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Quantitative Detection of Reverse Transcriptase–PCR Products by Means of a Novel and Sensitive DNA Stain

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We constructed a plasmid for the in vitro synthesis of a competitor RNA for use as an internal exogenous control during reverse transcriptase–PCR (RT–PCR) detection of epidermal growth factor receptor (EGFR) expression. The competitor RNA harbors a 32-base deletion compared with wild-type EGFR mRNA and generates a PCR product that is easily distinguished from the wild-type PCR product by agarose gel electrophoresis. We encountered the problem of heteroduplex formation during later stages of PCR, which could be solved by decreasing the PCR cycle number. This was accompanied by a significant loss of sensitivity. Sensitivity could be restored by using a novel and extremely sensitive DNA stain (SYBR Green I) instead of ethidium bromide.

Competitive reverse transcriptase–polymerase chain reaction (competitive RT–PCR) is being used increasingly for determination and quantification of gene expression, particulary when test sample size is limited and high sensitivity is required.[1,2] The use of internal control templates in competitive RT–PCR is necessary to ensure that the reverse transcription and the PCR have functioned as expected and to generate a standard curve from which the concen-

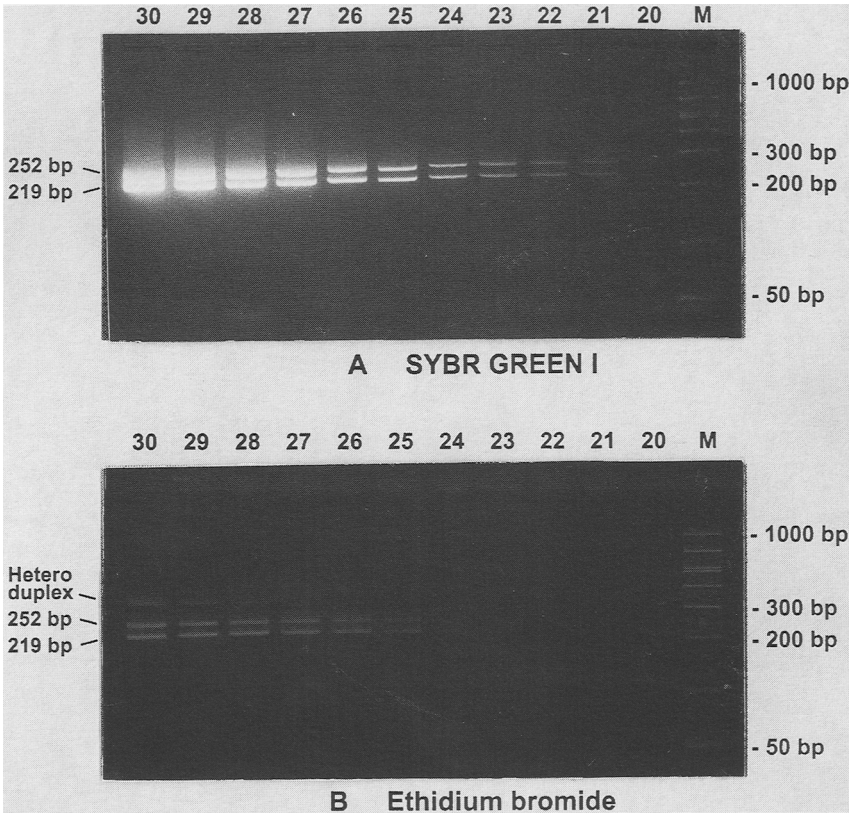

FIGURE 1 SYBR Green I- (*A*) and ethidium bromide-stained (*B*) agarose gel of competitive RT–PCR products (12 μl PCR product). (*A*) The extreme sensitivity of this DNA stain; (*B*) the effect of decreasing PCR cycle numbers on the formation of heteroduplexes. The total RNA (500 ng) extracted from a breast cancer sample, was reverse transcribed and amplified in the presence of 50 pg of EGFR–competitor RNA. Lanes *20–30* correspond to 20–30 PCR cycles; (lane M): Bio-Marker Low (Bioventures, Inc., Murfreesboro, TN).

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tration of the target sequence in the test sample can be deduced.[3–5] Internal controls can be exogenous mRNA added to the cDNA synthesis reaction or endogenous mRNA. Exogenous internal standards that share the same primer annealing sequences with the target allow calculation of the absolute amount of target mRNA.[6] However, two conditions must be met to use competitive RT–PCR: (1) The quantity of the competitor RNA (or DNA) must be known, and (2) the amplification efficiency of the competitor and target must be identical. This occurs often if the standard and the target possess the same primer binding sites.

To test these conditions, we constructed a plasmid (pEGFR-219) for the in vitro synthesis of a competitor RNA for use as an internal exogenous control during RT–PCR detection of epidermal growth factor receptor (EGFR) expression. The competitor RNA harbors a 32-base deletion compared with wild-type EGFR mRNA. Different amounts of competitor RNA and fixed amounts of total cellular RNA are reverse transcribed simultaneously in the same reaction tube, which allows the generation of a standard curve. Upon amplification of the synthesized cDNAs by using the same primer pair, the competitor RNA generates a PCR product that is easily distinguished from the wild-type PCR product by agarose gel electrophoresis without further manipulation.

During the course of experiments designed to optimize and establish the competitive RT–PCR method, we encountered formation of heteroduplexes during later stages of PCR. Heteroduplexes, identified by reduced electrophoretic mobility, were generated by cross-hybridization of competitor and wild-type sequences. In later amplification cycles, when the concentration of products is high, the probability of such a recombination event is increased. In particular, heteroduplexes formed in the last amplification cycle are not denatured and, therefore, form a significant portion of the final product profile.[3,7–9] We noticed that heteroduplex formation often interfered with the attainment of accurate quantitative results. The problem of heteroduplex formation can be resolved by decreasing the PCR cycle number from 30 to 25, which is accompanied by a significant loss of sensitivity. Sensitivity can be restored by using

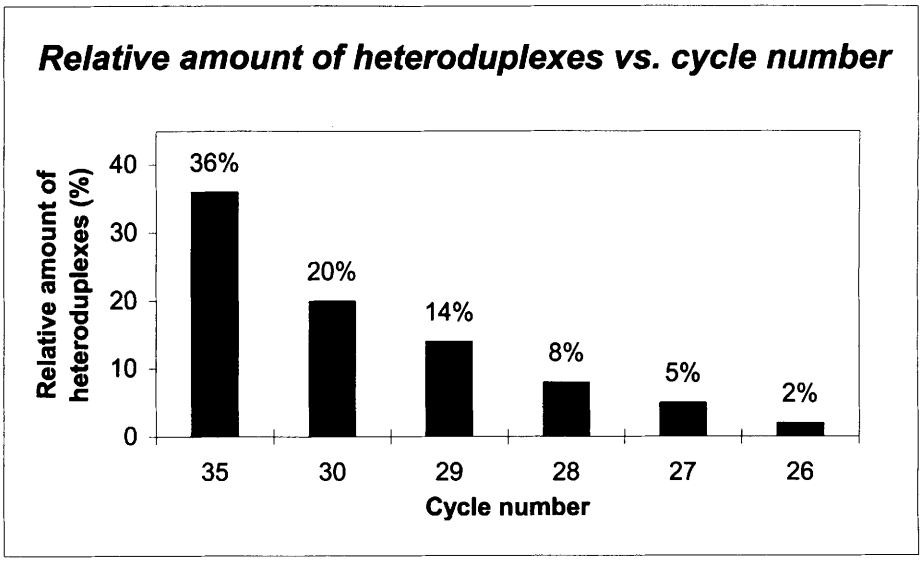


FIGURE 2 Relationship among total lane intensities (252-bp band + 219-bp band + heteroduplex band) and heteroduplex intensities. Quantitation was performed by densitometric scanning of the gels shown in Fig. 1B (lanes *26–30*) and Fig. 3B (lane *2*), using a CCD Video Camera Imaging System (Vilber Lourmat, Marne la Vallee, France).

a novel and extremely sensitive DNA stain (SYBR Green I; Molecular Probes, Inc., Eugene, OR) instead of ethidium bromide. The remarkable sensitivity of SYBR Green I can be attributed to a combination of unique dye characteristics. SYBR Green I exhibits exceptional affinity for DNA and a large fluorescence enhancement upon DNA binding—at least one order of magnitude greater than that of ethidium bromide. Also, the fluorescence quantum yield of the DNA/SYBR Green I complex is more than five times greater than that of DNA/ethidium bromide (Product information, Molecular Probes, Inc.).

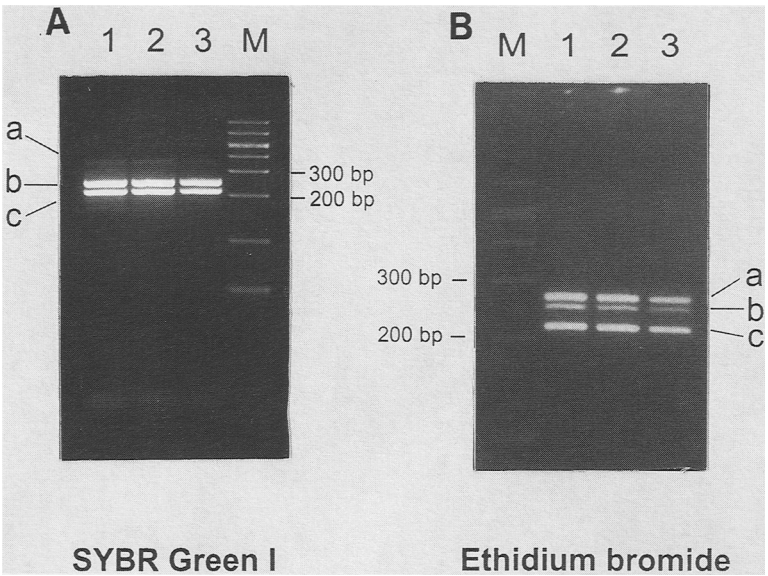


FIGURE 3 SYBR Green I- (*A*) and ethidium bromide-stained (*B*) agarose gels of a competitive RT–PCR experiment (35 PCR cycles). Staining was done by adding the dyes to the agarose solution prior to pouring the gel. (*a*) Heteroduplex band; (*b*) 252-bp band; (*c*) 219-bp band). (Lane *1*) 15-µl PCR product; (lane *2*) 10-µl PCR product; (lane *3*) 5-µl PCR product; (lane M) BioMarker Low.

MATERIALS AND METHODS

RNA Extraction and Reverse Transcription

Total RNA was extracted from cell lines and tumor tissue by isopycnic centrifugation as described.[10] cDNA was synthesized in 50 µl total volume containing the commercially available Random Primed Reverse Transcription Reaction Mix (ViennaLab, Vienna, Austria), 40 units of RNasin (Promega Madison, WI), 100 units of Mu-MLV reverse transcriptase (ViennaLab), and 30 µl of template RNA (competitor RNA and wild-type RNA). Reactions were incubated at room temperature for 10 min followed by 50 min at 37°C and 5 min at 95°C.

Construction of pEGFR-219

A 252 bp-EGFR–cDNA fragment was amplified using a tumor-derived cDNA as template, primer EGFR-5′ (position 1152–1170 of the EGFR cDNA sequence,[11] and primer EGFR-3′ (position 1386–1404 of the EGFR cDNA sequence.[11] *Mse*I (Boehringer Mannheim, Mannheim, Germany) digestion of the amplified sequence yielded fragments of 150, 69, and 33 bp, which were separated by agarose gel electrophoresis and isolated using an agarose-digesting enzyme (Gelase; Epicentre Technologies, Madison, WI). Fragments of 150 and 69 bp were ligated using T4 DNA ligase (Boehringer Mannheim). The ligated product was reamplified using primers EGFR-5′ and EGFR-3′ and purified by agarose gel electrophoresis. The resulting 219-bp fragment was cloned into plasmid pCR II, according to the manufacturer's instructions (TA Cloning System; Invitrogen, San Diego, CA) and named pEGFR-219. pEGFR-219 was used to transform *Escherichia coli* INVαF′ (Invitrogen). Positive clones were selected by their blue/white color and checked for the presence of the expected deletion mutant by PCR using primers EGFR-5′ and EGFR-3′.

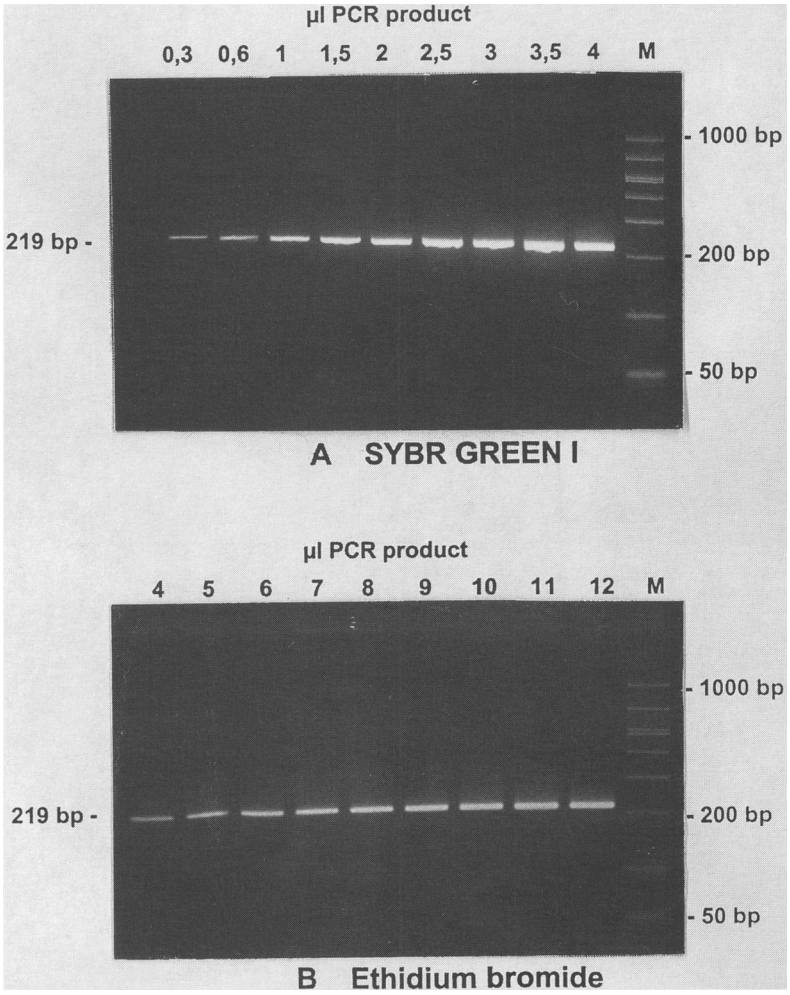


FIGURE 4 Dilution series of a 219-bp PCR fragment, stained either with SYBR Green I (*A*) or ethidium bromide (*B*). 100 fg competitor RNA (~5×10^5/molecules) was reverse transcribed, 1/50 of the cDNA was used for PCR (30 cycles). 1 µl PCR product corresponds to ~2000 molecules of competitor RNA. (Lane M) BioMarker Low.

In Vitro RNA Synthesis

Plasmid pEGFR-219 was purified by using a Qiagen Plasmid Kit (Qiagen, Inc., Studio City, CA) and linearized with *Bam*HI (Boehringer Mannheim). Competitor RNA was synthesized by using the AmpliScribe T7 Transcription kit from Epicentre Technologies. Template DNA was removed by digestion with RNase-free DNase (Epicentre Technologies). In vitro-synthesized RNA was recovered by ethanol precipitation, controlled and purified by polyacrylamide gel electrophoresis (5% acrylamide, 8 M urea), and quantified by measuring the absorbance at 260 nm.

DNA Amplification

PCR was performed on a Perkin-Elmer GeneAmp PCR System 9600 and carried out in a total volume of 50 µl containing 1–2 µl of cDNA template, 20 pmoles of primer EGFR-5′ (5′-CGTCCGCAAGTGTAAGAA-3′, ViennaLab), 20 pmoles of primer EGFR-3′ (5′-AGCAAAAACCCTGTGATT-3′; ViennaLab), 250 µM dNTPs, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.01% (wt/vol) gelatin, 1.5 mM $MgCl_2$, 0.1% Triton X-100, and 0.2 unit Hi-Taq DNA Polymerase (ViennaLab). Amplification cycles were as follows: 94°C for 30 sec, 50°C for 30 sec, and 72°C for 45 sec. The 20–30 amplification cycles were preceded by a primary denaturation step (94°C for 1 min).

PCR Product Analysis

PCR products were separated by agarose gel electrophoresis (3% NuSieve agarose/1% GTG agarose; FMC BioProducts, Rockland, ME). All agarose gels used for comparison of staining efficiency were of the same dimension and volume. Electrophoresis was performed at 10 V/cm for 30 min. Staining was done either by incubating the gel after electrophoresis in staining solution [1:10,000 dilution of the SYBR Green I stock reagent in 1× TAE (40 mM Tris-acetate, 1 mM EDTA) or 5 µg ethidium bromide/ml

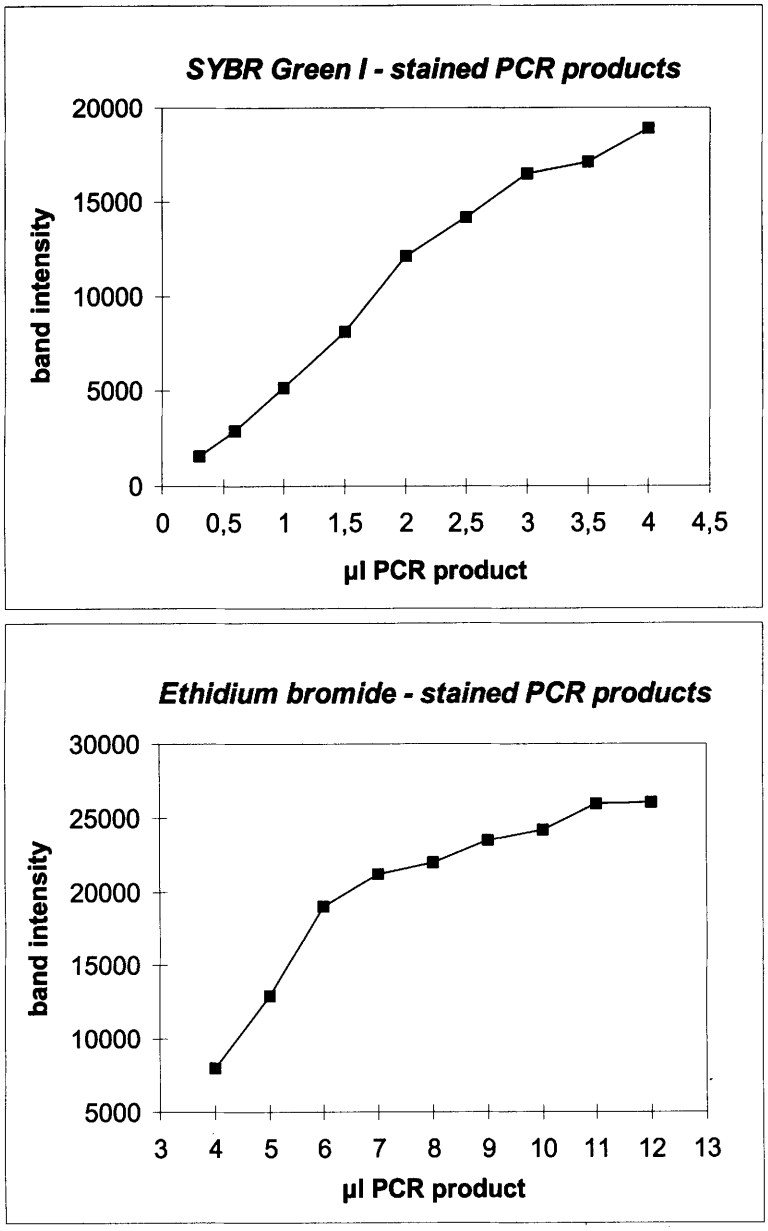
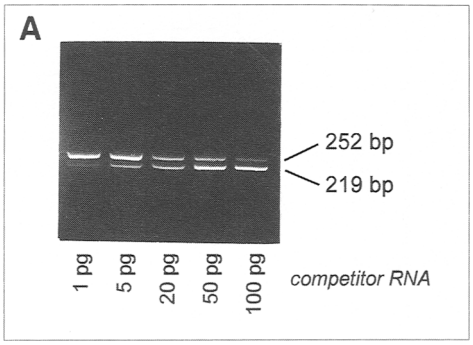


FIGURE 5 Relationship between fluorescence intensity and concentration of a 219-bp PCR product, stained with either SYBR Green I or ethidium bromide. Quantitative analysis was performed by densitometric scanning of the gels shown in Fig. 4, A and B, using a CCD Video Camera Imaging System (Vilber Lourmat, Marne la Vallee, France).

in 1× TAE] for 25 min or by performing electrophoresis on dye-containing gels, which were precast by diluting the dye into the gel solution just prior to pouring the gel (1:10,000 dilution of the SYBR Green I stock reagent or 5 µg ethidium bromide/ml). Ethidium bromide-stained gels were excited using 302 nm transillumination, whereas SYBR Green I-stained gels were excited using 254 nm transillumination. Gels were photographed with Polaroid 557 film, using a yellow (for the SYBR Green I gels) or red (for the ethidium bromide gels) gelatin filter.

RESULTS AND DISCUSSION

The problem of heteroduplex formation, which was associated with the use of our internal RT–PCR standard, was avoided by decreasing PCR cycle numbers (Fig. 1B.) Figure 2 shows the correlation of heteroduplex formation and cycle number. The relative amount of heteroduplexes decreased from 36% (35 cycles) to 2% (26 cycles). Because of these results, we decided to reduce the cycle number for the EGFR RT–PCR system to 25. To prove that SYBR Green I stains heteroduplexes, the PCR cycle number had to be extended to 35. As shown in Figure 3A and B, SYBR Green I stained the heteroduplex band but with much lower efficiency than ethidium bromide did. In addition, heteroduplexes showed much higher reduction of electrophoretic mobility in SYBR Green I gels compared with ethidium bromide gels. Although SYBR Green I can be used to stain double-stranded DNA with higher sensitivity than ethidium bromide, heteroduplexes would not necessarily yield the same sensitivity staining. We may well have discovered an unusual characteristic of this stain. It is known that the fluorescence quantum yield and fluorescence enhancement of SYBR Green I/RNA complexes is less than that of SYBR Green I/DNA complexes. It is possible that SYBR Green I either binds poorly to heteroduplexes compared with homoduplexes or yields a poor fluorescence quantum yield (compared with that observed with RNA) upon such binding. The loss of sensitivity, which is associated with the reduction of cycle numbers, can easily be offset by SYBR Green I staining instead of ethidium bromide staining (Fig. 1A). The sensitivity of the RT–PCR procedure is such that as little as 0.2 fg (~1000 molecules) of the competitor transcript can be detected easily by agarose gel electrophoresis combined with SYBR Green I staining (Fig. 4A). Quantitative analysis revealed a much better linear relationship between fluorescence intensity and amount of PCR product for SYBR Green gels as opposed to ethidium bromide gels (Fig. 5), as well as accurate quantitative results in PCR with SYBR Green I over ethidium bromide. Figure 6, A and B show an example of our quantitative EGFR analysis system: SYBR Green I was used to stain competitive RT–PCR products obtained by 25 PCR cycles (Fig. 6A). Quantitative analysis (Fig. 6B) of this gel demonstrated the quantitative power of SYBR Green I in determining gene expression by competitive RT–PCR. Taken together, SYBR Green I has proven to be an extremely useful reagent for qualitative as well as quantitative PCR applications where high sensitivity or reduction of PCR cycle numbers is required.



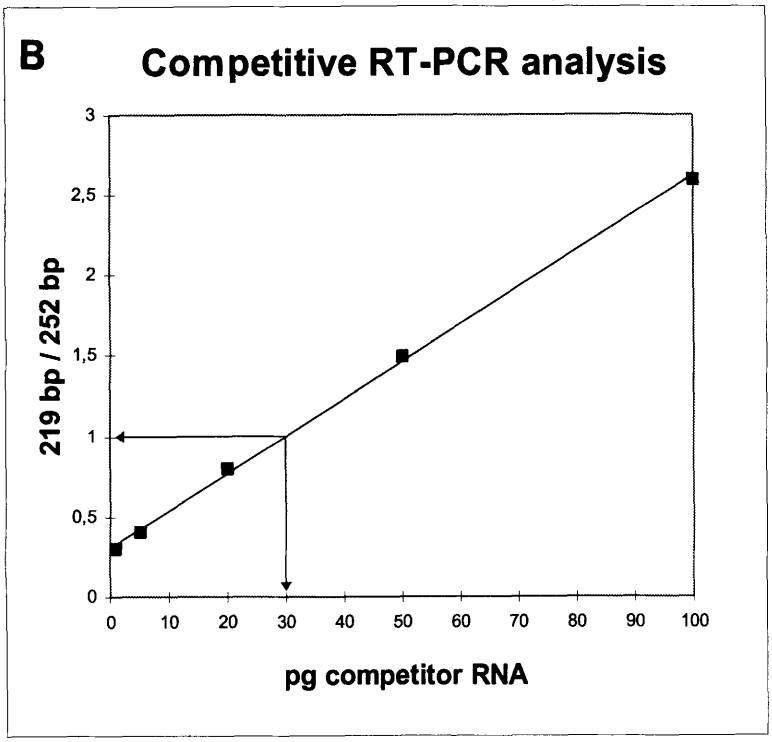


FIGURE 6 (*A*) Competitive EGFR RT–PCR: 500 ng total RNA, extracted from a breast cancer sample, was reverse transcribed and amplified in the presence of 1, 5, 20, 50, and 100 pg of EGFR–competitor RNA. Staining of amplified products (10-μl PCR products) was done by adding SYBR Green I to the agarose solution prior to pouring the gel. (*B*) Linear plot of data obtained by densitometric scanning of (*A*). Plotting of the concentration of EGFR–competitor RNA vs. the 219-bp/252-bp ratio, allows calculation of the concentration of EGFR–mRNA. At the theoretical equivalence point (219 bp/252 bp = 1) the amount of EGFR–competitor RNA equals the amount of EGFR–mRNA present in the analyzed sample.

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