

UKAS ISO: 15189 accreditation visit – A positive IHC experience

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Following our recent UKAS visit in May 2015 – no findings were attributed to the IHC section. The assessors were very happy with all our quality procedures and I actually enjoyed the assessment process. This isn't to say that I particularly enjoyed the period leading up to the big day as such a lot of work was required to bring our procedures up to standard. But it was all worth it in the end and the service has certainly improved as a result of it.

This short article highlights some of my experiences on the day of the visit and also describes the principles of our validation and verification procedures that have been put in place in the IHC section. I thought it could be useful and interesting to describe this 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 visit, the first question asked was 'Do you have any antibodies that you have to make up?' If only I could have said no, and that they were all RTUs (Ready to Use). But there was one – and once this was known, the rest of the days questions focused on this. The following questions/information was then required;

1. Do you use expired antibodies?
2. How do you make the antibody? What dilution? Can I see verification documentation?
3. Which pipettes do you use? Can I see them and their UKAS calibration certificates?
4. Who carries out this procedure? Can I see their pipette accuracy training records?
5. 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 standard?
6. How is the batch logged on the instrument and how do you know who has made it and which protocol is used for it?
7. Can I see your full training records for IHC?
8. What control tissue do you use for this antibody?
9. 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 your documentation contains the necessary information is important. This includes author, authoriser, active date, version number, department etc. We use a document management system called QPulse – everything about every antibody is kept on here, but our assessor was happy to see paper copies too.

Validation

All antibodies and probes are validated by the manufacturer prior to receipt in the histology 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 our 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 P. Maxwell et al (2014), and utilised in our verification studies, each test can be placed into one of 3 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 the table below, along with how 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 competency assessments. ER intra-scorer variability assessment.
Instrument performance	Annual preventative and reactive maintenance carried out by manufacturer, weekly/monthly maintenance carried out by BMS 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 carried out – only with pathologist approval and under strict quality management procedures
Sampling error	Adequate training, minimising cold ischaemic time, adequate fixation and processing.

The majority of antibodies offer a qualitative staining result (ie. it is either positive or negative). The only exceptions are oestrogen and progesterone receptors and the assessment of Her2 status, which both 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 has 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 is collected on a monthly basis, and with the aid of the online 'UK NEQAS ICC & ISH Audit Tool', the rate for each biomarker is calculated and displayed in a graph. This is audited every 3 months, to see if any rates fall outside of 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 ICC & ISH.

Immunohistochemistry Verification

Following a risk assessment of all IHC procedures/reagents, it is necessary to verify all new batches of the following reagents;

- All primary antibodies
- Detection kit

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 which 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 which is very important 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, for example 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 lab 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 in a paper by Fitzgibbons. P et al (2014). 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 dependant 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 3 groups, depending on its intended use:

- Diagnostic
- Prognostic
- Predictive

Diagnostic Markers – provides information enabling 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; i.e. 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 CK 5/6 above), the sample set should contain 10 invasive cancers (CK 5/6 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 intervals (CI).

Prognostic Markers – provides information on the likely course of the cancer/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, for instance – 10 low, 10 medium and 10 high expressors would be appropriate.
- For prognostic markers, a concordance of 95% is acceptable with 95% confidence intervals (CI).

Predictive Markers – are able to identify subpopulations of patients that are likely to respond to a particular treatment. For example, over expression of the oestrogen receptor (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 instance, ER expression is measured using the Allred scoring system on a scale from 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 which is recorded as one of four scores; 0, 1+, 2+ and 3+. 25 cases at each score, totalling a sample size of 100 would be appropriate. This would allow for up to 5 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 4µm section 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-square 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 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 the reagents and techniques are working appropriately. The tissue contains specific antigens/proteins/cellular components at known, stable levels. When performing special stains, immunocytochemistry, immunofluorescence and in-situ hybridisation, it is necessary to use positive control material to assure the quality of the staining and to verify results.

Control (n.) - A standard of comparison for checking or verifying the results of a scientific experiment.

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 constitutively expressed in a particular tissue type (ie. LCA will always stain lymphocytes in tonsil) this can also be known 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 involved the verification of all the protocols currently in use. Most machines work on a barcoded, closed platform which 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.
- Fridge / incubator / room temperature monitoring.
- Calibration of measuring devices (pipettes, thermometers)

References

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The views expressed in this article are those of the author, UK NEQAS ICC & ISH by publishing the article, does not endorse any of the methods and recommendations.

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