

HER2 testing: ASCO/CAP guidelines and beyond – implications for South Africa

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Abstract

HER2 status in breast cancer has major significance in terms of prognostication and prediction of response to chemotherapeutic agents, as well as being an absolute indicator for the use of trastuzumab. Recent guidelines for the testing and interpretation for HER2 overexpression have resulted in more accurate and concordant results. Excellent concordance between immunohistochemistry and in situ hybridisation techniques can be achieved if these recommendations and guidelines are followed. The recently developed diagnostic techniques of chromagenic in situ hybridisation (CISH) and brightfield dual in situ hybridisation (BDISH) combine the techniques of immunohistochemistry (IHC) and in situ hybridisation (ISH) and will revolutionise HER2 testing.

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Introduction

The presence of the HER2 protein, a member of the epidermal growth factor receptor (EGFR) family, has been associated with a poorer prognosis in breast carcinomas, and predicts the development of metastases in node negative patients.^{1,2,3,4,5,6,7,8} The discovery of humanised antibody-based chemotherapeutic agents such as trastuzumab (trastuzumab) has, however, resulted in the most important advance in breast cancer treatment of the past two decades.⁹ Two major forms of testing for HER2 overexpression by neoplastic cells exist. The cheaper and technically simpler test is immunohistochemistry (IHC) whereby overproduction of the HER2 protein on the cell membrane is demonstrated.¹⁰ This protein is the target for the antibody trastuzumab.

The other method for showing HER2 overexpression is demonstration of overexpression of the HER2 gene found on chromosome 17, by means of in situ hybridisation studies.¹¹ Currently fluorescence in situ hybridisation (FISH) is the most popular method for this, but chromagenic in situ hybridisation (CISH) and silver in situ hybridisation (SISH) techniques have become technically feasible and may rival or supplant the more cumbersome FISH testing.^{12,13}

In the early days of HER2 testing, discrepancies between IHC and FISH testing arose and many expert researchers stated that FISH testing, was the preferred modality for HER2 testing.¹⁴ In retrospect, the reason for this is that HER2 IHC testing criteria were slow to be refined and tested and only with expanding experience with testing and clinical trial results did robust and reproducible criteria evolve, with clinical significance.¹⁵

The initial clinical trials required positive IHC or FISH testing and initially the finding of a HER2 2+ or 3+ status on IHC was regarded as indicative of HER2 overexpression.^{16,17} With expanding knowledge and experience it became apparent that there was relatively poor concordance between IHC and FISH if both IHC 2+ and 3+ were regarded as positive and an initial refinement was accepting that only HER2 3+ should be regarded as positive for overexpression, and that HER2 2+ status was equivocal and indicated the need for further testing with FISH. Concordance data suggested that only 24% of 2+ cases were truly HER2 positive. IHC and FISH concordance was much improved, approaching 95% if only a IHC 3+ result was regarded as positive. Using this data an initial algorithm for HER2 was instituted (Figure 1). With this refinement it has become evident that 20% of breast cancers tested by IHC were truly positive for HER2 overexpression and 22.7% if tested by FISH.¹⁸ Although initially registered for use in the metastatic setting, five prospective and randomised international trials have shown that trastuzumab is effective in the adjuvant setting.^{19,20,21,22,23}

Determination of HER2 status has many clinical uses. As an indicator of prognosis, the knowledge of HER2 is incorporated into everyday clinical decision-making as to whether to institute adjuvant therapy or not. Furthermore HER2 status is predictive of response to other non-trastuzumab-based chemotherapeutic modalities. HER2 positive cancers have a relative but not absolute resistance to some chemotherapeutic drugs, particularly anti-oestrogens such as tamoxifen,²⁴ even if oestrogen-receptor-positive but probably not to aromatase inhibitors, influencing the use of the latter in ER+, HER2+ patients. In addition HER2 positive patients have a poorer response to non-athraclycline, non-taxane

treatment regimens,²⁵ but respond better to anthracyclines particularly if there is also overexpression of topoisomerase IIA as well.²⁶ Emerging data is that HER2 positive cancers probably respond to paclitaxel as well. Given the importance of the correct determination of the HER2 status of a breast cancer it is important that pathology laboratories can accurately and reproducibly provide data on HER2 status. In an ideal world such testing would have 100% sensitivity and specificity. Unfortunately there are grey areas in testing with both IHC and in situ hybridisation techniques. Biologically there are definitive cases which have HER2 gene over-amplification but due to upstream or downstream molecular events do not respond to trastuzumab. These may not exhibit 3+ membrane positivity. Similarly some rare cases may demonstrate HER protein overexpression in the absence of gene overexpression. In Genentech's trial [h0650G], three out of thirty IHC 3+ and FISH-cases showed some response to trastuzumab.¹⁷ Protein overexpression may not always be due to gene amplification.

These cases are fortunately quite rare but remain challenges for the testers and oncologists dealing with them. The challenge is to provide a robust and reproducible test result that will satisfactorily demonstrate HER2 status in the vast majority of cases.

ASCO/CAP guidelines

In an attempt to address deficiencies and shortcomings in HER2 testing, a joint position paper under the auspices of the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) was published ahead of print on December 11, 2006 addressing guidelines for HER2 testing in breast cancer.²⁷ This paper addressed many important issues in HER2 testing and introduced new and updated guidelines for the testing and interpretation of HER2 data. In addition to technical recommendations an updated algorithm was introduced in an attempt to achieve the best possible concordance between the varying methodologies for HER2 testing available.

Optimal testing algorithm for HER2 testing

Although it would appear to be self evident it must be stated that only invasive carcinoma should be assessed, a statement emphasised by the guidelines. Although there are some data suggesting that HER2 positive ductal carcinoma in situ (DCIS) is more likely to progress to invasive carcinoma,²⁸ at present the clinical relevance of HER2 testing pertains only to invasive carcinomas. Consequentially it is important that only definitive areas of invasive carcinoma are assessed for HER2 status. In contrast to a positive HER2 status of around 20% in infiltrative carcinomas, up to 60% of DCIS may be demonstrated to have HER2 overexpression and unless these areas are specifically excluded from assessment they may be interpreted to be positive erroneously giving a false positive diagnosis of HER2 positivity. Due to use of dark field microscopy, of necessity

a part of FISH testing, it is possible that such areas may be included in the field for FISH testing. Furthermore fine needle aspiration biopsy (FNAB) material is NOT suitable for HER2 testing unless the lesion is a metastasis, for the simple reason that it is not possible on FNA material from the breast to determine whether the tumour cells are derived from an invasive or non-invasive area.

The older previous algorithm (Figure 1) is compared with the updated ASCO/CAP recommended algorithms for IHC and FISH testing (Figures 2 and 3 respectively).

Figure 1: Previous HER2 testing algorithm (after Bilous²⁹)

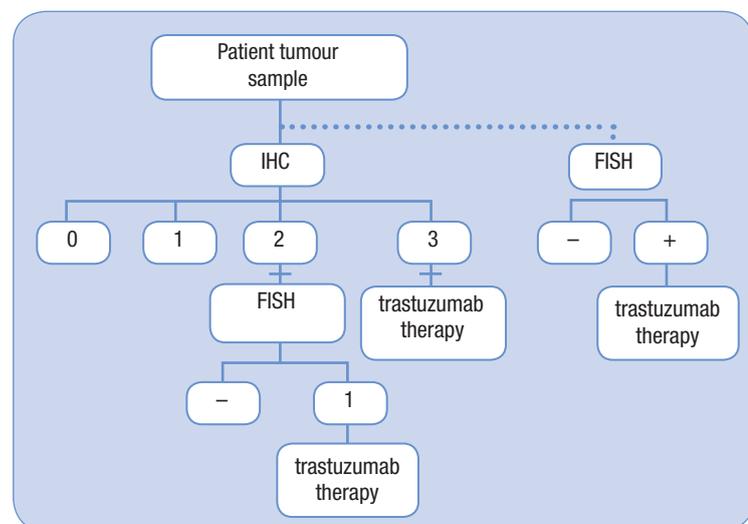


Figure 2: ASCO recommended testing algorithm for HER2 using immunohistochemistry (after Wolff²⁷)

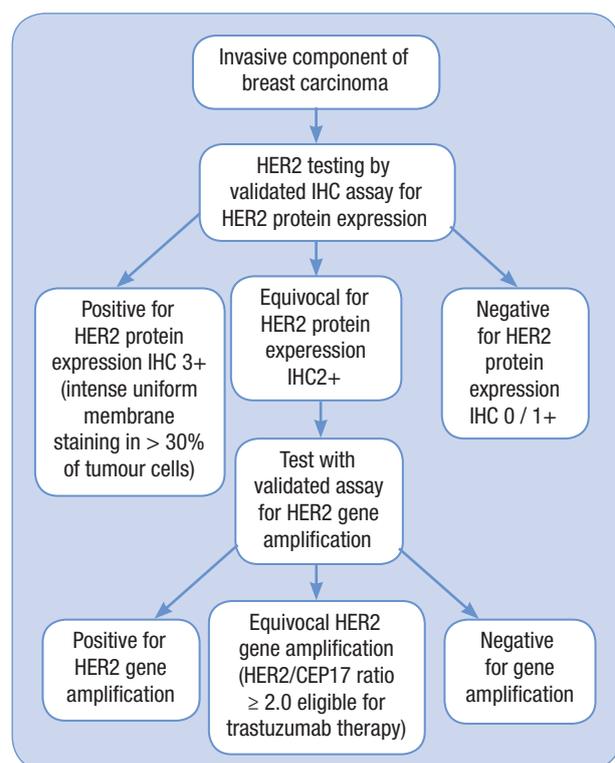
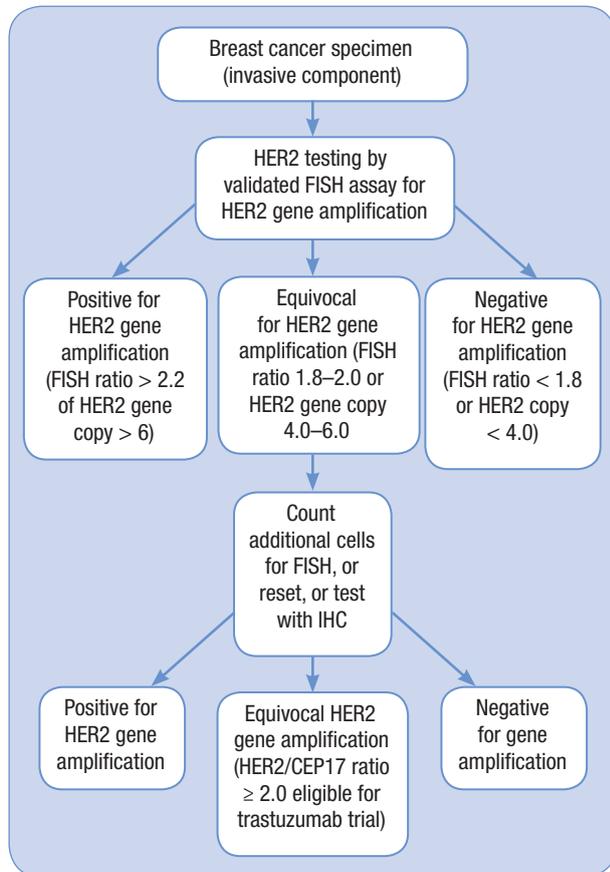
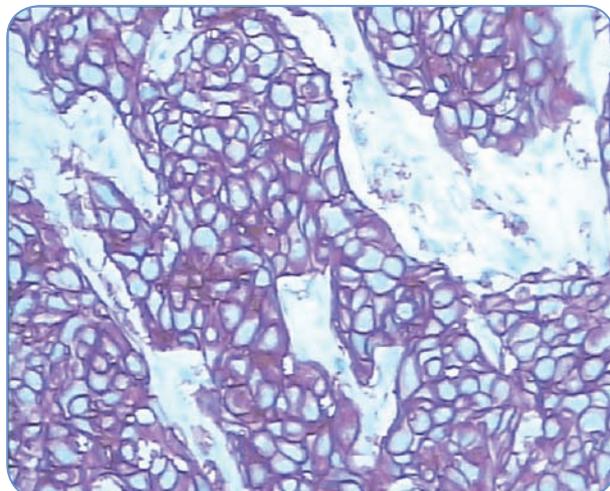


Figure 3: ASCO recommended testing algorithm for HER 2 for fluorescent in situ hybridisation (FISH) assay (after Wolff²⁷)



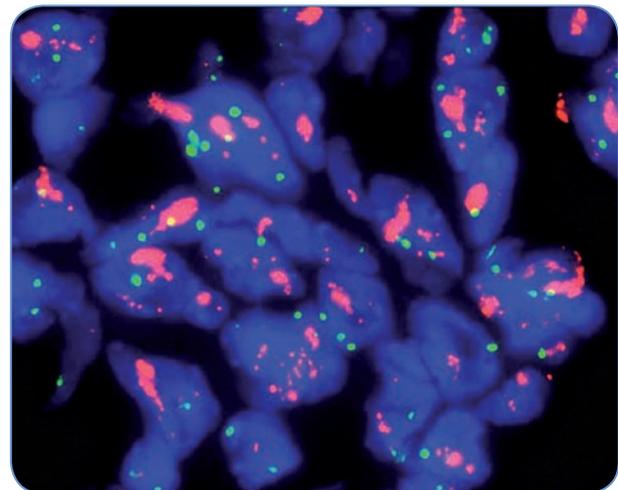
These algorithms clearly state the criteria for HER2 overexpression is > 30% cells showing strong and complete membranous positivity for HER2 immunohistochemical stains (Figure 4) or FISH HER2/CEP17 ratio of ≥ 2.2 or an absolute HER2 gene count of six copies per cell nucleus. The ratio is determined by assessing a minimum of 20 cells and calculating the ratio of HER2 genes to chromosome 17 copies.

Figure 4: Immunohistochemical stain showing diffuse complete strong (chicken-wire) membranous positivity for HER2 protein



The most important changes from the previous guidelines are that for IHC the threshold of positivity required has been raised from a 10% to a minimum of 30% of cells with complete and strong membranous staining. The intensity of staining is the same as was required previously to diagnose positivity, i.e. strong and complete membrane positivity. This does mean that there will be a percentage of cases which previously would have been regarded as positive which will now fall into the equivocal group (between ten and thirty percent of positive cells); these would now be required to be retested, with ISH. With regard to FISH testing, a HER2:CEP17 ratio of > 2.2 is regarded as being positive, Figure 5. Previously the threshold was 2.0. If no ratio is calculated then an absolute HER2 gene count of > 6 copies of the HER2 gene is required to diagnose positive HER2 gene overexpression, although theoretically, if no ratio assessment is done, some polysomic cases may then be counted as positive. An equivocal FISH test is now defined, i.e. HER2:CEP17 ratio of 1.8–2.2 or an absolute gene count between four and six. These equivocal cases require retesting.

Figure 5: Fluorescence in situ hybridisation showing overexpression of HER2 gene (red dot-like signals) compared to chromosome 17 marker – CEP17 (green dots). The ratio of HER2:CEP17 is $> 2.2:1$



Two important issues arising from these algorithms are:

1. HER2 3+ IHC does not require to be confirmed with any other tests.
2. It is acknowledged that FISH testing may be equivocal and this militates one of the following: either recounting or repeating the FISH test; or performing an alternative methodology, such as an IHC assay. There is no gold standard for determining HER2 status and thus one test cannot be definitively regarded to be superior to the other testing modality.

It is also important for pathologists and oncologists to recognise that, as with any biological assessment, there will always be a grey zone or equivocal test. For instance, although previous guidelines accepted a HER2:CH17 ratio of > 2 as being the cut-off, and most cases exhibit a clear-cut overexpression, there are always going to be heterogeneous cases with

variable expression. Selection of a small area of positivity in such cases may give a positive ratio, but if the overall ratio is calculated then these cases may fall below a positive ratio. In IHC tests such cases are regarded as negative because a minimum percentage of $\geq 30\%$ is required to ascribe such cases as positive. Polysomic cases may be IHC 3+ positive but on calculation the HER2/CEP17 ratio may be deemed negative. Some of these cases may, however, respond to trastuzumab treatment. The ASCO CAP guidelines also do not proscribe one method of testing as the gold standard. Many cases with a good clinical outcome were selected on older criteria, i.e. 10% 3+ IHC and may be discounted using the updated criteria. Does this mean data from these earlier trials are invalid or should be discounted?

Standardisation of testing methodology

The ASCO/CAP guidelines also set-out and describe the potential pitfalls for failure of the varying test modalities. These include a range of pre-analytical, analytical and post-analytical factors which may influence the test results; factors which testing laboratories need to take cognisance of should attempt to eliminate, as far as possible, as causes of variability.

Amongst the more important factors influencing the outcome of tests is fixation. The ASCO guidelines propose exclusion criteria for performance and/or interpretation of IHC, among which are listed the following: Use of fixatives other than neutral buffered formalin, needle biopsies fixed for less than one hour and excisional biopsies fixed for less than six hours or more than 48 hours, core biopsies with crush or retraction artefacts, and any tissue with strong internal membrane staining of normal epithelial elements. If any of these are identified, IHC testing should be abandoned and ISH testing undertaken or further tissue obtained. Similarly, exclusion criteria are specified for the performance and interpretation of FISH assays. These include the samples with minimal invasive tumour present (as these are extremely difficult to detect under UV light), use of fixatives other than buffered formalin, tissues fixed for more than 48 hours, the presence of non-uniform FISH signals or a sample in which the background obscures the signal.

It is clear that pre-analytical factors influence either protein or DNA-based tests, and that all laboratories offering HER2 tests should attempt to standardise these variables according to the ASCO recommendations. One factor that seems to play a role is the "ischaemic time", i.e. the time from removal to placing the specimen into formalin. This should be done as quickly as possible, but may be prolonged if there is prolonged intra-operative assessment.

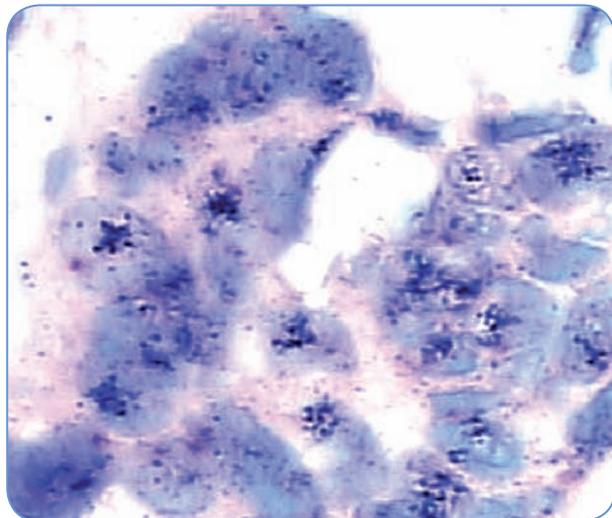
The ASCO CAP guidelines also carry strong recommendations for the analytical and post-analytical environment. These address laboratory methodology, laboratory good practice, quality control and proficiency testing. Such issues have been previously addressed, but the ASCO/CAP guidelines clarify these issues. The most important factors are:

1. The testing methodology should be based upon rigorous validation of the technique being offered with concordance of no less than 95% with another validated method/test. A minimum of between 25–100 cases should be run in parallel to demonstrate the concordance. Laboratories are required to keep detailed validation data.
2. Laboratories offering HER2 tests should perform stringent internal quality control (IQC) and pursue external quality assurance (EQA). The former includes inter alia running controls, and repeating any test with dubious or unexpected controls, questioning positive or negative tests results in certain situations, i.e. if a low grade tumour such as a tubular carcinoma is determined to be positive or conversely if a tumour which is expected to be positive shows a negative result. At least two external quality assurance events per annum should occur. Any failure to fall within the expected tolerance levels or IQC/EQA parameters should be addressed and corrected by the laboratory.
3. It is recommended that HER2 result reporting is best done in laboratories with a high volume and by select pathologists who are experienced and thus expert at interpretation (specialists). The minimum volume of cases previously recommended is 100 tests a month or 250 tests per annum.²⁹
4. The use of standardised methodologies is desirable and this is best done within an environment of laboratory accreditation. Any deviation from established standard operating procedures (SOPS) must be validated and stringently documented.
5. In the USA the utilisation of FDA-approved assays is preferred but not an absolute requirement, providing the assay used is stringently validated.
6. Ongoing competency assessment of technical staff and pathologists performing HER2 testing and interpretation must be adhered to.
7. Technical improvements in results may be achieved by using standardised control material and improvement in interpretation by utilising computer assisted image analysis systems.
8. Reporting elements for HER2 status must also be standardised.

Although molecular techniques such as FISH are thought by many oncologists and funders to be more reliable than IHC, it should be noted that for equivocal FISH results the guidelines recommend that IHC retesting is an acceptable methodology for retesting. FISH techniques are being supplanted in the USA and Europe by the easier to perform and more robust and permanent chromagenic and silver in situ hybridisation technologies (CISH and SISH) and studies have been performed which show excellent concordance with FISH technology, in the order of 96%.^{30,31} This is an important evolution as it places the molecular assessment firmly in the hands of the morphologist and it allows a marriage of morphological assessment and determination of ISH status of morphologically appropriate areas, which was not always possible on FISH. The technology to perform dual

staining for HER and CEP17 on histology slides allows for the same ratio assessment possible on FISH and is now readily available, and can be done in routine anatomical pathology laboratories, using automated staining machines. An example of BDISH can be seen in Figure 6. Techniques will very shortly become freely available allowing the pathologist to determine HER protein status and gene status on one slide using combined (automated) IHC and ISH techniques, allowing the pathologist to determine both parameters in one visualisation. This will allow for more rapid turnaround time and a more comprehensive HER2 assessment. Such technologies are relatively cheap and robust and easy to institute in South Africa, without laboratories needing to set up a dedicated molecular laboratory. Validation of such techniques will of course be necessary as stated by the ASCO/CAP guidelines.

Figure 6: BDISH overexpression of HER2 gene (black dot-like signals) compared to chromosome 17 marker (red signals). The ratio of HER2:CEP17 is $> 2, 2:1$



Conclusion and summary

Knowledge of HER2 status has become vital in breast cancer management. Testing laboratories are under pressure to produce reliable and reproducible results. These results can be easily produced if the guidelines are adhered to.

In summary, the implications for HER2 testing in South Africa are as follows:

- a) All pre-analytical variables should be standardised across laboratories by following these guidelines.
- b) All specimens should be placed in 10% buffered neutral formalin as soon as possible after removal from the patient and fixed for 6–48 hours. Novel processing techniques should be avoided, with specimens being routinely processed avoiding rapid and microwave techniques.
- c) Tests should be validated prior to being offered, and proficiency testing and rigorous IQC and EQA measures followed by these laboratories.

- d) Proficiency testing of technical staff and pathologists doing interpretation should form part of the IQC, EQA and accreditation environment.
- e) Testing is best performed in laboratories with high volumes of breast cancer specimens and by designated “specialists”.
- f) The algorithms proposed by ASCO/CAP, although not perfect, seem to be the best and most reproducible ones available and although 5% lack of concordance is allowable, we should be aiming for as close to 100% positive and negative predictive values. Pathologists must therefore attempt to give patients the benefit of the doubt and default to retesting if there are any doubts of either an IHC or ISH test.
- g) It is probable that one-stop testing (i.e. IHC and ISH tests on a single slide) will allow the morphologist to do the morphological and HER assessment, making the pathologist the best placed individual to assess the breast carcinoma specimen holistically.
- h) Pathologists and clinicians should, however, appreciate that there will be equivocal and contradictory results, an inherent limitation in any biological system, and that dealing with such issues both from a diagnostic and therapeutic point of view will constitute a challenge and may require further testing, discussion and a dollop of good-sense and practicality.

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Disclaimer

The author is a diagnostic pathologist in private practice and has no interests in any company marketing trastuzumab or any of the commercial tests for HER2 overexpression. He is a member of the Herceptin® advisory board, convened by Roche Pharmaceuticals.

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