We adapted competitive reverse transcription-polymerase chain reaction (RT-PCR) for quantitative evaluation of c-erbB-2 gene expression in breast cancer. A fixed amount of cDNA target was coamplified with dilutions of a nonhomologous DNA sequence used as an internal standard (IS). The IS and the target shared the same primer sequences but yielded PCR products of different sizes (348 and 340 bp, respectively). A fluorescent sense primer was used so that the PCR products separated on denaturing polyacrylamide gels could be quantified with an automated DNA sequencer. In human breast cancer cell lines, c-erbB-2 expression was found to range from 0.151 to 652 fmol/µg total RNA (i.e., 91 to 391 200 molecules/µg total RNA; 1 fmol = 10⁻¹⁵ amol), with the two highest values corresponding to the c-erbB-2 overexpressing cell lines MDA-MB-453 and SK-BR-3. In a series of 39 breast cancer biopsies, the concentrations ranged from 0.117 fmol/µg to 1.15 amol/µg total RNA (i.e., 70 to 690 000 molecules/µg total RNA). The c-erbB-2 oncoprotein (p185) was determined in 30 samples by an enzyme immunoassay. A close correlation was found between c-erbB-2 gene and oncoprotein expression (r² = 0.0067). Thus, this competitive RT-PCR method appears to be a reliable way to evaluate the expression of c-erbB-2 in small tumor samples.

The c-erbB-2 oncogene [1], also called HER2 [2], is the human homolog of the neu oncogene identified in DNA from rat neuroglioblastomas induced by ethyl-nitrosourea [3]. Located on chromosome 17q21 [2], the gene encodes a transmembrane protein (p185) with tyrosine kinase activity, which is closely related to epidermal growth factor receptor [4].

In human breast cancer, amplification [5] and overexpression [6] of the c-erbB-2 oncogene are observed in ~20% of the cases. These alterations have been associated with a poor prognosis [7–12] and a lower response to hormonal therapy [13, 14] and chemotherapy [15–18].

Until recently, conventional methodologies such as Southern or Northern transfer, requiring large amounts of nucleic acids, were used for evaluation of amplification or overexpression of c-erbB-2 in breast cancer. The small tumor samples were studied by immunohistochemistry, and the results largely depended on the specificity and sensitivity of the antibody used [19]. Development of the polymerase chain reaction (PCR) method [20] has permitted the analysis of small tumor samples. Determination of c-erbB-2 gene amplification by PCR, in fine-needle biopsies and in fresh and paraffin-embedded breast tumor samples, has been described by several groups [21–24]. Recently, Schneeberger et al. [25] developed a competitive reverse-transcription (RT)-PCR¹ assay for determination of c-erbB-2 gene expression.

Taking into account the fact that studies have reported a c-erbB-2 overexpression in breast cancer specimens despite the lack of gene amplification [26–28], we decided to develop an assay for quantifying c-erbB-2 gene expression by competitive RT-PCR, using a nonhomologous DNA competitor and an automated DNA sequencer. The method was developed with SK-BR-3, a c-erbB-2-overexpressing human breast cancer cell line, a fixed amount of cDNA target being coamplified with various concentrations of an internal standard (IS), which had the same primer binding sites as the target but gave a slightly larger PCR product. We applied the assay to human breast cancer cell lines already known to express c-erbB-2 [29] and to a series of 39 breast tumor biopsies. The results were compared with those obtained by a quantitative assay of p185, a good correlation between mRNA concentrations and p185 having been reported [27, 30].

Materials and Methods

CELL LINES
All the cell lines were purchased from the American Type Culture Collection and were grown at 37 °C in air enriched with 50 mL/L carbon dioxide. The MCF7 (passage

¹Nonstandard abbreviations: RT-PCR, reverse transcription PCR; IS, internal standard; EIA, enzyme immunoassay.
79), BT-20 (passage 313), T-47D (passage 105), and MDA-MB-231 (passage 393) cells were cultured in Minimum Essential Medium; the MDA-MB-453 (passage 355) and SK-BR-3 (passage 38) cells were cultured in RPMI 1640; and the MDA-MB-468 (passage 344) cells were cultured in Leibovitz’s L-15 medium. All these media contained 100 mL/L fetal calf serum. The MDA-MB-134 (passage 48) cells were cultured in Dulbecco Modified Essential Medium containing 100 mL/L fetal calf serum. The HBL 100 (passage 52) cells were cultured in Dulbecco Modified Essential Medium containing 50 mL/L fetal calf serum. All media were supplemented with 2 mmol/L glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin. The cells were collected at subconfluence.

**TUMOR SAMPLES**

With the agreement of the local Ethics Committee, 39 breast cancer samples (38 primary breast cancers and 1 metastatic) were obtained from patients operated on at Center Oscar Lambret between January and November 1994. The c-erbB-2 expression was determined by competitive RT-PCR in all 39 samples; the 30 samples for which sufficient biological material were available were also examined for expression of p185 by enzyme immunoassay (EIA).

**ISOLATION OF TOTAL RNA**

The RNA was isolated with use of Tri-Reagent from Sigma in a procedure based on the method of Chomczynski and Sacchi [31]. The cells were lysed directly on the culture dish in 1 mL of the Tri-Reagent per 10 cm² of culture plate surface area; the tumor samples were homogenized in 1 mL of the Tri-Reagent with a Potter homogenizer. Following the supplier’s recommendation, to minimize the possibility of DNA contamination when isolated RNA is to be used in RT-PCR, we performed two additional steps in the extraction procedure. First, the homogenates were centrifuged at 12 000 g for 10 min at 4 °C to remove the insoluble material. Second, the precipitation of RNA with isopropanol was performed in two steps. The RNA pellet was then washed with 750 mL/L ethanol, centrifuged, and allowed to dry for 5–10 min by air-drying. The amount of isolated RNA was quantified by measuring its absorbance at 260 nm. The extracted RNA was stored at −80 °C to −20 °C with ethanol, was then dissolved in water and stored at −80 °C until PCR.

**REVERSE TRANSCRIPTION OF RNA**

The cDNA was synthesized with the cDNA Cycle Kit for RT-PCR from Invitrogen (Leek, The Netherlands). Briefly, 1 µg of total RNA was incubated with 1 µg of random hexamers for 10 min at 65 °C. After 2 min at room temperature, the following reagents were added: 10 units of RNase inhibitor, 4 µL of 5× RT buffer (1× RT buffer is 100 mmol/L Tris-HCl, pH 8.3, 40 mmol/L KCl, 10 mmol/L MgCl₂, and 0.5 mmol/L spermidine), 1.25 mmol/L of each dNTP, 4 mmol/L sodium pyrophosphate, and 5 units of AMV (avian myeloblastosis virus) reverse transcriptase. After 1 h at 42 °C, the RNA–DNA hybrids were denatured at 95 °C for 2 min. The cDNA, extracted with phenol and precipitated overnight at −20 °C with ethanol, was then dissolved in water and stored at −80 °C until PCR.

**PCR**

**Primer.** The primers were purchased from Eurogentec (Seraing, Belgium) and had the following sequence: 5′-GAAGGTGAAGGTGCTTCGAG-3′ (residues 2337–2360; sense primer) and 5′-TAGCTCATCCCCCTG-GCAATCTGG-3′ (residues 2655–2678; antisense primer) as already described by Gordon et al. [32]. This defined an amplicon of 340 bp, corresponding to a sequence located in the tyrosine kinase domain of the c-erbB-2 oncogene. Tests of these primers for their ability to amplify genomic DNA of SK-BR-3 cells yielded no PCR product.

**Construction of the IS.** To construct the IS, we used the PCR Mimic construction kit from Clontech (Ozyme, Montigny le Bretonneux, France). A nonhomologous DNA fragment (BamHI:EcoRI fragment of v-erbB) was first amplified with composite primers constituted by the c-erbB-2 primer sequence attached to a short, 20-nucleotide stretch of sequence (provided by the supplier) designed to hybridize to opposite strands of the heterologous DNA fragment. The c-erbB-2 gene-specific primer sequences were thus incorporated during PCR amplification. A dilution of the first PCR reaction product was then amplified again, but with use of only the c-erbB-2 gene-specific primers. This ensured that all molecules of the IS have the complete gene-specific primer sequences. The composite primers used in this study were chosen to design a fragment of 348 bp. The IS was purified by chromatography on a Chroma spin +TE-100 column (Ozyme) and its quantity estimated by measuring the absorbance at 260 nm. The concentrated IS was used to prepare the working solution by diluting with the Mimic dilution solution (50 µg/mL ultrapure glycogen, provided by the supplier). The serial dilutions of IS were then obtained by diluting the working solution in water.

**PCR conditions.** The PCR reaction mixture (20 µL final volume) contained 1 µL of each patient’s cDNA (equivalent to 50 ng of starting total RNA), 1.5 mmol/L MgCl₂, 200 µmol/L of each dNTP (Pharmacia), 250 µmol/L of primers, 2 units of Taq DNA polymerase (Eurobio, Les Ulis, France), and various concentrations of IS DNA (250–1.25 amol/L). The PCR was performed as follows: initial denaturation at 94 °C for 5 min; 33 cycles of 94 °C for 30 s, 65 °C for 20 s, and 72 °C for 1 min; and a final extension at 72 °C for 8 min. Negative controls (sterile water) were included in each PCR run. The PCR amplifications were performed on a GeneAmp 9600 PCR System (Perkin-Elmer).
Quantification of the products. The sense primer was 5' fluorescently labeled with 6-carboxy-hexachlorofluorescein. After completion of the PCR, 1/60 of the PCR reaction mixture was electrophoresed through a denaturing 6% polyacrylamide–8 mol/L urea gel on a 373 DNA Sequencing System from Applied Biosystem (Perkin-Elmer). Molecular mass markers fluorescently labeled with 6-carboxy-X-rhodamine were provided from Applied Biosystem. The results were analyzed with Genescan 672 Software (Applied Biosystem).

Because plots of the ratios between the PCR products of the target and of the IS vs the logarithms of the initial concentrations of added IS were linear, the initial concentration of mRNA target was determined as the point on the plot where the concentrations of target and IS were equal.

**Quantification of Oncoprotein p185 in Membrane Extracts**

The expression of p185 in the cell lines and biopsies was measured with a monoclonal antibody-based EIA from Triton Diagnostics (Ciba-Corning). As advised by the supplier, protein was quantified by using the Pierce BCA Protein Assay Reagent (Interchim, Montluçon, France).

**Results**

**Validation of the Internal Standard**

We first tested the influence of cycle numbers on the amounts of both PCR products, as follows. We coamplified 0.2 μg of SK-BR-3 cDNA and 0.05 amol of IS for various numbers of PCR cycles, from 18 to 36. As shown in Fig. 1, the amplification took place in an exponential fashion between cycles 18 and 27. After 27 cycles, amplification for both of the PCR products reached the plateau phase. That the relative amounts of the two products remained constant throughout the reaction, even when the plateau phase was reached, demonstrated that the amplification efficiency of the IS was similar to that of the target. Because we found that, as already reported [33], the competitive RT-PCR was independent of cycle number, we performed the experiments at 33 cycles to obtain a detectable fluorescent signal in cells and biopsies that did not overexpress c-erbB-2.

In a second experiment, we verified that the initial ratio of target to IS was equal to the ratio of their amplification products. First, we assayed by competitive RT-PCR an unknown SK-BR-3 cDNA preparation. Then, we mixed different amounts of this preparation with IS so that their initial ratio (amol of target to amol of IS) varied from 0.015 to 5.8. After 33 PCR cycles, the PCR products were measured and their ratios were plotted against their initial ratios (Fig. 2). The ratios before and after amplification were identical, demonstrating that the target and the IS had very similar amplification efficiency.

**Assay Performance**

**Linearity.** In this experiment, we mixed increasing amounts of the previously assayed SK-BR-3 cDNA preparation (from 0 to 2 μg) with a constant amount of IS (0.025 amol). After 33 PCR cycles, the target to IS ratios were measured, and the log of the ratios was plotted against the log of the target amounts (Fig. 3). We obtained...
a linear correlation \( y = 0.797x - 0.267, R^2 = 0.98 \) for from 0.007 to 0.105 amol of target cDNA.

Reproducibility. c-erbB-2 gene expression was evaluated in six replicates of the same sample, during the same experiment. The amounts of target and IS products were variable (CV = 13% and 17.7%, respectively), confirming tube-to-tube variability. In contrast, the target to IS ratio showed a good repeatability (CV = 10.6%).

We also loaded onto one polyacrylamide gel six aliquots of the same sample to test the reproducibility of the detection and quantification system. The amounts of target and IS products were detected and quantified with high reproducibility (CVs of 5.8% and 5.2%, respectively, with a CV of the target to IS ratio of 1.6%).

Finally, we tested interassay reproducibility by comparing the results obtained for the same sample of SK-BR-3 cDNA after three different PCR amplifications, using a different IS working solution for each PCR. The extent of c-erbB-2 expression obtained in these 3 different experiments was highly reproducible (CV = 4.07%).

**Quantification of c-erbB-2 Gene Expression**

In breast cell lines. To test the ability of the assay to discriminate between different extents of c-erbB-2 gene expression, we determined c-erbB-2 gene expression in nine human breast cell lines and compared the results obtained by competitive RT-PCR with those already reported in the literature [29] by hybridization studies (Table 1). The c-erbB-2 expression ranged from 0.151 to 652 zmol/\( \mu \)g total RNA (i.e., 91 to 391 200 molecules/\( \mu \)g total RNA). The greatest expressions were indeed found in the c-erbB-2-overexpressing cell lines SK-BR-3 and MDA-MB-453. We also evaluated c-erbB-2 oncoprotein expression. The SK-BR-3 and MDA-MB-453 cell lines were found to present oncoprotein expression >200 units/\( \mu \)g protein, the cutoff value of overexpression chosen by Koscielny et al. [34] in a series of 1065 human breast tumors.

In breast tumor biopsies. We determined c-erbB-2 gene expression by competitive RT-PCR in a series of 39 breast tumor biopsies (Fig. 4). The c-erbB-2 gene expression ranged from 0.117 zmol/\( \mu \)g to 1.15 amol/\( \mu \)g total RNA (i.e., from 70 to 690 000 molecules/\( \mu \)g total RNA; median = 9.63 zmol/\( \mu \)g total RNA, or 5778 molecules/\( \mu \)g total RNA), corresponding to the range of expression found in human breast cancer cell lines (Fig. 5). The highest value observed in the biopsies was that from the metastatic breast cancer sample. Oncoprotein p185 expression was determined in 30 of these samples. Although discrepancies were observed in several samples between the quantities of c-erbB-2 mRNA and p185, the c-erbB-2 gene expression was closely correlated with the p185 oncoprotein expression (Fig. 6, \( P = 0.0067 \)).

**Discussion**

In this paper, we describe an adaptation of the competitive RT-PCR for the evaluation of c-erbB-2 gene expression
in breast tumor biopsies. A constant amount of target cDNA was coamplified with a titration of a competitor DNA (IS), as basically performed in competitive RT-PCR. A recent report, however, describes an alternative competitive technique termed rapid competitive PCR (RC-PCR) designed to study the relative expression of specific genes, instead of absolute quantities, in a large number of small tumor biopsies, with use of a single reaction tube per sample [35].

In our study, the IS shared the same primer binding sequences as the target but contained a different sequence and gave a PCR product slightly larger than the PCR product generated from the target sequence. This small difference in size (348 vs 340 bp) was sufficient for easy separation of the PCR products on denaturing polyacrylamide gels, followed by detection and quantification with an automated fluorescent DNA sequencer.

We demonstrated that the characteristics of the present competitive RT-PCR assay are compatible with clinical use. As a matter of fact, the IS amplified with equal efficiency and achieved the plateau phase simultaneously with the target, the ratios of the two being constant throughout the reaction. This ratio remained quite identi-
tical before and after PCR, confirming that the IS and target sequences amplified with very similar efficiencies despite their slight difference in size and their difference in primary sequences. These findings are consistent with other studies demonstrating that amplification efficiency of the IS and the target was identical when the IS was designed to contain the same primer binding sequences as the target, even though the sequence between the primer sites was different [36, 37].

A dose-dependent linear response was obtained by mixing increasing amounts of target with a constant amount of IS, indicating that the assay is a reliable method to quantify c-erbB-2 gene expression, even in samples exhibiting a wide range of c-erbB-2 mRNA concentrations. Finally, the intraassay reproducibility and the interassay reproducibility were good in this competitive RT-PCR method.

By competitive RT-PCR, the c-erbB-2-overexpressing MDA-MB-453 and SK-BR-3 cell lines have the highest values. This is in agreement with the results we obtained when assaying p185 by EIA and also with the results reported in the literature [25, 29]. The other cells were found to be negative by EIA (<200 units/mg protein), as has been described in hybridization studies [29] and for competitive RT-PCR by Schneeberger et al. [25].

We determined the absolute amount of c-erbB-2 expression in 39 tumor biopsy samples. All tumors but one were primary breast cancers. In such tissue, the epithelial component (cancer cells) is the major one, and the fat component is very low. The percentage of stromal cells varies as a function of the tumor type (being higher in carcinomatous fibrosis of the breast; however, our series did not contain such tumors). The tumor biopsy samples exhibited a wide range of c-erbB-2 expression, as we already observed in the cell lines. We also looked for a positivity threshold, and we hypothesized that the tumor samples with less mRNA than that found in the MDA-MB-468 cells were without overexpression. Consequently, in our population, at least 28 (72%) cases did not overexpress the c-erbB-2 gene. This is in line with the mean percentage of negativity obtained by Northern transfer and reported in the literature [9, 26, 27, 30, 38]. Current follow-up of the patients has not been long enough to determine the prognostic value of the RT-PCR c-erbB-2 concentrations.

In 30 of the 39 tumor biopsies, the c-erbB-2 oncoprotein (p185) concentration was determined. Some discrepancies between the mRNA quantity and the p185 were observed. Five cases exhibited overexpression by EIA that was not detected by RT-PCR. Moreover, of the 12 cases determined to have high expression of c-erbB-2 mRNA, 10 were assessed for p185 production by EIA; in all the cases but 1, a high result for mRNA was associated with protein overexpression. Several reasons can be evoked to explain these discrepancies. First, the primers used in this RT-PCR method define an amplicon corresponding to a sequence located in the tyrosine kinase domain of the c-erbB-2 oncoprotein, whereas the antibodies used in EIA are directed against the extracellular domain of the p185. Another possibility is that a partial degradation of RNA could happen in some samples, such that results are underevaluated by RT-PCR; the EIA method would be advantageous in these cases.

In conclusion, with these few discrepancies, we found a good correlation between the expression of c-erbB-2 assessed by RT-PCR and by EIA. This result fits well with the correlation described in the literature between c-erbB-2 mRNA expression analyzed by Northern transfer and p185 expression assessed by immunohistochemistry [27, 30]. Consequently, we consider clinically usable the competitive RT-PCR method described. More generally, this method could be used to assay several biological variables in the same biopsy.

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