Quantification of HER2/neu Gene Amplification by Competitive PCR Using Fluorescent Melting Curve Analysis

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Background: Molecular detection methods for HER2/neu gene amplification include fluorescence in situ hybridization (FISH) and competitive PCR. We designed a quantitative PCR system utilizing fluorescent hybridization probes and a competitor that differed from the HER2/neu sequence by a single base change.

Methods: Increasing twofold concentrations of competitor were coamplified with DNA from cell lines with various HER2/neu copy numbers at the HER2/neu locus. Competitor DNA was distinguished from the HER2/neu sequence by a fluorescent hybridization probe and melting curve analysis on a fluorescence-monitoring thermal cycler. The percentages of competitor to target peak areas on derivative fluorescence vs temperature curves were used to calculate copy number.

Results: Real-time monitoring of the PCR reaction showed comparable relative areas throughout the log phase and during the PCR plateau, indicating that only end-point detection is necessary. The dynamic range was over two logs (2000–250,000 competitor copies) with CVs <20%. Three cell lines (MRC-5, T-47D, and SK-BR-3) were determined to have gene doses of 1, 3, and 11, respectively. Gene amplification was detected in 3 of 13 tumor samples and was correlated with conventional real-time PCR and FISH analysis.

Conclusion: Use of relative peak areas allows gene copy numbers to be quantified against an internal competitive control in <1 h.

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The HER2/neu gene is amplified in 25–30% of primary breast cancers (1) and correlates with relapse and shorter survival time (1–4). In addition to its prognostic significance, HER2/neu status may also impact therapeutic decisions. Novel therapeutic strategies are being developed for tumors with HER2/neu amplification. Antisense oligonucleotides can be used to block the translation of messenger RNA in vitro (5). A monoclonal antibody against HER2/neu can inhibit tumor growth when combined with traditional chemotherapy such as doxorubicin and paclitaxel (6). This antibody, known as Herceptin® (trastuzumab), is being tested in clinical trials to determine safety and efficacy (7). Higher response rates were seen when Herceptin was combined with other chemotherapeutic agents, such as cisplatin (8, 9).

Because of its prognostic significance and potential in therapeutic decisions, HER2/neu gene quantification is becoming increasingly important. Currently, HER2/neu amplification is most commonly detected by protein expression (immunohistochemical detection). Molecular methods for HER2/neu have also been developed. The Food and Drug Administration has recently approved a fluorescence in situ hybridization (FISH) technique (10, 11). Several PCR techniques use a competitor as an internal control. The competitor is designed to be nearly identical to the target sequence, while still being distinguishable from the target. Reported competitors differ from the target sequence by ~20 bp (12–14) or by restriction site sequence alterations (15–17). The target and competitor are amplified together using the same PCR primer sets, then distinguished and visualized by gel electrophoresis. Differing band intensities corresponding...
Fluorescent hybridization probes can be used to detect a single base change, discriminating between alleles by probe melting temperature ($T_m$) using rapid-cycle PCR (18, 19). When the sample is heated slowly, the probe melts at a characteristic temperature. As the probe melts from the PCR product, a decrease in the resonance energy transfer is seen. A single base alteration within the region complementary to the probe decreases the probe’s stability and lowers its $T_m$. Derivative melting curve plots can easily distinguish different alleles by differences in $T_m$ (20–23). Peak areas can also be used to quantify gene dose (24, 25).

Fluorescent probes are also used to monitor product accumulation during PCR for real-time quantification. As product accumulates, fluorescence increases and the cycle at which the product is detected above background can be used for quantification (19, 26). Real-time quantitative PCR usually uses external standards with or without reference genes. However, several assumptions are made with these methods. External standards assume equal efficiencies between the samples and the standards (27). Methods that use a reference gene also usually assume equal efficiency between the target and the reference. These techniques require that all relevant PCR efficiencies are constant up to the fluorescent threshold for accurate quantification. In contrast, competitive PCR does not make these assumptions.

We have used melting curve analyses and peak area determinations to develop competitive PCR for HER2/neu quantification in the LightCycler®. To eliminate PCR efficiency differences, the competitor was designed to be as similar to the wild-type sequence as possible with a single base alteration. The competitor was amplified by the same primers as the target and was distinguished from the target by melting curve analysis. The peak areas of target to known copy numbers of competitor DNA allowed quantification of the target without any assumption about PCR efficiency of different targets and samples.

**Materials and Methods**

**Samples and Controls**

Assay characteristics were determined using two cell lines with increased HER2/neu gene copies: SK-BR-3 (ATCC no. HTB-30), derived from an adenocarcinoma cell line with a high HER2/neu copy number (~11 copies) (12, 28), and T-47D (ATCC no. HTB-133), a ductal carcinoma cell line with an increased, but lower HER2/neu copy number (~4 copies) (12, 29). The single-copy control was DNA from MRC-5 (ATCC no. CCL-171), a fetal lung cell line with a normal copy number of HER2/neu. DNA was prepared from cell lines using phenol-chloroform extraction and ethanol precipitation (30). DNA samples from fresh and frozen tissues were obtained under a research protocol from Penrose Hospital (Colorado Springs, CO) and Louisiana State University Medical Center (Shreveport, LA). The DNA concentration was 50 mg/L (as determined by spectroscopy) in 10 mmol/L Tris (pH 8.0) containing 0.1 mmol/L EDTA. All samples were boiled for 10 min to denature the DNA before the template was added to the reaction. HER2/neu FISH testing was performed by Penrose Hospital or Louisiana State University (31).

**Primer synthesis and labeling**

Primer synthesis and labeling were performed as described previously (19, 20, 32). The HER2/neu assay used a fluorescently labeled primer and a probe that hybridized to the region of the amplicon with the competitor base alteration (Fig. 1). The forward primer for HER2/neu was 5′-CCTCTGAGCTCCATCGTTC-3′. For fluorescent detection, this sequence was labeled 4 bp from the 3′ end with Cy5 (Amersham). The reverse primer sequence was 5′-CGGATCTTCTGCGGTGCG-3′ (12). The probe for HER2/neu was 5′-ACCAGCAGATGCAAAACCACGC-3′, with the 3′ end conjugated to fluorescein (Biogenix).

For conventional real-time PCR, portions of the β-globin (33) and HER2/neu genes were amplified and detected by two adjacent hybridization probe sets. The probe sequences for β-globin were 5′-CAACAGACACCATGGTGCACTGAGGATC-3′, with the 3′ end labeled with fluorescein, and 5′-TCTGCGTTACTGCCCTGTTGCAA-3′, with the 5′ end labeled with LCRed 705 (Roche Biochemicals) and the 3′ end blocked with a phosphate group. The probes for HER2/neu were 5′-CTTGTAGGATTCACACCAACCCACCAAGA-3′, with a fluorescein label, and 5′-ACCAGCAGATGCAAAACCACGC-3′, with a 5′ LCRed 640 label and a 3′ phosphate.

**Competitor Preparation**

The competitor was constructed by introducing the desired base alteration into a 44-bp oligonucleotide primer; its sequence was 5′-CCTCTGAGCTCCATCGTTCGCGGTGTTAGCATTGCTGCTTTG-3′. This primer was used to amplify genomic DNA to incorporate this alteration. The PCR product for the competitor template was amplified by rapid-cycle PCR (18). PCR was performed with 0.5 μM each primer, 200 μM each dNTP, 3.0 mm MgCl₂, 50 mM Tris (pH 8.3), 500 mg/L bovine serum albumin, 0.4 U of Taq polymerase (Boehringer Mannheim), and 250 ng of DNA per 50-μL total reaction volume in a thin-walled microcentrifuge tube. PCR con-
ditions were 94 °C for 30 s, 50 °C for 30 s, and 75 °C for 30 s for 30 cycles on a rapid air thermocycler (Rapid Cycler™, Idaho Technology, Salt Lake City, UT). The transition rate between 50 °C and 72 °C was 1.0 °C/s. The PCR product was visualized as a single band by agarose gel electrophoresis, purified by phenol-chloroform extraction, and precipitated with ethanol (30). Primers were removed from PCR products by microconcentrators (MicroCon 50; Millipore) and 5 min of centrifugation at 10 000g. Competitor concentrations were determined by spectroscopy. PCR product (40 ng) was sequenced in each direction by dye-terminator chemistry using an ABI 377 sequencer (Applied Biosystems) to confirm the base alteration in the competitor.

**PCR amplification**

Serial twofold dilutions of the competitor from 500 000 to 500 copies were coamplified with 50 ng of genomic HER2/neu from cell lines. DNA from breast tumor tissue was amplified with 15 000 copies of competitor. PCR reagents were as described above except that 0.25 mM Cy5-labeled forward primer, 0.1 μM reverse primer, and 0.1 mM fluorescein-labeled probe were used for HER2/neu. For β-globin, the forward primer concentration was 0.2 μM, with a reverse primer concentration of 0.5 μM. The fluorescein-labeled probes for real-time PCR were used at a concentration of 0.1 μM, and the LCRed 640 or 705 was used at 0.5 μM. Klentaq polymerase (4 U/10 μl; AB Peptides) and TaqStart antibody (0.16 – 0.32 μg/10 μl; Clontech) were used for amplification. PCR conditions were 94 °C for 0 s, 53 °C for 10 s, and 72 °C for 0 s for 40 cycles. The programmed transition rates were 20 °C/s from denaturation to annealing, 1 °C/s from annealing to extension, and 20 °C/s from extension to denaturation. Rapid cycling technology allows “0 s” temperature spikes at denaturation (94 °C) and annealing (53 °C) as described previously (18). Fluorescence was detected once per cycle at the end of the annealing stage.

After 30–40 cycles of amplification, the samples were denatured at 94 °C for 3 s, cooled to 65 °C, and held for 10 s, followed by 55 °C for 10 s and 45 °C for 3 min with transition rates of 20 °C/s. Samples were heated from 45 °C to 85 °C at 0.2 °C/s. Fluorescence was monitored at every 0.2 °C. Fluorescence vs temperature plots were converted into derivative fluorescent curves with respect to temperature (−dF/dT) for easy visualization of “melting peaks” and the T_m of the competitor and wild-type PCR products. The derivative curve was fit to the sum of two gaussian curves by nonlinear least-squares regression analysis, and the area of each gaussian curve was used as the area of each component (LightCycler Data Analysis software, Ver. 2.1.36).

Results from the internal competitor system were compared with conventional real-time PCR results using external standards. A portion of a single-copy gene (β-globin) was coamplified with HER2/neu in the same tube and analyzed by real-time PCR (19, 26). Monitoring of the reaction at each cycle detected the accumulation of PCR product as seen by fluorescent increase above background. Twofold serial dilutions of calibrators (prepared as described for competitor templates) ranging from 10^9 to 10^3 copies were used as a calibration curve (19).

**Analysis**

Assay variation was established by coamplifying the MRC-5 cell line with nine different competitor concentrations ranging from 250 000 to 500 copies (10 replicates each). The areas of the competitor and target peaks were determined at each competitor concentration, and a percentage of the competitor area to target area (A_C/A_T) was calculated. The log of the peak area percentages [log(A_C/A_T)] for each competitor concentration was plotted against the log of the competitor copy number (log[C]). A third-order polynomial (y = ax^3 + bx^2 + cx + d) was used to fit the competitor concentrations analyzed. When the MRC-5 cell line was analyzed with equal copy numbers of the competitor and target, the log(A_C/A_T) was 0.53. The copy numbers of unknown samples were determined by setting y = 0.53 (copy number equivalence) and solving for x. A two-point linear interpolation of the copy number was also obtained by choosing the nearest data point on each side of the equivalence line. Finally, a single-point estimate of the copy number was obtained by vertical translation of the third-order polynomial. Given the data point y = log(A_C/A_T) and x = log[C], and the best fit values for a, b, and c of the nearest calibration curve, the vertical offset of the translated curve (d’) was calculated and 0.53 = ax^3 + bx^2 + cx + d’ solved for x.

The HER2/neu copies for each tumor and cell line were compared with HER2/neu copies of the MRC cell line (single-copy control) to obtain the degree of amplification or gene dose. The gene dose was then compared with reported values for the cell lines (12, 28, 29). A gene dose >2.5 was considered amplified for HER2/neu. Gene doses of tumor samples were compared with conventional kinetic PCR with external standards and FISH.

**Results**

Melting curve analysis showed that the competitor and target amplicons were easily distinguished from each other (Fig. 2). The wild-type T_m was 70 °C, whereas the competitor T_m was 62 °C, a difference of 8 °C. As the amount of added competitor increased, the relative area of the competitor peak to the target peak (A_C/A_T) also increased.

To determine whether melting curve analysis is best done during the exponential or plateau phases of PCR, melting curves were acquired each cycle after the fluorescence significantly exceeded baseline (cycle 33) and until the reaction reached the plateau phase (cycle 36). The results are shown in Fig. 3. At 15 000 competitor copies, the competitor peak area from cycle 33 to cycle 36 was 74.1% ± 2.3% of the total area. The expected percentage with equal target and competitor copies would be 50%.
This discrepancy indicated that equal concentrations of target and competitor do not necessarily produce equal peak areas. However, the relative areas remained constant during amplification, although total peak areas increased with increasing cycles (as expected). Because competitive melting curve analysis did not strongly depend on the acquisition cycle, all subsequent experiments were performed in the plateau phase when the fluorescence signal was maximal. Real-time analysis of the PCR was not necessary.

Assay variation and dynamic range were established for replicates in different analytical runs using the MRC cell line and various competitor concentrations. The log($A_C/A_T$) mean and standard deviation at each competitor concentration are shown in Fig. 4A. A third-order polynomial, a second-order polynomial, and a line were fit to the average log($A_C/A_T$) values of each competitor concentration. The best fit was a third-order polynomial ($R^2 = 0.9978$) with a dynamic range of over two logs with CVs $<20\%$ (Fig. 4B). Higher order polynomials only slightly improved the fit ($R^2 = 0.9982$ for a fifth-order polynomial). The linear portion of the curve covered more than one log. The best estimate of the relative peak areas observed with equal copy numbers of target and competitor was $A_C/A_T = 3.4$ or log($A_C/A_T$) = 0.53.

Using the established equivalence of peak areas, [log($A_C/A_T$)] = 0.53, we determined the HER2/neu copy numbers of three cell lines by fitting to third-order polynomials (Fig. 5 and Table 1). Gene doses relative to the MRC-5 cell line are also shown. In addition to a third-order polynomial fit, two other analytical methods were used to estimate HER2/neu copy numbers. One method used two competitor concentrations per sample, with points above and below the equivalence line. The other method used a single competitor concentration of 15 000 copies. This concentration was chosen so that $A_C.A_T$ for MRC-5 cells. For HER2/neu-amplified samples, the relative area of the competitor peak decreased, allowing
relative HER2/neu quantification in the range of clinically relevant samples. The copy number was calculated using a vertical displacement of the known third-order polynomial curve closest to the single point. The results of all three methods are shown in Table 1. Although there was a decrease in the apparent gene dose with the single-point estimate, amplification was still apparent in the abnormal (T-47D and SK-BR-3) cell lines.

To test the potential clinical utility of the method, DNA samples from 20 frozen breast tumors, 2 cultures of healthy breast cells, and 8 white blood cell samples were amplified using a single competitor concentration. For the 10 nonpathological samples tested with a single-point estimate, amplification was still apparent in the abnormal (T-47D and SK-BR-3) cell lines.

Fig. 5. HER2/neu coamplification of cell lines with twofold dilutions of competitor (50 000–500 copies).

A third-order polynomial was used to determine the copy numbers of cell lines: MRC-5 (●), \( y = -0.07x^3 + 0.56x^2 - 0.99x - 1.6 \) \( (R^2 = 0.9994) \); T-47D (■), \( y = -0.07x^3 + 0.79x^2 - 2.3x + 0.71 \) \( (R^2 = 0.9988) \); SK-BR-3 (▲), \( y = -0.20x^3 + 3.1x^2 - 15x + 23 \) \( (R^2 = 0.9994) \). Bars, SD. ••••, equivalent competitor and target copy numbers (C = T).

Fig. 6. Correlation of single-tube competitive PCR amplification of tumor DNA to real-time kinetic PCR.

Twenty tumor samples were coamplified with 15 000 competitor copies. Gene doses were compared with gene doses calculated using real-time PCR and external standards. The correlation coefficient is 0.85.

Table 1. Comparison of three calculation techniques\textsuperscript{a} to quantify copy number and gene dose in cell lines.

<table>
<thead>
<tr>
<th>Analysis method</th>
<th>MRC-5</th>
<th>T-47D</th>
<th>SK-BR-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copy number\textsuperscript{b}</td>
<td>20 300 ± 5970</td>
<td>60 600 ± 14 400</td>
<td>242 000 ± 43 700</td>
</tr>
<tr>
<td>Gene dose</td>
<td>3.0 ± 0.71</td>
<td>2.8 ± 0.75</td>
<td>12.0 ± 2.2</td>
</tr>
<tr>
<td>Copy number (15 000 competitor copies)</td>
<td>19 600 ± 4590</td>
<td>42 800 ± 9840</td>
<td>171 000 ± 22 100</td>
</tr>
<tr>
<td>Gene dose</td>
<td>2.2 ± 0.50</td>
<td>8.7 ± 1.1</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Three calculation methods were analyzed using three cell lines: MRC-5, which contains a single dose of HER2/neu; T-47D, with a reported gene dose of 4; and SK-BR-3, with a reported dose of 11.

\textsuperscript{b} Values are reported as mean ± SD (n = 5).

Discussion

Current methods for HER2/neu detection include immunohistochemistry and FISH. Immunohistochemical techniques are subject to variations among antibodies, fixatives, and technical assessment. FISH allows analysis of individual cells and can detect whether amplification is the result of chromosome duplication or gene amplification (10, 11, 31). However, FISH is time-consuming and requires several hours for hybridization (~4 h) and washings, a fluorescent microscope, and significant technical time to count amplification in individual cells.

Quantitative PCR techniques have several advantages over FISH for assessing HER2/neu amplification. The speed and ease of PCR-based testing makes it more conducive to screening multiple samples at the same time. Furthermore, fluorescent PCR monitoring offers advantages over existing PCR methods for quantification. PCR amplification and quantification are performed in the

Gene dose (Competitive PCR)
same reaction tube to eliminate manual manipulation of the PCR product after amplification. This reduces the chance of PCR product contamination and simplifies sample tracking. It also eliminates the use of gels and mutagenic DNA dyes such as ethidium bromide for visualization. Rapid-cycle PCR also reduces amplification time and increases specificity over traditional PCR (18). A 40-cycle PCR and subsequent melting curve analysis can be completed in ~45 min. The use of sequence-specific probes ensures that nonspecific PCR products will not interfere with the analysis.

Fluorescent real-time HER2/neu detection has previously been described using an exonuclease system (26). In this method, the cycle in which target amplicon is detected above background is compared with a series of external standards. When external standards are used, real-time detection of product as it accumulates is more accurate than end-point analysis. Although this procedure has advantages over conventional methods, external standards do not control for standard-to-sample or sample-to-sample variation in PCR efficiency.

The use of internal competitors for quantitative PCR has several advantages over other quantification methods. Internal competitors control for amplification efficiency differences between samples and allow end-point analysis because the percentage of competitor relative to the target area is independent of cycle number (13). The use of a reference gene in quantification, amplified in either the same or a different reaction tube, also assumes equal amplification efficiencies between reference and target PCRs. Additionally, the use of a reference gene becomes problematic with tumor samples because many tumors have unstable chromosomes. Duplications or deletions of a chromosome or chromosome region containing the reference gene will distort the true HER2/neu gene dose.

HER2/neu amplification has been linked with DNA aneuploidy (34, 35), and therefore low gene amplification may be attributable to aneuploidy (duplication) of chromosome 17 rather than gene amplification on the same chromosome. In FISH analysis, the use of a reference gene near the centromere of chromosome 17 can differentiate between gene amplification and polysomy of chromosome 17 (10, 31). However, reports differ on the significance of chromosome 17 polysomy and the resulting HER2/neu overexpression (10, 31, 34).

Equal copy numbers of competitor and template did not produce equal peak areas on derivative melting curve plots. One explanation may be variations in the amounts of genomic DNA and/or competitor DNA in the reaction. The use of fluorescent DNA dyes such as Picogreen (Molecular Probes) or limiting dilutions (36) is an alternative to ultraviolet spectroscopy in determining DNA concentrations. However, in our hands, simple absorbance measurements were the most consistent. A second explanation may be that probes hybridize preferentially to one PCR product over the other, particularly if probe concentrations are limiting. Additionally, peak areas at lower temperatures are expected to be larger than at higher temperatures because of the temperature effect on fluorescence (19). Unequal peak areas for equal concentrations of DNA were also reported in a study detecting gene deletion and duplication by peak area using the LightCycler (24). In this study, peak areas for heterozygotes were not the predicted one-to-one ratio, and a known control was used to calculate a correction factor.

Testing each sample at multiple competitor concentrations and fitting all data points to a third-order polynomial should give the most accurate results and the widest dynamic range. However, good quantitative results can be obtained with only two competitor concentrations, using a linear fit to determine copy number. Repeat testing may be required to ensure competitor concentrations that flank the equivalence point. A single amplification at one competitor concentration can also be used to distinguish between amplified and nonamplified samples after the representative polynomial curves are known.

HER2/neu amplification status by competitive PCR correlated to conventional real-time PCR in 20 of 20 tumors. Eighteen of the 20 tumors correlated with FISH results. Of the two discrepant samples, one had a tetraploid DNA content. FISH analysis uses a chromosome 17 probe as a reference control and would not consider a tetraploid tumor amplified, a difference that is detected with PCR techniques. The second discrepant sample was amplified by FISH but not amplified by both PCR methods. A possible explanation is that the HER2/neu amplified genes were diluted in a background of nontumor cells. For this reason, samples containing a high percentage of tumor cells or microdissected samples are necessary for molecular analysis.

In evaluating this method, several sources of errors should be considered. One possible error is in calculating peak areas. A $T_m$ difference of 8 °C does not produce complete baseline separation between melting peaks. However, fitting a curve from the sum of two gaussian curves incorporates the minor melting curve overlap present into the calculation. Even the most stable mismatch can be resolved with melting curve analysis of hybridization probes (21). However, less stable mismatches improve $T_m$ separation, reduce the overlap of melting curves, and may increase the precision of relative area calculations. In choosing an appropriate base alteration for the competitor, all possible mismatches at the selected site were examined using nearest-neighbor thermodynamic estimates of DNA stability (37–40). These estimates are useful in predicting the $T_m$ for melting curve analysis (41, 42). C-A, C-T, and C-C mismatches gave similar predicted $T_{m}s$ of 63.2–63.3 °C. Other mismatches (G-G, G-T, G-A) were predicted to be more stable, with $T_{m}s$ of 64.4–65.5 °C.

A second potential error may exist in samples with low gene amplification. Quantitative PCR is estimated to be accurate only within fourfold (43). This may explain the
lower gene dose for the T-47D cell line than is reported in the literature. An alternative explanation may be that genetic alterations occurred through additional cell passages. FISH analysis of the T-47D cell line confirmed an average gene dose of 2.6, less than the reported value. In the clinical samples studied, a gene dose of 2.5 appeared to discriminate between amplified and nonamplified samples. This was confirmed using DNA from sources without HER2/neu gene amplification. Repeat analysis of gene doses between two and four may be useful to confirm low amplification.

In conclusion, this report describes a novel quantitative, competitive PCR application using fluorescent hybridization probes and melting curve analysis. Quantification by melting peak area using fluorescent hybridization probes provides a sensitive and practical PCR method for gene quantification. This method controls for sample to sample efficiency differences, without the need of external standards or an independent reference gene.

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References


