



HER2 testing in the UK: recommendations for breast and gastric in-situ hybridisation methods

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ABSTRACT

These guidelines supplement existing guidelines on HER2 testing by immunohistochemistry and in-situ hybridisation (ISH) methods in the UK. They provide a specific focus on aspects of guidance relevant to HER2 ISH testing methods, both fluorescent and chromogenic. They are formulated to give advice on methodology, interpretation and quality control for ISH-based testing of HER2 status in common tumour types, including both breast and gastric tumours. The aim is to ensure that all ISH-based testing is accurate, reliable and timely.

National and international guidelines and recommendations highlight the importance of quality control, accuracy and reproducibility in the determination of HER2 status in breast and gastric carcinomas.^{1–5} The value of HER2 in-situ hybridisation (ISH) testing is that it provides a robust, accurate and highly clinically informative measure of HER2 status in clinical samples. While debate continues as to the optimal testing regime and the relative importance of HER2 detection by immunohistochemistry versus ISH^{1–4} all guidelines recognise that for breast cancers with equivocal immunohistochemistry results HER2 testing by ISH is mandatory.^{1–5} While current National Institute for Health and Clinical Excellence guidelines recommend immunohistochemical detection of HER2 in gastric carcinoma, and therapy with trastuzumab only for patients with metastatic disease who have tumours with high (3+) immunodetectable HER2 expression, the current UK licence for herceptin in gastric cancer permits treatment of both immunohistochemistry positive (3+) and fluorescence in-situ hybridisation (FISH; immunohistochemistry 2+ amplified) positive cases as follows: 'Herceptin should only be used in patients with metastatic gastric cancer whose tumours have HER2 overexpression as defined by IHC2+ and a confirmatory SISH or FISH result, or by an IHC3+ result. Accurate and validated assay methods should be used.'

There is now a growing number of commercial kits using both chromogenic and fluorescent detection systems for the ISH analysis of HER2.^{6–8} Each has particular technical strengths and weaknesses that impinge upon their application for routine diagnostic laboratories. As experience with ISH methods has grown so has the recognition of specific methodological challenges that must be addressed in performing these techniques in a diagnostic setting. Also, as the numbers of cancers tested by ISH have risen exponentially over the

past 10 years, interest in reporting challenging cases, which fall outwith the conventional definition of gene amplification, has risen. National guidelines, with specific space constraints, may not address all these issues in detail. Therefore, we have sought to develop specific recommendations, relevant to diagnostic practice, particularly within the UK, which may improve consensus in the diagnostic practice of ISH and thereby improve patient care. In specific areas we are gathering data to inform the diagnostic community, particularly where there are gaps in current knowledge. Therefore, we expect, over the coming months and years, to inform and extend the knowledge on practice-based HER2 ISH testing. When appropriate we will distinguish between evidence led and consensus statements and those that are statutory requirements. We will also highlight areas where additional research would be of value. These guidelines supplement, but do not replace, UK guidelines for HER2 testing.^{3 9 10}

LOCATION OF TESTS AND PERFORMANCE

It is a regulatory requirement that all diagnostic ISH tests within the UK be performed in a laboratory accredited under the clinical pathology accreditation scheme. In addition, laboratories must participate in the UK national external quality assurance scheme for ISH (NEQAS–ISH).^{11 12} For clinical trials, in which centralised testing is performed to confirm HER2 status, testing may be performed in accredited laboratories that adhere to good clinical laboratory practice (accredited/inspected by either clinical pathology accreditation or medicines and healthcare products regulatory authority). Participation of all laboratories performing such testing in the UK NEQAS scheme is strongly recommended.

There is no strong evidence to mandate the restriction of testing to high volume ISH laboratories. Nonetheless, in order that staff (see below) maintain a high degree of expertise in performing and reporting ISH analyses, and to ensure that testing may be performed in a cost-effective manner, recommendations have suggested a minimum of 100 ISH tests (two per week) be performed in each centre per annum. However, there is increasing evidence¹³ that a subset of over 10% of breast cases present with challenging ISH patterns; for example, co-amplification of HER2/CEP17 (chromosome 17 centromere enumeration probe) or heterogeneity of gene amplification. Similarly, evidence suggests a high frequency of heterogeneity of amplification with complex

ISH patterns in gastric cancers.⁵ As staff will require expertise in the assessment of both straightforward and challenging cases it is reasonable to suggest that volumes of at least 150 ISH cases per annum, with a minimum of 100 breast and 50 gastric cases by ISH per annum, represent the optimal environment for the delivery of appropriate quality and cost effectiveness (see section below for internal quality assurance recommendations).

STAFF

There is sufficient evidence to show that, with appropriate training and/or supervision, pathologists, clinical and biomedical scientists can reliably perform, interpret and report the results of *HER2* ISH analyses.^{8 11 12 14–17} Staff performing diagnostic tests, including ISH, should be state registered (recognised by the appropriate professional body) as clinical/biomedical scientists, pathologists or genetic technologists (where cytogenetics departments are performing ISH-based analyses). In all cases results must be authorised by a state registered clinical/biomedical scientist or pathologist.

TRAINING

All staff involved in the routine performance or analysis of ISH methods should have adequate training that is appropriate to the tasks they are performing. Therefore, staff who are undertaking as well as analysing ISH should be trained in both aspects. Ideally these staff members should also be aware of the specific technical challenges of slide preparation and interpretation. Training should include participation in an accredited (by the Royal College of Pathologists/Institute of Biomedical Science or equivalent) ISH training course. In addition, we recommend that each of these staff should perform a minimum of 100 ISH evaluations in parallel with an experienced member of staff to ensure scoring consistency before working independently. A minimum concordance of 95% (taking into account the number of borderline cases) is recommended; this should include both concordance of diagnostic results (amplified and non-amplified status) and numerical results (for both *HER2* and *CEP17*, possibly using interclass correlation coefficients to assess any scoring bias). Annual performance for each practitioner should be monitored, preferably within an external quality assurance scheme, and results of both training and annual assessments entered in the individual's training file as evidence of continuing competence. A pilot assessment scheme is being developed.

Each centre offering ISH testing should ensure there are adequate numbers of trained staff to support the workload, balanced with ensuring individual expertise, at least two per department (to ensure there is cover for annual leave, sickness, etc.). Where multiple members of staff are actively involved in *HER2* ISH testing, each member should maintain a level of competence in all aspects of ISH for which they are responsible. This may require restricting the numbers of staff involved to ensure each scores a sufficient number of cases.

QUALITY CONTROL AND AUDIT

A proportion of cases should be audited by dual scoring (10% is recommended as the minimum), to ensure consistency between members of staff reporting diagnostic results. 'Difficult' cases should always be reviewed by a second scorer. Each member of staff should analyse NEQAS–ISH external quality assessment specimens, while involved in ISH evaluation. It is likely that, in the near future, a new scheme will be developed to monitor

performance by individual scorers and this is in line with good practice. It is recommended that each member of the laboratory involved in the interpretation and reporting of results should report at least 100 tests per annum (at least two per week) although the optimum number may be higher than this. While there is currently little evidence of the impact of experience in improving diagnostic quality, it is intuitive that this is the case. Evidence to support this should be gathered.

Internal audit can provide an additional means to ensure that laboratories are maintaining standards. A routine audit of results from internal controls, using numerical *HER2*, *CEP17* and ratio results, can provide early indications of any quality issues. An audit of results from test samples can provide a longer term means of quality assessment. Intra and interobserver results should be assessed routinely for all staff undertaking evaluation. Audit should include assessment of time taken to produce results and the frequency of assay failures. Success rates should, in competent laboratories, be greater than 95% unless samples are poorly fixed. Turnaround times should reflect the need to ensure results are available for discussion at multiple disciplinary meetings at which clinical management is decided. For patients eligible for neoadjuvant *HER2*-directed therapy or neoadjuvant clinical trials the result may be required within 1–2 weeks or less.

INTERNAL QUALITY CONTROLS

The inclusion of controls in every assay run is recommended to ensure test accuracy, this may be performed cost effectively by the use of a multiblock or tissue microarray.¹⁸ Internal quality controls should reflect the range of cut-off points including a known negative and a low level amplified case (see below for scoring). Cell line preparations containing samples of known *HER2* status are valuable. It is optimal to include, in addition, tissue in which *HER2* status has been validated by more than one method in all assay runs. These controls provide a means of assessing the success of the ISH method and consistency from run to run. Failures in the quality of control slides should be used to determine if a run is technically substandard. Low volume laboratories may find reagent costs for running controls equivalent to those required for performing tests, which provides one reason for ensuring sample numbers are maintained above the recommended threshold in ISH testing laboratories. Internal tissue controls are also important as the assessment of signal quality in the background 'normal cells' is important in the determination of assay success. This is especially critical in chromogenic-based assays in order to confirm successful enzyme/chromogen development and to assess the under or over development of signals.

SAMPLES

As with all diagnostic samples, adequate fixation is essential. ISH testing should not be performed on poorly fixed samples (see *HER2* guidelines).³ Due to the methods used to assess *HER2* status by ISH (counting 20–60 cells³ see below), there is little evidence to suggest that the type of biopsy sample (core, trucut, excision specimens or tissue microarrays) affect the accuracy of the *HER2* result. However, samples should allow the distinction between in-situ versus invasive disease (for both breast and gastric cancer) and therefore fine needle aspirates are not recommended.

There is now evidence to suggest that, for breast cancer at least and possibly for gastric cancer as well, there is the potential for *HER2* status to change during treatment and progression of

disease.^{19 20} Increasingly, this is being recognised by the multi-disciplinary team and re-biopsy of such recurrent lesions may be undertaken, partly to confirm the histopathological diagnosis but also the biomarker status, particularly the HER2 status, of the metastatic lesions.

SAMPLES (REFERRAL/SECTIONS)

Many centres receive samples not only from their own hospital, but also referrals from other units in the local cancer network and potentially from further afield. Samples should be checked, on arrival, for the presence of appropriately fixed invasive carcinoma. Referring centres should ensure that samples are representative of the tumour assessed for other biomarkers. In particular, if samples are referred due to equivocal (2+) immunohistochemistry results, it is strongly recommended that the same block, or sections from it, are provided to the ISH laboratory. When this is not the case it should be indicated, with reasons provided for the change.

ISH for breast cancer should be performed on 3–4 µm sections, while for gastric cancer 2–3 µm sections have been suggested to improve interpretation.⁵ Optimal practice is to cut sequential sections for haematoxylin and eosin, immunohistochemistry and ISH, maintaining orientation and placement on the slides. Slides recommended by manufacturers of ISH systems should be used, as other slides may interfere with ISH. It is also important to be aware that most dyes used to orientate surgical specimens autofluoresce and may interfere with FISH. While deterioration in antigenicity can affect immunohistochemistry, there are no published data on the effects of section storage on the DNA quality relevant to ISH analysis. However, it is recommended that whenever possible freshly cut sections are used and the use of sections stored for over 12 months is avoided (UK guidelines).

CHOICE OF ISH METHODS

While FISH has been used most extensively for the evaluation of *HER2* gene amplification and has the greatest evidence base, novel methods using colorimetric ISH have recently been evaluated and shown to provide accurate and consistent results. Each approach has its strengths and weaknesses, and each laboratory should ensure the method chosen is appropriately validated, quality controlled and robust in their hands.

SINGLE OR DUAL COLOUR ISH METHODS: THE ROLE OF CEP17

Dual colour ISH, in which both *HER2* copy numbers and the number of CEP17 (chromosome 17 centromere enumeration probe) copies are evaluated simultaneously are preferred. There is now strong evidence that, in order to determine the presence of amplification (defined as a *HER2*/CEP17 ratio ≥ 2.00), both probes are required.² Sequential testing of *HER2* and CEP17 in single colour ISH methods is acceptable; however, we no longer recommend the use of *HER2* ISH tests that do not assess CEP17 copy numbers. However, if *HER2* copy number-only methods are in use then all cases with copy numbers of three to eight must be tested for chromosome 17.²

A number of ISH methods have been evaluated in multicentre studies by different groups. Several have been evaluated by ring studies in UK reference laboratories. To date, none has been found that do not perform adequately with appropriate training, specific to the method of choice.¹⁵ Currently, we recommend the use of dual colour ISH methods from commercial sources that are CE marked. Dual colour chromogenic-based assays have recently gained popularity and there is good evidence to support

their use. However, strict quality assurance is mandatory to prevent the underdevelopment of the enzyme steps required for signal development. When using chromogenic-based assays, evaluation of the normal cellular components is essential, as recommended by the manufacturers; *HER2* and CEP17 copy numbers should demonstrate a 'normal' signal pattern, ie, one to two copies of both per cell. It is our opinion that a selection of background normal cells should be formally counted to confirm successful hybridisation, detection and visualisation as a baseline, before interpretation of the tumour. This is based on observations from NEQAS assessments and training courses in which it has been noted that inappropriate signal counts as a consequence of suboptimal Chromogenic In situ hybridisation (including all forms of non-fluorescent colorimetric detection) can lead to either under or over scoring.

DIGESTION METHODS AND CHECKING DIGESTION QUALITY

Most ISH systems have linked methods for preparing samples and probe hybridisation. Tissue digestion is the most critical stage in the preparation of samples for successful *HER2* ISH evaluation.²¹ Ensuring successful tissue preparation is essential for appropriate assay performance. A prehybridisation assessment of digestion^{21 22} can enhance the performance of *HER2* ISH methods. This is not applicable to fully automated systems. It is also essential that, as with all diagnostic methods, reagent quality is assessed before each assay run. An appropriate audit trail of all reagent batches, expiry dates, dates first used and of the staff performing assays is an essential component of the quality control of assay performance.

ASSAY FAILURES

Assay runs with inappropriate results from internal quality controls should be rejected. Individual samples where hybridisation has failed should be repeated at least once. Where problems persist a case by case assessment of digestion efficiency is imperative.

EVALUATION AND REPORTING OF RESULTS

Correct assessment of ISH requires appropriate training (see above) and experience in recognising histological features of breast or gastric carcinoma tissues. Before undertaking an evaluation of *HER2* ISH, all staff must receive relevant training. *HER2* status in the breast should only be reported on the invasive portion of a tumour. While ductal carcinoma in situ of the breast is frequently *HER2* amplified, it is irrelevant with regard to the selection of patients for treatment with *HER2*-targeted therapies. The therapeutic relevance of the *HER2* status of 'in-situ' disease in gastric carcinoma remains to be evaluated. Until this is done, it may still be appropriate to score carcinoma in situ/high grade dysplasia for *HER2* in cases of clinically established metastatic gastric carcinoma when the sample does not include overtly invasive disease.

As suggested above, trained biomedical and clinical scientists can assess ISH results, with support from consultant histopathologists for interpretation of complex histology.

Areas for evaluation of ISH should be identified by reviewing a serial haematoxylin and eosin stained slide and *HER2* immunohistochemistry (if available in breast and always for gastric), to identify any *HER2* immunohistochemistry hot spots, if present. This is of particular importance in gastric carcinoma, when the presence of ulceration and/or variation in pathological characteristics increases the difficulty in interpretation. In addition, the entire ISH slide should be reviewed on a 4× or 10×

objective to ensure no tissue has been lost, to assess the quality of ISH across the tumour area, and to select appropriate areas for analysis. After this review, the section should be screened to select appropriate areas for analysis (scoring) and analysed using at least a 40× objective.

Following a review of the entire slide, select three representative tumour areas (when possible), including both amplified and non-amplified areas if both are present. Count 20–60 cells (as appropriate) from these three separate areas, recording the location of each area using the microscope Vernier scale or equivalent. Counting is targeted to areas of tissue sections with a large proportion (>50%) of nuclei showing signals. The number of CEP17 and *HER2* signals counted in 20–60 non-overlapping invasive cancer cell nuclei, using at least three distinct tumour fields (when possible), should be recorded. Overdigested, mechanically damaged and truncated nuclei should be excluded.

While heterogeneity is relatively uncommon in breast cancer (*Am J Clin Pathol*, in press), reports on gastric cancer suggest a significant proportion of cases exhibit heterogeneity.⁵ In addition, amplified cells in gastric cancer may appear in smaller clumps or areas (of as few as three to five cells) within the section. Scanning the slide is therefore even more critical. From our recent audit of heterogeneity in breast cancer (*Am J Clin Pathol*, in press) the following approach has been formulated to manage heterogeneous *HER2* gene amplification in breast cancer:

‘In all cases where ISH is performed the entire slide should be scanned before counting, areas of apparent heterogeneity should be identified during this scan and/or by reference to an IHC stained slide. The number of chromosome 17 (CEP17) and *HER2* signals should be counted in 20–60 non-overlapping invasive cancer cell nuclei, using at least three distinct tumour fields. If there is evidence of heterogeneity between fields (or less frequently within fields) additional cells (at least 20 per field) and/or fields (up to 6) should be counted. The *HER2*/CEP17 ratio should be calculated for each field individually. Where the mean *HER2*/CEP17 ratio in any field is 2.00 or greater, the tumour should be regarded as amplified. Cases containing both amplified and non-amplified fields, using this definition, should be reported as exhibiting heterogeneous amplification. For all cases where the ratio is between 1.80 and 2.20 results should be based on counting at least 60 tumour cells, and in cases where heterogeneity is suspected this should be 60 cells per field. In rare cases where amplified and non-amplified tumour cells are intermingled in a single field, interpretation is difficult and evidence is lacking. We suggest that for such cases only the presence of clearly amplified cells, with multiple *HER2* signals, is considered evidence of heterogeneity, although evidence is lacking in this area. Current evidence does not support using the existence of small numbers of apparently amplified cells within an individual tumour field to identify heterogeneous amplification.’

For gastric cancer this approach should be taken but when reporting heterogeneity the presence of small numbers of amplified cells is regarded, according to current criteria, as clinically significant and therefore should be reported as such.

Only count cells in which the nuclear borders can be identified. When possible count all signals, if this is not possible (eg, clusters) then it may be possible to estimate the number of signals based on a single signal size in non-malignant cells. Count doublets as a single signal. It is strongly recommended that representative images are captured and archived for every ISH case.

In equivocal cases, ie, those with a *HER2*/CEP17 ratio of 1.80–2.20, additional cells should be counted when possible

(optimally a minimum of 60 per case), optionally this should include a dual count (from a second observer; either internally or to a second centre). The optimal approach to improving accuracy in this range is to increase the number of cells counted to 60–120 and/or repeat the test. A ratio of 1.80–1.99, after counting further cells and/or repeating the test, should be reported as borderline but not amplified and include a clear statement that the carcinoma is regarded as *HER2* negative. A ratio of 2.00–2.20 should be reported as borderline but amplified, and therefore regarded as *HER2* positive. Data on the response of patients to trastuzumab whose cancers fall within the borderline amplified category are not available; a statement to this effect can be included in reports.

While these guidelines are sufficient for the majority of cases, there are occasions when difficult cases should be referred to expert centres for guidance.

IMAGE ANALYSIS

Image analysis systems have recently become available for the interpretation of *HER2* FISH. They remain of variable quality and the phrase ‘caveat emptor’ still applies. However, some systems appear to provide diagnostic utility. A full training/validation period of image analysis should be performed, as for training of a human scorer. It is also recommended that all results are reviewed by an experienced scorer before issuing a report. When these systems are used for reporting they should be assessed by an ‘independent’ scorer within the NEQAS–ISH module.

Requesting additional tissue blocks

Additional tissue samples should be requested after repeated technical ISH failure for any reason (under fixation, over fixation, etc.). If there is an equivocal result on a core biopsy or excision, consider testing the second block. In the case of poor fixation of an excision specimen, consider testing lymph node metastasis if material is available.

ISH REPORTS

Reports should include the number of cells assessed and the numerical *HER2*/CEP17 ratio. In addition, the average *HER2* and CEP17 copy number for all cells should be reported. If there is anything ‘unusual’ this should be noted in the report, and when possible refer to difficult cases reviews (to be published) for specific guidance. If a dual count has been performed, report an overall summary of the two counts.

For heterogeneous cases with distinct subclones (in either gastric or breast cancers) report each subclone separately, specifying the mean *HER2*/CEP17 ratio for each, the number of cells counted and whether dual reporting was performed. It is important to be aware that, particularly in gastric carcinomas, small subclones with few assessable cells may be present; these should be noted even if fewer than 20 cells can be counted. For heterogeneous cases with intermixed amplified and non-amplified cells, in which distinct clones cannot be identified, count all tumour cells (both amplified and non-amplified) across three areas and report both the mean *HER2*/CEP17 ratio and the percentage of cells with amplification (see case reports by J. Starczynski, manuscript in preparation). Report the pattern of heterogeneous amplification as either distinct clones or intermixed amplified and non-amplified tumour cells.

Reporting ISH in the context of immunohistochemistry results

When both immunohistochemistry and ISH results are available both should be included on the report. For example, cases

Take-home messages

- ▶ These recommendations are a supplement to the best practice recommendations.³
- ▶ Workload: laboratories should test a minimum of 200 cases (combining both gastric and breast cancer cases) per annum by ISH.
- ▶ Staff: reporting can be performed by pathologists, clinical scientists, biomedical scientists and genetics technologists, state registered with the appropriate professional body.
- ▶ Training: all staff involved in *HER2* ISH reporting should have attended a Royal College of Pathologists/Institute of Biomedical Scientists approved training course and completed a 100-case training period alongside an experienced scorer.
- ▶ Quality assurance: all laboratories performing *HER2* ISH testing must participate in the NEQAS–ISH module. When available this should be extended to all staff.
- ▶ Audit: internal audit is essential to monitor quality and intra and interobserver concordance.
- ▶ Reporting: the whole tissue section must be carefully screened and 20–60 cells from three areas should be assessed.
- ▶ Borderline: cases with a *HER2*/CEP17 ratio between 1.80 and 2.20 should have at least an additional 40 cells (60 in total) counted either by the same scorer or by an independent scorer. Thereafter, a *HER2*/CEP17 ratio of 1.80–1.99 should be reported as borderline not amplified, *HER2* negative, and a ratio of 2.00–2.20 should be reported as borderline amplified and *HER2* positive.
- ▶ Heterogeneous amplification should be reported when clear evidence of separate cell populations is available.
- ▶ Reports should include the number of cells counted; the average *HER2*/CEP17 ratio; the average *HER2* copy number; the average CEP17 copy number; a summary statement describing the *HER2* status, for example *HER2* is amplified. If any unusual features are noted these should be included in the report.

(of either breast or gastric cancer), with immunohistochemistry 3+ or 2+ and with ISH results should have both annotated. In gastric cancer, however, specific attention should be given to the immunohistochemistry and ISH results with regard to the spatial orientation of the stained areas of the section, with attention drawn to areas considered positive for both ISH/immunohistochemistry and those ISH positive but immunohistochemistry negative or vice versa.

FISH SLIDE STORAGE

FISH-stained slides will fade with time. It is recommended that representative images are taken and that slides are stored at –20°C for a minimum of 12 months.

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