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A Method to Monitor mRNA Levels in Human Breast Tumor Cells Obtained by Fine-needle Aspiration

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A method based on the reverse transcriptase–polymerase chain reaction (RT-PCR) was developed that allows the determination of relative mRNA expression levels in fine-needle aspirates from human tumors. The method was developed for the *c-erbB-2* gene, using the porphobilinogen deaminase (*PBGD*) gene as an internal standard. It was validated for mRNA isolated from cell lines and for material obtained by fine-needle aspiration from human breast cancer. Gene expression levels were determined by measuring the activity of radiolabeled RT-PCR–amplified gene-specific bands with a phosphor imager. At least four points are measured on the log-linear part of the amplification cycle versus signal intensity curves, and subsequently the distance between the curves of the gene of interest and that of an internal standard gene is used to calculate the relative expression levels. The method worked equally well with the *BRCA1* gene, illustrating that it can be generalized to other genes. The method is suitable to measure or monitor semiquantitively gene expression levels in accessible human tumors in situ.

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Characteristics of both normal and malignant cells are determined by the specific repertoire of genes expressed. Knowledge of this repertoire may assist in clinical decision making. The expression of normal steroid hormone receptor molecules in breast cancer cells, for example, is strongly associated with clinical sensitivity to tamoxifen (5,15), and a high level of expression of the multidrug resistance gene *MDR1* can indicate resistance to doxorubicin (18). As the molecular mechanisms that lead to therapy sensitivity and resistance are progressively elucidated (4,16,19), the need arises to determine the role of such mechanisms in clinically encountered human cancer. In breast cancer, and in many other malignant disorders, crucial guidance could be obtained in the selection of therapeutic strategies if the clinician were able to monitor the emergence of resistance over time and to determine its specific cause.

Many types of chemotherapy resistance have been associated with abnormal expression levels of certain genes. Classic examples include dihydrofolate reductase overexpression (and amplification) in methotrexate resistance (1), high levels of thymidylate synthase expression in fluorouracil resistance (20), and low levels of topoisomerase II expression in resistance to topoisomerase II inhibitors (2,17). Some of the products of these genes can be detected using immunocytology, but this technique does not allow quantification. An average fine-needle aspiration (FNA), in clinical practice often the only material available, frequently does not yield a sufficient number of cells to use quantitative immunologic testing. As a result, changes in expression levels over time are difficult or impossible to establish.

Quantitative estimations of the expression of certain genes can be performed, at the mRNA level, using reverse transcriptase–polymerase chain reaction (RT-PCR) (7,12,14,21). Several limitations of this technique, however, must be controlled for. The amplification of cDNA

by PCR is log-linear for only a limited number of amplification cycles. In the presence of excess reagents, this number depends critically on the amount of input RNA in the reaction. The number of cells obtained by FNA of a human tumor is typically small but nevertheless highly variable. As a result, the amount of template RNA with which the reaction begins is highly variable as well, although it is invariably too small for direct measurement. Consequently, an internal control gene, ideally a household gene that is expressed independently from cell cycle perturbations, is required as an internal standard. Further, fine-needle aspirates may contain significant numbers of nonmalignant cells, such as stroma cells or inflammatory cells. Because the mRNA expression level measured by RT-PCR is essentially an average of all cells put into the reaction, the nontumor cells may compromise the reliability of the assay. If the percentage of nonmalignant cells is too high, a purification procedure may be performed (11).

We have developed a practical method to measure mRNA derived from breast cancer cell lines in a semiquantitative way, and we have validated the assay by comparing its results to that of standard Northern blot analysis. The assay is sufficiently sensitive to be used with FNA-derived material and was shown to yield reproducible results for *c-erbB-2* and *BRCA1* expression in breast cancer fine-needle aspirates.

MATERIALS AND METHODS

Cell Lines

The human breast cancer cell lines MCF7 and SKBr3 were obtained from the American Type Culture Collection. The cells were maintained in Dulbecco's Modified Eagle Medium, supplemented with 10% fetal calf serum, streptomycin, and penicillin (all Gibco BRL, Life Technologies, The Netherlands). The medium for the SKBr3 cells was complemented with 10 μg/ml insulin.

Processing of Tumor Cells Obtained by Fine-needle Aspiration

Aspiration of tumor cells from palpable breast tumors was performed using a pistol-grip (Cameco, Sweden), a 20-ml syringe and a 23-gauge needle. The aspirated cells were collected in 5 ml cold Dulbecco's Modified Eagle Medium, supplemented with 10% fetal calf serum and were kept on ice until further processing. The number of collected cells was estimated by counting a small portion (10 μl) in the presence of trypan blue, a marker for the viability of the cells. Usually, the total viable cell yield per FNA varied between 5×10^3 and 5×10^6 cells. The percentage of tumor cells was >80%.

mRNA Isolation

Polyadenylated mRNA was isolated from the cells using oligo$(dT)_{25}$-coated magnetic beads (Dynabeads oligo$(dT)_{25}$, Dynal AS, Norway). The cells, ~10,000 were washed once in cold phosphate buffered saline (PBS). The cell pellet was resuspended and lysed in 30 μl lysis buffer (10 m*M* Tris-HCl, pH 7.5; 0.14 *M* NaCl; 5 m*M* KCl; 1% Triton X-100) for 1 min on ice. Intact nuclei were separated from the cytoplasmic fraction by centrifugation at 12,000 rpm for 30 s in an Eppendorf centrifuge. The supernatant fraction, which contains the total cytoplasmatic RNA pool was added to 150 μg Dynabeads and incubated for 3–5 min at room temperature in 30 μl 2x binding buffer (20 m*M* Tris-HCl, pH 7.5; 1.0 *M* LiCl; 2 m*M* EDTA, 0.4% sodium dodecyl sulfate [SDS]). The polyadenylated mRNA fraction bound to the beads was separated from the unbound RNA fraction using a magnetic separator. The beads were then washed twice with 100 μl washing buffer (10 m*M* Tris-HCl, pH 7.5; 0.15 *M* LiCl; 1 m*M* EDTA; 0.1% SDS) and once with 100 μl elution buffer (2 m*M* EDTA, pH 7.5) in order to remove residual traces of SDS. The polyadenylated mRNA was finally eluted from the beads by incubation in 20 μl elution buffer for 2 min at 65°C.

cDNA Synthesis

First-strand cDNA synthesis was performed using a cDNA kit (GeneAmp RNA PCR, Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, NJ, U.S.A.). One to three microliters of the isolated mRNA were reverse-transcribed using random primers, in a final volume of 20 μl containing 5 m*M* $MgCl_2$, 50 m*M* KCl, 10 m*M* Tris-HCl (pH 8.3), 1 m*M* of each deoxyribonucleoside triphosphate (dNTP), 2.5 μ*M* random hexamers, 20 U RNase inhibitor and 30 U avian myeloblastosis virus (AMV). The mixtures were incubated for 10 min at room temperature, for 30 min at 42°C, for 5 min at 99°C, and chilled on ice.

PCR Amplification

A 0.5- to 2.0-μl aliquot of each cDNA sample was used in a PCR reaction with a final reaction volume of 50 μl containing 20 m*M* Tris-HCl (pH 8.4), 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 0.05% (v/v) W-1 detergent, 0.1 m*M* of each dNTP, 0.5 μ*M* of each primer, 1 U of *Taq*-polymerase (all reagents, Gibco BRL) and 0.5 μCi α-^{32}P-dCTP (3000 mCi/mmol, Amersham, Life Science, Ltd., U.K.). The reactions were run in a thermal cycler (Hybaid, U.K.). The following amplification conditions were used: 0.5 min at 95°C, 0.5 min at 60°C, and 1 min at 72°C. Different numbers of amplification cycles were performed. The tubes were taken out at the end of between 14 and 40 cycles.

Primers used for the amplification of a 200-bp cDNA

fragment of the human *c-erbB-2* gene were designed according to Cuossens et al. (3). A 253-bp cDNA fragment of the human porphobilinogen deaminase (*PBGD*) gene was amplified with primers designed according to Grandchamp et al (8). The primers were chosen in such a way that the optimal annealing temperatures were comparable. Two *BRCA1* cDNA fragments were amplified of 404 and 259 bp respectively (13). For primer sequences see Table 1.

Quantitative Measurement of PCR Products

After completion of the PCR reactions, the overlaying paraffin was removed from the PCR mixture by extraction with 25 μl chloroform. Ten microliters of the PCR product was mixed with 5 μl loading buffer (15% ficoll in H_2O with bromophenol blue and xylene cyanol). This was loaded on a 6% nondenaturing polyacrylamide gel in 1 × TBE buffer (89 m*M* Tris-borate; 2 m*M* EDTA, pH 8.3) and electrophoresed for 2 h at a constant voltage of 250 V and current of 40 mA. The gel was subsequently fixed in 10% acetic acid for 20 min at room temperature and dried under vacuum at 50°C for 2 h. To quantitate the amount of radiolabeled PCR fragment, a phosphor imaging plate (bas-III, Fuji Photo Film Co., Ltd., Tokyo, Japan) was exposed to the dried gel. The imaging plate was scanned in a phosphor imager (bas-2000, Fuji) and the intensity of each band was determined by volume integration with Tina software (Raytest Isotopenmeßgeräte, Germany).

The amount of PCR product was determined for at least four different cycle numbers. The logarithm of the intensity of the band was plotted as a function of the number of PCR cycles. *c-erbB-2* mRNA levels are given relative to the mRNA level of the internal standard *PBGD* with the same amount of input cDNA. All experiments were done at least in ninefold (cell lines) or twofold (fine-needle aspirates). Two tumor samples are analyzed only once.

Calculation of Expression Levels Relative to *PBGD* Expression

Two different methods were tested to calculate the relative expression levels of *c-erbB-2* and *PBGD* (or *BRCA1* and *PBGD*). The extrapolation method in theory should allow the estimation of the relative levels of input RNA for both the tested gene and the internal standard gene, with a correction for possible differences in amplification efficiency. To obtain the theoretical signal intensity after one cycle of amplification by PCR, a linear regression curve is determined that yields the signal intensity for a given number of amplification cycles. The following equation is derived by standard regression analysis:

$$\log(SI) = kN + C \quad (1)$$

in which SI is the signal intensity in cpm, and N is the number of amplification cycles. The constant C and slope k are calculated from the experimental data. Extrapolation of the curve allows the estimation of the theoretical signal intensity after one round of amplification, by substituting 1 for N. The expression of *c-erbB-2* relative to that of *PBGD* is then given by the ratio:

$$SI_{c\text{-}erbB\text{-}2}/SI_{PBGD} \text{ for } N = 1 \quad (2)$$

The distance of curves method involves the following steps:

The logarithm of the cpm value of each PCR product is plotted as a function of the number of PCR cycles, for both the internal standard and the gene of interest.

A linear regression line is calculated through these points.

For both lines the highest and lowest measurements (in terms of PCR cycles) that appear to be in the exponential phase are determined, with their cpm values: cpm(high) and cpm(low).

The two regression formulas are used to calculate the cycle number values for cpm(high) and cpm(low) for both curves. This yields four x values: $xL1$ and $xL2$ for the left curve, associated with cpm(low) and cpm(high), respectively, and $xR1$ and $xR2$ for the right curve, associated with cpm(low) and cpm(high), respectively.

The distance between the left curve and the right curve is given by the simple formula:

$$D(LR) = \frac{(xR1 + xR2)}{2} - \frac{(xL1 + xL2)}{2} \quad (3)$$

Assuming a PCR efficiency between 1.0 (no amplification) and 2.0 (precise doubling at each cycle), *eff*, the amount of gene product R relative to the amount of gene product L is

TABLE 1. *Oligonucleotide primers used*

Gene	Primers	
	Sense	Antisense
c-erbB-2	5′-ATG TGC GGC TCG TAC ACA GG	5′-TCA CTC TGG TGG GTG AAC CG
BRCA1 (exons 20–24)	5′-GCT GAA TGA GCA TGA TTT TGA AG	5′-GTG GCT GTG GGG GAT CTG G
BRCA1 (exons 2–5)	5′-GCT CTG GGT AAA GTT CAT TGG	5′-CTT TCT TGT AGG CTC CTT TTG G
PBGD	5′-TCT GGT AAC GGC AAT GCG GC	5′-CCA GGG CAT GTT CAA GCT CC

$$eff^{D(LR)} \quad (4)$$

eff is the mean efficiency calculated for both curves. Values of *eff* between 1.6 and 2.0 were considered acceptable for the exponential phase of the PCR. Reactions with *eff* values outside that range were considered unreliable and were not used for the calculation of results.

Northern Blot Analysis

Polyadenylated mRNA was isolated as described earlier but with different reaction volumes because of the higher concentration of cells. Approximately 3 μg mRNA was electrophoresed in a formaldehyde-containing 1% agarose gel and transferred to a nylon filter (Hybond N^+), using 20× standard saline citrate (SSC) buffer (3 *M* NaCl; 0.3 *M* $Na_3C_6H_5O_7 \cdot 2H_2O$, pH 7.0). Probes were made of PCR products (PCR as described earlier), purified with a Qiaex DNA gel extraction kit (Qiagen, The Netherlands), and labeled by ^{32}P random priming with the addition of antisense primer. These probes were hybridized to the filter in hybridization mixture (48% formamide; 5× SSC; 0.02 *M* Tris, pH 7.6; 1× Denhardt's; 10% dextran sulfate; and 0.1% SDS) at 42°C overnight. After hybridization, the filters were washed at 50°C in 2× SSC/0.1% SDS and subsequently in 0.1× SSC/0.1% SDS. The signal was visualized and quantified employing a phosphor imager (Fuji).

RESULTS

mRNA of Breast Cancer Cell Lines MCF7 and SKBr3

To determine the mRNA expression levels of selected mammalian genes, the human breast cancer cell lines MCF7 and SKBr3 were harvested in exponential phase, and mRNA was isolated. Multiple aliquots of the two mRNA preparations were frozen at −70°C to be used for the validation of the quantitative RT-PCR and for the Northern blots.

Calculation of (Relative) mRNA Expression: Extrapolation Method versus Distance of Curves Method

RNA isolated from SKBr3 was tested for *c-erbB-2* expression relative to *PBGD* expression in nine independent experiments. The RT-PCR–amplified products were run on an acrylamide gel and the radioactively labeled bands were scanned and visualized by a phosphor imager (Fig. 1C). In all cases, the signal intensities of the reaction products were integrated and plotted at several numbers of amplification cycles (at least four). A typical plot, representing one of the nine independent data sets, is shown in Figure 1D. For further calculations, all points that appeared to lie within the linear range of the reaction were used, as indicated by the boxed areas. All points upward of this range were excluded. The decision as to which amplification cycle number still belonged to the

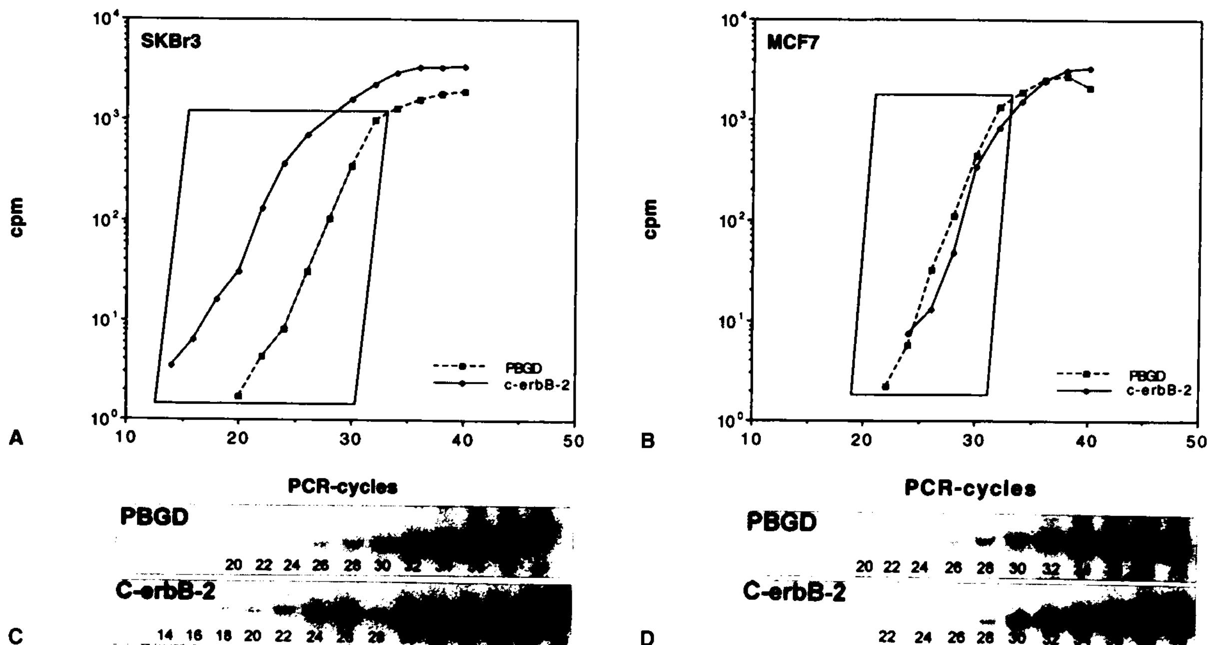

FIG. 1. Signal intensity versus amplification cycle curves for *c-erbB-2* and *PBGD*. Graphical representation of *c-erbB-2* versus *PBGD* expression in the cell lines SKBr3 **(A)** and MCF7 **(B)**. The logarithm of the cpm of the radiolabeled PCR product is plotted as a function of the PCR cycle number. The phosphor imaging results of the RT-PCR–amplified products run on acrylamide gels are shown in **(C)** and **(D)** with their cycle number.

log-linear phase could invariably be made with ease by visual inspection of the log-linear plots (see Fig. 1).

The results of the two techniques of calculating relative mRNA expressions are shown in Table 2 (see Materials and Methods for details). Both methods demonstrate relatively high expression levels for *c-erbB-2*, which was expected because of the known amplification of that gene in SKBr3. A similar set of experiments was done to determine the *c-erbB-2/PBGD* ratio for the MCF7 cell line in which *c-erbB-2* is not overexpressed (Fig. 1B). The results of 10 independent experiments are shown in Table 3.

Although the distance of curves method does not correct for possible differences in PCR efficiency between the two different templates, it is clearly more reproducible than the extrapolation method. The wide variability of results from the extrapolation method results from the experimental error that is made when the PCR efficiency is determined from the available measurements, each of which had used 20 or more cycles of PCR. This experimental error is greatly amplified when the theoretical signal intensity at one PCR cycle is calculated, the value on which the method is based. It follows from equation 1 that the theoretical signal intensity at the first round of PCR (when $N = 1$) is e^{k+C}, whereas the signal intensity after 25 PCR rounds is e^{25k+C}. Thus, any experimental error becomes $e^{(N-1)k}$ as large when the logically correct extrapolation procedure is used. For a typical experiment with 25 PCR cycles and a k value of 0.26, this would lead to an error that is 512 times larger than the experimental error.

Using the distance of curves method revealed that *c-erbB-2* is expressed 33 times higher in SKBr3 than in MCF7 (see Tables 2 and 3).

BRCA1 Expression

The levels of *BRCA1* expression were determined in the mRNA preparations of both cell lines, by identical methods as for *c-erbB-2* and using the primers listed in Table 1. The results of two independent measurements are shown in Table 4. The mRNA expression level of *BRCA1* is 0.4 in SKBr3 relative to MCF7.

TABLE 2. *Comparison of two methods to calculate the* c-erbB-2/PBGD *gene expression ratio in exponentially growing SKBr3 cells measured by RT-PCR*

	c-erbB-2/PBGD ratio	
Data set number	Extrapolation method	Distance of curves method
1	136	28
2	37	13
3	909	29
4	151	17
5	11	11
6	335	19
7	758	19
8	336	30
9	122	17
Mean	311	20
Range	11–909	11–30

TABLE 3. *Comparison of two methods to calculate the* c-erbB-2/PBGD *gene expression ratio in exponentially growing MCF7 cells measured by RT-PCR*

	c-erbB-2/PBGD ratio	
Data set number	Extrapolation method	Distance of curves method
1	0.3	0.6
2	2	0.4
3	0.6	0.9
4	0.1	0.7
5	0.1	0.4
6	0.02	0.2
7	0.03	0.2
8	0.06	0.4
9	0.7	0.9
10	0.3	0.9
Mean	0.4	0.6
Range	0.02–2	0.2–0.9

Northern Blotting

The expression levels of *c-erbB-2*, *BRCA1*, and *PBGD* in the same mRNA preparations of SKBr3 and MCF7 as used for the RT-PCR were also determined using standard Northern analysis. A resulting Northern blot is shown in Figure 2; the areas used for scanning of band intensities are indicated. The signal intensities support the finding that *c-erbB-2* expression in SKBR3 is amplified, whereas it is not in MCF7. The expression of *BRCA1* was seen in either cell line. Table 5 shows the corresponding relative expression levels. The relative expression levels between the two cell lines are in good agreement with those determined with the RT-PCR method (Table 6). These levels are calculated by first determining expression levels relative to PBGD expression for each cell line separately and subsequently dividing the ratio of *c-erbB-2/PBGD* for the cell line SKBr3 by the ratio of *c-erbB-2/PBGD* for MCF7.

Feasibility in Fine-needle Aspiration Samples

Fine-needle aspiration samples were obtained from palpable breast tumors of four mastectomy specimens immediately after having been received at the pathology laboratory. All tumors were classified as infiltrating duct carcinomas. Two larger tumors (coded tumors C and D) were subjected twice to FNA sampling, aspirating material from distant sites of the tumors. mRNA expression levels of *c-erbB-2* and *BRCA1* were determined for each FNA (one or two independent experiments, as indicated). Relative expression levels were calculated by the dis-

TABLE 4. *Expression levels of* BRCA1 *relative to the internal standard* PBGD *in cell line mRNA isolated during exponential growth measured by RT-PCR*

Cell line	*BRCA1/PBGD* ratio	
	First measurement	Second measurement
SKBr3	0.1	0.2
MCF7	0.5	0.3

TABLE 5. *Expression levels of* c-erbB-2 *and* BRCA1 *relative to the internal standard* PBGD *in cell line mRNA isolated during exponential growth measured by Northern blot analysis*

Cell line*	*c-erbB-2/PBGD* ratio	*BRCA1/PBGD* ratio
SKBr3	27	0.2
MCF7	0.7	0.5

* For both cell lines, in two independent experiments, the intensity of the signal of the *c-erbB-2* or *BRCA1* probe is divided by the signal of the *PBGD* probe.

tance of curves method. All tumors show relatively high expression of *c-erbB-2* (ratio, 3–33) and low expression of *BRCA1* (ratio, 0.2–0.6). Moreover, paired FNA sampling of one tumor gives comparable results (Table 7).

DISCUSSION

In theory, RT-PCR should allow the molecular genetic analysis of the minute amounts of material that can be obtained by FNA from accessible tumors of patients. In order to both maintain acceptability for the patient and ensure conditions for quantitative or at least semiquantitative analysis, a number of technical issues must be addressed. First, the amount of nucleic acids that can be obtained from a single FNA is small but quite variable; thus an internal rather than external standard needs to be used. Secondly, some of the sample has to be available for routine microscopical analysis to confirm the representativeness of the sample. Finally, and most importantly, the method needs to make sure that the PCRs of both the target gene and the internal standard gene are in their log-linear phases and have comparable efficiencies.

We have developed and validated a method that meets these requirements. As an internal standard gene, we selected the *PBGD* gene, a household gene that is expressed in all mammalian cells in a non-cell cycle–dependent manner (6,10). Although we cannot be sure that the level of expression of *PBGD* is precisely the same in all cells irrespective of cell type, it is reasonable to assume that it is fairly constant in a given cell type. Thus, the level of *PBGD* expression should be a suitable surrogate for the number of cells that are put into the reaction. Ideally, the amplification of a perfect internal control should be carried out in the same test tube as the gene of interest in order to avoid small variabilities of amplification between the tubes. In our experience, this resulted in undesirable competition and nonspecific amplification products. In a triplicate (or more) experiment, we were able to show that the ratios of expression are not substantially different between different experiments in the linear phase of amplification.

The quantification of mRNA expression using RT-PCR is far from straightforward. The amount of product yielded by PCR is exponentially related to the number of PCR cycles, but this is only true below a certain number of amplification cycles, and only if the conditions of the reaction are suitably selected. Although one might expect

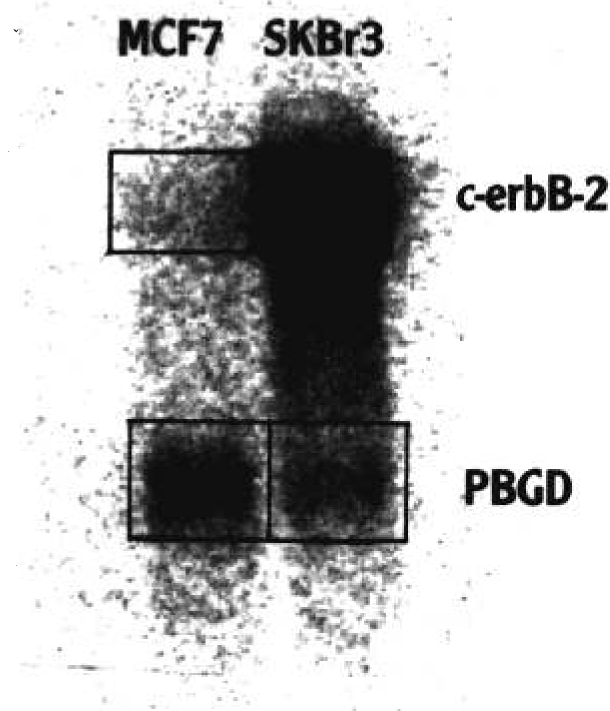

FIG. 2. Expression levels of *c-erbB-2* and *PBGD.* Northern blot with 3 μg mRNA of MCF7 (lane 1) and SKBr3 (lane 2), hybridized with ^{32}P-labeled *c-erbB-2* and *PBGD* probes simultaneously. The boxes indicate the measured bands analyzed by phosphor imaging.

TABLE 6. *Comparison of the RT-PCR assay with Northern blot analysis*

	Ratio of *c-erbB-2/PBGD* ratios (SKBr3/MCF7)	Ratio of *BRCA1/PBGD* ratios (SKBr3/MCF7)
RT-PCR*	33	0.4
Northern blot analysis†	39	0.4

The relative expression levels are calculated by dividing the ratio of *c-erbB-2/PBGD* or *BRCA1/PBGD* for the cell line SKBr3 by this ratio for MCF7.
* See Tables 2, 3, and 4.
† See Table 5.

TABLE 7. *Gene expression levels in fine-needle aspirates of human breast cancer specimens measured by RT-PCR*

FNA specimen	c-erbB-2/PBGD ratio	BRCA1/PBGD ratio
Tumor A	4	
Tumor B	16	
Tumor C, FNA 1*	4/6	0.4/0.5
Tumor C, FNA 2*	3/5	0.4/0.6
Tumor D, FNA 1*	15/25	0.2/0.3
Tumor D, FNA 2*	9/33	0.2/0.6

* Two independent experiments.

each amplification cycle to result in a precise doubling of the DNA fragment defined by the primer set, PCR is usually less efficient. In practice, efficiencies of 1.6–2.0 are common. Differences in efficiency may result in part from differences in size or purine content of the template. This is particularly troubling when an internal standard is used because a relatively small difference in efficiency can result in very large differences in product ratios after many amplification cycles. However, the final products formed are small (253 and 200 bp) and the guanine–cytosine content is comparable (52% and 58%). Thus, it is not likely that there will be a difference in amplification efficiency as shown by our experiments. Because the ^{32}P-labeled CTPs are randomly distributed in both the control and the product of interest, the overall incorporations are comparable. We do not consider it likely that many PCR products are unfinished, even without final extension. Moreover, including such a final extension step for each cycle number did not alter our results (data not shown). Because of this we decided to omit the final extension step.

At first instance we did attempt to control for these efficiency variations by calculating the actual PCR efficiencies in each reaction and to correct for them using linear regression analysis (extrapolation method). As Tables 2 and 3 show, this method yielded nonreproducible results. The reason for this is that the error in determining the efficiency is relatively large (see Results). As a result, this method had to be abandoned, and the relative signal intensities had to be calculated from the distance of the two amplification curves (distance of curves method). Clearly, any difference in amplification efficiency is disregarded by this method. We arbitrarily chose, however, only to use experiments in which the calculated efficiency of each experiment separately was between 1.6 and 2.0. The resulting values are reasonably reproducible, with a standard deviation between experiments of only 28%.

One generally accepted standard for the quantification of mRNA expression continues to be the Northern blot analysis. Northern blots probed for *PBGD*, *c-erbB-2*, and *BRCA1* show that the expression levels in our assay are comparable to those using Northern analysis. Our assay, however, yields gene expression levels relative to an internal standard gene. Clearly, this ratio is not identical in the Northern assay because it depends on the relative sizes and activities of the probes and on the hybridization conditions employed. The degree of difference between the values obtained for MCF7 and SKBr3, however, should be similar in both systems. This is the case, as shown that the assay is feasible when the input is not measurable mRNA but instead the minute quantity of cells obtained by typical FNA (see Table 7).

In summary, we have shown that a relatively straightforward RT-PCR assay can measure gene expression levels of at least two genes, *c-erbB-2* and *BRCA1*, relative to the internal standard gene *PBGD*. To obtain reproducible results, at least four points on the log-linear part of the amplification cycle versus signal intensity curve must be determined. Using these points, the amplification per cycle efficiency must be between 1.6 and 2.0. The relative expressions must be calculated using a method such as the distance of curves method, rather than a method based on linear extrapolation. The quantitativeness of the method was validated for *c-erbB-2* with mRNA isolated from two cell lines and from tumor cells obtained by FNA of four tumor specimens. The use of similar methods for a second gene, *BRCA1*, immediately resulted in reproducible and valid results. It is reasonable to assume that these results can be generalized to any mRNA species for which suitable primers can be obtained. □

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