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PLEASE SCROLL DOWN FOR TEXT.
The Reliability of Rabbit Monoclonal Antibodies in the Immunohistochemical Assessment of Estrogen Receptors, Progesterone Receptors, and HER2 in Human Breast Carcinomas

Anthony Rhodes, PhD, Julia Sarson, MSc, Emma E. Assam, MSc, Sarah J.R. Dean, BSc (Hons), Edward C. Cribb, BSc (Hons), and Andrew Parker, FRCPA

Key Words: Rabbit monoclonal antibodies; Immunohistochemistry; Fluorescence in situ hybridization; FISH; Silver in situ hybridization; SISH; Estrogen receptors; Progesterone receptors; HER2

Abstract

The reliability of the rabbit monoclonal antibodies SP1, SP2, SP3, and 4B5 was immunohistochemically assessed on a range of 96 invasive breast and carcinomas and the results compared with those achieved with established antibody markers for estrogen receptors (6F11), progesterone receptors (PgR636), and HER2 (polyclonal A0485 and clone CB11), with HER2 status validated by fluorescence in situ hybridization (FISH) and silver in situ hybridization. Optimal results depended on the duration of microwave antigen-retrieval time and the use of a high pH buffer for rabbit and mouse estrogen receptor antibodies (SP1 and 6F11), although only on antigen-retrieval duration for the progesterone receptors, SP2 and PgR636. The highest rate of concordance between HER2 overexpression and HER2 gene amplification was with the rabbit monoclonal antibodies (SP3 and 4B5) and FISH. Rabbit monoclonal
antibodies are reliable alternatives to established antibody markers for the immunohistochemical testing of estrogen receptors, progesterone receptors, and HER2 in breast cancer.

The immunohistochemical assessment of estrogen receptors (ERs), progesterone receptors (PRs), and the human epidermal growth factor receptor 2 (HER2) is now required for all patients with newly diagnosed breast cancer to determine the most effective treatment regimens. A large proportion of these patients will have invasive breast carcinomas that are positive for hormonal receptors or HER2 and, therefore, be eligible for treatment with hormone therapy or trastuzumab (Herceptin), respectively. These therapies bring significant benefits with respect to overall survival at 5 years and are associated with minimal morbidity compared with cytotoxic chemotherapy alone. However, recent events show that erroneous ER and PR results can have devastating effects; patients are denied appropriate therapy, and this can lead to ensuing legal action resulting in million-dollar lawsuits and, in some cases, government inquiries.

To help prevent such catastrophes, it is vital that information be readily available on the optimal testing method and is the reason that the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) and similar professional bodies worldwide have devised extensive evidence-based guidelines on HER2 testing and hormonal receptor testing. Accrual of evidence is an ongoing process in order that new and potentially improved reagents and methods for the determination of hormonal receptors and HER2 are continually assessed. In this respect, the current study provides new evidence on the reliability of rabbit monoclonal antibodies to hormonal receptors and HER2, when compared with established antibody markers, in determining the receptor status of invasive breast carcinomas.

Rabbit monoclonal antibodies are reputed to be potentially superior immunohistochemical reagents for clinical use, with claims that they retain the high affinity of rabbit polyclonal antisera and the high specificity achieved with monoclonal antibodies. The reputed higher affinity has allowed some researchers to use the antibodies at much higher dilutions than comparable mouse monoclonal antibodies and, in some cases, without the need to resort to heat-induced antigen retrieval, which is an essential requirement for optimal results when using mouse monoclonal antibodies to ER, PR, and HER2. However, a recent study also reported potentially false-positive PR staining...
and suggested that this may be due to the use of inappropriate antigen retrieval with this rabbit monoclonal antibody.\textsuperscript{18}

To address these issues, we investigated the reliability of rabbit monoclonal antibodies to ERs (clone SP1),\textsuperscript{16} PRs (SP2),\textsuperscript{17} and HER2 (clones SP3 and 4B5)\textsuperscript{19,20} on a series of breast carcinomas arranged in a tissue microarray (TMA) in comparison with established antibody markers to ERs (clone 6F11),\textsuperscript{21} PRs (clone PgR636),\textsuperscript{22} and HER2 (polyclonal antisera A-0485 and clone CB11).\textsuperscript{23,24} In the evaluation of ER and PR, we compared the results obtained with 3 commonly used antigen-retrieval buffers at different pH values (pH 6.0, pH 8.0, and pH 9.0) and increasing microwave antigen-retrieval times. In the evaluation of antibodies to HER2, we compared the immunohistochemical assessment of HER2 protein expression with an evaluation of HER2 gene status on the same cases, determined by fluorescence in situ hybridization (FISH) and silver-labeled in situ hybridization (SISH).\textsuperscript{25}

Materials and Methods

Breast Carcinomas

We selected 120 invasive breast cancers from patients treated between 2005 and 2007 from the FileMaker Pro archive (FileMaker, Santa Clara, CA) at the Department of Histopathology, John Radcliffe Hospital, Oxford, England. The main criteria for selection were that the tumors were greater than 15 mm in diameter and more than 1 tissue block was available for future diagnostic, teaching, or research purposes. Tissues were fixed in neutral buffered formalin, embedded in paraffin wax, and classified according to main histologic type into the following carcinomas: invasive ductal or ductal/mixed, 107; invasive lobular, 5; tubular, 1; mucinous, 3; medullary, 1; papillary, 1; metaplastic, 1, and apocrine, 1.

TMA Construction

TMAs were constructed by using a TMA Builder (LabVision, Fremont, CA); each TMA block contained twenty-four 2-mm cores of breast tissue. Duplicate tissue cores were taken from each tissue block to compensate for potential loss of cores or missing areas of invasive disease in the TMA construction process. Tissue sections were cut at 3 µm and mounted on Snowcoat Micro Slides (Surgipath, Richmond, IL).
locations for all commercial products mentioned YES CORRECT | One section from each TMA tissue block was stained with H&E for review to ensure that the cores contained adequate invasive disease. Whenever possible, adjacent normal breast ducts were included to provide for an internal control. After adjusting for cores lost during microtomy and excluding unsuitable cases, eg, cores containing excessive necrotic material and cores composed of primarily ductal carcinoma in situ or normal mammary glands, 96 cases were available for HER2 analysis. Of these, 55 were selected for hormonal receptor analysis.

Immunohistochemical Studies

The HER2 clone 4B5 was stained on a Ventana BenchMark following the manufacturer’s guidelines (Ventana Medical Systems, Tucson, AZ). All other antibodies Table 1 were incubated for 60 minutes in primary antibody at optimized dilutions, followed by 30 minutes in each of secondary biotinylated antibody and avidin-biotin complex with horseradish peroxidase label (Vector Labs, Peterborough, England). Visualization was achieved by using a hydrogen peroxide substrate and a diaminobenzidine chromogen (DAKO, Ely, England). Nuclei were counterstained with Harris hematoxylin.

Antigen Retrieval

HER2 Antibodies

For all but the 4B5 clone, antigen retrieval comprised heating the sections in 750 mL of 0.01 mol/L sodium citrate (pH 6.0) buffer in a 750-W microwave oven for 30 minutes. At 30 minutes, the plastic container containing buffer and slides was removed from the microwave and immediately cooled to room temperature by running cold water into the plastic container.

ER and PR Antibodies

Three antigen retrieval buffers were Used: sodium citrate (pH 6.0), EDTA (pH 8.0), and tris(hydroxymethyl)aminomethane (Tris)-EDTA |Au 2: Correct for Tris throughout?YES CORRECT| (pH 9.0). To identify the influence of heating time on the results, the duration of heating in a 750-W microwave oven was regulated at 0, 5, 10, 20, and 30 minutes for each assay using each of the buffer solutions. Each time, the plastic antigen-retrieval slide container was filled with the same volume (750 mL) of antigen-retrieval
solution, using the same number of slides to ensure standardization and equivalency of the heating. After heating, the slides were washed and cooled to room temperature in running tap water and rinsed in Tris-buffered saline before proceeding with the immunostaining.

**Silver In Situ Hybridization**

SISH (INFORM HER2 DNA probe, Ventana) staining was performed on the Ventana BenchMark XT series following the manufacturer’s guidelines.

**Fluorescence In Situ Hybridization**

FISH was carried out by using the Vysis PathVysion HER-2/neu DNA Probe Mix (Abbott Molecular, Abbott Park, IL) using a method described by Ventura et al. The HER-2/neu probe comprised a locus-specific identifier, HER-2/neu 190-Kb SpectrumOrange, specific for the HER-2/neu locus (17q11.2-q12). The Ch17 [Au 3: Does Ch17 mean chromosome 17 here and later in the sentence? YES CORRECT] probe was a chromosome enumeration probe 17 (CEP17), 5.4-Kb SpectrumGreen, for the α satellite centromeric DNA sequence of Ch17 (17p11.1-q11.1) (Ventana). The FISH pretreatment step used Pretreat FISH (DAKO, Glostrup, Denmark) for 10 minutes at 100°C in a steam microwave (Sixth Sense, Philips Whirlpool, Guildford, England) and allowed to cool.

Slides were washed in tap water and subjected to pepsin digestion (DAKO, Glostrup) for 5 minutes at 37°C. After rinsing in buffer, the sections were dehydrated, and 10 µL of Vysis PathVysion HER-2/neu DNA probe mix was applied to each slide. Slides were incubated in a prewarmed humidified hybridization chamber (DAKO, Glostrup) to allow hybridization of the probe mix. The hybridizer was programmed at 82°C melting temperature for 5 minutes followed by 37°C hybridization temperature for 48 hours, following which the slides were rinsed in posthybridization buffer at 65°C for 10 minutes. Slides were mounted with Vectashield mounting medium containing 4’6 diamidine-2-phenyliadole (DAPI, Vector Laboratories, Burlingame, CA) and visualized by using a fluorescence microscope (Zeiss, Axioskop, Göttingen, West Germany) and a Vysis multi-bandpass filter set (DAPI/Orange dual bandpass, DAPI/Green dual bandpass, and DAPI/Green/Orange triple bandpass filters) under oil immersion with a 100× objective. A representative area from each of the TMA tissue cores stained with FISH and SISH were photographed using a Hamamatsu Digital Camera (Hamamatsu, Hertfordshire, England).
Scoring of Slides

In all cases, only invasive tumor was evaluated, and to ensure consistency, a proportion of all slides was counterscored by a second scorer (A.R. or A.P.). Consecutive tissue sections from the same TMA cores were used when comparing the results for the different antibodies and different antigen-retrieval buffers and when comparing HER2 immunohistochemical scores with the \textit{HER2} gene amplification status as determined by FISH and SISH.

\textbf{ER/PR Immunohistochemical Studies}

The Allred scoring system was used; a score for intensity, 0 through 3, was assigned for none, weak, moderate, and strong nuclear staining, respectively, and a score for the proportion of nuclear staining, 0 through 5, was assigned for no staining, less than 1%, 1% to 10%, 11% to 33%, 34% to 66%, and 67% to 100% nuclear staining. The score for intensity was then added to the score for proportion, resulting in a score ranging from 0 (no staining), through 8 (strong staining of 67%-100% tumor nuclei). Invasive tumor with an Allred score of more than 2 was considered to be positive for hormonal receptors. When cores were receptor-negative, adjacent normal glands in the same core or adjacent cores were checked for appropriate positivity.

\textbf{HER2 Immunohistochemical Studies}

Slides were scored as 3+ when showing uniform intense membrane staining in more than 30% of cells, 2+ when membrane staining was present in at least 10% of cells, 1+ for weak or incomplete staining in more than 10% of cells, and 0 when there was no staining or weak incomplete membrane staining in fewer than 10% of cells.

\textbf{Fluorescence In Situ Hybridization}

Each tissue core was scored by using Vysis signal enumeration guidelines (Abbott Molecular, Maidenhead, England).

\textbf{Silver In Situ Hybridization}

Slides were scored by using the Ventana “Quantitative Method 2” and the “Additional Quantitative Method” (Ventana). For the FISH- and SISH-stained slides, an HER2/chromosome 17 ratio greater than 2.2 was designated as showing \textit{HER2} gene amplification, a ratio of 1.8 to 2.2 as equivocal results, and a ratio less than 1.8 designated as no \textit{HER2} gene amplification.
Statistical Analysis

Data were recorded on Excel (Microsoft, Redmond, WA) files and transferred to an SPSS version 17 program (SPSS, Chicago, IL) for statistical analysis. The Mann-Whitney U test was used for comparing the hormonal receptor results achieved by using different antigen-retrieval times, buffers, and antibodies. The Cohen κ statistic was used to establish the level of agreement between HER2 gene amplification as determined by FISH and SISH and between HER2 gene amplification and protein expression determined by each of the HER2 antibodies.

Results

The influence of duration of heating, antigen-retrieval buffer, and choice of antibody on results for ERs and PRs is described in the following sections.

Influence of Heating Time

Microscopic assessment of the results for ERs and PRs showed that a 30-minute duration of antigen retrieval gave the highest mean Allred score with all of the antigen-retrieval buffers and antibodies tested Figure 1 and Table 2. The time taken to reach boiling for each of the buffers was 9 minutes. Heating beyond 30 minutes was found to be detrimental to tissue morphologic features.

Influence of Antigen-Retrieval Buffer

At the optimal heating time of 30 minutes, significantly higher mean Allred scores were achieved by using the Tris hydrochloride, pH 9.0, and Tris-EDTA, pH 8.0, buffers than when using citrate buffer, pH 6.0, for the 6F11 and SP1 clones. However, the choice of antigen-retrieval buffer had little bearing on the results for PRs, with no significant difference in scores when different antigen-retrieval buffers were used Table 3.

Influence of Antibody Clone (Mouse Monoclonal vs Rabbit Monoclonal)

There were no significant differences between the Allred scores for the ER clones 6F11 and SP1 when using Tris-EDTA buffer, pH 9.0, and a 30-minute antigen-retrieval time (Mann-Whitney U, 1,064; P = .552) Image 1, nor were there significantly different results between PR clones PgR636 and SP2 when using these optimal antigen-retrieval parameters (Mann-Whitney U, 1,178; P = .292) Image 2. When using an Allred score of more than 2 as the threshold to define hormone receptor positivity, under these antigen-retrieval conditions, the highest proportion...
of ER+ cases was recorded using the 6F11 clone: 36 (71%) of 51 vs 34 (69%) of 49 for the SP1 clone. The highest proportion of PR+ cases was recorded using the PgR636 clone: 24 (48%) of 50 vs 22 (47%) of 47 for the SP2 clone.

**HER2 Results**

The total level of agreement for HER2 gene status in the 96 evaluable cases, as determined by FISH and SISH, was 89 (93%) of 96 with a κ value of 0.770 [Table 4†]. The highest level of agreement between HER2 protein expression (as determined immunohistochemically) and HER2 gene status (as determined by FISH or SISH) was between FISH and the rabbit monoclonal antibodies SP3 (93%; κ = 0.725), 4B5 (91%; κ = 0.722), and the rabbit polyclonal antiserum A0485 (90%; κ = 0.705) [Table 5† and Table 6†]. In addition, all of the cases designated as 3+ by the 4B5 rabbit monoclonal and the rabbit A0485 polyclonal antiserum had HER2 gene amplification or equivocal HER2 gene status, as determined by FISH. Similarly, for these 2 antibodies, all cases scored as 0 or 1+ had no HER2 gene amplification or an equivocal HER2 gene status, as determined by FISH (Table 5). The poorest level of agreement was between the CB11 antibody and FISH (83%; κ = 0.558) and SISH (82%; = κ 0.559). With respect to clarity of immunohistochemical staining, the rabbit monoclonal antibodies produced less cytoplasmic staining than did the A0485 and CB11 antibodies [Image 3†].

**Discussion**

Previous studies have emphasized the increased sensitivity achieved by using rabbit monoclonal antibodies for the immunohistochemical demonstration of ERs, PRs, and HER2.14,16,17 The presumed hypothesis is that this is due to the greater affinity of rabbit monoclonal antibodies than mouse monoclonal equivalents, and, therefore, these antibodies may be effectively used at higher dilutions and, in some cases, without the need for heat-induced antigen retrieval.17 A recent article suggested that this increased sensitivity resulted in inappropriate staining with the PR clone SP2 on a breast cancer previously identified as receptor-negative and used as part of a national quality assurance program; the authors suggested this apparent nonspecific staining of the tumor was the result of the use of inappropriate antigen retrieval with this clone.18 In the current study, to address these issues, we investigated the use of the most commonly used antigen-retrieval buffers: citrate (pH 6.0), EDTA (pH 8.0), and Tris-EDTA (pH 9.0) with established antibody markers and rabbit monoclonal antibodies to hormonal receptors. In addition, we tested
the use of no antigen retrieval at all and then at increasing time intervals up to 30 minutes in a microwave oven for all 3 buffers.

Shi et al., in their seminal study that demonstrated the increased sensitivity achieved using high-pH antigen-retrieval buffers for ERs compared with citrate buffer (pH 6.0), used a relatively short microwave antigen-retrieval time of just 10 minutes. Subsequent articles highlighted the need for adequate microwave antigen-retrieval time or the use of superheating as achieved by pressure cookers.

In the present study, we sought to address both of these issues and determine whether the use of a high-pH antigen-retrieval buffer could compensate for heating time (duration) in the optimal demonstration of established mouse monoclonal antibodies and the more recent rabbit monoclonal antibodies to ERs and PRs. It is interesting that significantly higher Allred scores were achieved at microwave times of 10 minutes with the rabbit monoclonal SP1 than with the mouse monoclonal 6F11. However, the duration of microwave heating time was still critical in achieving optimal results, with the longer times of 20 and 30 minutes giving the highest mean Allred scores, irrespective of the choice of antigen-retrieval buffer or antibody clone used. At the optimal heating time of 30 minutes’ duration, the use of the high-pH antigen-retrieval buffers (EDTA, pH 8.0, and Tris-EDTA, pH 9.0) gave significantly higher scores compared with sodium citrate buffer for the ER antibodies tested, indicating that adequate duration of heating and the use of high-pH buffers are critical, regardless of whether using rabbit or mouse monoclonal antibodies to ERs. It is interesting that this was found not to be the case for the PR antibodies, with no significant improvement in sensitivity achieved by the use of a high-pH buffer at the optimal microwave antigen-retrieval time. Consequently, for all PR antibodies tested, adequate heating, ie, duration of microwave antigen-retrieval time, was the critical factor to ensure optimal results, not the choice of antigen-retrieval buffer. Moreover, no inappropriate staining was observed with any of the antibodies.

With respect to reports that the rabbit monoclonal antibodies are more sensitive than comparable mouse monoclonal antibodies or, in some cases, give rise to false-positive results, the current study found no evidence of this, with very similar results achieved for rabbit and mouse monoclonal antibodies in terms of appropriate nuclear staining and in the proportion of cases staining positively for ER and PR. While it is appreciated that many laboratories will use automated immunohistochemical platforms with custom-designed onboard antigen retrieval, the latest survey available from the UK NEQAS Scheme for Immunocytochemistry shows that the majority of laboratories
participating in the Breast Hormonal Receptor quality assurance module use a nonautomated platform for hormone receptor immunohistochemical studies. In addition, many of the automated antigen-retrieval systems are likely to incorporate the use of high temperature and/or antigen-retrieval buffers similar to those used in the present study.

HER2 expression analysis by immunohistochemical studies and HER2 gene amplification analysis by FISH are now recognized as producing a proportion of equivocal results, ie, neither assay can be considered the “gold standard” for HER2 analysis. Because unequivocal HER gene amplification as determined by FISH and unequivocal HER2 protein expression as determined by immunohistochemical studies predict a similar response to the drug trastuzumab or combinational therapy in invasive breast carcinomas, either assay is considered suitable for clinical use. However, it is recommended that in both cases, the assays be validated against each other to ensure a high degree of concordance. The use of in situ hybridization systems using a chromogenic signal instead of a fluorescent label have gained increasing popularity owing to the obvious advantages of permanently stained preparations that can be readily viewed under the light microscope and yet retain the quantitative nature of the FISH assay. However, they have yet to be fully accepted as a replacement for FISH in clinical analysis of HER2 gene amplification status. In the current study, FISH and a chromogen-based in situ hybridization system (SISH) were compared with each other and with each of the antibody assays investigated. It is interesting that the highest concordance rates between the immunohistochemical assays and in situ–based systems were between the rabbit monoclonal antibodies and FISH. Arguably, the most reliable results were obtained with FISH and the rabbit monoclonal antibody 4B5, as all 3+ cases with this antibody gave HER2 gene amplification by FISH (plus 1 equivocal result), while all 0 and 1+ cases showed no HER2 gene amplification.

The quality and reliability of immunohistochemical results achieved with rabbit monoclonal antibodies to ERs and PRs on a range of invasive breast carcinomas was comparable to that achieved with established mouse monoclonal antibodies. In addition, in the assessment of HER2, the rabbit monoclonal antibody 4B5 and FISH gave the most reliable results.

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Supported by Ventana Medical Systems (4B5 and SISH staining) and LabVision (provision of SP1, SP2 and SP3 antibodies).

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References


Dietel M, Ellis IO, Hofler H, et al. Comparison of automated silver enhanced in situ hybridization (SISH) and fluorescence ISH (FISH) for the validation of HER2 gene status in breast carcinoma according to the guidelines of the American Society of Clinical Oncology and the College of American Pathologists. Virchows Arch. 2007;451:19-25.


<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Supplier</th>
<th>Epitope</th>
</tr>
</thead>
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<tr>
<td>Clone 6F11</td>
<td>Mouse monoclonal</td>
<td>Novocastra Laboratories, Newcastle upon Tyne, England</td>
<td>Estrogen receptor α</td>
</tr>
<tr>
<td>Clone SP1</td>
<td>Rabbit monoclonal</td>
<td>LabVision, Runcorn, England</td>
<td>Estrogen receptor α</td>
</tr>
<tr>
<td>Clone PgR636</td>
<td>Mouse monoclonal</td>
<td>DAKO, Ely, England</td>
<td>Progesterone receptor (A and B)</td>
</tr>
<tr>
<td>Clone SP2</td>
<td>Rabbit monoclonal</td>
<td>LabVision</td>
<td>Progesterone receptor (A and B)</td>
</tr>
<tr>
<td>Anti–c-erbB2 (A0485)</td>
<td>Rabbit polyclonal</td>
<td>DAKO</td>
<td>HER2 intracellular domain</td>
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<tr>
<td>Clone CB11</td>
<td>Mouse monoclonal</td>
<td>Novocastra Laboratories</td>
<td>HER2 intracellular domain</td>
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<tr>
<td>Clone SP3</td>
<td>Rabbit monoclonal</td>
<td>LabVision</td>
<td>HER2 extracellular domain</td>
</tr>
<tr>
<td>Clone 4B5</td>
<td>Rabbit monoclonal</td>
<td>Ventana Medical Systems, Tucson, AZ</td>
<td>HER2 intracellular domain</td>
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Table 2

Comparison of Mean Allred Scores for Each Hormone Receptor Antibody at 10 and 30 Minutes’ Microwave Antigen-Retrieval Time

<table>
<thead>
<tr>
<th>Antibody/Antigen-Retrieval Buffer</th>
<th>Microwave Antigen-Retrieval Time (min)</th>
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<tr>
<td></td>
<td>10</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>Mean Allred Score (SEM)</td>
<td>N</td>
<td>Mean Allred Score (SEM)</td>
</tr>
<tr>
<td>6F11</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Sodium citrate pH 6.0</td>
<td>49</td>
<td>2.4 (0.4)</td>
<td>48</td>
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<tr>
<td>EDTA pH 8.0</td>
<td>47</td>
<td>3.8 (0.5)</td>
<td>48</td>
</tr>
<tr>
<td>Tris-EDTA pH 9.0</td>
<td>49</td>
<td>3.4 (0.5)</td>
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<tr>
<td>SP1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Sodium citrate pH 6.0</td>
<td>52</td>
<td>5.4 (0.4)</td>
<td>49</td>
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<td>EDTA pH 8.0</td>
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<tr>
<td>Tris-EDTA pH 9.0</td>
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<td>PgR636</td>
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<td>SP2</td>
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<td>Sodium citrate pH 6.0</td>
<td>50</td>
<td>2.2 (0.1)</td>
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<tr>
<td>Tris-EDTA pH 9.0</td>
<td>50</td>
<td>2.5 (0.5)</td>
<td>52</td>
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N, number of evaluable cases; Tris, tris(hydroxymethyl)aminomethane.
### Table 3

<table>
<thead>
<tr>
<th>Antibody</th>
<th>With Sodium Citrate Buffer, pH 6.0/Mean Allred Score (SEM)</th>
<th>Alternative Antigen-Retrieval Buffer</th>
<th>Mean Allred Score (SEM)</th>
<th>Mann-Whitney U</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>6F11</td>
<td>5.7 (0.4)</td>
<td>EDTA, pH 8.0</td>
<td>6.3 (0.4)</td>
<td>889</td>
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<td>757</td>
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<td>SP1</td>
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<td>EDTA, pH 8.0</td>
<td>6.3 (0.4)</td>
<td>967</td>
<td>.049</td>
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<td></td>
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<td>6.5 (0.4)</td>
<td>900</td>
<td>.014</td>
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<tr>
<td>PGR636</td>
<td>3.7 (0.5)</td>
<td>EDTA, pH 8.0</td>
<td>4.4 (0.5)</td>
<td>1133</td>
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<td>3.7 (0.5)</td>
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<td>1131</td>
<td>.100</td>
</tr>
</tbody>
</table>

Tris, tris(hydroxymethyl)aminomethane.

### Table 4

**Correlation of HER2 Gene Status of 96 Invasive Breast Carcinomas as Determined by FISH and SISH**

<table>
<thead>
<tr>
<th>HER2 Gene Status (SISH)</th>
<th>Amplified</th>
<th>Equivocal</th>
<th>None Amplified</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER2 Gene Status (FISH)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplified</td>
<td>12</td>
<td>1</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>Equivocal</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>None amplified</td>
<td>1</td>
<td>3</td>
<td>76</td>
<td>80</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>5</td>
<td>76</td>
<td>96</td>
</tr>
</tbody>
</table>

FISH, fluorescence in situ hybridization; SISH, silver in situ hybridization.

*HER2 gene status refers to the ratio of HER2/chromosome 17 signals per nucleus. Amplified, ratio >2.2; equivocal, ratio 1.8-2.2; none amplified, ratio <1.8.

Concordance (κ), 0.770 (95% confidence interval, 0.607-0.934).
Table 5

Correlation of HER2 Expression by Immunohistochemical Studies Using HER2 Antibodies A0485, CB11, SP3, and 4B5

With HER2 Gene Status as Determined by FISH

<table>
<thead>
<tr>
<th>FISH Gene Status</th>
<th>Immunohistochemical Expression</th>
<th>CB11</th>
<th>SP3</th>
<th>4B5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A0485 (Polyclonal)</td>
<td>3+</td>
<td>2+</td>
<td>0/1+</td>
</tr>
<tr>
<td>Amplified</td>
<td>12</td>
<td>1</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Equivocal</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>None amplified</td>
<td>0</td>
<td>5</td>
<td>73</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>6</td>
<td>74</td>
<td>13</td>
</tr>
<tr>
<td>Concordance (κ)</td>
<td>0.705 (0.521-0.888)</td>
<td>0.558</td>
<td>0.725</td>
<td>0.722</td>
</tr>
<tr>
<td></td>
<td>(0.361-0.756)</td>
<td>(0.529-0.549)</td>
<td>(0.921)</td>
<td>0.895)</td>
</tr>
</tbody>
</table>

FISH, fluorescence in situ hybridization.

HER2 gene status refers to the ratio of HER2/chromosome 17 signals per nucleus. Amplified, ratio >2.2; equivocal, ratio 1.8-2.2; none amplified, ratio <1.8.

Concordance is given as κ statistic (95% confidence interval).

NB: Please see original/attached file for appropriate format for table 5 and 6 i.e. the 2nd column needs to the exact same width as the other columns, Concordance values, ideally on one line as in first column.

Table 6

Correlation of HER2 Expression by Immunohistochemical Studies Using HER2 Antibodies A0485, CB11, SP3, and 4B5

With HER2 Gene Status as Determined by SISH

<table>
<thead>
<tr>
<th>SISH Gene Status</th>
<th>Immunohistochemical Expression</th>
<th>CB11</th>
<th>SP3</th>
<th>4B5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A0485 (Polyclonal)</td>
<td>3+</td>
<td>2+</td>
<td>0/1+</td>
</tr>
<tr>
<td>Amplified</td>
<td>12</td>
<td>1</td>
<td>2</td>
<td>11</td>
</tr>
</tbody>
</table>
**Image 1** An invasive ductal carcinoma immunohistochemically stained for estrogen receptors using a 30-minute microwave antigen-retrieval time, 3 different antigen-retrieval buffers, and the mouse monoclonal antibody 6F11 (A, Sodium Citrate ×20; B, EDTA ×20; and C, Tris-EDTA×20) and the rabbit monoclonal antibody SP1 (D, Sodium Citrate ×20; E, EDTA ×20; and F, Tris-EDTA×20). [Au 7: Please indicate the quantitative magnification for the image parts, as indicated by blanks]

**Image 2** An invasive ductal carcinoma immunohistochemically stained for progesterone receptors using a 30-minute microwave antigen-retrieval time, 3 different antigen-retrieval buffers, and the mouse monoclonal antibody PgR636 (A, Sodium Citrate ×20; B, EDTA ×20; and C, Tris-EDTA×20) and the rabbit monoclonal antibody SP2 (D, Sodium Citrate×20; E, EDTA ×20; and F, Tris EDTA×20).

**Image 3** An invasive ductal carcinoma showing overexpression of HER2 when immunohistochemically tested using rabbit polyclonal anti-cerbB2 A0485 (A, ×40), mouse monoclonal CB11 (B, ×40), rabbit monoclonal SP3 (C, ×40), and rabbit monoclonal 4B5 (D, ×40). The same case shows HER2 gene amplification by fluorescence in situ hybridization (E) and silver in situ hybridization (F).

**Figure 1** The influence of microwave antigen-retrieval time on the mean immunohistochemical (IHC) Allred score for estrogen receptors on 55 invasive breast carcinomas when using the mouse monoclonal 6F11 (A Sodium Citrate, B EDTA, C Tris-EDTA buffers) and the rabbit monoclonal SP1 (D Sodium Citrate, E EDTA, F Tris-EDTA buffers, respectively). Error bars show the 95% confidence intervals. [Au 8: Correct that the “error bars” show the 95% CIs? YES]