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# Evaluation of HER2 Gene Status in Breast Cancer Samples with Indeterminate Fluorescence *in Situ* Hybridization by Quantitative Real-Time PCR Method

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Administration of drugs targeting HER2 (official name *ERBB2*) is an important component of therapy for breast cancer patients with HER2 amplification/overexpression as determined by *in situ* hybridization (ISH) and immunohistochemistry (IHC). In approximately 5% of breast cancers, ISH assays fail. In these cases, HER2 protein expression is evaluated by IHC alone that may yield false negatives/positives for poor-quality samples. Therefore, we developed a method that was based on quantitative real-time PCR applicable for DNA from formalin-fixed, paraffin-embedded tissue samples. Its limit of detection was determined with breast cancer cell lines and validated with 223 breast cancer patient samples. On the basis of comparisons with fluorescent ISH (FISH) and IHC data, the sensitivity of the new method was 94.2% and 95.1%, its specificity was 100% and 99.1%, and overall concordance between results obtained with the quantitative real-time PCR method and FISH/IHC was 97.6% for both methods. The quantitative real-time PCR method was then used to evaluate the HER2 status of 198 of 3696 breast cancer tissues that yielded indeterminate FISH results. The HER2 copy number was successfully determined in 69.2% of these indeterminate samples. Thus, the DNA-based technique appears to be a specific, sensitive method for determining HER2 copy numbers when the FISH assay fails, which may complement IHC tests. *(J Mol Diagn 2015, ■: 1—10; http://dx.doi.org/10.1016/j.jmoldx.2015.03.007)*

Q4 The human epidermal growth factor receptor 2 gene (HER2, official name *ERBB2*) is located on chromosome 17q and amplified in 15% to 20% of breast cancer patients. HER2 is a prognostic biomarker associated with poor prognosis, early recurrence, and reduced progression-free survival.[1,2] HER2 expression is also a predictor of responses to drugs that target HER2 (including trastuzumab, lapatinib, and pertuzumab) that are currently approved by regulatory agencies for treating HER2-positive breast cancer patients.

Various methods can be used to determine the HER2 copy number or abundance of the corresponding protein in a tissue sample, including fluorescence (FISH), chromogenic, or silver *in situ* hybridization, immunohistochemistry (IHC), Southern or Western blot analysis, slot blot analysis, PCR, reverse-transcription PCR, and enzyme-linked immunosorbent assays.[3,4] IHC and ISH are widely accepted as the gold standards for evaluating HER2 status. IHC is the primary recommended screening method, with ISH being used to confirm IHC results. Patients with an IHC (HercepTest) score of 3+ are eligible for HER2-targeted therapy, whereas patients with 0 or 1+ scores do not overexpress HER2 and are therefore unsuitable for the

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**Table 1** Characteristics of HER2, GCS1, *DCK*, and *EPN2* Primers/Probes

| Gene | Primer/probe | DNA sequence | Product size (bp) |
|---|---|---|---|
| HER2 | Forward | 5′-AGAGTCACCAGCCTCTGCAT-3′ | 138 |
| | Reverse | 5′-GCAACTCCCAGCTTCACTTT-3′ | |
| | Probe | BHQ1-CTCCTTTTCACACTCTTGCCGACGTC-FAM | |
| GCS1 | Forward | 5′-CAGGTGACCCTGAAAATTCC-3′ | 133 |
| | Reverse | 5′-CTTCAGCATGGCTCTCCAG-3′ | |
| | Probe | BHQ1-AAATCAAGCCCTGCCAAGACTGGC-HEX | |
| *DCK* | Forward | 5′-CCGCCACAAGACTAAGGAAT-3′ | 109 |
| | Reverse | 5′-CGATGTTCCCTTCGATGGAG-3′ | |
| | Probe | BHQ1-AGAAGCTGCCCGTCTTTCTCAGCC-HEX | |
| *EPN2* | Forward | 5′-CCGCCACAAGACTAAGGAAT-3′ | 130 |
| | Reverse | 5′-CGATGTTCCCTTCGATGGAG-3′ | |
| | Probe | BHQ1-AGAAGCTGCCCGTCTTTCTCAGCC-HEX | |

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treatment. Samples from patients with equivocal result (IHC 2+) must be retested for HER2 gene copy number by using the ISH assay.[2,5]

However, in approximately 5% of breast cancer samples, HER2 copy numbers cannot be evaluated with FISH because of poor tissue quality, which may be due to incorrect handling, especially in cases in which inappropriate fixation methods are used or degradation occurs.[6,7] Similarly, the use of different fixation methods can adversely affect IHC but not gene amplification and FISH results,[8] necessitating use of a DNA-based method to determine the HER2 gene copy number.

We therefore designed a novel method that is based on quantitative real-time PCR (qPCR) for determining HER2 gene copy numbers. The method involves three duplex qPCR amplifications to compare copy numbers of the HER2 gene with those of three reference genes: glucosidase I (GCS1; official name *MOGS*), deoxycytidine kinase (*DCK*), and epsin 2 (*EPN2*). These genes were selected because their copy numbers rarely change in breast cancers.[9]

## Materials and Methods

### Cell Lines

The CALU3 and MDA-MB-231 breast cancer cell lines used for determining the limit of detection (LOD) of qPCR method were purchased from ATCC (Rockville, MD). The HER2 gene is amplified and constitutively activated in the CALU3 cell line, which was used as a positive control. HER2 assays with the use of both FISH and IHC showed that cells of this line contained 20 copies of the HER2 gene per nucleus and had an IHC score of 3+. The MDA-MB-231 cell line was used as a negative control because it has a normal physiologic HER2 copy number (two copies) as determined by both FISH and IHC. CALU3 cells were diluted with MDA-MB-231 cells to create a dilution series with 1%, 5%, 10%, 15%, 20%, and 50% HER2-positive cell contents. Total DNA was extracted from samples of each suspension of the dilution series and analyzed by qPCR.

### Tissue Samples

A consecutive retrospective cohort of 181 formalin-fixed, paraffin-embedded (FFPE) invasive breast cancer samples collected in 2006 was used to assess the specificity and sensitivity of the qPCR method. For each sample, the HER2 gene copy number was determined by both FISH and qPCR, and the level of HER2 protein expression was determined by IHC. Samples with at least 10% of tumor tissue and both FISH and IHC data were included to the study. The consecutive retrospective cohort was further enriched for 60 samples with equivocal IHC result (2+) and complete FISH data collected in 2007. The final validation set consisted of 223 samples. The validation set was used for all statistical analyses.

Then, qPCR and IHC methods were used in parallel to analyze HER2 expression/copy number in 198 of 3696 patient samples prospectively collected between 2007 and 2012 that yielded indeterminate FISH results. The percentage of HER2-positive tumors in our study is much higher than other institutions and the literature. The reason is that mainly positive samples are sent from local laboratories to us for confirmatory testing, because our institution serves as a central/reference laboratory.

### IHC

HER2 protein was immunohistochemically detected in 4-μm FFPE sections by using the US Food and Drug Administration-approved HercepTest (DakoCytomation, Glostrup, Denmark) according to the manufacturer's instructions. The standard HercepTest scoring system was used in all cases to obtain scores of 0, 1+, 2+, or 3+, indicating no staining or incomplete faint membrane staining of $<10\%$ of tumor cells, incomplete faint membrane staining of $\geq 10\%$ of tumor cells, incomplete and/or moderate membrane staining of $\geq 10\%$ of tumor cells, and complete intense membrane staining in $\geq 10\%$ of tumor cells, respectively.[2]

### FISH

FFPE sections (4 μm) were baked overnight at 56°C on microscope slides, deparaffinized with xylene, dehydrated with

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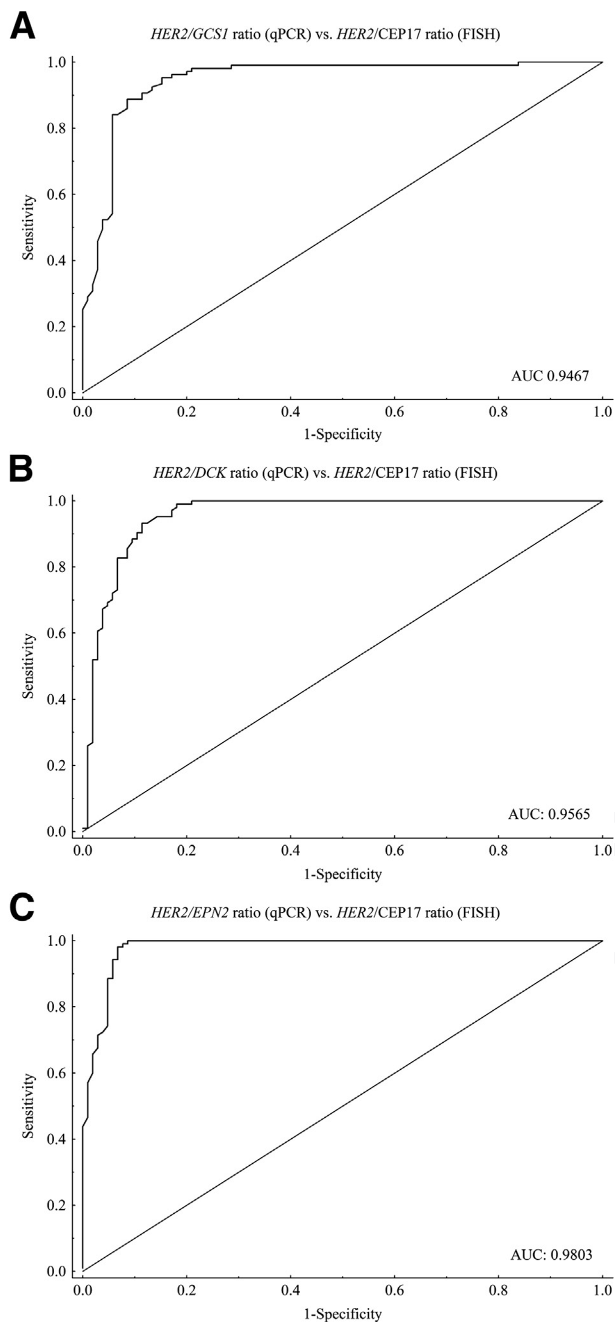


Q9 **Figure 1** ROC curves for HER2/reference gene ratios (qPCR) compared with gold standard FISH (HER2/CEP17 ratio) in the validation set. Shown are the ROC curves for the three reference genes separately: GCS1 (**A**), *DCK* (**B**), and *EPN2* (**C**). AUC, area under curve; CEP17, chromosome enumeration probe 17; FISH, fluorescence *in situ* hybridization; qPCR, quantitative real-time PCR; ROC, receiver operating characteristic.

ethanol, then chemically and enzymatically treated and co-denatured (2 minutes, 85°C) by using the PathVysion HER-2 DNA Probe Kit (Vysis, Downers Grove, IL) or Her-2/neu FISH Kit (IntellMed, Olomouc, Czech Republic), approved by the US Food and Drug Administration and/or Conformité Européenne, *In Vitro* Diagnostics. The sections were then incubated overnight at 37°C. Unannealed probe molecules were subsequently removed by washing the sections with 0.4× saline-sodium citrate followed by a 2× saline-sodium citrate solution that contained 0.1% Nonidet-P40. The samples' nuclei were then counterstained with DAPI.

HER2 and chromosome 17 signals were counted in 100 non-overlapping nuclei in each patient sample by using a fluorescent microscope (Olympus BX-51; Olympus America, Center Valley, PA). HER2 clusters of ≥20 signals were scored as 20 HER2 copies. The mean HER2/chromosome 17 ratio was calculated for each sample, using the scoring criteria recommended by the American Society of Clinical Oncologists and the College of American Pathologists. Samples were classified as amplified if the ratio was ≥2.0 and/or the mean HER2 copy number was ≥6, equivocal if the ratio was <2.0 and the mean HER2 copy number was 4 to 6, and negative if the ratio was <2.0 and the mean HER2 copy number was <4.

## DNA Extraction and qPCR

DNA was extracted from four 4-μm FFPE sections from each patient sample or from a million cells from each suspension of the CALU3/MDA-MB-231 cell line concentration series, using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. FFPE sections were deparaffinized with a routine series of xylene and ethanol washes before DNA extraction.

PCR was performed with 50 ng of total DNA and Thermo-Start DNA polymerase (ThermoScientific, Waltham, MA) with PCR primers and probes listed in Table 1. PCR conditions consisted of 95°C for 15 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. [T1]

DNA isolated from FFPE normal (noncancerous) breast tissue samples that had physiologic HER2 gene copy number and showed no evidence of HER2 amplification and expression (as determined by FISH and the HercepTest) was used as a standard. The standard DNA was also tested by qPCR for no evidence of copy number changes of control genes (GCS1, *DCK*, and *EPN2*). From the standard DNA, a dilution series of 20, 30, 40, 50, 70, and 100 ng/reaction mixture was prepared to produce standard curves by plotting DNA concentrations against cycle threshold values. Relative gene copy numbers of HER2 and the reference genes were calculated from the standard curves, assuming that normal cells have two copies of the gene and approximately 7 pg of DNA.[10] The results obtained were expressed as ratios of the HER2 copy numbers to those of the reference genes. A cutoff value of 2.2 for amplification was selected on the basis of receiver operating characteristic curve analysis (Figure 1), and the HER2 gene was considered to be amplified in samples in which the ratio of the HER2 copy number to that of at least two of the three references gene exceeded this threshold. [F1]

## Statistical Analysis

Receiver operating characteristic curves were generated from data of validation set and to compare HER2 copy

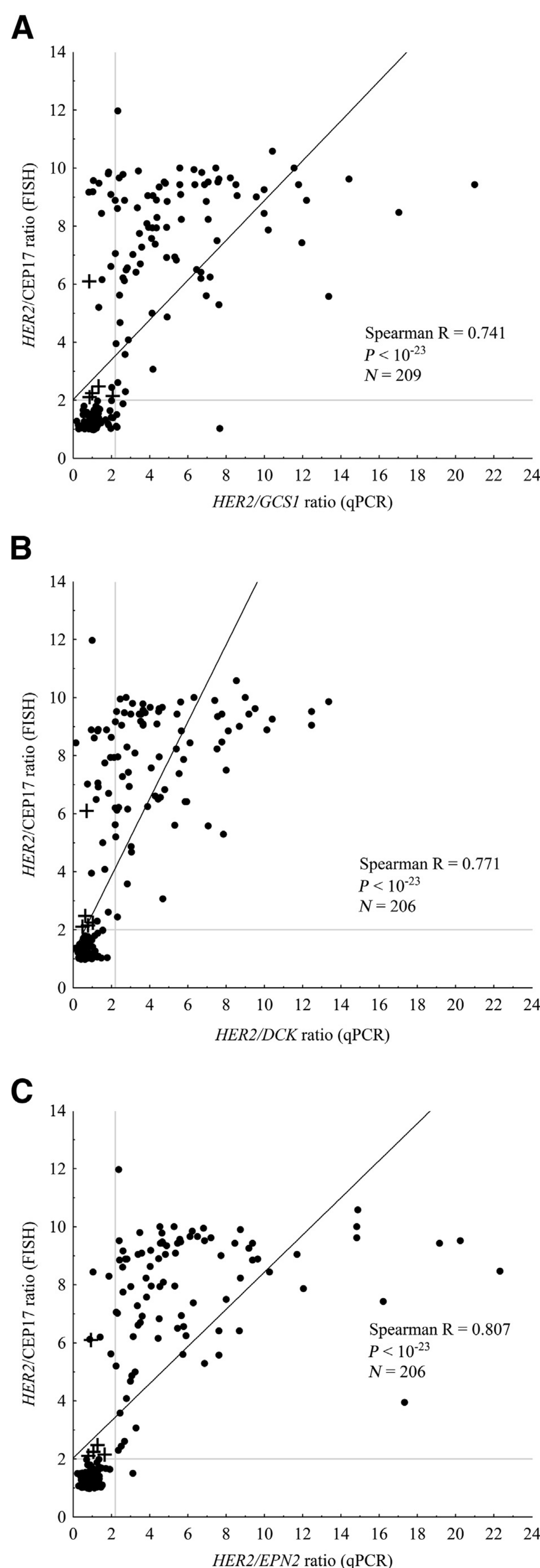


**Figure 2** Correlation of HER2/reference gene ratios (qPCR) and HER2/CEP17 ratio (FISH) in the validation set. Scatterplots with regression line (orthogonal fit − total least squares regression) and reference lines for cutoffs ($x = 2.2$, $y = 2.0$) are shown for the three reference genes separately: GCS1 (**A**), *DCK* (**B**), and *EPN2* (**C**); $P < 10^{-23}$. Five false-negative samples (qPCR negative/FISH positive) are marked with a cross. CEP17, chromosome enumeration probe 17; FISH, fluorescence *in situ* hybridization; qPCR, quantitative real-time PCR.

number detected by qPCR with gold standard FISH (HER2/chromosome enumeration probe 17 ratio) (Statistica 12.0; StatSoft, Inc., Tulsa, OK). Cutoff values were calculated with receiver operating characteristic curve analysis (software R, package OptimalCutpoints) as points with 85% sensitivity. We selected cutoff 2.2 as the mean for all three reactions.[11] Nonparametric methods were used to analyze HER2 gene copy number data obtained from the qPCR and FISH experiments and to compare the HER2 gene/protein levels determined by qPCR and IHC. Spearman rank coefficients were applied to measure correlations between qPCR and FISH. These values as well as the *P* values of the significance test are presented in scatterplots (Figure 2). Post hoc multiple comparisons of mean ranks for all groups were performed, and *P* values (two-sided significance levels with a Bonferroni adjustment) associated with each comparison were calculated for significance by Kruskal-Wallis tests (Statistica 12.0; StatSoft, Inc.). Sensitivity, specificity, concordance, and Cohen κ values were calculated for data from all qPCR assays, using the FISH and IHC results as references (R Development Core Team, *http://www.r-project.org*, last accessed February 6, 2015). IHC score 3+ was considered positive indicator of HER2 expression, whereas scores of 0 and 1+ were considered negative indicators in all statistical analyses. IHC score 2+ was considered positive indicator of HER2 expression if the FISH was positive only.

## Results

### Detection Limit of the qPCR Method

The LOD for the method was determined with the CALU3/MDA-MB-231 cell line dilution series. The HER2 gene copy numbers in six samples with different amplified/nonamplified DNA contents were analyzed relative to those for the three reference genes by using qPCR. Six replicates were run for each reference gene. The qPCR method reliably detected HER2 gene amplification in samples that contained approximately 5% of strongly positive cells (Table 2).

### Sensitivity and Specificity Assessments of the qPCR Assay versus FISH and IHC Methods in the Validation Set

To validate the cell line data obtained with the novel qPCR method, a cohort of 223 invasive breast cancer samples (Table 3) was selected and used to assess its sensitivity and

**Table 2** Evaluation of the Detection Limit of the qPCR Method

| HER2 amplified cells (%)* | HER2/GCS1 gene ratio (95% CI)† | HER2/*DCK* gene ratio (95% CI)† | HER2/*EPN2* gene ratio (95% CI)† |
|---|---|---|---|
| 1 | 1.23 (1.118–1.342) | 0.78 (0.652–0.900) | 1.52 (1.220–1.829) |
| 5 | 2.43 (2.167–2.686) | 2.01 (1.880–2.133) | 4.83 (4.116–5.550) |
| 10 | 5.12 (4.096–6.149) | 6.06 (5.396–6.730) | 14.32 (12.994–15.638) |
| 15 | 5.03 (4.283–5.781) | 5.23 (4.939–5.515) | 13.56 (12.605–14.516) |
| 20 | 8.69 (7.766–9.609) | 12.25 (11.659–12.851) | 24.23 (22.570–25.892) |
| 50 | 16.94 (15.366–18.524) | 30.05 (27.198–32.902) | 58.99 (55.153–62.824) |

*The percentage of HER2-amplified cells refers to the percentage of CALU3 cells (>20 HER2 copies/nucleus) in suspensions of the dilution series.
†Average value from six replicates.
qPCR, quantitative real-time PCR.

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specificity. The HER2 gene copy number and protein expression in all of these samples were measured in parallel by using FISH, IHC, and qPCR. HER2/GCS1, HER2/*DCK*, and HER2/*EPN2* products were successfully amplified in 212, 209, and 209 of the 223 samples, giving amplification successes of 95.1%, 93.7%, and 93.7%, respectively.

The amplification success/conformity (frequency of the identical positive/negative result) of HER2 status determined with the following combinations of reference genes HER2/GCS1 + *DCK*, HER2/GCS1 + *EPN2*, HER2/*DCK* + *EPN2*, and HER2/GCS1 + *DCK* + *EPN2* were 92.8% (207 of 223)/76.7% (171 of 223), 92.8% (207 of 223)/84.3% (188 of 223), 92.4% (206 of 223)/82.1% (183 of 223), and 91.9% (205 of 223)/74.4% (166 of 223), respectively. With the use of a single gene, we were able to analyze from 93.7% to 95.1% samples with sensitivity from 74.3% to 90.4%. However, the sensitivity improved substantially (91.7% to 93.2%) by using the combination of two reference genes with analyzable samples range from 76.7% to 84.3%. With the use of the combination of all three reference genes, the sensitivity was not superior to combinations of two genes (91.2%), nonetheless the number of analyzable samples decreased to 74.4% only.

On the basis of these data, we have established threshold criterion for qPCR HER2 positivity as a HER2/reference gene copy ratio ≥2.2 for at least two reference genes, to compromise high sensitivity of the qPCR assay with reasonable percentage of evaluable tissue samples. With the use of this criterion, we were able to determine the HER2 gene status of 210 of 223 samples (94.2%) from the validation set with sensitivity 94.2% and high concordance with FISH/IHC results (97.6%/97.6%). Sensitivity, specificity, and κ values for all combinations are summarized in Table 4. [T4]

## Comparison of FISH and qPCR Results

FISH data indicated that 102 of 223 patient samples (45.7%) were HER2 negative, 95 (93.1%) of which also gave negative qPCR results, whereas results for the other 7 samples (6.9%) were indeterminate because of qPCR amplification failure. Thus, all HER2 FISH-negative samples gave negative (or indeterminate) results in the qPCR analysis. FISH equivocal result was found in 12 cases (5.4%). Eleven cases (91.7%) were qPCR negative; one sample (8.3%) was indeterminate. Of the 223 patient samples tested, 109 (48.9%) were FISH positive and 98 of these 109 (89.9%) were also found to be positive by the qPCR analysis, whereas the qPCR results were indeterminate for six samples (5.5%), again because of amplification failure. Negative results were obtained from the qPCR method for 5 of the 109 FISH-positive samples (4.6%) (Table 5). [T5] In two of these false-negative cases, samples were heterogenic and yielded low amplification levels with three to eight gene copies per nucleus (HER2/chromosome 17 ratios were 2.11 and 2.25), together with strong complete immunohistochemical membrane staining in 10% and 15% of cells (IHC 3+), respectively.

Similarly, the other two false-negative samples yielded low amplification level with HER2/chromosome 17 ratio 2.15 and 2.48 and IHC score 1+ and 2+. In these borderline cases, the amplification levels were below the qPCR method's LOD. High-level amplification with 15 HER2 copies per nucleus, together with moderate membrane staining in 50% of tumor cells (IHC 2+) was found in the last false-negative case. qPCR method detected physiologic copy number of HER2 gene in all of the three qPCR reactions. The most probable reason for the qPCR failure could be the borderline percentage of tumor cells in the specimen (approximately 10%), leading to critical dilution of tumor DNA.

**Table 3** Histopathology of 223 Invasive Breast Cancer Samples Used to Validate the qPCR Method

| | | IHC (HercepTest), *n* | | | FISH, *n* | | |
|---|---|---|---|---|---|---|---|
| Tumor type | *N* (%) | 0/1+ | 2+ | 3+ | Nonamplified | Equivocal | Amplified |
| Ductal | 203 (91.0) | 51 | 64 | 88 | 84 | 12 | 107 |
| Lobular | 16 (7.2) | 14 | 1 | 1 | 15 | 1 | 0 |
| Mixed ductal/lobular | 4 (1.8) | 3 | 0 | 1 | 3 | 0 | 1 |

FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry; qPCR, quantitative real-time PCR.

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**Table 4** Specificity and Sensitivity of Duplex qPCR Amplifications of HER2 with GCS1, *DCK*, *EPN2*, and Their Combinations as Reference Genes, in Samples of the Validation Set

| Methods | Genes (qPCR) | Specificity | Sensitivity | κ (95% CI) | Analyzable samples, *N* |
|---|---|---|---|---|---|
| qPCR vs FISH | HER2/GCS1 | 0.963 | 0.829 | 0.792 (0.711–0.874) | 212 |
| | HER2/*DCK* | 1.000 | 0.743 | 0.742 (0.654–0.830) | 209 |
| | HER2/*EPN2* | 1.000 | 0.904 | 0.904 (0.847–0.962) | 209 |
| | HER2/GCS1 + HER2/*DCK* | 1.000 | 0.917 | 0.927 (0.870–0.984) | 171 |
| | HER2/GCS1 + HER2/*EPN2* | 1.000 | 0.932 | 0.936 (0.885–0.986) | 188 |
| | HER2/*DCK* + HER2/*EPN2* | 1.000 | 0.925 | 0.933 (0.880–0.986) | 183 |
| | HER2/GCS1 + HER2/*DCK* + HER2/*EPN2* | 1.000 | 0.912 | 0.924 (0.865–0.984) | 166 |
| | Min. 2 genes | 1.000 | 0.942 | 0.943 (0.898–0.988) | 210 |
| qPCR vs IHC | HER2/GCS1 | 0.963 | 0.845 | 0.811 (0.732–0.889) | 212 |
| | HER2/*DCK* | 0.991 | 0.748 | 0.741 (0.652–0.830) | 209 |
| | HER2/*EPN2* | 0.991 | 0.912 | 0.904 (0.846–0.962) | 209 |
| | HER2/GCS1 + HER2/*DCK* | 1.000 | 0.930 | 0.939 (0.887–0.992) | 171 |
| | HER2/GCS1 + HER2/*EPN2* | 1.000 | 0.943 | 0.946 (0.900–0.993) | 188 |
| | HER2/*DCK* + HER2/*EPN2* | 0.990 | 0.936 | 0.933 (0.879–0.986) | 183 |
| | HER2/GCS1 + HER2/*DCK* + HER2/*EPN2* | 1.000 | 0.925 | 0.937 (0.882–0.991) | 166 |
| | Min. 2 genes | 0.991 | 0.951 | 0.943 (0.898–0.988) | 210 |
| IHC vs FISH | | 1.000 | 0.982 | 0.982 (0.957–1.007) | 223 |

FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry; Min. 2 genes, the criterion for amplification (a ratio of 2.2 in at least two of the three reference genes); qPCR, quantitative real-time PCR.

Although qPCR assessments for 5 of the 223 samples were not correct (according to FISH data), a highly significant positive correlation was found between the FISH results and all three duplex qPCR analyses ($P < 10^{-23}$). They all effectively detected elevated HER2 copy numbers, and the Spearman correlation coefficients for analyses by using GCS1, *DCK*, and *EPN2* as the reference genes were 0.74, 0.77, and 0.81, respectively (Figure 2). All of the qPCR analyses exhibited similar, high levels of specificity (96.3%, 100%, and 100%) and sensitivity (82.9%, 74.3%, and 90.4%). The overall sensitivity and specificity for the qPCR assay were 94.2% and 100%, respectively. The overall concordance between the qPCR and FISH results was 97.6%, with a κ value of 0.943 (95% CI, 0.898–0.988) (Table 4).

### Comparison of IHC versus Both DNA-based Methods (qPCR and FISH)

We also compared the performance (relative to IHC) of the IHC and FISH/qPCR methods, which assess HER2 status by using different principles. IHC gave negative indications (HercepTest scores of 0 or 1+) for 68 of 223 tested samples (30.5%). Of these 68, 59 (86.8%) were also FISH and qPCR negative, two (2.9%) were FISH equivocal and qPCR negative, five (3.0%) were FISH negative but qPCR indeterminate. One sample (1.5%) was qPCR negative and FISH positive. The qPCR results indicated that the remaining one IHC-negative sample (1.5%) was qPCR positive, and correspondingly strong HER2 amplification (>20 copies/nucleus) was found for this sample by using FISH analysis.

Strong IHC positivity (3+) was detected in 90 of all tested samples (55.2%). Both the FISH and qPCR methods gave positive results for 85 of these 90 samples (94.4%); qPCR failed for three FISH-positive samples (3.3%). Two of the IHC-positive samples (2.2%) were judged to be HER2 negative on the basis of the qPCR analysis alone. FISH positivity was detected in both samples. These samples were heterogeneous (see above). An equivocal IHC 2+ score was obtained for 65 samples (29.1%). Of these 65 IHC 2+ specimens, 36 (55.4%) were both FISH and qPCR negative, 9 (13.8%) were FISH equivocal/qPCR negative, and 12 (18.5%) were both FISH and qPCR positive. qPCR failed for three FISH-positive (4.6%), one FISH-equivocal (1.5%), and two FISH-negative (3.1%) samples. FISH positivity/qPCR negativity was found in two IHC 2+ cases (3.1%) (Table 5). One sample was heterogeneous; tumor DNA was probably diluted in the second false-negative case (see above).

Significant correlations were observed among all three qPCR ratios and the immunohistochemical scores ($P < 10^{-7}$)

**Table 5** HER2 Status of the 223 Breast Cancer Samples in the Validation Set

| FISH | IHC | qPCR Positive | qPCR Negative | qPCR Indeterminate |
|---|---|---|---|---|
| Positive (*N* = 109) | 3+ | 85 | 2* | 3 |
| | 2+ | 12 | 2*† | 3 |
| | 0/1+ | 1 | 1* | 0 |
| Equivocal (*N* = 12) | 3+ | 0 | 0 | 0 |
| | 2+ | 0 | 9 | 1 |
| | 0/1+ | 0 | 2 | 0 |
| Negative (*N* = 102) | 3+ | 0 | 0 | 0 |
| | 2+ | 0 | 36 | 2 |
| | 0/1+ | 0 | 59 | 5 |

*False negative, heterogeneous sample.
†False negative, borderline percentage of tumor cells in the specimen.
FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry; qPCR, quantitative real-time PCR.

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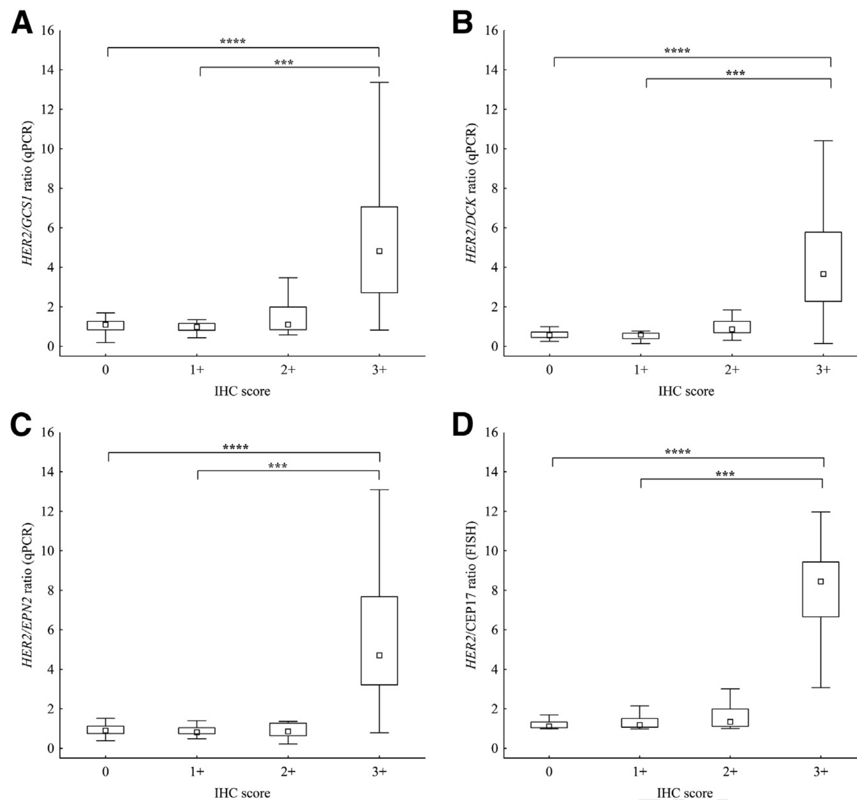


**Figure 3** Box plots show the distribution of the HER2 copy number detected by qPCR and FISH according to the immunohistochemical score in the validation set. Comparisons are shown for the HER2/reference gene ratios: GCS1 (**A**), *DCK* (**B**), and *EPN2* (**C**) detected by qPCR and HER2/CEP17 ratio (FISH) (**D**). Rectangle boxes indicate the 25% to 75% percentiles, small inner squares represent the median, whiskers show non-outlier range. Statistical differences between the pairs of groups (according to multiple comparisons) are indicated by asterisks; ***$P < 10^{-7}$ and ****$P < 10^{-23}$. CEP17, chromosome enumeration probe 17; FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry; qPCR, quantitative real-time PCR.

[F3] (Figure 3). All of the qPCR reactions exhibited similar, high levels of specificity (96.3%, 99.1%, and 99.1%) and sensitivity (84.5%, 74.8%, and 91.2%). The overall levels of sensitivity and specificity for the qPCR assay were 95.1% and 99.1%, respectively. The overall concordance between the FISH/qPCR and IHC results was 97.6%/97.6%, with a κ value of 0.982/0.943 (the 95% CI for the κ value was 0.957/0.898 to 1.007/0.988). No significant difference in concordance was found with the IHC data between the qPCR and FISH results (Table 4).

## HER2 Quantification in Poor-Quality Samples

A total of 3867 breast cancer tissue samples were prospectively collected between 2007 and 2012 for reference HER2-FISH testing in a central laboratory (joint facility of the Institute of Molecular and Translational Medicine and the Department of Clinical and Molecular Pathology, Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech [F4] Republic) to confirm their HER2 status (Figure 4). Of these specimens, 171 (4.4%) that were unsuitable for the analysis because of the absence of tumor cells in sections were excluded from the study. The HER2 gene and chromosome 17 copy numbers in the remaining 3696 samples were then determined by FISH. The FISH analyses were successful for 3498 of these specimens (94.6%); amplification was detected in 1523 (43.5%) of them, 159 (4.6%) were considered equivocal and 1816 (51.9%) gave negative results. High frequency of HER2-amplified breast cancers in the cohort was due to enrichment for positive tumors sent from local laboratories for confirmatory testing to the central laboratory. One hundred ninety-eight specimens (5.4%) could not be evaluated by FISH because of poor sample quality.

The qPCR method that used three reference genes was applied to determine the HER2 gene status of the 198 poor-quality tissue samples that yielded indeterminate FISH results. The qPCR amplification failed for 61 of 198 samples (30.8%) but was successful for the other 137 samples (69.2%). Among the 137 successfully amplified samples, 107 (78.1%) were found to have physiologic HER2 gene copy numbers. Of these 107, 72 (67.3%) were also IHC negative,

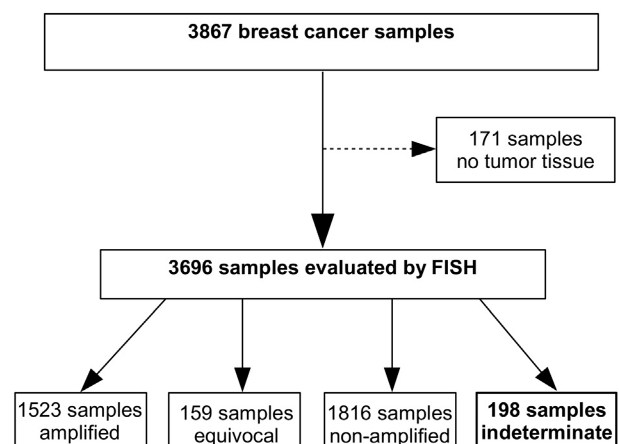


**Figure 4** Consort diagram of samples analyzed in the study. FISH, fluorescence *in situ* hybridization.

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**Table 6** HER2 Status of the 198 Poor-Quality Breast Cancer Samples in the Prospective Cohort

| HER2 status | N (%) | IHC (HercepTest) 3+ | 2+ | 0/1+ | Indeterminate |
|---|---|---|---|---|---|
| qPCR | | | | | |
| Positive | 30 (15.2) | 12 | 6 | 12 | 0 |
| Negative | 107 (54) | 16 | 17 | 72 | 2 |
| Indeterminate | 61 (30.8) | 6 | 16 | 36 | 3 |

IHC, immunohistochemistry; qPCR, quantitative real-time PCR.

17 (15.9%) were equivocal (IHC 2+), whereas the other 16 (15.0%) were IHC positive. IHC failed in two samples (1.9%). qPCR positivity was detected in 30 cases (21.9%) of which 12 (40%) also gave positive IHC result, 6 samples (20%) gave equivocal IHC result, whereas the other 12 samples (40%) were IHC negative. The results of qPCR and IHC differ in 28 of 137 cases (20.4%) (Table 6).

## Discussion

The assessment of HER2 status is fundamentally important for selecting therapies for breast cancer patients, because anti-HER2 agents significantly increase survival rates in both palliative and adjuvant settings. However, the therapeutic effect is limited to patients with tumors that overexpress or amplify the HER2 gene. Thus, assessment of HER2 status may serve as a paradigm for the role of laboratory medicine in the multidisciplinary management of cancer patients.[12]

The ISH and IHC assays are recommended by the American Society of Clinical Oncologists and the College of American Pathologists.[2,5] These methods are highly concordant because gene amplification is the most common mechanism of HER2 overexpression; to date, no strong evidence for other mechanisms of HER2 overexpression in breast cancer is presented.[8,13] However, the efficacy of trastuzumab therapy is linked to FISH HER2 positivity rather than IHC positivity,[14] and discrepancies between HER2 test results obtained with FISH and IHC are observed. Between 5% and 22% of all IHC 3+ breast cancers do not reportedly exhibit HER2 amplification, whereas between 2% and 11.5% of all IHC 0/1+ breast cancers show HER2 amplification.[8,14–17] False-positive or -negative IHC results are particularly common in samples with poor tissue quality.[7,18] False-positive IHC results arising from nonspecific antibody binding can occur in tissues with crush artifacts (needle biopsy specimens), tissue borders, or cautery artifacts.[8] False-negative IHC results can arise from delayed fixation and the use of inappropriate fixatives. In poor-quality samples, tissue damage that causes indeterminate FISH results also reduces the likelihood of IHC positivity.[8,19,20] Unlike the FISH assay, in which poor-quality samples can be readily identified because of the lack of a hybridization signal, it is often not possible to detect an artificial lack of staining because of poor sample quality when evaluating IHC results. This creates risks of obtaining false-negative results,[7] which may have serious consequences because a patient who could benefit from anti-HER2 treatment will not receive it.

Here, FISH failed in 5.4% of the tested specimens, similar to the rate reported in the literature.[8] Delays between sample resection and fixation lead to poor nuclear resolution, vague cellular outlines, and weak, nonuniform signals, thereby seriously compromising the quality of samples used in HER2 FISH assessments,[6] which are therefore evaluated with IHC alone. Thus, the FISH failures in the present study were probably because of poor sample quality arising from either inappropriate handling before fixation or use of an inappropriate fixation method. However, given the high rate of false positives and negatives observed with IHC, a robust alternative DNA-based method would be valuable for confirming HER2 copy numbers in samples that cannot be analyzed by FISH.

Therefore, we developed a method that can be applied to determine HER2 gene copy numbers in FFPE samples, using three duplex qPCR reactions in which the HER2 copy number are compared with those of three reference genes located on chromosomes 2, 4, and 17: GCS1, *DCK*, and *EPN2*, respectively. These reference genes were selected because their copy numbers rarely change in breast cancer. Both the sensitivity and κ values increased with combinations of reference genes (Table 4). The use of three independent reference genes also decreased rates of false positives and negatives. The amplification success for each duplex reaction ranged from 93.7% to 95.1%. These results are wholly consistent with those presented in the literature, because the amplification efficacy for products of this length reportedly ranges from 69% to 100%.[21–23] However, the amplification success decreased with the number of parallel-evaluated genes. To maintain high specificity, sensitivity, and performance (amplification success) of the qPCR method, we finally evaluated samples as HER2 amplified if HER2-to-reference gene copy number ratios were ≥2.2 for at least two of the reference genes.

Surprisingly, although qPCR techniques are often used to detect HER2 mRNA,[24–26] DNA-based PCR is not widely used to determine HER2 gene status, except by the LightCycler HER2/neu DNA Quantification kit (Roche, Mannheim, Germany).[23,27–30] However, this kit only uses a single reference gene (not specified) located on chromosome 17, for which copy numbers frequently change in human cancers,[31] and use of a single reference gene can clearly impair the reliability of HER2 analysis and amplification performance.

Our qPCR method proved to be highly sensitive and specific on the basis of comparisons with FISH data (94.2% and 100%, respectively) and IHC data (95.1% and 99.1%, respectively) obtained for the validation set. The overall concordance of the FISH and qPCR results was 97.6%, and it was higher than levels reported for the LightCycler HER2/neu DNA Quantification Kit, which range from 80% to 92%.[27,28,30] The overall concordance of the qPCR and IHC results was 97.6%, also much higher than rates (80% to

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91%) reported in previous studies.[27,28] We hypothesize that the high concordance of our qPCR method is due to the use of three (rather than one) more appropriate reference genes. Furthermore, in contrast to the FISH results, the qPCR analysis yielded no false-positive results and five false-negative results. In four of these cases, the amplification levels were probably below the method's LOD. The likely causes of failure were either a dilution effect that resulted from the presence of other tissue elements or tissue necrosis. The use of laser microdissection to isolate specific regions of interest within a sample may be beneficial in such cases.[23] The qPCR failed in the last false-negative case. Although there was high-level HER2 amplification detected by FISH and moderate membrane staining in 50% of tumor cells (IHC 2+), the qPCR assay was unsuccessful, most probably because of borderline percentage of tumor cells in the specimen.

Forty-nine samples were found to be qPCR negative but IHC positive. However, 47 of these were only equivocal IHC 2+, and no HER2 amplification is observed relatively often in such samples; the concordance between IHC and FISH results in 2+ cases ranges from 12% to 48%.[15,32–35] In two qPCR-negative/IHC 3+-positive samples the amplification levels were probably below the method's LOD as described above. One sample was found to be qPCR positive and IHC negative, but HER2 amplification was confirmed by FISH analysis in this sample, indicating that the immunohistochemical result was a false negative.

We then used our qPCR method to evaluate the HER2 copy number in 198 of 3696 tissue samples, obtained in a prospective study, for which FISH analysis failed. We were able to determine the HER2 gene status in 137 (69.2%) of these 198 samples. When the HER2 gene and protein detection by qPCR versus IHC were compared, the data differed in 20.4% cases (28 of 137). qPCR result was negative for HER2 amplification in at least 47% of the IHC 3+ tumors (16 of 34 cases). Conversely, 12 of 120 (10%) IHC 0/1+ tumors were qPCR positive. Despite the high disagreement with commonly used IHC, we do not assume that the problem is the qPCR method, considering the high concordance of qPCR with both IHC/FISH in the validation cohort. Most likely, the performance of the IHC was heavily impaired because of a high level of sample degradation that resulted in disruption of antigenic epitopes to cause false negativity, or a nonspecific antibody binding to damaged tissues to cause false positivity.[8] Unfortunately, we are not able to evaluate the results of qPCR in discrepant samples by other independent DNA technique because of FISH analysis failure. Nevertheless, we found six discordant/equivocal patients who were successfully tested by FISH with the use of different tissue block. Interestingly, five patients were both FISH/qPCR negative (three patients IHC 2+, two patients IHC 3+) and one was FISH/qPCR positive (IHC 2+). Although we were not able to prove directly the HER2 gene status in all qPCR versus IHC nonconforming samples, the data obtained from six patients having non-degraded parallel biopsies available suggest reliability of the qPCR method. Because no data obtained with the LightCycler HER2/neu DNA Quantification Kit for poor-quality samples are available, the presented qPCR method is the only technique reported to date that is capable of determining HER2 gene copy numbers in samples in which FISH fails.

Given the high concordance between our triple duplex qPCR technique and currently gold standard techniques, together with its high specificity and sensitivity demonstrated in tests with the validation set, we believe it is a promising method for determining HER2 gene copy numbers, especially in samples in which FISH fails. The main disadvantage of our method is its inability to distinguish HER2 status between invasive versus noninvasive components of tumors. The problem could be also tumor heterogeneity, which is, however, relevant also for ISH and IHC. Unlike the qPCR, the ISH and IHC techniques are capable to visualize molecular status in the context of tissue architecture, percentage of malignant cells, and presence of necrosis and to identify even small proportions of amplified cells. Samples analyzed by qPCR can fail in these circumstances. However, this is a relatively rare problem that can be easily resolved by tissue dissection. However, ISH and IHC are known to be affected by human factor and prone to subjective evaluation.

In summary, ISH is a gold standard technique that is generally capable of evaluating HER2 gene status in tumor architecture contexts. The triple duplex qPCR method presented here proved to be a highly concordant, specific, and sensitive tool for determining HER2 copy numbers in FFPE samples and could be recommended as an alternative DNA-based technique for samples in which the ISH fails.

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