USA; Kit No. N808-0069). Reverse transcription was performed with 18.1 amole pG4TH+I RNA, the indicated amount of pG4TH RNA or P815 total RNA, 15 pmole TPH6 primer (5′ATCTTCTCCTTTGCATCTTCA3′), 560 μM each of dCTP, dGTP, dTTP, and dATP, 90 mM KCl, 1 mM $MnCl_2$, 10 mM Tris, pH 8.3, 2.5 μCi [α-^{32}P]dCTP and 5 units rTth in a volume of 20 μl at 70°C for 10 min. Following this reverse transcription step, 8 μl of 10X chelating buffer (1 M KCl, 100 mM Tris-HCl, pH 8.3, 7.5 mM EGTA, 0.5% Tween 20, 20% (v/v) glycerol), 15 pmole TPH5 primer (5′AAACTGGCAACGTGCTACTT3′) in 1 μl, 5 μl 25 mM $MgCl_2$ and 66 μl water were then added. Samples were incubated at 95°C for 2 min, amplified for the indicated number of cycles, each cycle consisting of 1 min at 95°C and 1 min at 56°C. Thermocycling was followed by a 6 min extension step at 56°C. A multiblock robocycler (Stratagene, LaJolla, CA, USA), that allowed instantaneous changes in block temperature, was used to amplify samples. Primers, TPH5 and TPH6, amplified a 239 bp fragment of tryptophan hydroxylase (TPH) mRNA. This amplified fragment includes the 3′ region of exon 8, exon 9 and the 5′ region of exon 10 (7). Amplification of pG4TH+I amplified a 294 bp fragment.

Gel electrophoresis. Amplified products were electrophoresed as indicated. Gels were dried and exposed to phosphorimaging screens for 24 h. Screens were visualized with a Model 400 PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

RNA purification. Total RNA was harvested using the acid guanidinium thiocyanate-phenol-chloroform extraction procedure (8)with modifications (9).

RESULTS

Appearance of an additional band in gel electrophoresis of RT–PCR products. Amplification of either total P815 RNA containing TPH mRNA or synthetic pG4TH RNA in the presence of the pG4TH+I internal standard resulted in the formation of three labeled bands as shown in Figure 1. The lower band was of the predicted size for the amplification of pG4TH and TPH mRNA and the middle band the correct size for pG4TH+I RNA. As can be seen after non-denaturing gel electrophoresis, an additional band was observed above these primary bands of interest. Based on a predictable mobility, we presumed that this band was a heteroduplex between DNA strands of the pG4TH or TPH product and the pG4TH+I product. This heteroduplex band was observed when 30, 40 or 50 cycles of amplification were performed on the P815 total cellular RNA. When only 4.1 amole of pG4TH RNA was amplified the interfering band was observed after 40 and 50 cycles of amplification and no product was observed in the band corresponding to the primary amplicon.

Analysis of RT–PCR products by denaturing gel electrophoresis. To determine the origin of the interfering band, we examined the RT–PCR products by electrophoresis under denaturing conditions (Figure 2). When samples were denatured and electrophoresed in a denaturing gel alongside a sequencing ladder, two single stranded products were observed of the predicted sizes. No other interfering bands were found under the denaturing conditions. This result demonstrated that the interfering band seen in Figure 1 was due to the formation of heteroduplexes between products of the internal standard and either pG4TH or TPH. Additional studies in our laboratory using native gels electrophoresed at 90°C gave similar results to that seen with the denaturing sequencing gel (data not shown).

When the PCR products depicted in Figure 2 were run under nondenaturing conditions, three prominent bands were obtained for pG4TH and P815. Calculated values based on the assumption that the third band was a heteroduplex were in close agreement (average error = 1.37%/band) with the values obtained under denaturing conditions.

DISCUSSION

Presentation of electrophoretograms. Unless specific precautions are undertaken, heteroduplex formation may be a problem associated with RT–PCR whenever an internal standard

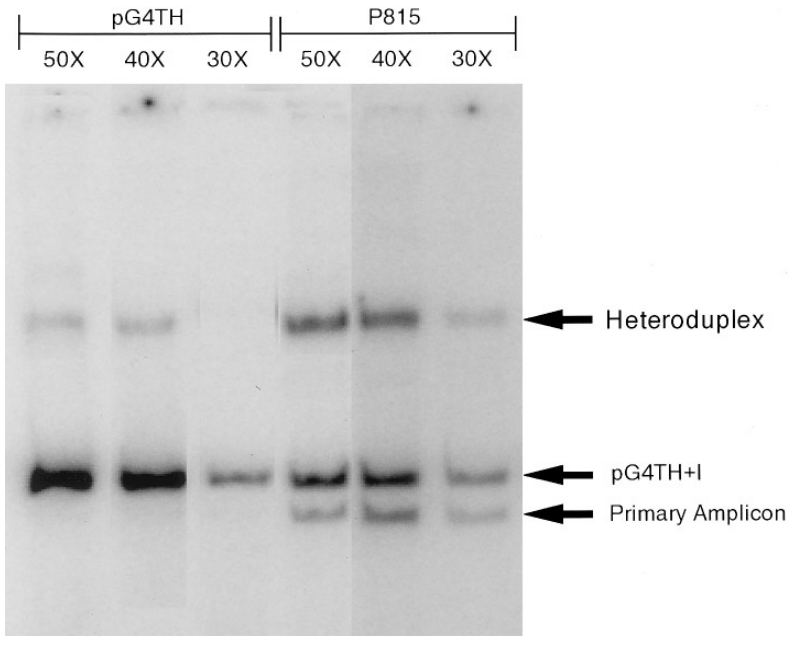

FIG. 1. Non-denaturing polyacrylamide electrophoresis of RT–PCR products. Amplified products were run in a 5% polyacrylamide/TBE gel at 150V for 45 min. Each lane represents separately amplified products obtained from the same assay. Each sample was prepared from an identical master mix containing 18.1 amole of pG4TH+I per sample (294 bp). The primary amplicon (239 bp) was either 4.1 amole of pG4TH RNA or 50 ng total P815 RNA as indicated. Amplifications were terminated after either 30 (30X), 40 (40X), or 50 (50X) cycles of PCR.

that is similar to the primary amplicon is employed. While the enhanced imaging capabilities of the Phosphor-Imager were helpful in identifying additional bands in our gels, other examples of problems with heteroduplex formation can be found (2,10). Although the widespread use of ethidium bromide to stain gels containing RT–PCR products has the potential to limit detection of heteroduplex bands, we have been able to observe these bands in ethidium bromide stained gels. Therefore, it is important that the whole gel be presented. The practice of presenting only primary bands of interest limits an evaluation of the extent of this problem. Scintillation counting of excised bands also has the potential to overlook the formation of heteroduplexes.

Accuracy of competitive RT–PCR. The accuracy of competitive RT–PCR depends on similar amplification kinetics of the primary amplicon and internal standard throughout the amplification process. Identification of an additional band with a predictable mobility during non-denaturing gel electrophoresis presumably revealed a heteroduplex incorporating a 1:1 ratio of DNA strands from the primary amplicon and internal standard. As such, competitive PCR in which the internal standard and the primary target are identical in concentration may not adversely affect quantitation. Since equimolar concentrations of primary amplicon and internal standard would be removed, the ratio of product would not be distorted. This is supported by a recent study in which accurate determinations were evident only when internal standard and primary amplicon contents were equivalent (3). When internal standard and primary amplicon contents differ, accurate quantification of amplified products is likely to be compromised if analyzed by non-denaturing gel electrophoresis. Even if cross-hybridization occurs in a 1:1 ratio, significant alinearity is predictable. If the primary amplicon is in greater abundance than the internal standard, equimolar removal of each amplicon will cause the primary amplicon to be overestimated. Conversely,

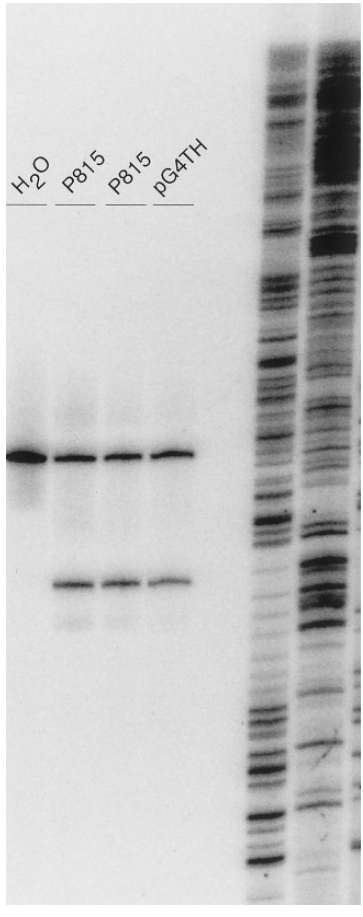

FIG. 2. Denaturing electrophoresis of RT–PCR products. Samples amplified 40 cycles were run in a 6% polyacrylamide/7M urea gel at 55 watts for 75 min. in a sequencing gel apparatus. Samples were incubated at 95°C for 2 min. in 22% formamide, 0.08% EDTA, pH 7.0, 0.07% bromophenol blue and 0.07% xylene cyanol FF prior to loading. Each lane represents separately amplified products obtained from the same assay. Samples were prepared from an identical master mix containing 18.1 amole of pG4TH÷I per sample. Water (Lane 1), 50 ng total P815 RNA (Lanes 2&3) or 16.5 amole pG4TH RNA (Lane 4) were added as indicated. The A and T lane of a sequencing reaction were run simultaneously as size standards.

when the internal standard is in excess, the primary amplicon will be underestimated as was observed in Figure 1 (pG4TH, 40 and 50 cycles).

Preventive measures. A number of approaches may minimize the negative impact of heteroduplex formation on the quantitative aspects of competitive RT–PCR. These approaches include:

1) Reducing sequence similarity between competing amplicons—Internal standards heterologous to the primary amplicon would decrease heteroduplex formation. The presence of identical primer sites on competing amplicons that have heterologous internal sequences may provide an optimal strategy to maximize the similarity of amplification kinetics between competing amplicons while minimizing heteroduplex formation.

2) Closely balancing the content of competitive amplicons—As noted above, maintaining internal standard content close to that of the primary amplicon is likely to minimize quantitative distortions due to heteroduplex formation.

3) The use of longer primers—Longer primers allow a higher anneal temperature possibly diminishing interfering hybridization between extended amplicons during thermocycling.

4) Minimizing the amount of amplified product—This approach requires a highly sensitive detection method. Sensitivity demands compromise the utility of ethidium bromide stained gels for quantitation of PCR products.

5) Full documentation of competitive RT–PCR assays—Presentation of complete gels and examination of heteroduplex formation will aid in optimization of these assays.

Heteroduplex formation can result in either the over- or underestimation of mRNA content and a substantial loss of sensitivity. However, such problems can be eliminated by a thoughtful design of assay parameters. Subsequent studies in this laboratory, utilizing an otherwise identical RT–PCR protocol, have been able to obtain an approximate 1000-fold increase in sensitivity when a linked PCR protocol (11) was used to synthesize an internal standard with identical primer sites to that of the primary amplicon and a nonidentical sequence between primer sites.

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