

# ISO 15189 assessment: a positive experience for immunohistochemistry

Accreditation terminology does not fit comfortably in the cellular pathology lexicon, as many of its reports are subject to interpretation, so how can it satisfy ISO 15189 standards? Julie Terry reports on a positive assessment exercise, and provides some relevant guidance.

Following a United Kingdom Accreditation Service (UKAS) assessment visit last May, no findings were attributed to the immunohistochemistry (IHC) section of our laboratory. The assessors were very happy with all the quality procedures and I actually enjoyed the process. That's not to say that I particularly enjoyed the period leading up to the big day – such a lot of work was required to bring our procedures up to standard. However, it was all worth it in the end and the service has certainly improved as a result. This short article highlights some experiences on the day of the assessment and also describes the principles of the validation and verification procedures that have been put in place in the IHC section of cellular pathology at the Calderdale Royal Hospital in Halifax. I thought it could be useful to describe a positive experience.

We all know that the terminology used in the ISO 15189 standards is not necessarily geared towards a cellular pathology laboratory. We do not deal with numbers, and many of our observations are subject to interpretation, so how can we satisfy the standards?

On the day of the assessment, the first question was "Do you have any antibodies that you have to make up?" If only I could have said no, and that they were all Ready to Use (RTU). There was just one, and once this was known then the remainder

of the day's questions focused on this aspect of the process, and answers to the following questions were sought:

- Do you use expired antibodies?
- How do you make the antibody?
  - What dilution? Can I see the verification documentation?
- Which pipettes do you use? Can I see them and their UKAS calibration certificates?
- Who carries out this procedure?
  - Can I see their pipette accuracy training records?
- Where is the antibody kept? Can I see evidence of temperature monitoring?
  - Can you provide evidence that the measuring devices have been calibrated to UKAS standards?
- How is the batch logged on the instrument? How do you know who has made it? Which protocol is used for it?
- Can I see your full training records for IHC?
- What control tissue do you use for this antibody?
- How do you verify the control tissue?
  - How do you store your controls? What are the expected staining patterns for the antibody?

Simple steps, like making sure all documentation contains the necessary information, are important. This includes

author, authoriser, active date, version number, department etc. We use a document management system called Q-Pulse; everything about each antibody is kept on here, but the assessor was also happy to see paper copies.

## Validation

All antibodies and probes are validated by the manufacturer prior to receipt in the histopathology laboratory. Validation is the process of demonstrating, through the use of specific laboratory investigations, that the performance characteristics of an analytical method are suitable for its intended analytical use. Each antibody/probe is accompanied by a detailed datasheet, which displays the following information:

- intended use
- characterisation
- instructions for use (including recommended protocol)
- quality control procedures (including recommended control tissue)
- interpretation of results
- sensitivity
- specificity
- reproducibility.

Using this information, it is possible to verify each reagent for use within the laboratory. This takes into account pre-analytical factors such as tissue fixation and processing. It is necessary to measure the degree of uncertainty that exists within each laboratory test. This is determined through establishing the accuracy, specificity and reproducibility of each test. In a model suggested by Maxwell *et al.* and utilised in our verification studies, each test can be placed into one of three levels, based on the level of knowledge available for each antibody (ie the information contained in the datasheet).

**Level 1:** Antibody is very well characterised and its specificity is fully understood. Publications and external QA support its diagnostic utility.

**Level 2:** Slightly less is understood about this antibody. Further examination of its target expression is needed.

**Level 3:** Antibody specificity has not been identified. A rigorous assessment is required of its utilisation.

Potential contributors to uncertainty are displayed in Table 1, along with the means to control for these factors.

The majority of antibodies offer a qualitative staining result (ie it is either positive or negative). The only exceptions are oestrogen receptor (ER), progesterone receptor (PR) and the assessment of HER2 status, all of which provide a semi-quantitative result. These require an assessment of the staining pattern and scoring according to a specific scale, as detailed in their respective SOPs/ documentation. There is low uncertainty associated with the scoring of these tests, due to the semi-quantitative nature of the results. Therefore, it is not possible to calculate parameters such as range, mean or standard deviation.

The final step in verification is reproducibility, making sure the same result occurs over multiple runs. This is also something that is carried out and confirmed by the manufacturer, but is also carried out in-house. Each reagent is labelled with a unique lot number and any lot-to-lot variability needs to be addressed. This is described in more detail later. Lot-to-lot verification enables continuous proof of sensitivity, specificity and reproducibility. Additional assessment of lot variation is carried out on the breast biomarkers ER, PR and HER2. Data are collected on a monthly basis, and with the aid of the online UK NEQAS Audit Tool, the rate for each biomarker is calculated and displayed on a graph. This is audited every three months, to see if any rates fall outside the expected positivity rates. An annual audit is also carried out on the biomarker rates and the results from

these are compared to national averages obtained from UK NEQAS.

### Immunohistochemistry verification

Following a risk assessment of all IHC procedures/reagents, it is necessary to verify all new batches of primary antibodies and detection kits.

#### Primary antibodies (existing stock)

- Lot details of the new antibodies are added to an 'Antibody lot quality checklist'. These need to be verified before they are put into routine use.
- The associated Process Control Record (PCR) must also be updated on Q-Pulse; this involves documenting the new lot number as a new action under the 'Method Verification' section.
- Whenever it is convenient, the new antibody lot should be tested on the appropriate control for that particular test. A section of the positive control tissue is placed on a slide that already contains a section from the IHC multiblock (composite control containing appendix, colon cancer, kidney and tonsil). This ensures a range of antigen expression can be assessed, and negative controls are present to confirm antibody specificity.
- Evaluate the quality of the staining alongside the slide stained with the previous antibody lot.
- If the new antibody lot does not stain as it should, a non-conformance (NC) needs to be initiated on Q-Pulse
- The slide is evaluated with a pathologist.
- The protocol is amended as required and any changes must be approved and signed off by a pathologist before amending for routine use.
- If large changes are required, this may need re-verification with a number of cases. If only small changes are made (eg to incubation time or antigen retrieval time) then a couple of negative and positive cases are sufficient to re-verify.

- All stained slides are kept in the main laboratory in the drawer labelled 'Verification slides'. These are boxed after one year and stored off-site.

#### Introducing new primary antibodies

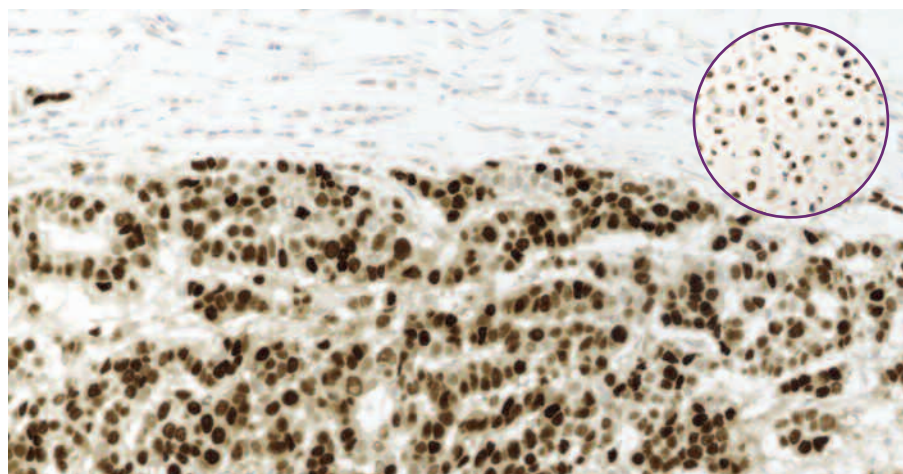
Before new antibodies can be introduced into the laboratory, they have to undergo vigorous verification procedures. The procedures outlined below have been recommended by Fitzgibbons *et al.* The extent of the verification depends on a number of factors, primarily the antibody's intended use. Verification should be carried out on tissue that is fixed and processed in an identical manner to clinical samples. Other factors include whether the antibody is a dependent marker (used as part of a panel) or an independent marker (used alone), how often it is likely to be used, and the complexity of its interpretation. Antibodies can be split into three groups, depending on intended use, as follows: diagnostic, prognostic and predictive

**Diagnostic markers:** provide information that enable a diagnosis to be made. For example, lack of CK5/6 expression in the myoepithelial cells surrounding breast ducts indicates a diagnosis of invasive ductal carcinoma.

- Diagnostic markers generally offer a qualitative result (ie the outcome is either positive or negative) therefore range and reference ranges are not applicable.
- When verifying a new antibody, the sample set would contain equal numbers of both outcomes. For example, if a new marker was to be introduced that was able to differentiate between non-invasive and invasive ductal carcinoma (as CK5/6 above), the sample set should contain 10 invasive cancers (CK5/6 would be negative) and 10 non-invasive cancers (CK5/6 would be positive).
- For diagnostic markers, a concordance of 95% is acceptable, with 95% confidence interval (CI).

**TABLE 1. POTENTIAL CONTRIBUTORS TO UNCERTAINTY, ALONG WITH MEANS TO CONTROL FOR THESE FACTORS.**

Source of uncertainty	Solution
Environment	Temperature monitoring, appropriate storage
Lot variation	Lot verification of 'risk reagents'
Human error	Adequate training and competence assessments. ER intra-scorer variability assessment
Instrument performance	Annual preventative and reactive maintenance carried out by manufacturer, weekly/monthly maintenance carried out by biomedical scientist staff, Thermopad temperature verification, vortex mix test, decontamination
Antigen degradation	Use freshly cut sections, store control slides appropriately. Adequate fixation and processing
Protocol	Minimal protocol alterations, only with pathologist approval and under strict quality management procedures
Sampling error	Adequate training, minimising cold ischaemic time, adequate fixation and processing



**Infiltrating ductal carcinoma of the breast showing semi-quantitative high oestrogen receptor . Inset: standardised cell line control slide.**

**Prognostic markers:** provide information on the likely course of the disease. For example, high expression of the Ki67 antigen is associated with high levels of proliferation and disease progression.

- Prognostic markers can offer a qualitative or semi-quantitative result (ie the outcome is either positive or negative, or there may be a range of expression such as low, medium and high). This means that range and reference ranges are not applicable.
- The sample set should reflect the number of possible outcomes (eg 10 low, 10 medium and 10 high expressors would be appropriate).
- For prognostic markers, a concordance of 95% is acceptable, with 95% CI.

**Predictive markers:** are able to identify subpopulations of patients likely to respond to a particular treatment. For example, over-expression of ER indicates that the patient is likely to respond well to hormone therapy.

- Predictive markers usually provide a semi-quantitative result (ie there may be a range of expression on a scale). For example, ER expression is measured using the Allred scoring system on a scale of 0–8. This means that range and reference ranges are not applicable.
- Predictive markers require a greater level of confidence, therefore the sample set is increased greatly. The larger the sample, the narrower the 95% CI range will be, which provides greater confidence that the assay is performing as expected.
- Acceptable concordance rates are increased to 95% for these markers.
- No specific guidelines exist on sample size for prognostic markers, but it should reflect the range of expression that exists within the new antibody. For example, the HER2 antibody can exhibit expression that is recorded as one of four scores: 0, 1+, 2+ and 3+.

Twenty-five cases at each score, totalling a sample size of 100, would be appropriate. This would allow for up to five cases to be discordant and to still achieve the requirements for its approved use in the laboratory.

- Once the sample size has been agreed and suitable cases found, each case should be anonymised by randomly allocating a number to it. The test outcome should be noted with the allocated number
- A section (4  $\mu\text{m}$ ) is cut from each case and this is labelled only with the anonymised number.
- All slides are stained with the new antibody and given to a pathologist for scoring. Depending on the antibody's intended use, a number of pathologists may need to assess the slides to account for intra-observer variability.
- The scores are compared to the original scores, and calculations are made to determine if the desired level of concordance has been reached. This involves the production of a contingency table and  $\chi^2$  analysis.
- If concordance levels are not met, advice must be sought from a pathologist and/or laboratory manager. This may result in the staining of additional samples, or the antibody may not be approved for routine use.
- If the required concordance is achieved, change management procedures are instituted, along with all other necessary documentation, including setting up a routine protocol on the IHC computer and the identification of appropriate positive control material.

### Control tissue verification

Positive control material is used to check that the reagents and techniques are working appropriately. The tissue contains specific antigens/proteins/cellular components at known, stable levels.

When performing special stains, immunohistochemistry, immunofluorescence and *in situ* hybridisation, it is necessary to use positive control material to ensure the quality of the staining and to verify results.

Positive control material can be taken from positive archived blocks/tissue or from cut-up specimens with approval from a pathologist. This is the preferred method as it ensures that pre-analytical factors such as fixation and processing are identical to diagnostic tissue. If a protein is expressed constitutively in a particular tissue type (eg leucocyte common antigen [LCA] will always stain lymphocytes in tonsil) this can also be used as a tissue process control. This type of tissue will control for both the staining process and the pre-analytical steps mentioned above.

### Instrument verification

Staining machines are verified at installation by the manufacturer, which also involves the verification of all the protocols currently in use. Most machines work on a barcoded, closed platform that minimises user error and ensures high accuracy and reproducibility. Further checks are carried out throughout the year as detailed below:

- heat pad temperature verification
- vortex mix test
- annual preventative maintenance by the manufacturer
- reactive maintenance by manufacturer as required
- refrigerator/incubator/room temperature monitoring
- Calibration of measuring devices (pipettes, thermometers) .

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### Further reading

- Bertheau P, Cazals-Hatem D, Meignin V *et al.* Variability of immunohistochemical reactivity on stored paraffin slides. *J Clin Pathol* 1998; **51** (5): 370–4.
- Fitzgibbons PL, Bradley LA, Fatheree LA *et al.*; College of American Pathologists Pathology and Laboratory Quality Center Principles of analytic validation of immunohistochemical assays: guideline from the College of American Pathologists Pathology and Laboratory Quality Center. *Arch Pathol Lab Med* 2014; **138** (11): 1432–43.
- Leong A, Cooper K, Leong F. *Manual of Diagnostic Antibodies for Immunohistology* 2nd edn. Cambridge University Press, 2003.
- Maxwell P *et al.* The management of uncertainty: increasing stringency for validation and verification of immunohistochemistry in tissue diagnostics. UK NEQAS ICC & ISH Immunocytochemistry Run 106/35, 2014: 5–6.