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Immunocytochemistry

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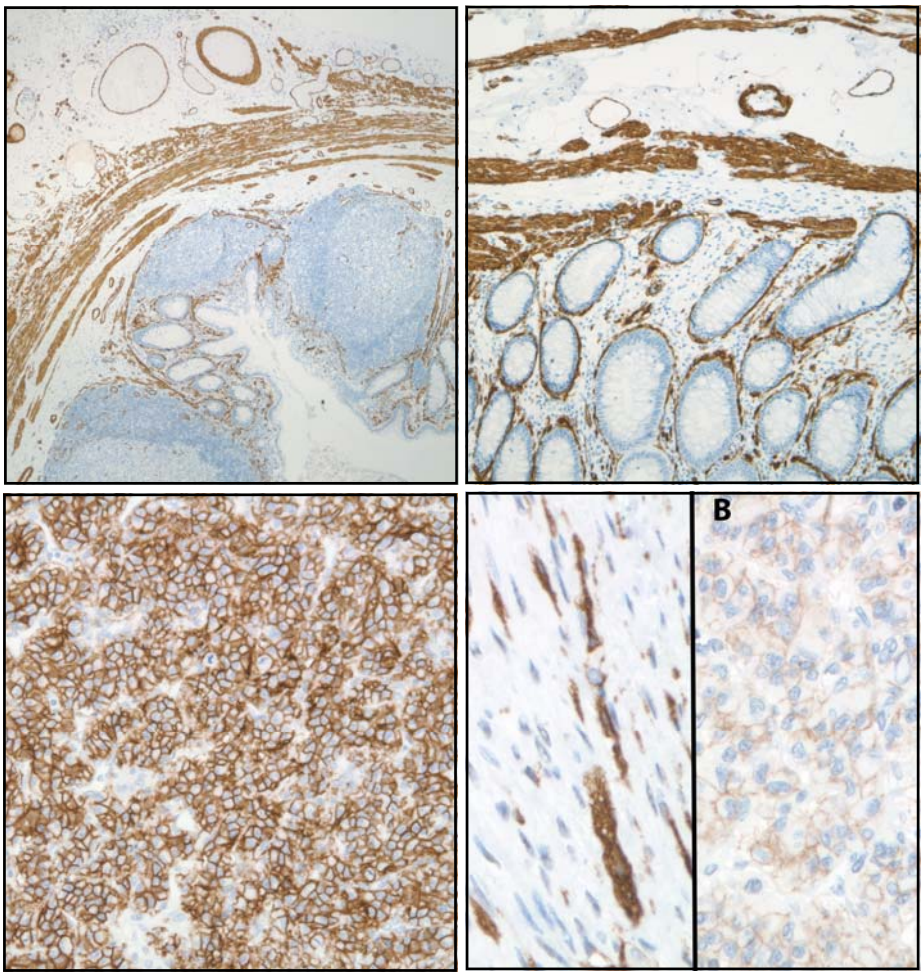
Assessment Dates: 21st September — 8th October 2015



Cover Photo: Taken from the General Module:

Top Left:: Optimal SMA staining on the NEQAS appendix sample
Top Right: Excellent SMA demonstration from an in-house appendix section
Bottom Left: Optimal CD56 staining on a neuroendocrine tumour NEQAS sample
Bottom Right: Good appendix staining, weak neuroendocrine tumour (B) NEQAS CD56

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UK NEQAS International Quality Expertise

CPA Accredited EQA Scheme Reference No: 044

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ASSESSORS

UK NEQAS ICC has over 100 Assessors (scientists and pathologists) from the UK, Australia, Canada, Denmark, Germany, Hungary, Ireland, Portugal, Slovenia, South Africa, Sweden and Switzerland.
The list below shows assessors who took part in the current assessment.

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Mr C Abbott, Bath
Dr N Atkey, Sheffield
Mr D Allen, London
Prof M Arends, Edinburgh
Mr N Bilbe, London
Mr D Blythe, Leeds
Ms A Brown, London
Ms A Clayton, Preston
Ms A Cramer, Manchester
Mr D Fish, Warwick
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Mr P Maxwell, Belfast
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Dr G Orchard, London
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Ms S Parry, London
Ms A Patterson, Belfast
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Dr E Provenzano, Cambridge
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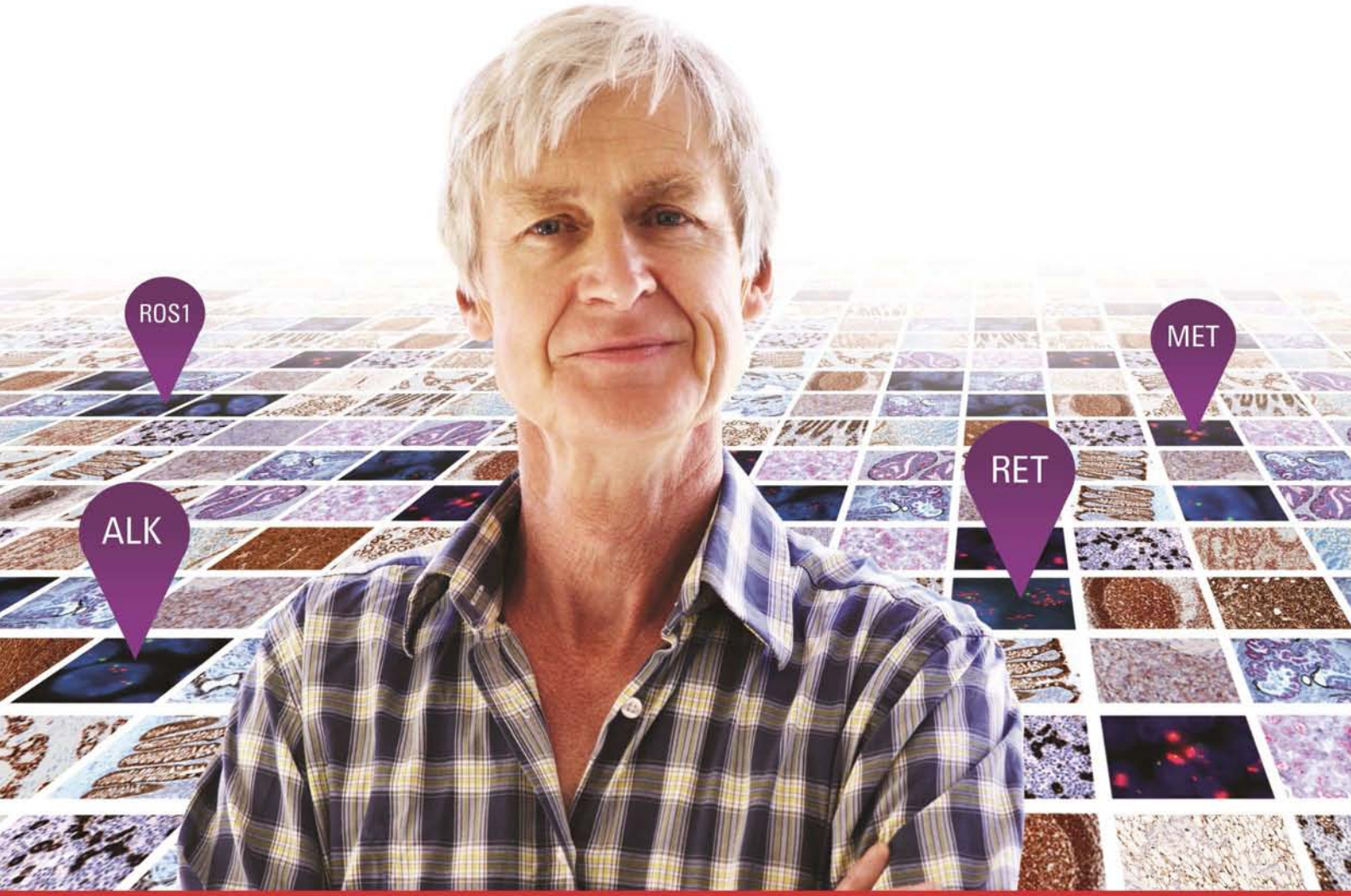

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UK NEQAS ICC & ISH Participants Meeting 2015

Neil Bilbe

UK NEQAS ICC & ISH Participant Meeting November 6th

UK NEQAS ICC & ISH welcomed around 90 delegates from the UK, Eire, and overseas, from over 60 individual laboratories, to its Participant Meeting held in Mander Hall at Hamilton House, the same venue as for the previous meeting in September 2014.

Five external speakers, in addition to UK NEQAS ICC & ISH staff, contributed to a full day's programme. The meeting was complimented by the presence of 4 trade stands. Further support was provided by another two companies, who sent representatives on the day.

The morning session was chaired by Keith Miller, the UK NEQAS ICC & ISH Scheme Director.

A range of disease entities were covered, alongside several scheme related talks. The initial session (3 talks) focused on lung:

Prof Andrew Nicholson, consultant histopathologist from The Royal Brompton and Harefield Hospitals opened proceedings. His talk was titled: Lung: diagnosis, ICC, ALK and PD-L1 which gave an overview of the classification and sub-typing of lung tumours, before going on to outline the role of ICC in their diagnosis. The final part of the presentation focused on the biology, diagnostic and prognostic uses of ALK and PD-L1.

Dr Merdol Ibrahim, UK NEQAS ICC & ISH Scheme Manager, continued with a talk on the recently established NSCLC ALK IHC module. Starting with the preliminary survey to participants, the setting up of the module, the pre-pilot assessments and subsequent validation. He concluded by giving a review and summary of the results of the 4 completed runs analysing the protocols and methodologies used and their success rates.

Mr David Allen, Laboratory Services Manager at UCL-AD gave a list of the current slide based biomarkers used in pathology, before concentrating on the plethora of markers (11) currently available for lung. After emphasising the importance of 'personalised medicine' he gave a summary of most of these markers, but with particular attention to ALK, ROS1, NTRK1, RET and EGFR and their use on NSCLC cases.

Dr Manuel Rodriquez-Justo, consultant histopathologist at UCLH. His title was: Are there any new biomarkers in GI cancers? Starting with 'old friends' HER2, BRAF, and FGFR delving into the HER family giving details about HER2 antibodies, platforms, and scoring systems relating to CRC cases. He then expanded on the role of HER3 (new kid) and BRAF, again comparing primaries and platforms. Mention of checkpoint inhibitors/immune modulators (rising stars), MMR, FGFR2, and PD-L1/PD1 rounded off the talk.

Prof Chas Mangham, consultant histopathologist, spoke on ICC in soft tissue and bone tumours, an overview and illustrative cases. He began with the incidences, tumour types, organisation of services, and classifications for these pathologies. He then outlined the handling of samples, the list of, and most useful of the antibodies employed, and their diagnostic use, adding that other techniques, especially molecular should be used alongside ICC. He concluded with a series of illustrative cases.

This concluded the morning session, and five excellent presentations on the diagnostic aspects of ICC and biomarkers.

Unfortunately, for reasons beyond our control the lunch was both late and the amount insufficient for the number of attendees. This also impacted upon the proposed visit to the UK NEQAS ICC & ISH offices. We apologise to those that had registered to do the tour.

The afternoon session was chaired by Suzanne Parry, the Assistant Scheme Manager, and focused primarily on topics closer to home, starting with two presentations from UK NEQAS ICC & ISH support scientists, **Neil Bilbe and Dawn Wilkinson**. Neil covered the new website which went live earlier this year, showing the old site and the need for a modern 21st century one and its features; he then drew attention to the Immunocytochemistry e-Journal which the scheme produces after each run, and concluded with a review of the 2015 User Survey, highlighting the disappointing low return rate (20%), and how previous survey feedbacks have been used by the scheme for improvement and advancement, with a series of examples.
Dawn gave a presentation on the recent single slide survey. She started by outlining the reasons for the switch to single slide EQA: regulatory (ISO), scheme (assessments and feedback) as well as reduced reagent costs. She then addressed the adhesion problems some labs had experienced with the new slides. Interestingly 63% of UK/Eire, but only 22% of overseas labs reported section adhesion problems. Modules, draining and drying protocols were analysed, before explaining hydrophobic and hydrophilic slide types. She concluded with some scheme news.

Julie Williams, Laboratory Manager at QAH, Portsmouth, and a long standing Lead Assessor for the scheme, continued with 'Day in the life of a UK NEQAS assessor' starting with a brief history of the ICC & ISH scheme, recent participant numbers, the role of both lead and other assessors, what's needed to be an assessor, how assessments are organised and conducted, from initiation to completion, outlining the scoring criteria with the help of some recent examples from the General module. Julie concluded by mentioning poor performance and some recent developments.

Dr Merdol Ibrahim, gave a second presentation on 'Uncertainty in Cellular Pathology', starting with the impact of both UKAS and NEQAS, ISO standards, *measurement uncertainty* contained in the standards, and the importance of good documentation. He then gave a review of a workshop where 53 delegates (scientists and pathologists) completed a matrix board where they indicated uncertainties (10 specific areas) in their own labs and showed the results. The final part focused on antibody validation and the thorny issue of out of date antibodies.

The final speaker was **Keith Miller**, who spoke about the CADQAS laboratory he has helped to set-up in Dorset. He started with a very brief overview of the genesis of ICC and his work at UCL, before expanding on the location (Poundbury Cancer Institute) and the role of CADQAS as a centre for education, R&D, and the validation and testing of antibodies produced in conjunction with new targeted therapies. He concluded by outlining the staff involved at the centre and some of the recent work produced.

This completed the programme of formal presentations. Unfortunately, due again to the late running we were unable to hold a Q&A session.

Dr Ibrahim giving a talk

Delegates listening intently!

The talks can be downloaded from the website:
http://www.uknegasiccish.org/wp/wp-content/uploads/2015/11/talks_final_web-2.pdf

UKAS ISO: 15189 accreditation visit – A positive IHC experience

Julie Terry
Specialist Biomedical Scientist – Immunohistochemistry Histology Department, Calderdale Royal Hospital

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.

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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.

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- 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

1. P. Maxwell et al (2014). The management of uncertainty: Increasing stringency for validation and verification of immunohistochemistry in tissue diagnostics. *UK NEQAS ICC & ISH Immunocytochemistry* Run 106/35; pgs 5-6.
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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.

Please contact Julie Terry if you have any views or wish to enquire about any of the methods contained in this article.

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Report on the Cytology EQA Module: Review of sub-optimal immunostaining in Runs 106—109

Neil Bilbe and Irena Srebotnik Kirbis

Introduction

This small study looks at the outcomes of the 4 Cytology Runs for the 2014/2015 EQA year, with particular emphasis on the levels and reasons for slides with sub-optimal marks.

Overview

There were a maximum of 79 laboratories registered with the scheme for the Cytology module during the year, from 15 individual countries, of which approximately 60% were from the UK and Ireland. For three out of the four runs the submission rate* was 100% (107, 108, 109), but with some labs withdrawing, and some new registrants at the start of the EQA year, the submission rate for run 106 was 97%.

* When a participant returns at least one slide for a run.

Table 1: Overview of submissions and slides with sub-optimal staining (unacceptable)

Run	Markers	No of participants/ slides	Failed slides (%)	Neqas slide - R	In house slide -S	Neqas slide - T	In house slide - U
106	CD45, Ki67	73/285	8 (2.8 %)	3	2	2	1
107	CD45, Calretinin	79/305	10 (3.6 %)	0	5	2	3
108	CD45, Melanoma	78/303	7 (2.3 %)	1	3	1	2
109	CD45, CK	79/310	3 (1 %)	1	1	1	0

The majority of participants in the UK NEQAS ICC & ISH Cytology module are able to produce a good quality of ICC staining on standard NEQAS preparations, as well as on in-house slides (82% had no issues). However, there were a small number of slides where the quality of ICC staining was sub-optimal and assessed with low marks (4 - 9/20). In order to find any specific problems or common themes, we analysed the frequency and reasons for low marks during 2014 - 2015.

There were a total of 28 slides assessed with low marks by the assessors.

- 11 NEQAS slides
  - 5 - Gold CD45 (R)
  - 6 - 2nd antigen (T)
- Preparations
  - 10 cytopsin preparations
  - 1 cell block section
- 17 in-house slides
  - 11 - CD45 (S)
  - 6 - 2nd antigen (U)
- Sample type (in-house)
  - 6 FFPE
  - 5 cell block sections
  - 3 cytospins
  - 2 liquid basec cytology samples
  - 1 FNA
- 17 individual participants
  - 6 UK or Ireland labs
  - 9 from an EU country
  - 2 from outside of the EU

As CD45 was the Gold antigen for the year, (requested 4 times) numerically, you would expect this to have the largest number of low scoring slides; but in fact this was only true for the in-house submissions (S = 11, U = 6) and the number of failed slides for the NEQAS CD45 Gold samples was slightly less than those for the 2nd antigens (R = 5, T = 6).

Reasons for low marks

The main or primary reason/comments given by assessors for low marks were (all slides n=28):

- Background and/or non-specific and diffuse staining 12 (43%)
- Weak or inappropriate demonstration of antigen 13 (46%)
- Uneven staining 1 (4%)
- Poor quality or inappropriate in-house control material 2 (7%)

Sub-optimal immunostaining on NEQAS slides

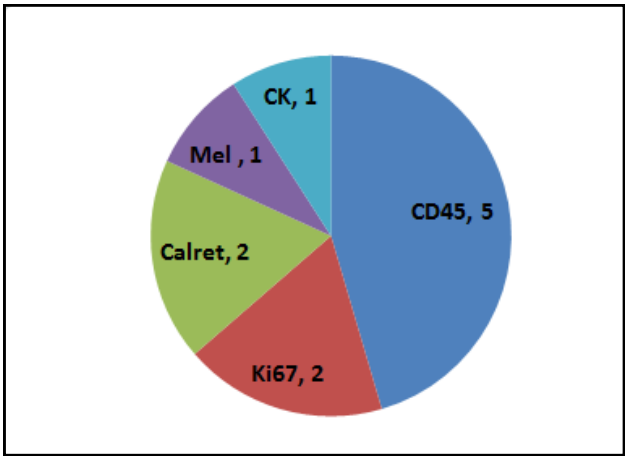
Cytospin (CS) v Cell Block (CB) samples

From Run 107 onwards, labs were able to choose between receiving a cytospin (CS) or cell block (CB) slide.

All cytology module participants were surveyed prior to the Run (107) to ask for their preferred preparation. Numbers have remained fairly consistent with 63% of participants submitting CB and 37% CS slides (average = 49 and 29 labs respectively), for the three runs.

The most interesting finding was that after the introduction of the cell block samples the number of labs failing the Gold (CD45) dropped from a total of 3 labs for Run 106 to only 2 labs for the rest of the year, and these were all on CS slides. Indeed out of the 6 slides assessed as unacceptable for Runs 107 - 109 only one was on a cell block section, and 5 were on cytospins.

Graph 1: NEQAS slides with sub-optimal staining by antigen



These figures represent levels of only:

1% Melanoma and Cytokeratin
2% CD45
3% Ki67 and Calretinin

With a range of between 70 - 79 slides submitted for R and T.

Table 2: Methodolgies resulting in sub-optimal staining on NEQAS slides:

	Run	Primary	Dilution 1:	Platform
CD45	106	Dako monoclonal	500	Bond Max
CD45	106	Dako monoclonal	10	Dako Autostainer
CD45	106	Dako monoclonal	RTU	Dako Autostainer
CD45	108	Dako monoclonal	RTU	Dako Autostainer
CD45	109	Dako monoclonal	RTU	Ventana Benchmark
Ki67	106	Dako MIB1	200	Ventana Benchmark
Ki67	106	Dako MIB1	50	Leica Bond III
Calret	107	Novocastra	100	Dako Autostainer
Calret	107	Cell Marque	40	Lab Vision Autostainer
Melanoma	108	Dako Melan A	100	Dako Autostainer
CK	109	Dako AE1/AE3	200	Dako Autostainer

For the CD45 Dako monoclonal, some labs did not give a dilution. This was listed as RTU in the above table.
N.B. Antigen retrieval details are often unreliable, participants often ignoring this field in data entry, or giving conflicting data, therefore it is not included in the above table (see page 4).

Table 3: Reasons for sub-optimal staining on NEQAS slides

Run	Antigen	Reason	Sample
106	CD45	Background and non specific staining	CS
106	Ki67	Excess background and insufficient block	CS
106	Ki67	Non specific staining	CS
106	CD45	Very weak demonstration	CS
106	CD45	Antigen not demonstrated	CS
107	Calretinin	Diffuse and non specific staining	CS
107	Calretinin	Very weak demonstration	CS
108	Melanoma	Very weak demonstration	CS
108	CD45	Backkground and weak counterstain	CS
109	CK	Very weak demonstration	CB
109	CD45	Very weak demonstration	CS

Images of sub-optimal staining slides on NEQAS samples

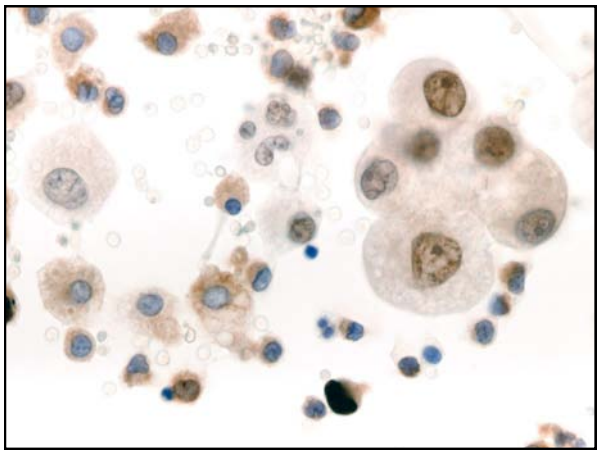


Fig 1: Failed Ki67 on the NEQAS cytospin Run 106 T - there is pronounced cytoplasmic and other non-specific staining

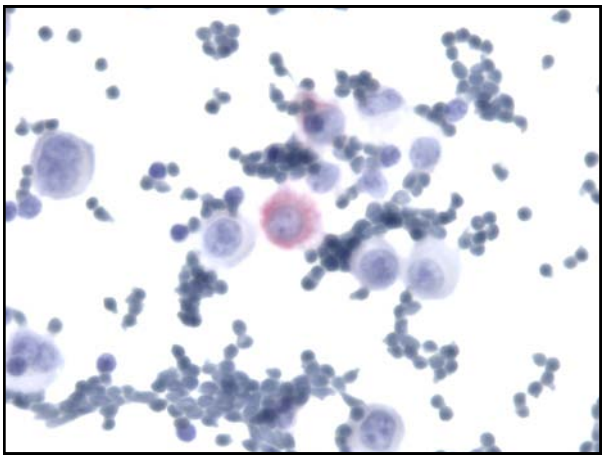


Fig 2: Failed Calretinin on the NEQAS cytospin Run 107 T - staining is very weak with only the occasioanl cell stained

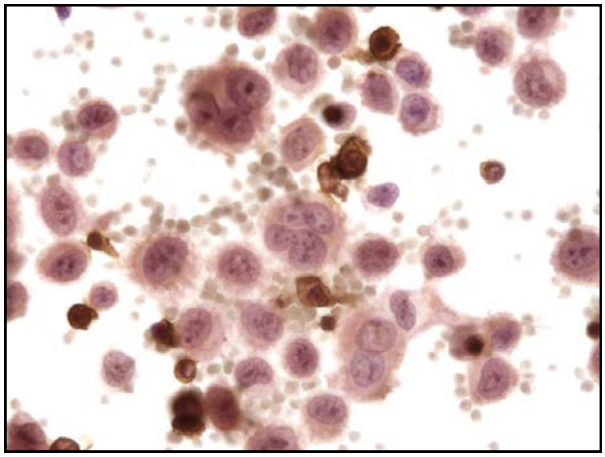


Fig 3: Failed CD45 on NEQAS cytospin Run 108 T – excessive background and non-specific staining of epithelial cells

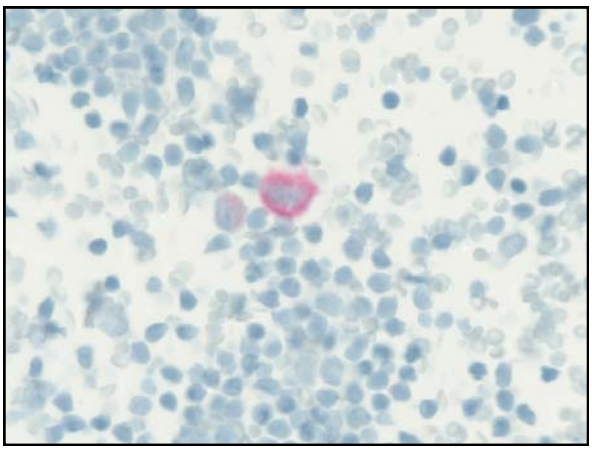


Fig 4: Failed CK on NEQAS cell block section Run 109 R - poor demonstration of the antigen

Sub-optimal immunostaining on in-house slides

Although in-house samples are not included in participant (UK) poor performance reviews, slides are assessed and scored in exactly the same way as for the NEQAS slides. The main difference is that feedback is also provided on the choice or quality of submitted in-house controls.

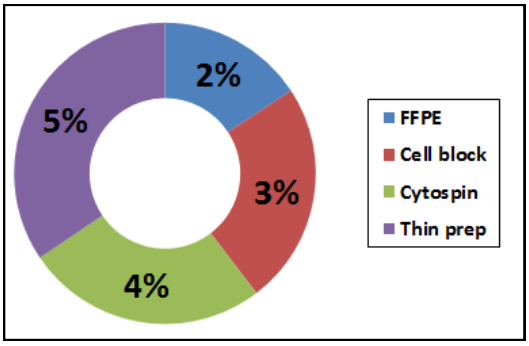
Furthermore, marks are not deducted if non-cytological in-house samples are submitted, even though cytological material is preferable, as long as labs are running the same control that is routinely used.

Table 4: Type of submitted in-house control slides (S & U)

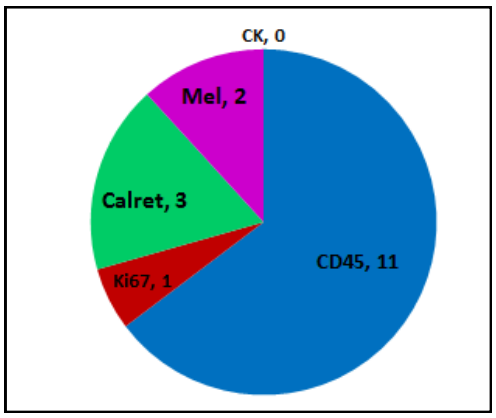
	106	107	108	109	Total
FFPE	58 (41%)	71 (48%)	71 (48%)	69 (45%)	269
Cell block	34 (24%)	37 (25%)	35 (24%)	42 (28%)	148
Cytospin	33 (24%)	24 (16%)	27 (18%)	25 (16%)	109
TP/LBC	9 (6%)	12 (8%)	10 (7%)	10 (7%)	41
Smear	6 (4%)	5 (3%)	4 (3%)	6 (4%)	21
Totals	140	149	147	152	588

The most notable trend seen here is the slight increase in the use of FFPE and cell block slides, and the reduced levels of cytospins after Run 106. Levels of smears or liquid based samples remained relatively unchanged.

Graph 2: Type of in-house slides with sub-optimal staining



Graph 3: In-house slides with sub-optimal staining by antigen



Just looking at antigen numbers does not really tell us much about samples, and reasons for these poor scores. Much more of interest is the combination of sample type, antigen and reasons for low marks.

Table 5: Reasons for sub-optimal staining on in-house slides

Run	Antigen	Letter	Comment /Reason	Sample type
106	CD45	S	Very weak	FFPE
106	CD45	S	Uneven staining	CB
106	Ki67	U	Weak and inappropriate control	CB
107	CD45	S	Poor material and morphology	CS
107	Calretinin	U	Very weak	FFPE
107	CD45	S	Diffuse and background staining	CB
107	Calretinin	U	Poor control material	CB
107	CD45	S	Diffuse and poor quality	LBC
107	CD45	S	Background and insufficient PT	CS
107	CD45	S	Background and poor tissue quality	FFPE
107	Calretinin	U	Weak, background, and poor quality	FFPE
108	Melanoma	U	Very weak	FFPE
108	CD45	S	Backkground and non specific staining	CB
108	CD45	S	Background and weak counterstain	CS
108	CD45	S	Diffuse staining and poor quality of material	LBC
108	Melanoma	U	Weak and poor control material	FFPE
109	CD45	S	Weak and poor control material	FNA

There were 9 comments concerning 'poor in-house material'. Two of these were given as the as the primary reason for failing the slide. In practice though, where a slide has been given a low score for weak or absence of staining, this is also likely to be attributable to the quality and type of in-house control sample used.

Images of in-house samples

The majority of images taken of in-house slides in the Journals are of good examples. We use these to illustrate what can be achieved from a variety of samples, given that we have no direct comparisons, as we do for the NEQAS slides which we assess against the validation slides.

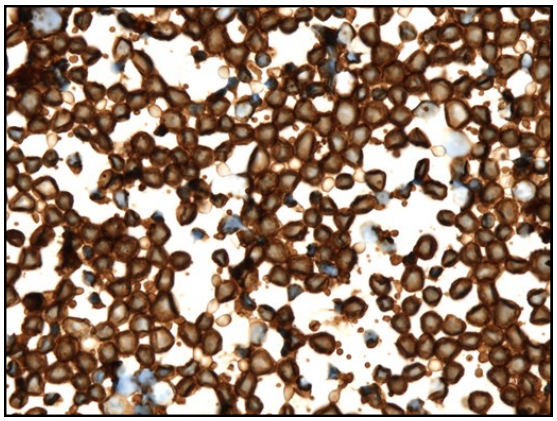


Fig 5: Example of a sub-optimal CD45 staining on an in-house cytospin Run 108 S

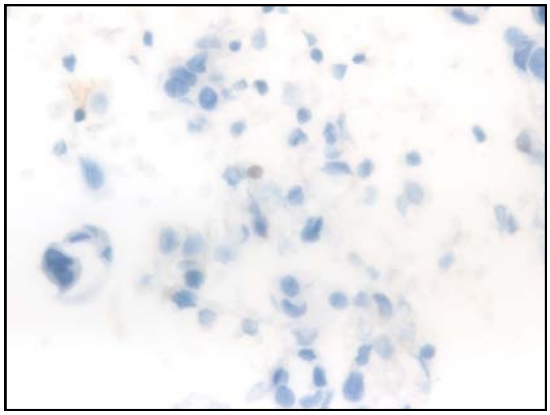


Fig 6: Sub-optimal Ki67 in-house CB section –staining is very weak and only a few cells are stained Run 106 U

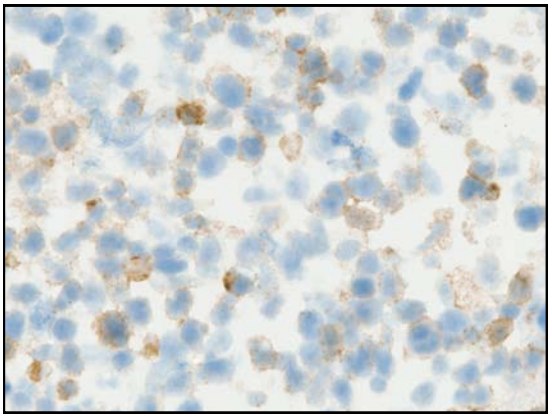


Fig 7: Sub-optimal CD45 in-house FNA sample –staining is very weak and the morphology is poor Run 109 S

Summary

This report confirms that the majority of participants in the Cytology module are able to demonstrate at least acceptable quality of immunostaining on the NEQAS, as well as on in-house slides, irrespective of the type of slides, the requested antigen, or origin of participants (UK, EU, OS).

A very important finding is that sub-optimal immunostaining was only a single or sporadic event for the majority of participants receiving low marks, indicating that participants took appropriate measures to improve the quality of their immunostaining.

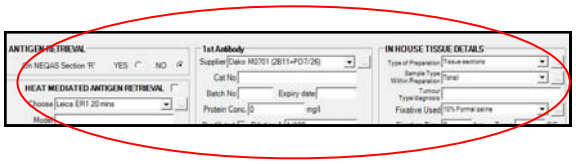
The NEQAS distributed slides (R & T) which were subsequently returned and then assessed as inadequate for diagnosis, were though, more likely to be on cytospin requested samples. As we have reported in previous Journals *sub-optimal methodologies /protocols* on cytospin samples, where no retrieval is employed, are more prone to non-specific staining of some cells in the sample 'cocktail' which contains a variety of cell types, which stain both positively and negatively for the chosen antigen.

For the NEQAS slides, the actual reasons for low marks were split between weak or absence of staining (6), and background/non-specific staining (5).

Of the 4 individual labs that received low marks for Run 106 (all received cytospins) the two that subsequently changed to cell blocks, did not produce a low score thereafter, whereas both the labs who continued to receive cytospins submitted slides with sub-optimal staining for at least one further run.

Given that the development of antibodies and their intended use is targeted towards FFPE and tissue samples, this is also a factor in these findings. But it is worthy of mention that we consistently see a high standard of immunocytochemistry staining on cytospins, and for some of the markers requested, the ***best examples*** of antigen demonstration have indeed been on cytospins.

In terms of any protocols relating to antigen retrieval, as mentioned above, we find the data to be unreliable. For the slides in Table 3, all labs had indicated that no retrieval had been used during the staining. Given that most of these are cytospins this is understandable. But when looking at the data for other labs using cell block sections and a FFPE in-house control, for example on CD45, where the recommended retrieval is 20 minutes, the button for: ANTIGEN RETRIEVAL ON NEQAS SECTION is often left as NO (see below). This is something that UK NEQAS ICC & ISH is addressing.



Further reading

- Journals Run 106 -109 Cytology Summary
- Journal Run 107 Cytology Summary: cell block v cytospin comparison
- Cytology cell block trial Journal Run 102

If you have any comments or queries email: n.bilbe@ucl.ac.uk

Julie Williams and Suzanne Parry

	Gold Standard	Second Antibody
Antigens Assessed:	SMA	CD56
Tissue Sections circulated:	Appendix and Leiomyosarcoma	Appendix and Neuroendocrine tumour
Number of Registered Participants:	330	
Number of Participants this Run	318 (96%)	

Introduction

Gold Standard: SMA

The alpha-isoform of smooth muscle actin (αSMA) belongs to a group of cytoplasmic actins, of which there are six major different isoforms (Roholl et al.). It is useful for the demonstration of myogenic differentiation. Antibodies to SMA label smooth muscle cells, which are found in vascular walls, intestinal muscularis, and muscularis propria and in the stroma of various other tissues (Skalli et al., Mason & Gatter, Rizeq et al.). It also reacts with myoepithelial cells in tissues such as breast and salivary glands. The main diagnostic use of SMA is in a panel of antibodies to demonstrate leiomyomas and leiomyosarcomas (Oda et al.). It is also used to identify the loss of myoepithelial cells around the ducts in invasive breast carcinomas (Lazard et al.).

Features of Optimal Immunostaining:
Appendix: (See Figs 1 & 6)
- Intense staining of the smooth muscle layers in the muscularis propria
- Staining of the delicate fibres extending into the epithelial crypts of the appendix
- The smooth muscle of the numerous blood vessels permeating the lamina propria should also be intensely stained
- No background staining

Leiomyosarcoma: (Fig 3A)
- Moderate to strong cytoplasmic staining of the tumour cells
- Minimal background staining

Features of Sub-optimal Immunostaining: (Figs 2, 3B & 4)
- Weak, uneven or no staining
- Non-specific staining of cell types not expected to stain, e.g. lymphocytes and epithelial cells (often due to excessive antigen retrieval)
- Excessive background staining (particularly in the connective tissue)

References:
1. Roholl PJM, Elbers HRJ, Prinsen I, Claessens JAJ, van Unnik JAM. Distribution of actin isoforms in sarcomas: an immunohistochemical study. Hum Pathol 1990;21:1269-74.
2. Skalli O, Ropraz P, Trzeciak A et al. A monoclonal antibody against α-smooth muscle actin: a new probe for smooth muscle differentiation. The Journal of Cell Biology. 1986; 103:2787-2796.
2. Mason D and Gatter K. (1987) The role of immunocytochemistry in diagnostic pathology. Journal of Clinical Pathology 40 :1042.
4. Rizeq MN, van de Rijn M, Hendrickson MR, Rouse RV. A comparative immunohistochemical study of uterine smooth muscle neoplasms with emphasis on the epithelioid variant. Hum Pathol 1994;25:671-7.
5. Oda Y, Miyajima K, Kawaguchi K, Tamiya S, Oshiro Y, Hachitanda Y, Oya M, Iwamoto Y, Tsuneyoshi M. Pleomorphic leiomyosarcoma: clinicopathologic and immunohistochemical study with special emphasis on its distinction from ordinary leiomyosarcoma and malignant fibrous histiocytoma. Am J Surg Pathol. 2001 Aug;25(8):1030-8.
6. Lazard D, Sastre X, Frid MG, et al. Expression of smooth muscle-specific proteins in myoepithelium and stromal myofibroblasts of normal and malignant human breast tissue. Proc Natl Acad Sci U S A 1993;90:999–1003.

Second Antigen: CD56

CD56, also known as N-CAM or Leu-19, is a membrane glycoprotein. It has multiple isoforms which are generated by alternative splicing from a single gene located on chromosome 11. It is expressed on the surface of neurons, glia, skeletal muscle and natural killer (NK) cells. Normal cells that stain positive with CD56 include activated T cells, natural killer cells, brain, cerebellum and neuroendocrine tissues. It is therefore widely expressed in the central nervous system, peripheral nerves and skeletal muscles. Uterine smooth muscle cells and osteoblasts are also positive with CD56. Tumours that are positive for CD56 include myeloma, myeloid leukaemia, neuroendocrine tumours, Wilms' tumour , neuroblastoma, NK and T-cell lymphomas, small cell lung carcinoma, pancreatic acinar cell carcinoma, pheochromocytoma and Ewing's sarcoma (Tsan et al., Schol et at., Mooi et al.).

Features of Optimal Immunostaining:
Appendix (Fig 7, 11A, 12A)
- Strong staining in virtually all of the peripheral nerves
- No background staining

Neuroendocrine tumour: (Figs 8 & 12B)
- Moderate to strong nuclear staining in virtually all of the tumour cells
- Minimal background staining

Sub-optimal Immunostaining: (Figs 9, 10 & 11B)
- Weak, uneven or no staining
- Diffuse staining
- Non-specific staining of cell types not expected to stain
- Excessive background staining

References:
1. Tsang WY, Chan JKC, Ng CS, Pau MY. Utility of a paraffin section-reactive CD56 antibody (123C3) for characterization and diagnosis of lymphomas. Am J Surg Pathol 1996;20:202-10.
2. Schol DJ, Mooi WJ, van der Gugten AA, Wagenaar S Sc, Hilgers J. Monoclonal antibody 123C3, identifying small cell carcinoma phenotype in lung tomours, recognizes mainly, but not exclusively, endocrine and neuron-supporting normal tissues. Int J Cancer 1988;Supplement 2:34-40.
3. Mooi WJ, Wagenaar S Sc, Schol D, Hilgers J. Monoclonal antibody 123C3 in lung tumour classification immunohistology of 358 resected lung tumours. Mol Cel Prob 1988;2:31-7.

Assessment Summary:

Overall the staining of the chosen gold standard **SMA** was good with 83% of labs receiving an acceptable pass, and a further 13% achieved a borderline pass (scores of 10-12/20). However, 10 labs (3%) failed the assessment, and this was due to either very weak staining (with little expression of actin filaments) or due to excessive background. The borderline passes were mostly due to non-specific staining, with one particular lab showing inappropriate nuclear staining in the lymphocytes of the appendix (Fig 4B). The Dako 1A4 clone was the most popular choice of antibody and this showed a pass rate of 88%. Participants mostly chose appendix and/or bowel for their in house controls.

The second antibody assessed for this run was **CD56** which showed slightly lower pass rates than that of the SMA assessment. Again, the reason for failure was due to very weak staining. This was most apparent in the distributed neuroendocrine tumour section, and several labs achieved very acceptable staining on the appendix, but little or no staining in the tumour (example shown in Fig 11). The most popular choice of antibody was the Leica/Novocastra 1B6 antibody, which had a pass rate of 76%. Similarly to SMA, many labs used appendix as their in house control, and several labs also used a neuroendocrine tumour.

Selected Images showing Optimal and Sub-optimal Immunostaining

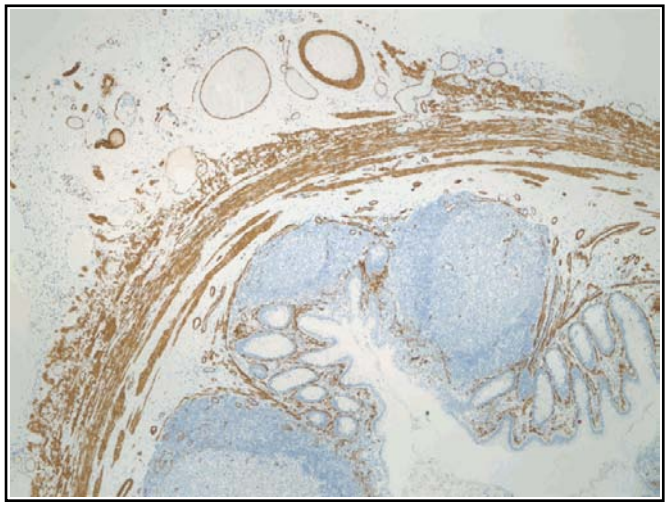


Fig 1. Optimal demonstration of SMA in the UK NEQAS appendix section, showing strong staining of the smooth muscle layers in the muscularis propria and muscualris mucosae. Even at low power the delicate fibres can be clearly identified. Section stained on the Dako Omnis platform, Dako 1A4 RTU antibody with 30 minutes antigen retrieval.

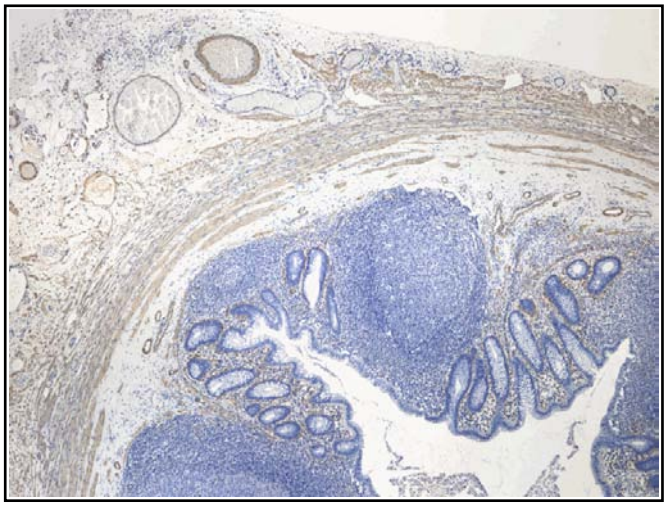


Fig 2. Weak demonstration of SMA in the UK NEQAS appendix section (compare to Fig 1). Additionally, the counterstain is excessive. The section was stained with the Dako 1A4 antibody, 1:150, on the Dako Autostainer with high pH antigen retrieval.

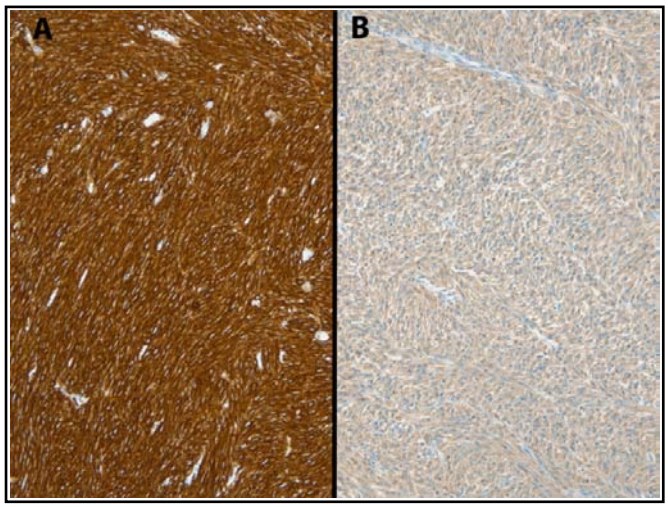


Fig 3. Two examples of staining in the UK NEQAS distributed leiomyosarcoma section: (A) Optimal crisp staining (same protocol as Fig 1), (B) Very weak staining. This section was stained using the Novocastra asm-1 antibody with the Menarini Intellipath platform and no antigen retrieval.

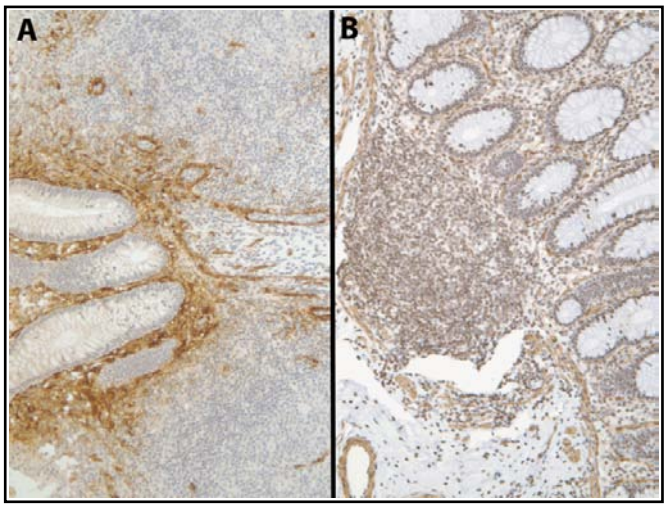


Fig 4. Two examples of sub-optimal staining in the UK NEQAS appendix: (A) Diffuse staining and (B) Non-specific staining in the lymphocytes and some background staining. Both sections were stained with the Dako 1A4 antibody and no antigen retrieval.

Fig 5. The image demonstrates an example of poor section quality: The participants' in house control shows chattering and creases.

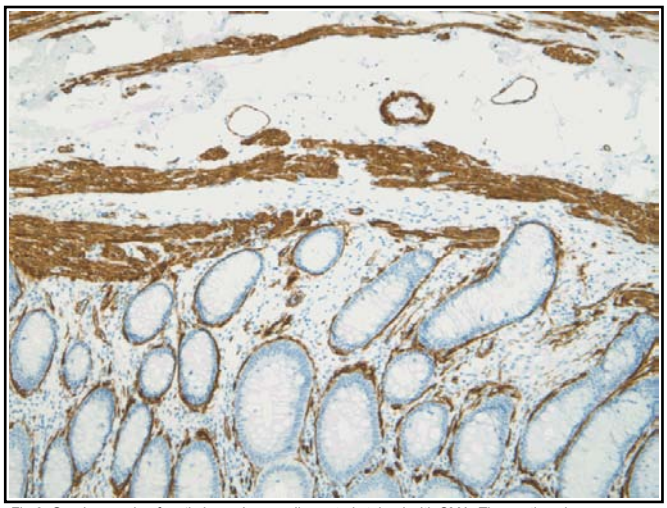


Fig 6. Good example of an 'in house' appendix control stained with SMA. The section shows strong specific staining with a clean background. (Same protocol as Figs 1 & 3A).

Selected Images showing Optimal and Sub-optimal Immunostaining

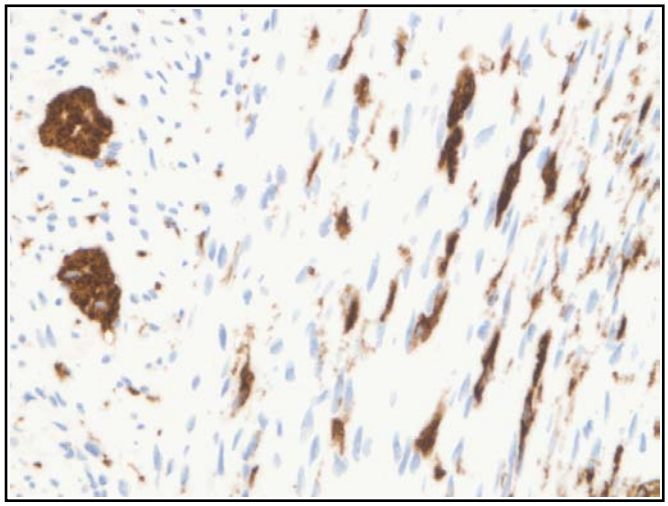


Fig 7. Optimal demonstration of CD56 in the UK NEQAS appendix section. The example shows strong staining in the peripheral nerves with a clean background. Stained with the Novocastra 1B6 antibody, 1:100, on the Leica Bond III with 20 minutes antigen retrieval ER2.

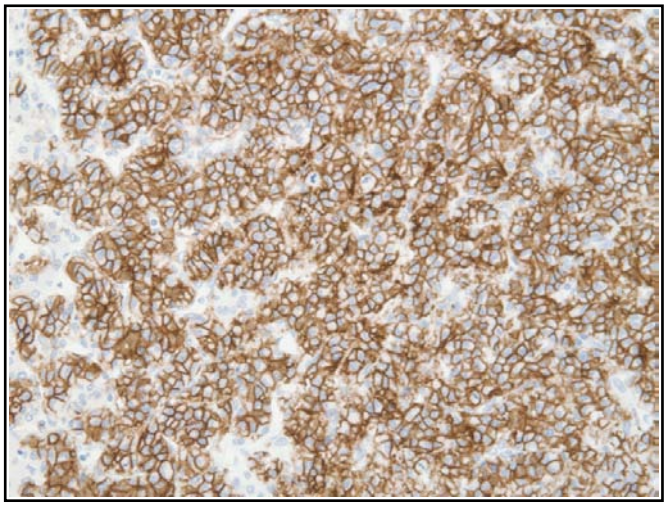


Fig 8. Optimal demonstration of CD56 in the UK NEQAS distributed neuroendocrine sample. The example shows strong distinct membranous staining of the tumour cell. (Same protocol as Fig 7).

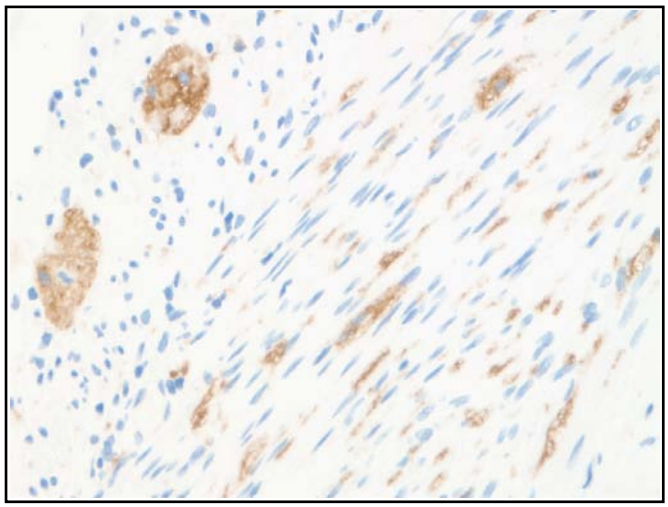


Fig 9. Sub-optimal CD56 staining in the UK NEQAS distributed appendix sample (Compare to Fig 8). The staining is weaker than expected. Interestingly the staining protocol was very similar to that used in Figs 7 & 8; the only difference being a dilution of 1:75 with the Novocastra 1B6 antibody.

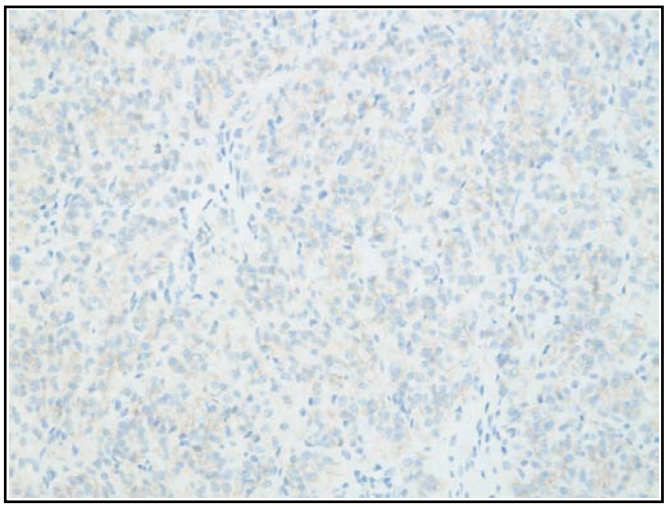


Fig 10. Unacceptable CD56 staining in the UK NEQAS distributed neuroendocrine tumour. Stained using the same protocol as in Figs 7 & 8. The example shows very little staining of the tumour and was therefore given a failed score of 8/20 at assessment.

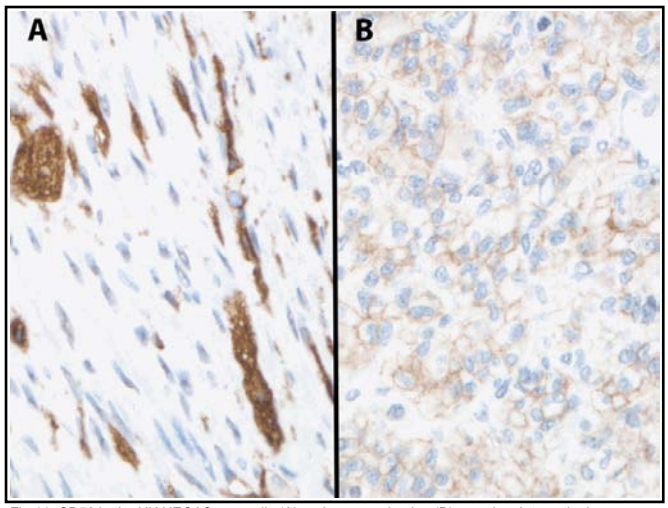


Fig 11. CD56 in the UK NEQAS appendix (A) and neuroendocrine (B) samples: Interestingly, although the samples were placed next to each other on the slide and therefore stained at the same time under the same conditions, the appendix shows acceptable staining, but the tumour is much weaker than expected.

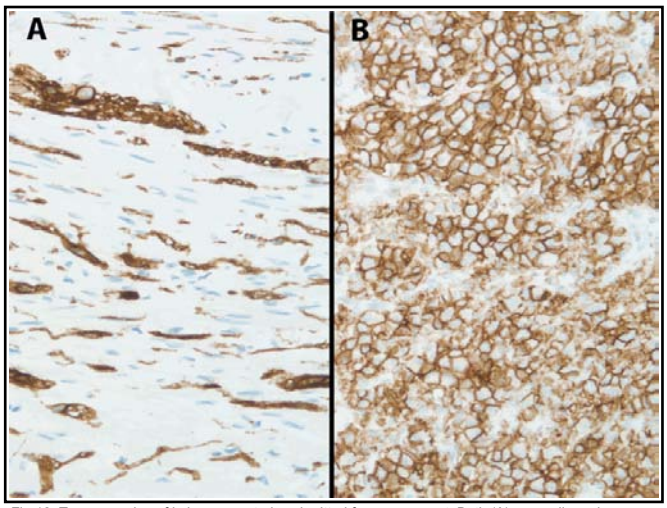
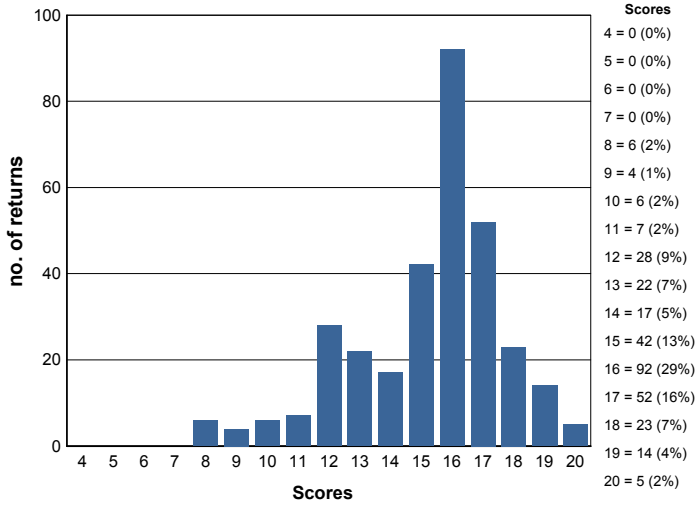


Fig 12. Two examples of in house controls submitted for assessment. Both (A) appendix and (B) neuroendocrine tumour (B) show strong distinct staining. (A) Stained with the Dako 123C3 antibody, and (B) Stained with the Leica 1B6 antibody.

GRAPHICAL REPRESENTATION OF PASS RATES

RUN 111A Smooth Muscle Actin (SMA) on NEQAS Sections

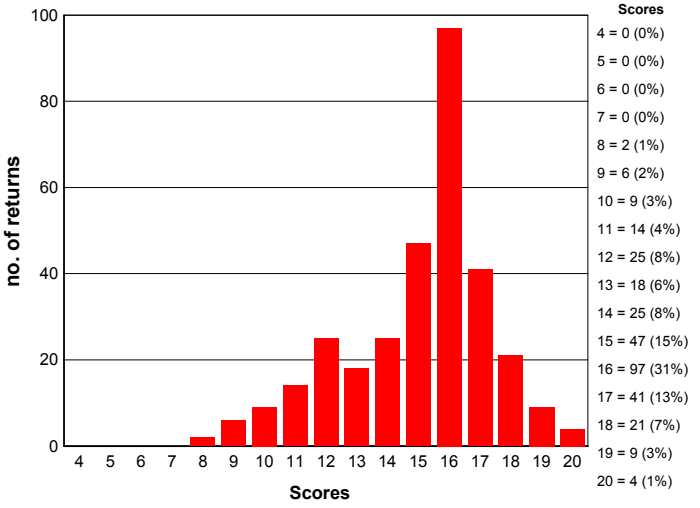


Individual Scores
4 = 0 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 0 (0%)
8 = 6 (2%)
9 = 4 (1%)
10 = 6 (2%)
11 = 7 (2%)
12 = 28 (9%)
13 = 22 (7%)
14 = 17 (5%)
15 = 42 (13%)
16 = 92 (29%)
17 = 52 (16%)
18 = 23 (7%)
19 = 14 (4%)
20 = 5 (2%)

Summary
16 - 20 = 186 (58%)
13 - 15 = 81 (25%)
10 - 12 = 41 (13%)
0 - 9 = 10 (3%)

Median = 14.00

RUN 111B Smooth Muscle Actin (SMA) on in-house Sections

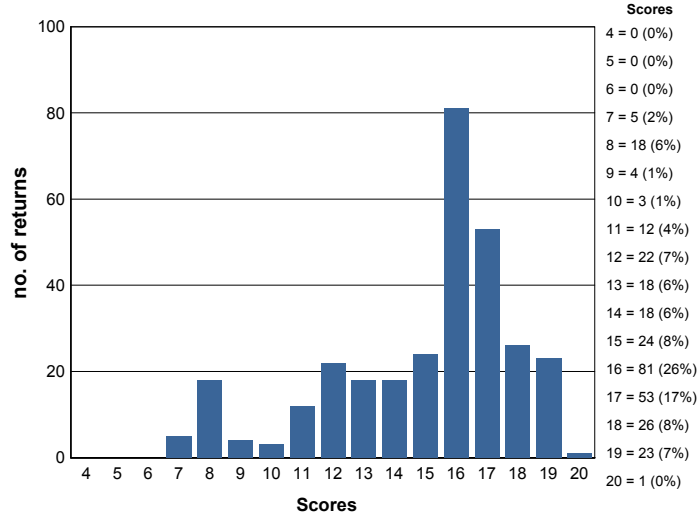


Individual Scores
4 = 0 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 0 (0%)
8 = 2 (1%)
9 = 6 (2%)
10 = 9 (3%)
11 = 14 (4%)
12 = 25 (8%)
13 = 18 (6%)
14 = 25 (8%)
15 = 47 (15%)
16 = 97 (31%)
17 = 41 (13%)
18 = 21 (7%)
19 = 9 (3%)
20 = 4 (1%)

Summary
16 - 20 = 172 (54%)
13 - 15 = 90 (28%)
10 - 12 = 48 (15%)
0 - 9 = 8 (3%)

Median = 14.00

RUN 111C CD56 on NEQAS Sections

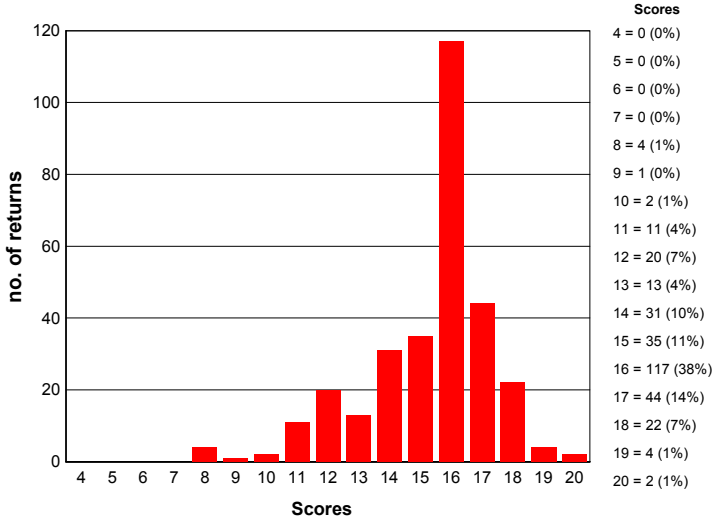


Individual Scores
4 = 0 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 5 (2%)
8 = 18 (6%)
9 = 4 (1%)
10 = 3 (1%)
11 = 12 (4%)
12 = 22 (7%)
13 = 18 (6%)
14 = 18 (6%)
15 = 24 (8%)
16 = 81 (26%)
17 = 53 (17%)
18 = 26 (8%)
19 = 23 (7%)
20 = 1 (0%)

Summary
16 - 20 = 184 (60%)
13 - 15 = 60 (19%)
10 - 12 = 37 (12%)
0 - 9 = 27 (9%)

Median = 13.50

RUN 111D CD56 on in-house Sections



Individual Scores
4 = 0 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 0 (0%)
8 = 4 (1%)
9 = 1 (0%)
10 = 2 (1%)
11 = 11 (4%)
12 = 20 (7%)
13 = 13 (4%)
14 = 31 (10%)
15 = 35 (11%)
16 = 117 (38%)
17 = 44 (14%)
18 = 22 (7%)
19 = 4 (1%)
20 = 2 (1%)

Summary
16 - 20 = 189 (62%)
13 - 15 = 79 (26%)
10 - 12 = 33 (11%)
0 - 9 = 5 (2%)

Median = 14.00

ANTIBODIES AND OTHER TECHNICAL PARAMETERS EMPLOYED BY PARTICIPANTS IN THE GENERAL PATHOLOGY MODULE

The following tables record the number of participants (N) using each primary antibody and the percentage (%) of these participants achieving acceptable staining (scores 12/20) on UK NEQAS sections.

General Pathology Run: 111

Primary Antibody : Smooth Muscle Actin (SMA)

Antibody Details	N	%
A. Menarini MP-001C/Px (1A4)	1	100
Biogenex AM128-5M/10M (1A4)	1	100
Cell Marque 201M (Clone HHF35)	1	0
Cell Marque 202M (Clone 1A4)	10	80
Dako M0635 SMA (Clone HHF35)	4	75
Dako M0851 SMA (Clone 1A4)	185	88
Dako RTU (Clone 1R611)	10	90
Invitrogen 18-0106 (1A4)	3	100
Leica/Novocastra PA0943 (asm-1)	19	100
Novocastra NCL-SMA (asm-1)	16	63
Novocastra RTU-SMA (asm-1)	2	100
Other	8	100
Sigma (1A4) A2547	7	100
Sigma IMMH2-1KT (1A4)	4	50
Thermo Scientific/Neomarkers MS 113-P (1A4)	3	67
Ventana (HUC1-1) 760-2502	2	0
Ventana 760 2833 (1A4)	33	67
Ventana 760-2601 (HHF35)	4	100

General Pathology Run: 111

Primary Antibody : CD56

Antibody Details	N	%
Cell Marque 156R-94,95,96,97,98 (MRQ-42)	11	82
Cell Marque CMA 361 (clone 123C3 D5)	7	71
Dako M7304 (123C)	31	74
Dako RTU FLEX Link IR628 (123C3)	19	100
Dako RTU FLEX Plus IS628 (123C3)	2	50
Medite MOB 261 (clone 123C3 D5)	1	100
Monson MOH9006-1 (clone 123C3 D5)	3	33
Neomarkers MS 1149 (clone BC45C04)	3	67
Neomarkers MS 204 (clone 123C3 D5)	4	50
Novocastra/Leica NCL-CD56 (clone 1B6)	96	76
Novocastra/Leica NCL-CD56-504 (CD564)	26	88
Novocastra/Leica RTU PA0191(CD564)	20	95
Other	6	83
Vector VPC 360 (clone 1B12)	11	91
Ventana 760 (clone IB6)	5	60
Ventana 760-4596 (MRQ-42)	19	95
Ventana 790 4465 (NCAM) (clone 123C3)	26	69
Zymed 18 0152	5	40

General Pathology Run: 111

Heat Mediated Retrieval	CD56 N	CD56 %	Smooth Muscle Actin (SMA) N	Smooth Muscle Actin (SMA) %
_Microwave Oven	0	0	1	100
Biocare Decloaking Chamber	2	50	1	0
Dako Omnis	5	100	5	100
Dako Pascal	1	100	1	100
Dako PTLink	46	91	42	95
Lab vision PT Module	5	60	3	100
Leica ER1 10 mins	2	100	8	88
Leica ER1 20 mins	37	84	4	100
Leica ER1 30 mins	17	76	4	75
Leica ER2 10 mins	1	100	7	100
Leica ER2 20 mins	28	82	17	82
Leica ER2 30 mins	7	86	1	100
Leica ER2 40 mins	1	100	0	0
Microwave	0	0	4	75
None	0	0	106	90
Other	2	100	3	100
Pressure Cooker	6	83	4	75
Pressure Cooker in Microwave Oven	1	100	1	100
Steamer	3	100	3	100
Ventana CC1 16mins	4	50	4	75
Ventana CC1 20mins	3	67	1	100
Ventana CC1 24mins	6	83	5	80
Ventana CC1 32mins	22	73	2	50
Ventana CC1 36mins	9	78	5	40
Ventana CC1 40mins	2	100	1	100
Ventana CC1 48mins	2	100	1	100
Ventana CC1 56mins	2	100	0	0
Ventana CC1 64mins	21	67	10	50
Ventana CC1 72mins	1	100	0	0
Ventana CC1 76mins	1	0	1	100
Ventana CC1 8mins	1	0	19	79
Ventana CC1 92mins	1	100	1	100
Ventana CC1 extended	3	100	1	100
Ventana CC1 mild	19	58	12	58
Ventana CC1 standard	29	79	9	56
Ventana CC2 mild	1	0	0	0
Ventana CC2 standard	1	100	2	50
Water bath 68 OC	1	100	1	100
Water bath 95-98 OC	0	0	1	100

General Pathology Run: 111

Enzyme Mediated Retrieval	CD56 N	CD56 %	Smooth Muscle Actin (SMA) N	Smooth Muscle Actin (SMA) %
AS PER KIT	6	100	5	60
NOT APPLICABLE	107	85	164	83
Other	0	0	1	100
VBS Bond Enzyme 1	0	0	2	100
Ventana Protease	0	0	1	100
Ventana Protease 1 (760-2018)	0	0	1	100

General Pathology Run: 111 Detection	CD56 N	CD56 %	Smooth Muscle Actin (SMA) N	Smooth Muscle Actin (SMA) %
A Menerini Polymer (MP-XCP)	1	0	1	0
AS PER KIT	29	76	20	95
BioGenex SS Polymer (QD 420-YIKE)	0	0	1	100
Dako EnVision FLEX (K8000/10)	4	100	12	100
Dako EnVision FLEX+ (K8002/12)	30	90	31	87
Dako Envision HRP/DAB (K5007)	5	100	9	89
Dako Envision+ HRP mouse K4004/5/6/7	2	100	2	50
LabVision UltraVision LP HRP (TL 125 HLJ)	1	0	1	100
LabVision UltraVision ONE Polymer (TL-012/5-HDJ/T	0	0	1	100
Leica Bond Polymer Define (DS9713)	0	0	1	100
Leica Bond Polymer Refine (DS9800)	75	83	89	94
MenaPath X-Cell Plus (MP-XCP)	1	100	2	50
None	3	67	4	50
NOT APPLICABLE	2	100	1	100
Novocastra Novolink PDS (RE7-140/150/280/290-K)	1	100	1	100
Other	8	88	7	57
Vector Elite Universal ABC (PK-6200)	1	100	0	0
Vector ImmPRESS Universal (MP-7500)	1	0	1	100
Ventana iView system (760-091)	5	80	7	86
Ventana OptiView Kit (760-700)	37	78	29	76
Ventana UltraView Kit (760-500)	71	72	89	75

General Pathology Run: 111 Automation	CD56 N	CD56 %	Smooth Muscle Actin (SMA) N	Smooth Muscle Actin (SMA) %
BioGenex GenoMX 6000i	0	0	1	100
Dako Autostainer	3	33	4	75
Dako Autostainer Link 48	42	93	42	93
Dako Autostainer plus	2	100	4	75
Dako Autostainer Plus Link	3	100	2	100
Dako Omnis	5	100	4	100
LabVision Autostainer	4	50	6	83
Leica Bond Max	40	78	51	96
Leica Bond-III	55	85	49	92
Menarini - Intellipath FLX	3	67	3	33
None (Manual)	4	100	10	80
Other	1	100	1	100
Shandon Sequenza	1	100	3	100
Ventana Benchmark GX	5	100	7	86
Ventana Benchmark ULTRA	75	72	70	70
Ventana Benchmark XT	51	71	59	78

General Pathology Run: 111 Chromogen	CD56 N	CD56 %	Smooth Muscle Actin (SMA) N	Smooth Muscle Actin (SMA) %
A. Menarini Liquid Stable DAB kit	1	100	1	0
AS PER KIT	39	85	29	90
BioGenex Liquid DAB (HK153-5K)	0	0	1	100
DAKO DAB+	1	100	1	0
Dako DAB+ Liquid (K3468)	3	33	5	100
Dako EnVision Plus kits	8	100	5	100
Dako FLEX DAB	35	91	41	90
Dako REAL EnVision K5007 DAB	5	100	9	89
LabVision DAB	0	0	1	100
Leica Bond Polymer Refine kit (DS9800)	80	80	89	94
menapath xcell kit DAB (MP-860)	1	100	2	50
Other	14	86	12	67
Sigma DAB (D5637)	1	100	1	100
Sigma DAB (D5905)	1	100	1	100
Ventana DAB	17	76	16	69
Ventana Enhanced Alk. Phos. Red Detection Kit	0	0	1	100
Ventana iview	7	71	6	83
Ventana Ultraview DAB	83	69	94	73

BEST METHODS - Gold Standard Antibody

A selection from just a few of the best methods employed by participants

Smooth Muscle Actin (SMA) - Method 1

Participant scored 18/20 (UK NEQAS Slide) and 16/20 (In House slide) using this method.

Primary Antibody: Leica/Novocastra PA0943 (asm-1) , 15 Mins, Room ºC Prediluted

Automation: Leica Bond-III

Method: AS PER KIT

Main Buffer: Bond Wash Buffer (AR9590)

HMAR: None

EAR:

Chromogen: Leica Bond Polymer Refine kit (DS9800), Room ºC., Time 1: 10 Mins

Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins, Room ºC Prediluted

Smooth Muscle Actin (SMA) - Method 2

Participant scored 20/20 (UK NEQAS Slide) and 20/20 (In House slide) using this method.

Primary Antibody:	Dako M0851 SMA (Clone 1A4) , 15 Mins, 32 ºC Dilution 1: 200
Automation:	Dako Omnis
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis
EAR:	
Chromogen:	Dako FLEX DAB, 32 ºC., Time 1: 5 Mins
Detection:	Dako EnVision FLEX (K8000/10) , 20 Mins, 32 ºC

Smooth Muscle Actin (SMA) - Method 3

Participant scored 20/20 (UK NEQAS Slide) and 18/20 (In House slide) using this method.

Primary Antibody:	Dako M0851 SMA (Clone 1A4) Dilution 1: 1:200
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	AS PER KIT
HMAR:	None
EAR:	
Chromogen:	AS PER KIT
Detection:	Leica Bond Polymer Refine (DS9800)

Smooth Muscle Actin (SMA) - Method 4

Participant scored 20/20 (UK NEQAS Slide) and 18/20 (In House slide) using this method.

Primary Antibody:	Dako RTU (Clone 1R611) , 20 Mins, RT ºC Prediluted
Automation:	Dako Omnis
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis, Buffer: EnVFLEX TRS HIGH
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, RT ºC., Time 1: 5 Mins
Detection:	Dako EnVision FLEX (K8000/10) , 20 Mins, RT ºC Prediluted

BEST METHODS - Secondary Antibody

A selection from just a few of the best methods employed by participants

CD56 - Method 1

Participant scored 19/20 (UK NEQAS Slide) and 17/20 (In House slide) using this method.

Primary Antibody:	Novocastra/Leica NCL-CD56 (clone 1B6) , 15 Mins, RT ºC Dilution 1: 100
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins, Buffer: 0, PH: 9
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), RT ºC., Time 1: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, RT ºC Prediluted

CD56 - Method 2

Participant scored 19/20 (UK NEQAS Slide) and 20/20 (In House slide) using this method.

Primary Antibody:	Zymed 18 0152 , 24 Mins, RT ºC Dilution 1: 100
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 30 mins, Buffer: ER2
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), RT ºC., Time 1: 9 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, RT ºC Prediluted

CD56 - Method 3

Participant scored 20/20 (UK NEQAS Slide) and 16/20 (In House slide) using this method.

Primary Antibody:	Novocastra/Leica NCL-CD56 (clone 1B6) , 30 Mins, 20 ºC Dilution 1: 100
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer, PH: 7.6
HMAR:	Dako PTLink, Buffer: Dako target retrieval High pH, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 20 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 15 Mins, 20 ºC Prediluted

CD56 - Method 4

Participant scored 19/20 (UK NEQAS Slide) and 18/20 (In House slide) using this method.

Primary Antibody:	Ventana 760 (clone IB6) , 32 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 36mins
EAR:	
Chromogen:	Ventana Ultraview DAB, Time 1: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins

The Breast Hormonal Receptor Module Run 111

Keith Miller and Merdol Ibrahim

Antigen Assessed:	Oestrogen Receptor (ER)
Tissue Sections circulated:	Composite slide consisting of 3 breast carcinomas with different levels of receptor expression. Most slides also included normal tonsil.
Number of Registered Participants:	289
Number of Participants This Run	273 (94%)

Circulated Tissue

The table below shows the staining characteristics of the tissue sections circulated during Run 109. This composed of three infiltrating ductal carcinomas (IDCs) with differing levels of receptor expression, along with sections of normal breast and tonsil. The staining of the breast tumours were characterised using the Leica 6F11, Ventana SP1 and Dako EP1 clones.

Sections	% Positivity	Expected Staining Intensity	Allred / Quick Score
A. IDC	>95%	High	8
B. IDC	40-60%	Medium	5-6 (depending on the serial section received)
C. IDC	0%	Negative	0
E. Tonsil	1-5%	Weak to Medium	0 (Negative)

Please Note: Multiple tissue blocks are compiled from the same cases, however, there may on occasion be variability in staining from the 'Allred / Quick score' shown above. This is taken into consideration when scoring participants slides and also noted prior to assessments when staining every 50th sample for quality control purposes.

General Guideline Used in The Assessment of slides:

Score	STAINING PATTERN
0	Slide not returned by participant.
1 or 2	**Unacceptable:** E.g. False positive / false negative / non-specific or inappropriate staining / lack of staining **Clinically Unacceptable.**
3	**Borderline Acceptable:** Staining weaker than expected / background staining / weak/strong counterstain, **Clinically still readable but technical improvements can be made**
4 or 5	**Acceptable:** Demonstration of the expected proportion of nuclei stained in the invasive tumours, with roughly the expected staining intensity.

Marks are also deducted when correct clinical interpretation of staining may be hindered due to factors such as:
- Excessive cytoplasmic or diffuse nuclear staining
- Excessively strong or weak haematoxylin counterstain
- Excessive antigen retrieval resulting in morphological damage
- Poor quality/inadequate choice of in-house control tissue (poor/inadequate fixation, damaged cell morphology, over retrieval etc.)

In-HouseTissue Recommendations:

Participants in-house control tissue MUST consist of composite breast tissue placed onto a single slide (cell line controls are an acceptable alternative). Commercial kit/assay controls are not accepted as the participants in-house control, and therefore will not be assessed.

The required composite control should consist of the following samples:

1. >80% tumour positivity with high intensity (Allred/ Quick score 7-8)
2. 30-70% tumour positivity with low-moderate intensity (Allred/Quick score 3-6)
3. Negative tumour, ideally with normal positively stained glands (Allred/Quick score '0')

Participants NOT using a composite control are scored a 'borderline' pass (10-12/20).

Introduction

Oestrogen receptor alpha (ER-α) plays a vital role in both the prognosis and predictive response of patients who may be considered for hormone therapy. Following the work of Harvey and colleagues[1], immunohistochemistry has now become the recognised 'gold standard' for determining patient ER status. It is therefore crucial that not only the antibodies are correctly validated prior to patient-tissue use, but also proper control tissues are used to gauge the sensitivity of the test. An incorrect assay can lead to false ER staining[6,7], which can have a direct impact on patient treatment regime. Furthermore, the UK NHS Breast Screening Programme (www.cancerscreening.nhs.uk/breastscreen/index.html) recommends using the Quick score (Allred)[1,2] to semi quantify the proportion and intensity of nuclear staining, thus further standardising the scoring criteria.

Choice of Tissue for Assessments

This assessment consisted not only of invasive breast tumour (samples A-C), but most slides also consisted of normal tonsil, which was included to help gauge the sensitivity of the assay.

ER Staining Within the Tonsil

The distributed tonsil sections showed ER expression in 1-5% of cells. This has also been indicated in the datasheets from the commercial suppliers, such as the Dako (EP1) and Ventana SP1 clones. Although not noted on the datasheet of the Leica 6F11 clone, staining was also seen in the tonsil of slides stained with this antibody. The staining was observed in some of the epithelial cells, but was mainly seen within the germinal centre lymphocytes.

Quality Control of NEQAS Samples

The NEQAS tissue samples were tested by staining every 50th section from the relevant tissue blocks using the Leica 6F11 clone. This made sure that any heterogeneity in the tissue samples did not result in a participant being penalised. Furthermore, samples were also tested by the relevant commercial companies to further verify the expected level of staining, including the Leica (clone 6F11), Dako (clone EP1) and Ventana (clone SP1).

Assessment Results

Features of Optimal Immunostaining: (Figs 1-6)

- Staining of the expected proportion of invasive tumour nuclei with the anticipated staining intensity
- Intense nuclear staining of the appropriate distribution in normal glands
- Cytoplasmic staining is not excessive
- No background staining of connective tissues or inappropriately localised staining

Features of Sub-optimal Immunostaining: (Figs 7-11)

- Inappropriate non-specific nuclear staining in the negative tumour
- Weak or lower expression of nuclear staining of the oestrogen receptor positive tumours
- Excessive cytoplasmic & background staining
- Excessive antigen retrieval
- Inappropriate staining of some cells in the tumour sections e.g. lymphocytes, fibroblasts
- Inappropriate non-specific staining in the normal breast/ tonsil.

NEQAS Slide Results

Results for the ER assessment on the NEQAS tissue showed an acceptable pass rate of 74% (scores of $\geq$13-20/20), a further 15% (n=41) received a borderline score of between 10 -12/20, and 30 labs (11%) failed the assessment. Overall this was a lower pass rate than the previous assessment where 84% received an acceptable pass. The borderline and failed marks for this assessment Run 111 were mostly due to weak staining, particularly in the mid-expressing tumour. A few labs failed due to inappropriate staining in the negative expressing tumour.

The Ventana SP1 clone was the most popular antibody, used by 109 (40%) of participants. This clone showed an acceptable pass rate of 84%. The Leica 6F11 and Dako EP1 were also popular clones and showed acceptable pass rates of 52% and 88% respectively.

In-House Tissue Results

99% of participants also submitted their in-house controls for assessment. Overall these showed a slightly higher acceptable pass rate of 81%, and a further 18% (N=48) received a borderline pass. Only 3 labs (1%) failed on their in-house tissue. Several labs that received a borderline pass lost marks because they did not provide a composite control consisting of high, mid and negative-expressing tumours as required by UK NEQAS IHC.

Comparing NEQAS Sample Scores and In-house Control Scores.

The NEQAS samples and in house material are scored differently, so participants should be cautious when comparing their UK NEQAS score directly to that of in-house scores. The two samples are scored on different factors:

- The **UK NEQAS** distributed samples are scored against the expected levels of staining as defined by; 1) initial patient diagnosis for ER; 2) re-tested and validated staining of every 50th section from the relevant tissue blocks using the Leica 6F11 clone; 3) re-tested and validated samples by the relevant commercial companies to further verify the expected level of staining. Such companies include the Novocastra/Leica (clone 6F11), Dako (clone EP1) and the Ventana (clone SP1) antibodies.
- The **In-house** samples are scored on the staining quality, readability of the samples, the correct choice of tissue, and good morphological preservation. If the in-house control samples do not comprise of the required composite breast tissue, containing a high (Allred 7-8), mid- (Allred 3-6) and a negative (Allred 0) ER-expressing tumour, participants are given a maximum score of 12/20. The presence of normal staining glands is also encouraged.

It is not possible to know the expected staining levels of the participants' in-house slides and whether there are cases showing false-positive/negative staining, however assessors do try and highlight to participants where there are conflicting observations between 'self-assessment' scores compared to the staining observed by the UK NEQAS assessors.

Participants who are having difficulty in producing acceptable staining results are encouraged to contact UK NEQAS ICC so that further help and advice can be provided.

Assessment Summary

The main observations for sub-optimal results on the NEQAS samples was due to weak staining of the mid-expressing tumour or false positive inappropriate staining in the tumour known to be negative.

Several labs contacted UK NEQAS ICC to highlight that they were having issues with their in-house control section lifting during staining. This problem seems to have occurred just recently since the scheme changed slides and now asks that labs cut and place their own in-house control sections together on the same slides as the UK NEQAS sections. The in-house sections are therefore stained together on the same slide and at the same time as the NEQAS sections. Any participant with such issues was asked to send in a separate in-house control slide. Despite a few labs having adhesion problems, this method of assessing has proven to be very helpful at assessment, enabling the assessors to review both sections at the same time.

Most laboratories received a similar score for both their in-house and NEQAS samples. However, there were a few labs that received lower scores on the NEQAS tissue. This was mostly due to weak and low expression of the mid-expressing tumour. Several reasons may have caused this, for instance, slides may not have been stained soon after receipt, and therefore the expression level of ER is reduced due to loss of antigenicity. UK NEQAS advises that slides are stained as soon as possible on receipt to the laboratory: For example, if the NEQAS section has been sitting at room temperature before the in-house section has been cut onto the slide, this may explain why the staining in the NEQAS section is much

lower than expected , and the in-house the control is much stronger/optimal.

Another factor to take into consideration is that the sensitivity of your assay may be acceptable, in that it is acceptable for your own tissue sections locally and you have validated your assay, However it can indicate that the sensitivity of your assay may not be optimal. A protocol should be in place that will stain effectively on both the NEQAS samples and the labs own samples.

UK NEQAS ICC and ISH would like to reassure participants that we have validated our new one slide approach and will be continually monitoring this approach to ensure our samples are of appropriate quality and will be further implementing quality improvements where deemed necessary.

References

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Selected Images showing Optimal and Sub-optimal Immunostaining

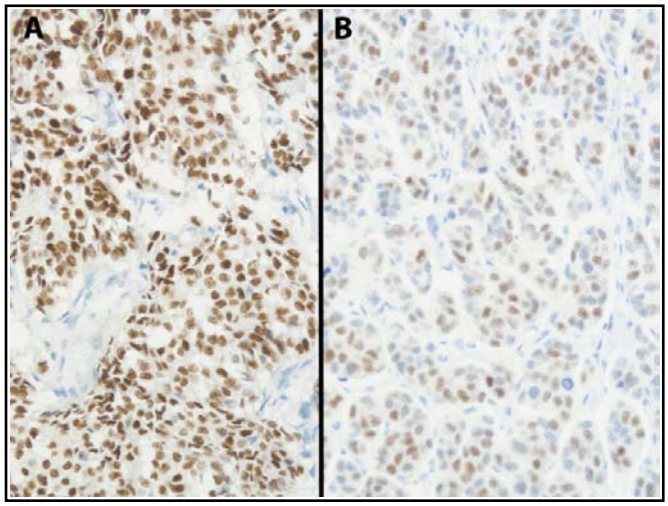


Fig 1. Optimally stained UK NEQAS distributed samples: (A) high expressing ER tumour showing intense staining in over 95% of neoplastic cells, while (B) the mid-expressing tumour shows varying intensity of positive staining in over 60% of neoplastic cells. Stained with the Dako EP1 antibody, 1:75 on the Leica Bond III, ER2 for 20 minutes.

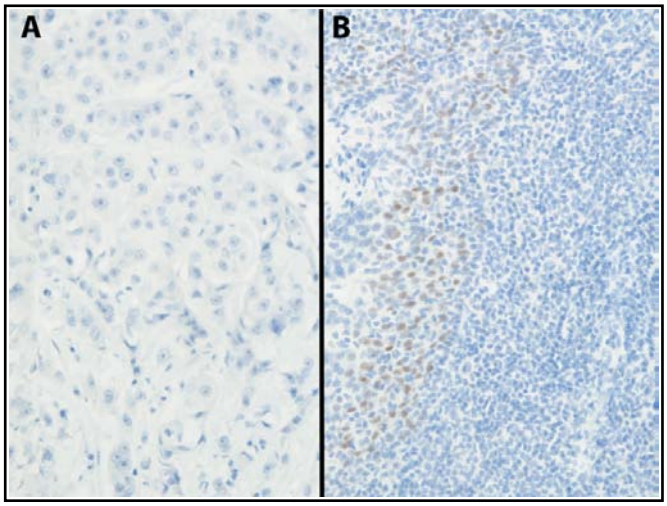


Fig 2. Optimal demonstration of ER in the UK NEQAS distributed samples. The ER negative tumour (A) remains unstained, and as expected a small percentage of lymphocytes are staining positive in the tonsil section (B).(Same protocol as Fig 1).

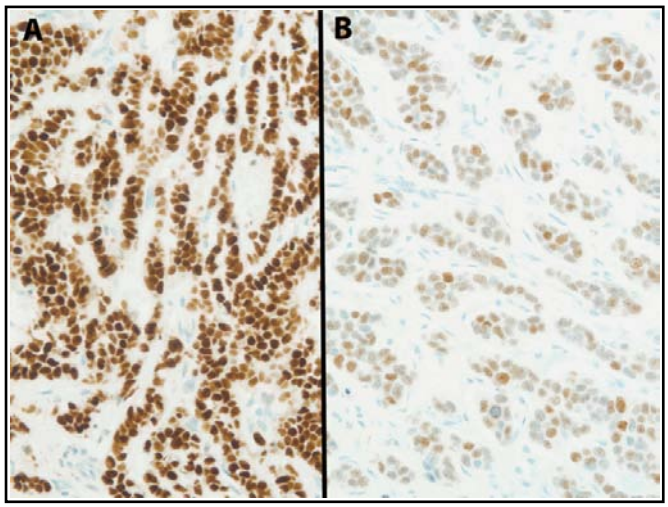


Fig 3. Optimal demonstration of ER in the UK NEQAS (A) high and (B) mid ER expressing tumours. Both sections demonstrate the expected level of staining. The sections were stained with the Ventana SP1 pre-diluted antibody on the ULTRA platform with CC1 retrieval for 64 minutes and UltraView detection kit.

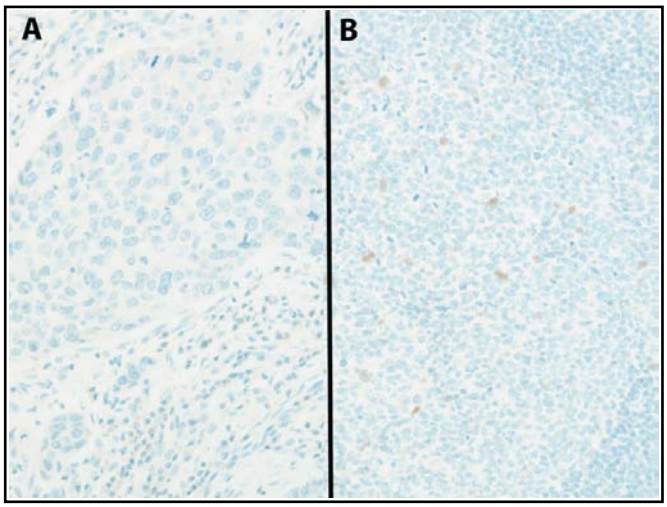


Fig 4. The example shows the expected result on both distributed (A)ER negative tumour and (B) normal tonsil section, which shows less than 5% of lymphocytes staining positive for ER. Same protocol as Fig 3.

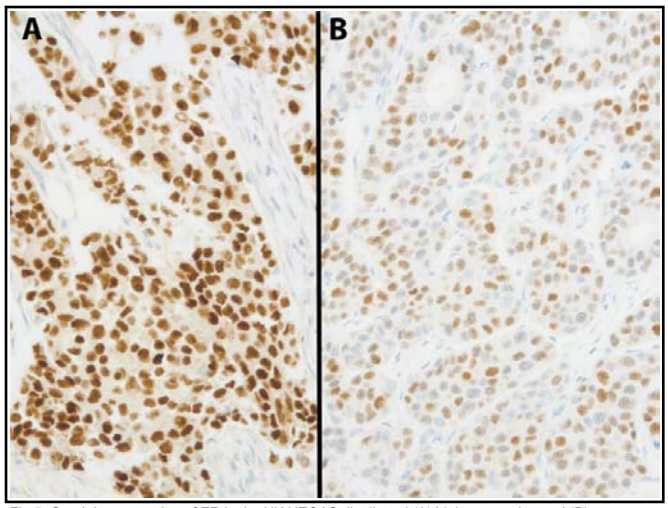


Fig 5. Good demonstration of ER in the UK NEQAS distributed (A) high-expressing and (B) mid-expressing tumours. The slide was stained with the Leica 6F11 antibody on the Ventana Benchmark XT with CC1 standard pretreatment. Both sections show the expected level of expression.

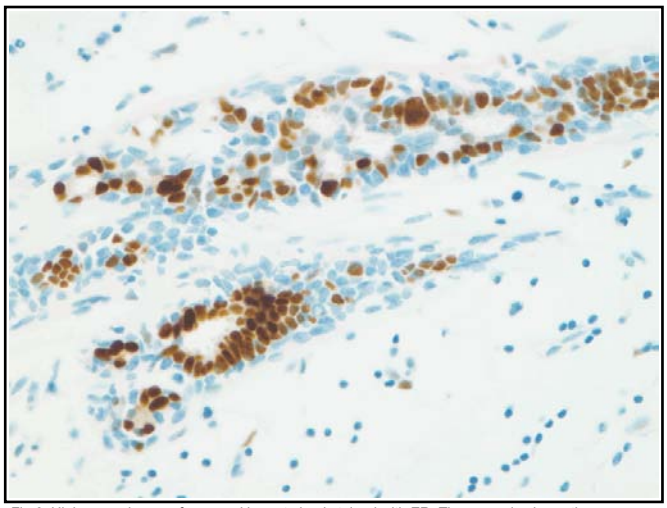


Fig 6. High power image of a normal breast gland stained with ER. The example shows the expected percentage of positive staining for ER in the normal gland. The section was stained with the Ventana prediluted SP1 antibody on the Benchmark GX and Optiview detection.

Selected Images showing Optimal and Sub-optimal Immunostaining

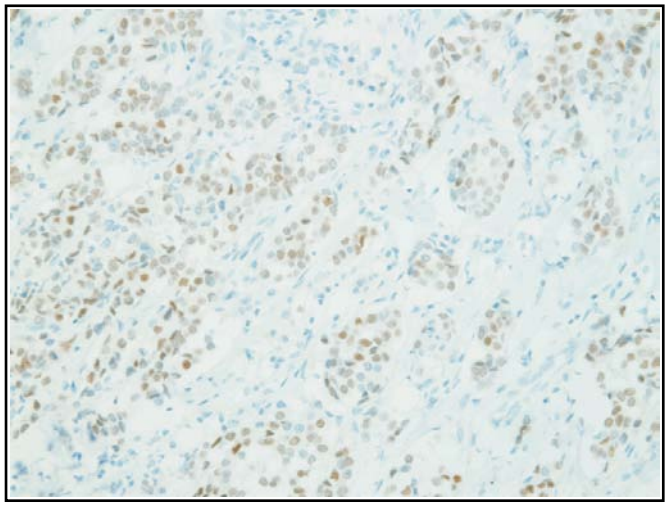


Fig 7. Unacceptable demonstration of ER in the UK NEQAS high-expressing tumour (compare to Figs 1A,3A & 5A).The staining intensity is weak with fewer than expected neoplastic cells staining positive The section was stained with the Ventana pre-diluted SP1 antibody.

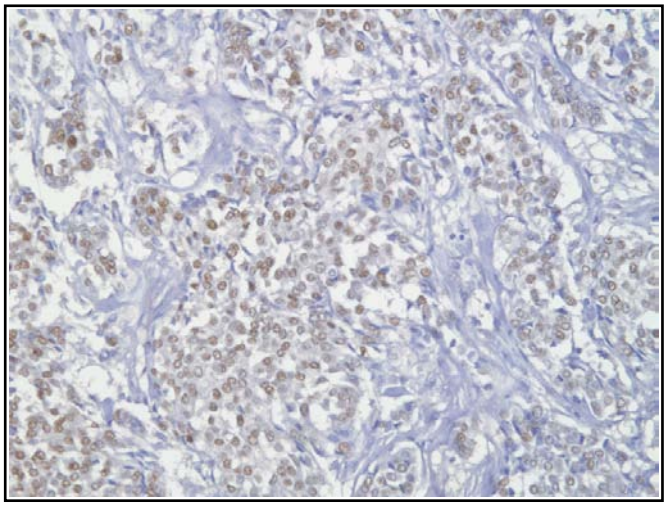


Fig 8. Poor demonstration of ER in the UK NEQAS high-expressing tumour. Although the expression level of ER in the tumour cells is as expected, the counterstain is excessive which hinders the contrast of staining between the tumour cells and stromal elements.

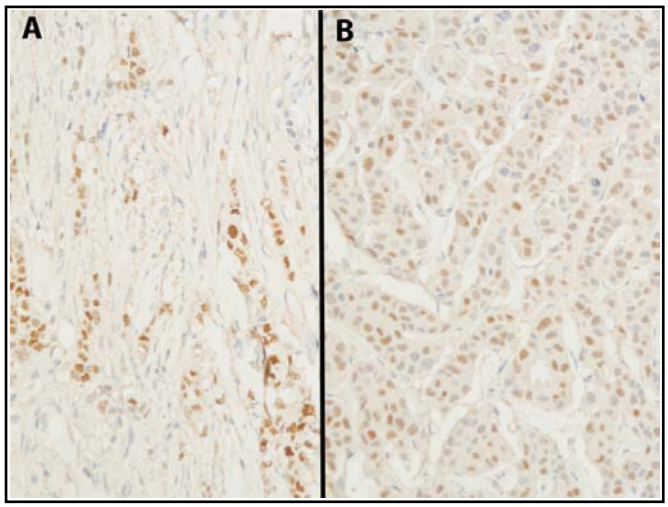


Fig 9. Example images show excessive background staining in the UK NEQAS (A) high-expressing and (B) mid-expressing tumours. Both sections stained with the Dako EP1 antibody, 1:80, on the Leica Bond III with ER2 antigen retrieval for 20 minutes.

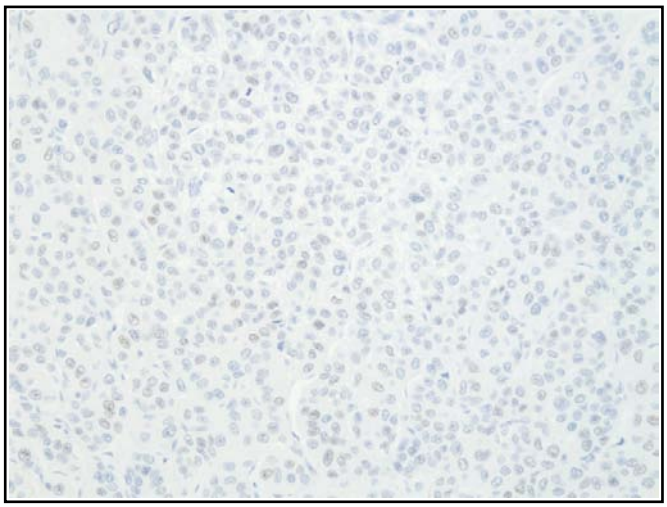


Fig 10. Unacceptable demonstration of ER in the UK NEQAS mid-expressing tumour (compare to Figs 1B,3B & 5B).The staining intensity is very weak with less cells staining positive than expected. The section was stained with the Ventana pre-diluted SP1 antibody.

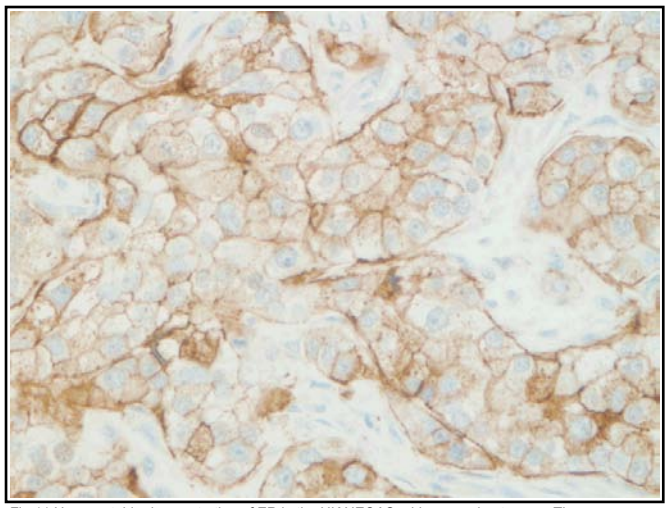


Fig 11 Unacceptable demonstration of ER in the UK NEQAS mid-expressing tumour. The staining is membranous, resembling a HER2 stain rather than a nuclear ER stain. This sample was not assessable, particularly as the high-expressing section on the same slide showed the expected nuclear stain.

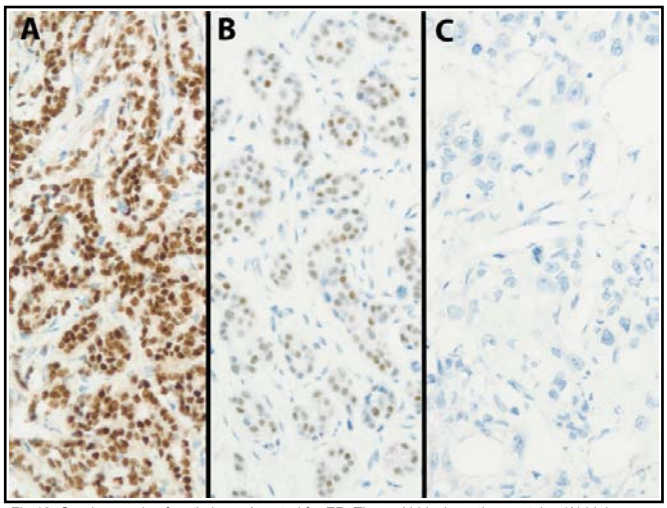
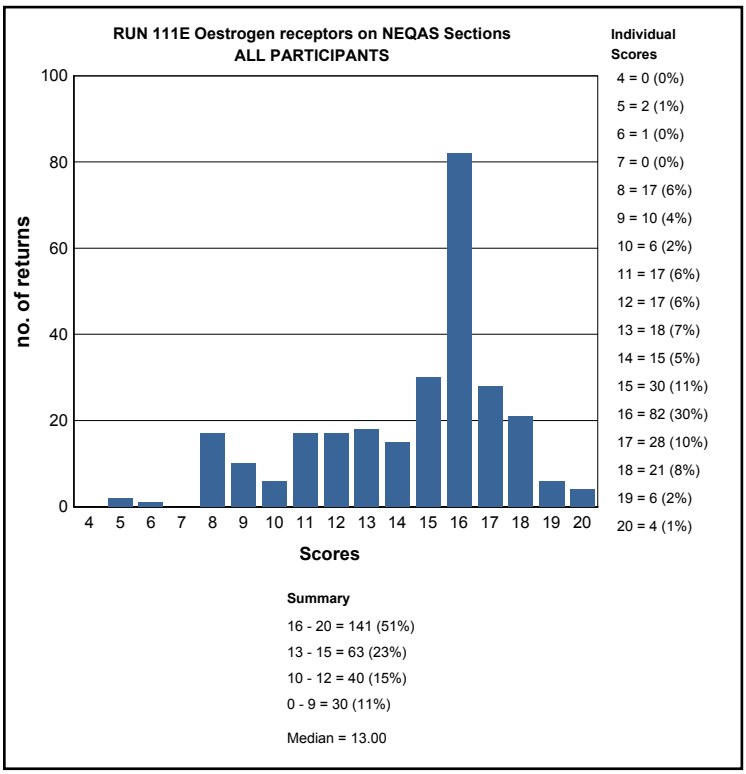
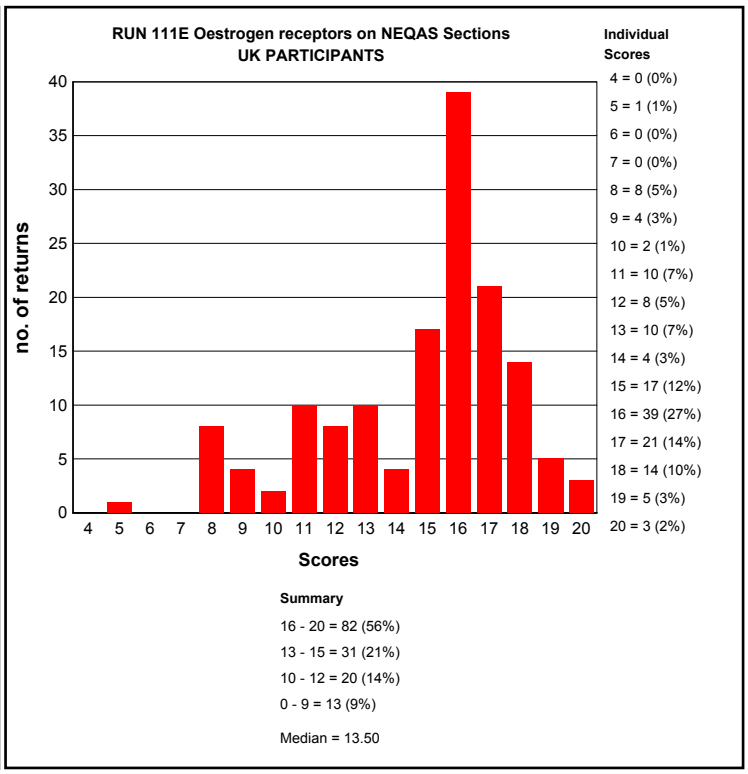
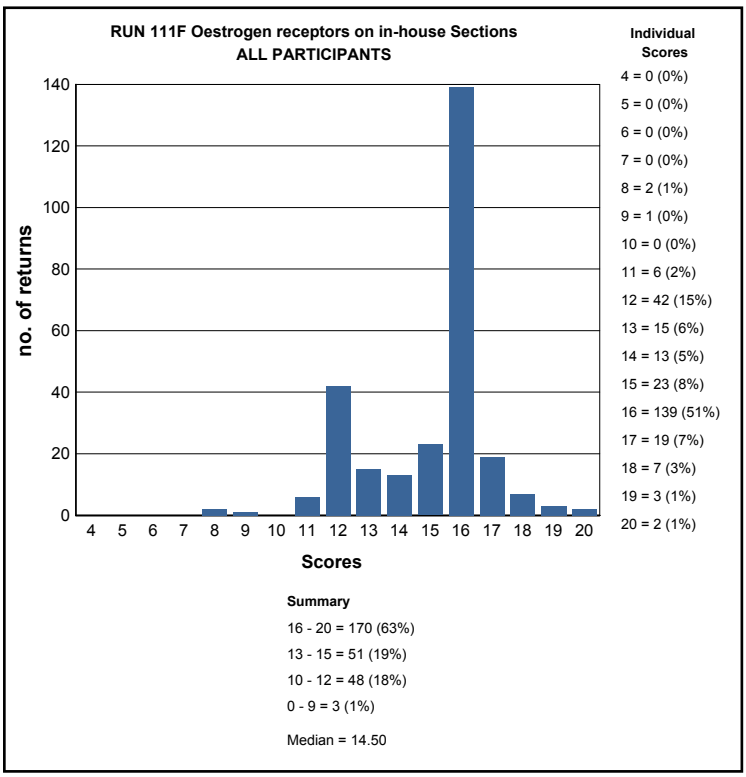


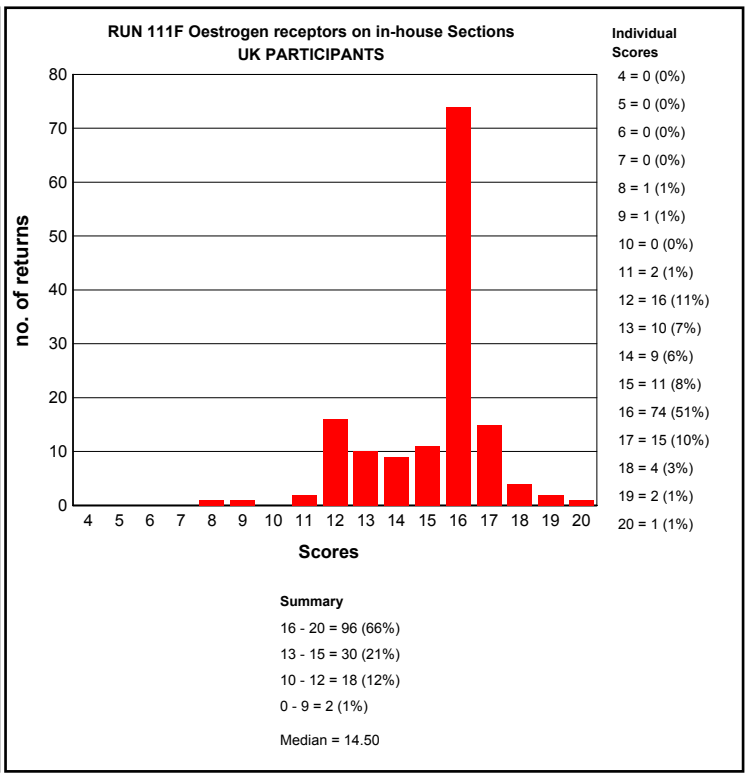
Fig 12. Good example of an 'in house' control for ER. The multi-block section contains (A) high (B) and (C) negative-expressing tumours. A control of known differing expression levels is important to gauge the sensitivity of the assay. Same protocol as Figs 1 & 2.

GRAPHICAL REPRESENTATION OF PASS RATES









ANTIBODIES AND OTHER TECHNICAL PARAMETERS EMPLOYED BY PARTICIPANTS IN THE BREAST STEROID HORMONE RECEPTOR MODULE

The following tables record the number of participants (N) using each primary antibody and the percentage (%) of these participants achieving acceptable staining (a score ≥12/20) on UK NEQAS sections.

Breast Steroid Hormone Receptor Run: 111

Primary Antibody : Oestrogen receptors

Antibody Details	N	%
Cell Marque 249-R (SP1)	3	100
Dako (EP1) M3643	32	84
Dako (EP1) RTU FLEX IR084	24	92
Dako FLEX (1D5) IR/IS657	1	100
Dako IR151 Autostainer Link (SP1)	2	100
Dako M3634 (SP1)	5	100
Dako M7047 ER (1D5)	4	25
Dako N1575 (1D5)	1	100
Dako SK310 pharmDX kit (Link) (1D5 + ER-2-123)	1	100
Leica Bond PA0151 (6F11)	4	50
Leica/Novocastra NCL-ER-6F11 (6F11)	10	50
Leica/Novocastra NCL-ER-6F11/2	11	64
Leica/Novocastra NCL-L-ER- 6F11	34	53
Leica/Novocastra RTU-ER-6F11	2	50
Menapath MP-093-CM1	1	0
Other	3	67
Thermo Fisher/ Neomarkers RM 9101-S (SP1)	15	60
Vector VP-E613/4 (6F11)	6	50
Ventana 790-4324 (SP1)	75	77
Ventana 790-4325 (SP1)	35	91

Breast Steroid Hormone Receptor Run: 111

Automation	Oestrogen receptors N	Oestrogen receptors %
Dako Autostainer	2	0
Dako Autostainer Link 48	34	82
Dako Autostainer plus	2	50
Dako Autostainer Plus Link	3	100
Dako Omnis	4	100
LabVision Autostainer	3	67
Leica Bond Max	32	59
Leica Bond-III	42	69
Menarini - Intellipath FLX	2	50
None (Manual)	3	100
Other	1	0
Shandon Sequenza	2	100
Ventana Benchmark GX	12	58
Ventana Benchmark ULTRA	74	80
Ventana Benchmark XT	56	80

Breast Steroid Hormone Receptor Run: 111

Heat Mediated Retrieval	Oestrogen receptors N	Oestrogen receptors %
Biocare Decloaking Chamber	2	0
Dako Omnis	4	100
Dako Pascal	2	100
Dako PTLink	35	83
Lab vision PT Module	3	67
Leica ER1 20 mins	10	10
Leica ER1 30 mins	15	60
Leica ER1 40 mins	5	20
Leica ER2 10 mins	5	80
Leica ER2 20 mins	27	89
Leica ER2 30 mins	8	63
Leica ER2 40 mins	2	100
Microwave	1	100
Other	2	100
Pressure Cooker	7	71
Ventana CC1 16mins	4	50
Ventana CC1 24mins	2	50
Ventana CC1 32mins	5	60
Ventana CC1 36mins	18	78
Ventana CC1 40mins	1	100
Ventana CC1 48mins	1	0
Ventana CC1 52mins	5	80
Ventana CC1 56mins	3	100
Ventana CC1 64mins	28	82
Ventana CC1 76mins	1	100
Ventana CC1 88mins	1	100
Ventana CC1 8mins	2	50
Ventana CC1 extended	5	80
Ventana CC1 mild	23	70
Ventana CC1 standard	38	89
Ventana CC2 mild	1	100
Ventana CC2 standard	1	0
Water bath 95-98 OC	3	67

Breast Steroid Hormone Receptor Run: 111

Enzyme Mediated Retrieval	Oestrogen receptors N	Oestrogen receptors %
AS PER KIT	6	50
NOT APPLICABLE	162	77
Ventana Protease 1 (760-2018)	2	50

Breast Steroid Hormone Receptor Run: 111

Detection	Oestrogen receptors	
	N	%
AS PER KIT	9	78
Dako EnVision FLEX (K8000/10)	8	88
Dako EnVision FLEX+ (K8002/12)	23	83
Dako Envision HRP/DAB (K5007)	5	80
Dako REAL HRP/DAB (K5001)	1	0
LabVision UltraVision LP HRP (TL 125 HLJ)	1	0
Leica Bond Polymer Refine (DS9800)	69	65
MenaPath X-Cell Plus (MP-XCP)	1	0
None	3	100
NOT APPLICABLE	1	100
Novocastra Novolink PDS (RE7-140/150/280/290-K)	2	100
Other	6	100
Vector Elite Universal ABC (PK-6200)	1	0
Vector ImmPRESS Universal (MP-7500)	1	0
Ventana iView system (760-091)	6	100
Ventana OptiView Kit (760-700)	15	60
Ventana UltraView Kit (760-500)	117	79

Breast Steroid Hormone Receptor Run: 111

Chromogen	Oestrogen receptors	
	N	%
AS PER KIT	21	81
Dako DAB K3468	1	0
DAKO DAB+	2	100
Dako DAB+ Liquid (K3468)	2	50
Dako DAB+ REAL Detection (K5001)	1	0
Dako EnVision Plus kits	4	100
Dako FLEX DAB	29	83
Dako REAL EnVision K5007 DAB	3	100
Dako REAL K5001 DAB	1	0
Leica Bond Polymer Refine kit (DS9800)	66	65
menapath xcell kit DAB (MP-860)	1	0
Other	7	57
Sigma DAB (D5905)	1	100
Ventana DAB	7	71
Ventana Enhanced Alk. Phos. Red Detection Kit	1	100
Ventana iview	6	100
Ventana Ultraview DAB	119	77

BEST METHODS

A selection from just a few of the best methods employed by participants

Oestrogen receptors - Method 1

Participant scored 16/20 (UK NEQAS Slide) and 16/20 (In House slide) using this method.

Primary Antibody: Leica/Novocastra NCL-L-ER- 6F11 , 15 Mins, RT ºC Dilution 1: 50
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER1 30 mins, Buffer: 0, PH: 6
EAR:
Chromogen: Leica Bond Polymer Refine kit (DS9800), RT ºC., Time 1: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins, RT ºC Prediluted

Oestrogen receptors - Method 2

Participant scored 20/20 (UK NEQAS Slide) and 20/20 (In House slide) using this method.

Primary Antibody: Dako (EP1) RTU FLEX IR084 , 20 Mins, 20 ºC Prediluted
Automation: Dako Autostainer Link 48
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako PTLink
EAR: NOT APPLICABLE
Chromogen: Dako EnVision Plus kits
Detection: Dako EnVision FLEX+ (K8002/12)

Oestrogen receptors - Method 3

Participant scored 20/20 (UK NEQAS Slide) and 20/20 (In House slide) using this method.

Primary Antibody: Ventana 790-4324 (SP1) , 16 Mins, 36 ºC
Automation: Ventana Benchmark ULTRA
Method: Ventana UltraView DAB
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 64mins
EAR:
Chromogen: Ventana Ultraview DAB
Detection: Ventana UltraView Kit (760-500)

The Breast HER2 ICC Module Run 111

Suzanne Parry and Merdol Ibrahim

Antigen Assessed:	HER2
Sections Circulated:	Composite slide consisting of 4 breast carcinoma cell lines (see table below)
Number of Registered Participants:	342
Number of Participants this Run	292 (85%)

Specific Guidelines Used in The Assessment of Slides:

The immunohistochemical results were evaluated by UK NEQAS assessors scoring independently using an adapted method initially devised by the Clinical Trials Assay. Due to the nature of cell lines, overall percentage staining criteria can not be accurately applied when scoring cell lines. Communication with Leica Microsystems, with whom UK NEQAS ICC & ISH developed the cell lines, indicate that the expected level of membrane staining for a given cell line may range from 30-90% from the viable cell line population. For this reason when cutting sections, every 50th section is stained as a reference point to gauge the expected level of staining throughout the cell block/s.

The table below demonstrates the staining patterns looked for in cell lines and in-house tissue sections during assessments.

Cell line position (slide label end)	Assessment of Cell Line Staining Pattern	Assessment of In-House Tissue Sections* *The ASCO/CAP guidelines by Wolff et al., (2013) are not currently used for scoring in-house controls
A: 3+	Strong complete cell membrane staining	Strong complete cell membrane staining in >30% of tumour cells
B: 2+	Weak to moderate complete cell membrane staining	Weak to moderate complete cell membrane staining in >10% of tumour cells
C: 1+	Faint barely perceptible incomplete membrane staining	Faint barely perceptible incomplete membrane in >10% of cells staining
D: Negative	No staining in the '0' control cell line	No staining in the '0' control cell line

Validation of Distributed UK NEQAS ICC & ISH Cell Lines

IHC Validation using: Leica Oracle, Ventana Pathway 4B5 and Dako HercepTest
ISH Validation using: FISH (Abbott Vysis) and DDISH (Roche)

Updated Assessment and Scoring Procedure

UK NEQAS Specific Membrane Scoring Algorithm:

UK NEQAS ICC & ISH devised an EQA specific algorithm for scoring the cell lines so as to provide participants additional technical feedback. As well as taking into account the expected range (30-90% see above) of cell line membrane staining, the acceptable staining levels of each of the cell lines are described as follows:

Table below illustrates the expected level of staining for each of the circulated breast carcinoma cell lines along with the UK NEQAS ICC scoring criteria used in assessments ONLY.

Cell line	Score	Acceptable Level/s of Staining During Assessments	Description of Staining Pattern Used By the Assessors
SK-BR-3	**3+**	3+ only	The 3+ cell line has a wide threshold of complete membrane staining showing strong staining. Only this level of membrane staining is deemed acceptable for this cell line.
MDA-MB-453	**2+**	2+ or 1+/2+ or 2+/3+ or 2+/1+ or 3+/2+	i) 1+/2+ or 2+/1+: Staining is slightly weaker than expected with membrane showing more 1+ compared to 2+ (1+/2+) or 2+ membrane staining is present but also showing 1+ staining (2+/1+). ii) 2+/3+ or 3+/2+: Staining is slightly weaker than expected with membrane showing more 2+ compared to 3+ (2+/3+) or 3+ membrane staining is present but also showing 2+ staining (3+/2+).
MDA-MB-175	**1+**	1+ or 0/1+ or 1+/0	i) 0/1+: Staining is more towards the weaker end of 1+ staining but still acceptable. ii) 1+/0: Staining is more towards the weaker end of 1+ staining but still acceptable.
MDA-MB-231	**Negative**	0 or 0/1+ or 1+/0	0/1+ or 1+/0 = Cells are starting to show very weak membrane staining

'**U'/Uninterpretable Scores**
Assessors may also give a score of '**U**' which indicates that the cell lines were 'uninterpretable' due to the reasons set out below.
Borderline Pass: A score of U/x e.g. U/3+ or U/2+ or U/1+ or U/0 indicates a borderline uninterpretable scores indicating that the staining is just about readable and further improvements are required.

Numerical Scoring Criteria

Once the team of assessors have assessed the membrane interpretation for each of the NEQAS samples, they will award a potential score out of 5 marks; based on the interpretability of the membrane staining and technical staining quality. An overall pass mark is then awarded by combining the four assessors scores to give scores out of 20. See table below:

Score and Interpretation	Interpretation
16-20/20: Excellent	Overall the staining is at the expected level for each of the samples.
13-15/20: Acceptable	Some slight technical issues noted by some of the assessors, but overall the staining is suitable for interpretation
10-12/20: Borderline Acceptable	Overall the samples are borderline interpretable (still clinically relevant) indicating that technical improvements need to be made. Marks may have been deducted due to: Weaker/stronger staining than expected, cytoplasmic staining, morphological damage etc.
4-9/20: Unacceptable	Overall the samples are of unacceptable quality for clinical interpretation and technical improvements need to be made. Marks may have been deducted due to: False positive/negative membrane staining, excessive cytoplasmic staining, excessive morphological damage, staining of normal glands

Introduction

HER2 immunohistochemistry has been routinely used as a predictive marker in breast cancer for more than 10 years. Patients with HER2 positive tumours generally have a poor overall prognosis. However, patients with metastatic disease showed an improved rate of survival when the humanized monoclonal antibody Trastuzumab (Herceptin) was given alone or added with chemotherapy treatment (Slamon et al., 1998). Herceptin, in the metastatic setting was made available in the UK in 2000 by the UK National Institute for Clinical Excellence (NICE). In 2005, Herceptin in the adjuvant setting was also shown to be effective in primary breast cancers by reducing the risk of recurrence and mortality (see articles by Wolff et al., (2006) and Walker et al., (2008), and in 2006 NICE approved primary breast screening for HER2. In the UK alone this has resulted in approximately 40,000 breast cancers per year being tested for HER2.

With so many more cases now being tested worldwide, it is imperative that correct protocols and methodologies are followed and adhered to. The UK NEQAS HER2 IHC module assesses the technical quality of staining achieved by laboratories and provides feedback to laboratories to help improve the analytical phase of patient diagnosis.

Recommended HER2 testing guidelines are: The ASCO/CAP guidelines by Wolff et al.(2007), the UK guidelines by Walker et al., (2008), the ASCO/CAP guidelines by Wolff et al., 2013. , and the updated UK guidelines by Rakha et al., (2015). These publications provide invaluable guidelines covering interpretation, tissue fixation and antibody validation. The articles by Walker et. al. and Rhaka et al. also provide guidelines on the minimum number of tests per year that labs should be carrying out in order to provide a robust HER2 IHC service. The recommendations are 250 tests for HER2 IHC and 100 tests for HER2 ISH.

Participants who continue to have difficulty in producing acceptable staining results should be aware that the members of staff at UK NEQAS ICC are always willing to assist any laboratory struggling to meet the required standard of HER2 immunostaining, and would be happy to receive requests for assistance via e-mail in the first instance (see front of journal or the UK NEQAS ICC & ISH website for contact details).

In-House Control Tissue Recommendations

Correct choice of in-house control tissue and good morphological preservation is paramount to gauge the sensitivity of the HER2 test, which can have an impact on clinical interpretation. UK NEQAS ICC & ISH therefore recommend the following:

In-house control tissue (or cell lines) must include 3+, 2+ and 1+/0 invasive breast cancer cases. However, it has become quite apparent that as patient tumour size and respective biopsies become smaller laboratories may be having difficulty finding appropriate invasive control material. It is therefore acceptable, if laboratories are having problems in finding appropriate invasive control material, to submit DCIS tissue showing differing levels of membrane staining as an acceptable alternative. Laboratories must indicate on their datasheet which component they have scored otherwise the invasive component (if present) will be assessed.

Assessment Summary:

Pass rates for the assessment are shown in the subsequent summary graphs. The most popular antibody was the Ventana 4B5, used by 57% (N=165) of participants. 24 labs (8%) are using the Dako HercepTest, and 24 labs (8%) are using the Leica Oracle kit. The rest of the participants are using lab devised methods with a variety of antigen retrieval methods and platforms.

Important: The Ventana OptiView detection system has not been validated for use with the 4B5 HER2 antibody for IHC. Any laboratory using this system for breast HER2 testing should be aware that they are doing so 'off label usage'.

References

1. Slamon D, Leyland-Jones B, Shak S, et al. Addition of Herceptin (humanised anti-HER2 antibody) to first line chemotherapy for (HER2+/MBC) markedly increases anticancer activity: a randomised, multinational controlled phase III trial. Proc ASCO 1998;17:98a.
2. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med 353:1659-1672, 2005
3. Bartlett JM, Ibrahim M, Jasani B, et al. External quality assurance of HER2 FISH testing: results of a UK NEQAS pilot scheme. J Clin Pathol 2007 60 (7):816-819.
4. Walker RA, Bartlett JM, Dowsett M, Ellis IO, Hanby AM, Jasani B, Miller K, Pinder SE. HER2 testing in the UK: further update to recommendations. J Clin Pathol. 2008 61(7):818-824.
5. Wolff AC, Hammond MEH, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol 2007;25:1–28.
6. Wolff AC, et al. Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/ College of American Pathologists Clinical Practice Guideline Update. J Clin Pathol. 2013; 31 (31):3998-4013.
7. Rakha EA, et al. Updated UK rRecommendations for HER2 assessment in breast cancer. J Clin Pathol. 2015;68:93-99.

Acknowledgments

We would like to thank Dako, Ventana and Leica for agreeing to stain the UK NEQAS Breast HER2 ICC 'Gold standard' samples with their respective kits/assays.

Selected Images showing Optimal and Sub-optimal Immunostaining

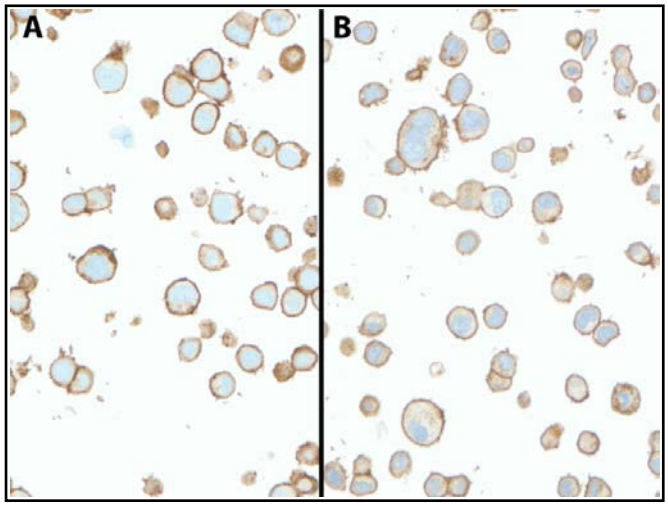


Fig 1. Appropriate staining of the UK NEQAS SK-BR3 (3+) cell line. Examples (A & B) show strong and complete circumferential membrane staining. (A) Stained with the Ventana Pathway 4B5 (32 mins), CC1 mild retrieval on the Benchmark XT with iView kit. (B) Stained using the Leica Oracle kit, on a Bond III as per recommendations.

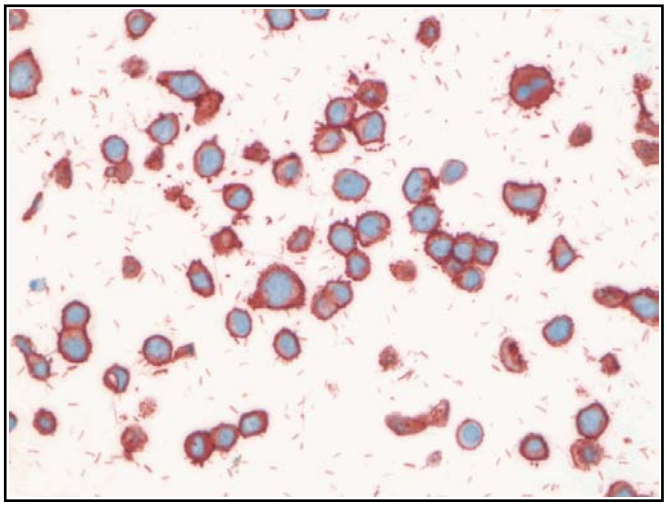


Fig 2. Sub-optimal demonstration of HER2 IHC on the UK NEQAS SK-BR3 (3+) cell line. There is not only excessive cytoplasmic staining, but also bacterial contamination on the slide, possibly due to the water bath not being cleaned. Stained manually with the Dako polyclonal antibody, 1:200, waterbath pre-treatment, and Dako REAL detection with red chromogen.

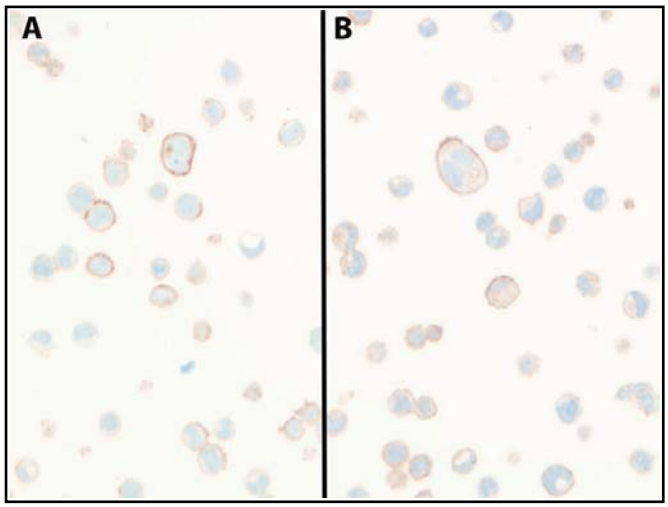


Fig 3. Appropriate level of HER2 staining of the MDA-MB-453 (2+) cell line (A & B): The majority of cells show weak to moderate complete membrane staining. (A) stained with the Ventana Pathway 4B5 (12 mins) on the ULTRA with 36 mins CC1 retrieval and UltraView detection. (B) Stained using the Leica Oracle kit, on a BondMax as per recommendations.

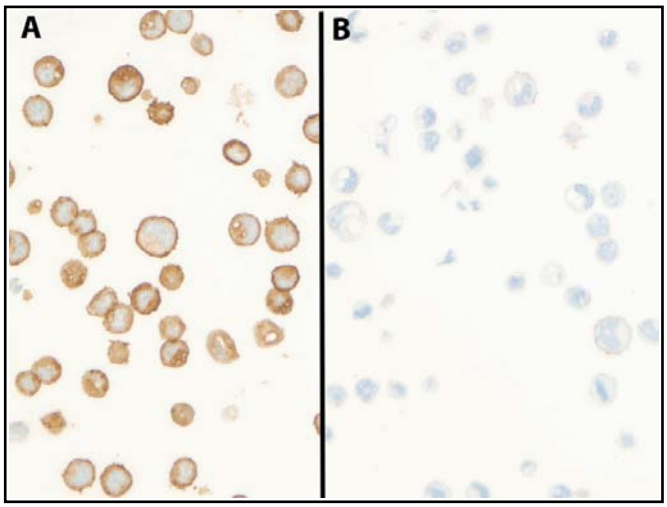


Fig 4. Two examples of unacceptable HER2 staining on the UK NEQAS MDA-MB-453 (2+) cell line. (A) Staining is much higher than expected, and therefore more representative of 3+ expression. (B) staining is too weak, and looks more like 1+ staining.

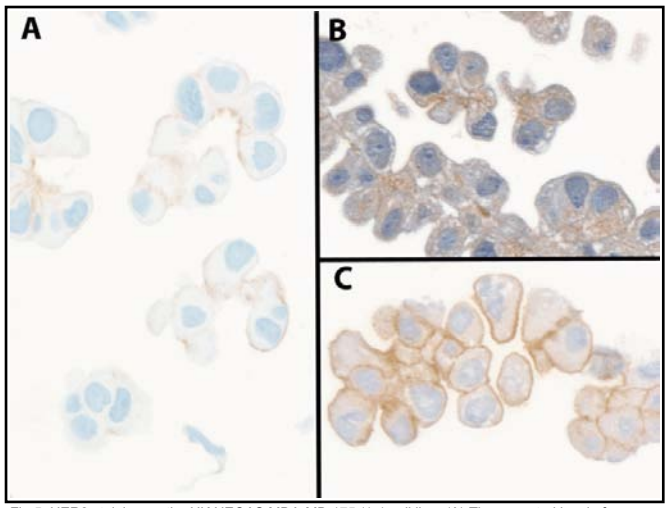


Fig 5. HER2 staining on the UK NEQAS MDA-MB-175 (1+) cell line. (A) The expected level of staining, showing incomplete membrane staining in Over 10% of the tumour cells. (B) Unacceptable excessive cytoplasmic staining, making it difficult to read the membrane staining. (C) Staining is too strong and is representative of 2+ staining.

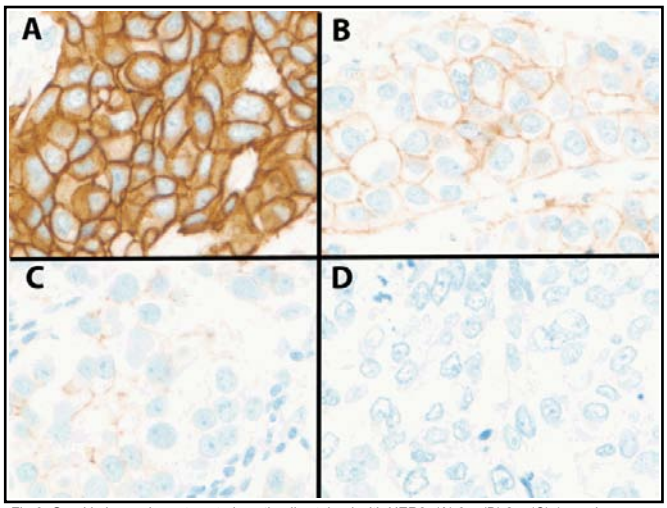
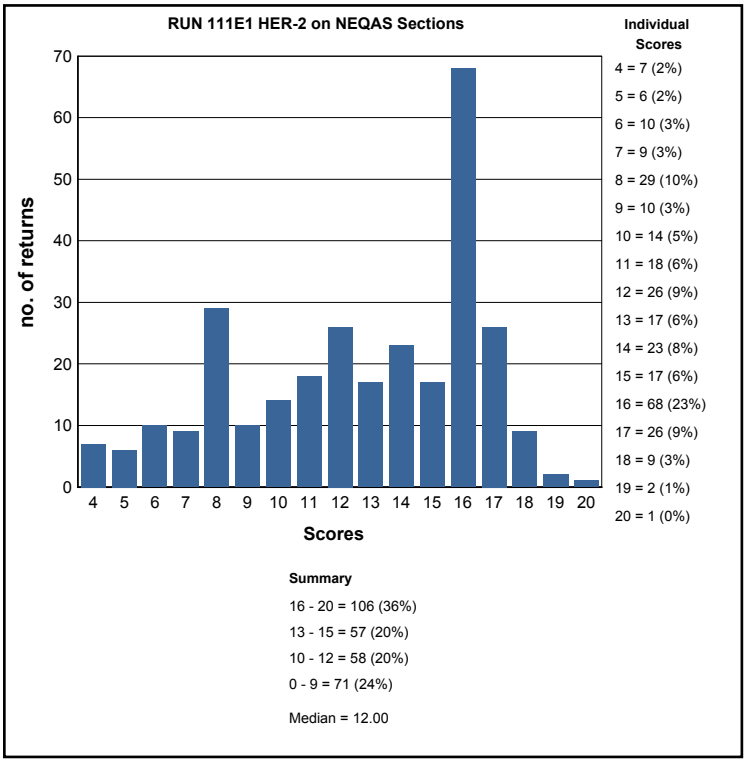
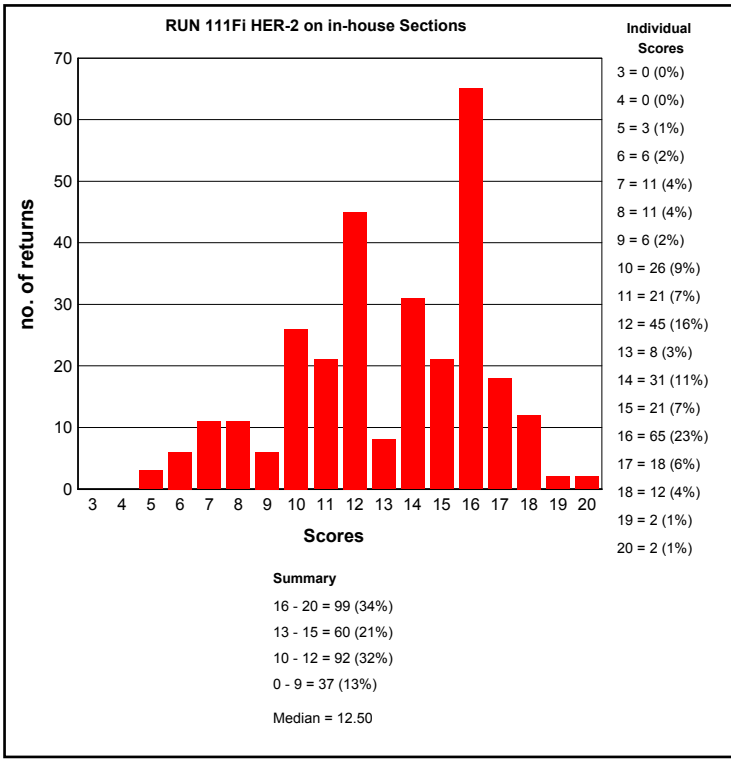


Fig 6. Good in house breast controls optimally stained with HER2. (A) 3+, (B) 2+, (C) 1+ and (D) negative. Same protocol as Fig 3A.

GRAPHICAL REPRESENTATION OF PASS RATES





ANTIBODIES AND OTHER TECHNICAL PARAMETERS EMPLOYED BY PARTICIPANTS IN THE BREAST HER2 ICC MODULE

The following tables record the number of participants (N) using each primary antibody and the percentage (%) of these participants achieving acceptable staining (Score >12/20) on UK NEQAS sections.

Breast HER2 ICC Run: 111

Primary Antibody	N	%
Biocare CME 342 A,B (EP1045Y)	3	33
BioGenex (EP1045Y) rb mono	4	25
Biogenex AM134-10M (CB11)	1	0
Biogenex AM134-5M (CB11)	2	0
Biogenix D608 (CB11)	1	0
Cell Marque 237R (SP3)	5	0
Cell Marque CMA 601 (CB11)	1	0
Dako A0485 C-erB-2 (poly)	27	15
Dako HercepTest K5204 (poly)	4	50
Dako HercepTest K5205 (poly)	1	100
Dako HercepTest K5207 (poly)	5	0
Dako Link HercepTest SK001 (poly)	14	36
Labvision / Neomarkers RM-9103 (SP3)	7	0
Leica Oracle HER2 Bond IHC (CB11)	20	45
Novocastra NCL-L-CB11 (CB11)	7	14
Novocastra NCL-L-CBE356 (10A7)	1	0
Novocastra RTU-CB11 (CB11)	1	0
Novocastra RTU-CBE-356 (10A7)	1	0
Other	10	20
Ventana Confirm 790-4493 (4B5)	39	74
Ventana Pathway 790-100 (4B5)	9	78
Ventana Pathway 790-2991 (4B5)	117	83

Breast HER2 ICC Run: 111

Automation	N	%
BioGenex GenoMX 6000i	3	0
Dako Autostainer	4	0
Dako Autostainer Link 48	23	26
Dako Autostainer plus	3	0
Dako Autostainer Plus Link	4	50
Dako Omnis	1	0
LabVision Autostainer	2	0
Leica Bond Max	19	26
Leica Bond-III	18	44
Menarini - Intellipath FLX	1	0
None (Manual)	22	5
Other	5	40
Shandon Sequenza	1	0
Ventana Benchmark GX	18	56
Ventana Benchmark ULTRA	76	80
Ventana Benchmark XT	79	80

Breast HER2 ICC Run: 111

Heat Mediated Retrieval	N	%
Biocare Decloaking Chamber	9	0
Dako Omnis	1	0
Dako Pascal	1	0
Dako PTLink	29	31
Lab vision PT Module	2	0
Leica ER1 10 mins	3	33
Leica ER1 20 mins	8	0
Leica ER1 25 mins	18	56
Leica ER1 30 mins	2	50
Leica ER1 40 mins	1	100
Leica ER2 20 mins	2	0
Microwave	8	0
None	2	100
Other	4	25
Pressure Cooker	7	14
Steamer	1	0
Ventana CC1 16mins	2	100
Ventana CC1 20mins	5	60
Ventana CC1 24mins	1	100
Ventana CC1 32mins	11	64
Ventana CC1 36mins	37	78
Ventana CC1 40mins	1	0
Ventana CC1 48mins	1	0
Ventana CC1 52mins	3	67
Ventana CC1 56mins	5	100
Ventana CC1 64mins	7	86
Ventana CC1 8mins	2	0
Ventana CC1 extended	1	0
Ventana CC1 mild	69	78
Ventana CC1 standard	17	88
Ventana CC2 16mins	1	100
Ventana CC2 36mins	1	100
Ventana CC2 standard	1	100
Water bath 95-98 OC	11	9

Breast HER2 ICC Run: 111

Detection	N	%
AS PER KIT	28	50
Biocare SLAB (STU HRP 700H,L10)	3	0
BioGenex HRP (HK 519-06K)	1	0
BioGenex SS Polymer (QD 420-YIKE)	1	0
BioGenex SS Polymer (QD 430-XAKE)	2	0
Dako HerCep Test (K5204)	2	50
Dako Duet St.ABC (K0492)	1	100
Dako EnVision FLEX (K8000/10)	9	0
Dako EnVision FLEX+ (K8002/12)	8	13
Dako Envision HRP/DAB (K5007)	7	0
Dako Envision+ HRP mouse K4004/5/6/7	1	0
Dako Envision+ HRP rabbit K4008/9/10/11	1	0
Dako HerCep Test Autor (K5207)	3	0
Dako HerCep Test Autor (SK001)	7	43
LabVision UltraVision ONE Polymer (TL-012/5-HDJ/T	1	0
Leica Bond Polymer Refine (DS9800)	20	35
None	2	100
Other	12	17
Ventana iView system (760-091)	6	83
Ventana OptiView Kit (760-700)	8	75
Ventana UltraView Kit (760-500)	146	77

Breast HER2 ICC Run: 111

Enzyme Retrieval	N	%
AS PER KIT	16	69
Enzyme digestion + HIER	1	0
NOT APPLICABLE	136	59
Other	1	0
Ventana Protease	2	0

Breast HER2 ICC Run: 111

Chromogen	N	%
A. Menarini Liquid Stable DAB kit	1	0
AS PER KIT	48	58
BioGenex DAB (QD430)	1	0
BioGenex Liquid DAB (HK153-5K)	2	0
BioGenex liquid DBA (HK-124-7K)	2	0
DAKO DAB+	2	0
Dako DAB+ Liquid (K3468)	4	0
Dako DAB+ REAL Detection (K5001)	1	0
Dako EnVision Plus kits	3	0
Dako FLEX DAB	18	22
Dako REAL EnVision K5007 DAB	5	0
LabVision DAB	1	0
Leica Bond Polymer Refine kit (DS9800)	21	29
Other	20	30
Sigma DAB (D5905)	1	0
Ventana DAB	5	80
Ventana iview	4	75
Ventana Ultraview DAB	141	77

BEST METHODS

A selection from just a few of the best methods employed by participants

HER-2 - Method 1

Participant scored 20/20 (UK NEQAS Slide) and 18/20 (In House slide) using this method.

Primary Antibody:	Ventana Pathway 790-2991 (4B5) , 32 Mins Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana iView Kit
Main Buffer:	AS PER KIT
HMAR:	Ventana CC1 mild
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	Ventana iView system (760-091) Prediluted

HER-2 - Method 2

Participant scored 16/20 (UK NEQAS Slide) and 17/20 (In House slide) using this method.

Primary Antibody:	Leica Oracle HER2 Bond IHC (CB11) , 30 Mins, 22 ºC Prediluted
Automation:	Leica Bond-III
Method:	Other
Main Buffer:	Bond Wash Buffer (AR9590), PH: 7.6
HMAR:	Leica ER1 25 mins
EAR:	
Chromogen:	AS PER KIT, 22 ºC., Time 1: 10 Mins
Detection:	AS PER KIT , 10 Mins, 22 ºC Prediluted

HER-2 - Method 3

Participant scored 18/20 (UK NEQAS Slide) and 20/20 (In House slide) using this method.

Primary Antibody:	Ventana Pathway 790-2991 (4B5) , 12 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 36mins
EAR:	
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500) Prediluted

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The Gastric HER2 ICC Module Run 111

Merdol Ibrahim and Suzanne Parry

Antigen Assessed:	**HER2 for IHC**
The NEQAS distributed sections consisted of a composite slide containing 4 surgical /resections of intestinal gastric carcinoma:	The expected expression levels of each tissue sample were: **A.** 0 **B**. 2+ or 1+ depending on the serial section received **C**. 2+ **D**. 3+
Number of Registered Participants	78
Number of Participants this Run	65 (83%)

Table: 1 Gastric HER2 ICC Membrane Scoring Guidelines: Hoffman et al., (2008) and Rüschoff et al., (2010)

Score	Surgical / resections As used in NEQAS assessments	Biopsies
0 (negative)	No staining in < 10% of tumour cells	No staining in any of the tumour cells
1+ (negative)	Faint barely perceptible incomplete membrane staining in >10% of cells staining	Tumour cells with faint barely perceptible incomplete membrane staining, irrespective of percentage of tumour cells stained
2+ (equivocal*)	Weak/ moderate complete, basolateral or lateral membrane reactivity in ≥ 10% of tumour cells	Tumour cells with weak/ moderate complete, basolateral or lateral membrane reactivity irrespective of % tumour cells stained
3+ (positive)	Strong complete, basolateral or lateral membrane reactivity in ≥ 10% of tumour cells	Tumour cells with strong, basolateral or lateral membrane reactivity irrespective of percentage of tumour cells stained
*** Equivocal cases should be refluxed to ISH testing. Note: in the UK, NICE guidelines recommend that only 3+ cases are put forward for Trastuzumab treatment, see http://guidance.nice.org.uk/TA208**		

Validation of Distributed Samples

IHC Validation of Distributed Samples

The NEQAS tissue sections are validated prior to sending out: Gold standard slides are stained at every 25th serial level as part of the tissue quality control and to assess the HER2 expression level throughout the blocks. The slides are also stained with the Ventana Pathway 4B5 and Dako HercepTest commercial kits and further assessed for gene expression using the Dako IQ FISH and Ventana/Roche DDISH (see the table below for HER2 ISH status).

Please Note: The variability and heterogeneity of HER2 expression throughout the block is taken into consideration when scoring participants slides.

Table 2: HER2 IHC staining and & ISH results

Section Label From slide Label End	Staining Pattern with IHC	HER2 status by ISH
A	**0**	Non-Amplified
B	**2+ or 1+**	Amplified or Non-Amplified depending on the serial section received
C	**2+**	Amplified
D	**3+**	Non-Amplified

Assessment Procedure

Assessment is carried out around a multi-header microscope with each slide being assessed by 4 independent assessors and a microscope lead. Each of the NEQAS samples (A-D) (as well as in-house samples where submitted) are individually assessed, with each assessor providing membrane interpretation (see table 3), and an individual score out of 5 (see table 4) based on interpretability of membrane staining and technical feedback. The four assessors scores are then combined to give an overall score out of 20 and it's interpretation (see table 4).

Table 3: UK NEQAS Specific Membrane Scoring Criteria: UK NEQAS ICC & ISH uses an EQA specific scoring criteria when scoring the tissue sections, so as to provide participants additional technical feedback.

Expected membrane staining	Scoring criteria used by UK NEQAS ICC & ISH
3+	i) 3+: as expected ii) 2+/3+ or 3+/2+: Staining is slightly weaker than expected with membrane showing more 2+ compared to 3+ (2+/3+) or 3+ membrane staining is present but also showing 2+ staining (3+/2+).
2+	i) 1+/2+ or 2+/1+: Staining is slightly weaker than expected with membrane showing more 1+ compared to 2+ (1+/2+) or 2+ membrane staining is present but also showing 1+ staining (2+/1+). ii) ii) 2+/3+ or 3+/2+: Staining is slightly weaker than expected with membrane showing more 2+ compared to 3+ (2+/3+) or 3+ membrane staining is present but also showing 2+ staining (3+/2+).
1+	i) 0/1+: Staining is more towards the weaker end of 1+ staining but still acceptable. ii) 1+/0: Staining is more towards the weaker end of 1+ staining but still acceptable.
Neg.	0/1+ or 1+/0 = Staining starting to show very weak membrane staining
'U' = Uninterpretable: Assessors may also give a score of 'U' which indicates that the cell lines / tissue sections were 'uninterpretable' due to the reasons set out below. U/x = Borderline interpretable. A score of U/x e.g. U/3+ or U/2+ or U/1+ or U/0 indicates that the staining is just about readable and further improvements are required. Any other membrane score other that assigned for each of the expected scores are deemed as unacceptable	

Table 4: Numeric Scoring Criteria used to Calculate the Overall Pass Mark

Individual Assessor Score Out of 5 Marks	Combined Assessor Scores Out of 20 Marks	Overall Score Interpretation
0	**0**	Slide not submitted for assessment
1 & 2	**4-9 = Unacceptable**	**Overall the samples are of unacceptable quality for clinical interpretation and technical improvements need to be made**. Marks may have been deducted due to: • False positive / negative membrane staining • Excessive cytoplasmic staining • Excessive morphological damage • Excessive staining of normal glands
3	**10-12 = Borderline**	**Overall the samples are borderline interpretable indicating that technical improvements need to be made**. Marks may have been deducted due to: • Weaker / stronger than expected membrane staining • Some cytoplasmic staining • Morphological damage
4 & 5	**13-15 = Acceptable** **16-20 = Acceptable/ Excellent Standard**	**Overall the samples show acceptable membrane staining and are suitable for interpretation.**
Further comments are also provided on individual participant reports. Other factors which may lead to a low score include: excessive/ insufficient haematoxylin staining; poor quality of in-house control tissue; poor/inadequate choice of control tissue; poor/inadequate fixation; excessive antigen retrieval etc.		

Introduction

Immunohistochemical testing of HER2 status is now routinely used in breast cancer testing and is recognised as a prognostic and predictive marker, generally used alongside breast hormonal receptor markers ER/PR. Patients who are HER2 positive (IHC 3+ and IHC 2+/ISH+) have been shown to benefit from Herceptin (Trastuzumab) therapy and increased overall survival rate. More recently the Trastuzumab for Gastric Cancer (ToGA) study, which investigated Trastuzumab in HER2 positive advanced gastric cancer (Bang et al., 2010) showed overall median survival of nearly 3 months. Similar to breast cancer, the ToGA trial showed an increased benefit from Trastuzumab treatment for patients showing 3+ IHC and IHC2+/FISH+ expression. The initial development of the HER2 scoring criteria was developed as a precursor to the ToGA trial (Hoffman et al., 2008) with the study group modifying the breast HER2 IHC scoring algorithm to compensate for the incomplete membrane staining and greater tumour heterogeneity seen in gastric cancers. A different scoring system was also established for resection and core biopsies as illustrated in table 1. A more recent article by Rüschoff et al., (2010) has validated the scoring procedure further with a detailed approach to 'stepwise' HER2 IHC scoring in gastric cancers. The article also compared the Ventana 4B5 assay with the HercepTest and indicated "a tendency towards higher sensitivity for 4B5 detecting positive HER2 amplification" but "there was no difference between both test platforms with respect to the ISH-negative cases,

with the one equivocal case testing negative at some sites and as positive at others". Rüschoff and colleagues also used both fluorescent (Dako PharmDX or PathVysion Vysis) and chromogen (Ventana DDISH) to confirm their IHC findings.

Assessment Results

Features Of Acceptable Staining: (Figs 1– 4)

- Membrane staining of the invasive tumour with the expected expression level
- Cytoplasmic staining not excessive
- No background staining of stromal tissues or inappropriately localised staining

Features Of Sub-optimal or Unacceptable Staining: (Figs 5 -6)

- Weaker or stronger staining than the expected expression level of membrane staining in the invasive tumour
- False positive or negative membrane staining
- Excessive cytoplasmic staining
- Excessive background staining or inappropriately localised staining
- Morphological damage
- Excessive staining of normal glands

Additional Comment:

The Ventana 4B5 is known to stain intestinal metaplasia (not illustrated) and participants are not penalised when this staining is observed.

Pass Rates

The pass rates for the UK NEQAS distributed samples were reasonably good, but slightly lower than the previous assessment (Run 110): 82% of participating labs received an acceptable pass and a further 5 labs (8%) achieved a borderline pass. However, 7 labs (11%) failed the assessment, and this was due to either weak membrane staining or inappropriate over-staining, which therefore resulted in an incorrect Her2 score. This was mostly attributable to inappropriate antigen retrieval methods. Labs that received a borderline score were also mostly marked down due to either a slightly higher or lower level of HER2 membrane staining than expected.

All participants apart from 1 lab also submitted their in-house controls. The overall pass rate was similar to that seen on the Neqas samples, with 77% receiving an acceptable pass, and a further 17% receiving a borderline score. Several laboratories that received a borderline pass did not submit the required composite control material, consisting of a 3+, 2+ and 1+/0 expressing gastric/breast control tumour sample, and therefore these labs were given a maximum score of 12/20, i.e. 3/5 from each assessor. Other labs were marked down for poor tissue quality or fixation. 4 labs (6%) failed the assessment, again this was mostly due to poor tissue quality.

Methodologies

Most labs are using the Ventana 4B5 standardised kit for their gastric HER2 testing: In this particular assessment run the Ventana assay was used by 72% of participants and showed an acceptable pass rate of 87% on the UK NEQAS distributed section. All 6 labs that are using the standardised Dako HercepTest also received acceptable passes. The Leica Oracle kit was used by 2 labs; one of these labs received a borderline pass and the other Leica user failed the assessment due to very weak and low expression of HER2 than was expected.

Control Tissue and Recommendations

UK NEQAS ICC recommends that gastric tissue samples are used as an ideal in–house control for gastric HER2 testing. However, laboratories were given the choice to submit either gastric or breast control material for their in-house control assessment and therefore laboratories were not penalised if they submitted breast carcinomas instead of gastric tissue. Whether gastric or breast tissue is used, laboratories should still submit in-house controls showing 3+, 2+ and 1+/0 levels of membrane expression.

Due to the heterogenic nature of many gastric tumours, it is acceptable for labs to submit a heterogeneous in-house control with areas of both 3+ and 2+ membrane expression for example, as long as the participant clearly indicates the areas where the assessment should be carried out.

Important: The Ventana OptiView detection system has not been validated for use with the 4B5 HER2 for IHC, and therefore any laboratory using this system for gastric HER2 testing should be aware that they are doing so 'off label usage'.

References:

1. Hofmann M, Stoss O, Shi D et al. Assessment of a HER2 scoring system for gastric cancer: results from a validation study. Histopathology. 2008 52 (7):797-805.
2. Rüschoff J, Dietel M, Baretton G etc al. HER2 diagnostics in gastric cancer-guideline validation and development of standardized immunohistochemical testing. Virchows Arch. 2010 457(3):299-307.
3. Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, Lehle M, Rüschoff J, Kang YK. ToGA Trial Investigators. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. Lancet. 2010 376(9742):687 -97

Acknowledgments

We would like to thank Dako and Ventana for agreeing to stain the UK NEQAS Gastric HER2 ICC 'Gold standard' samples with their respective kits/assays and also for providing the ISH.

Selected Images showing Optimal and Sub-optimal Immunostaining

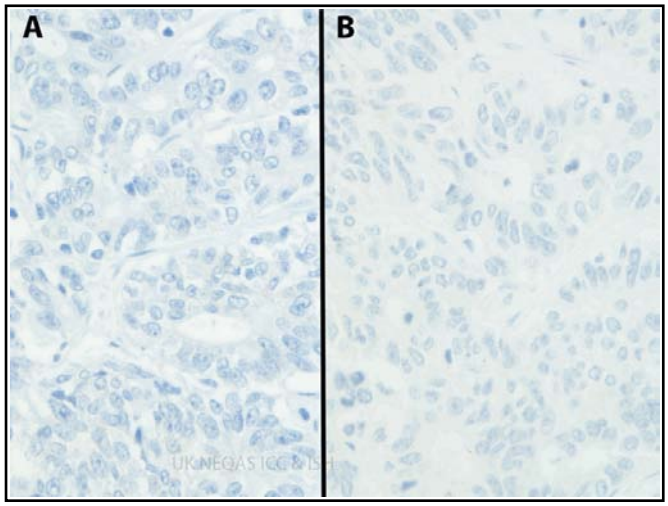


Fig 1. (A, B) Two good examples of negative HER2 IHC gastric samples from the NEQAS distributed sample 'A'. (A) Stained with the Dako HercepTest on the Autostainer 48, 40 mins pre-treatment in the PT Link. (B) Stained with the Ventana 4B5 (16 mins), CC1 mild retreival on the Benchmark XT and UltrView detection.

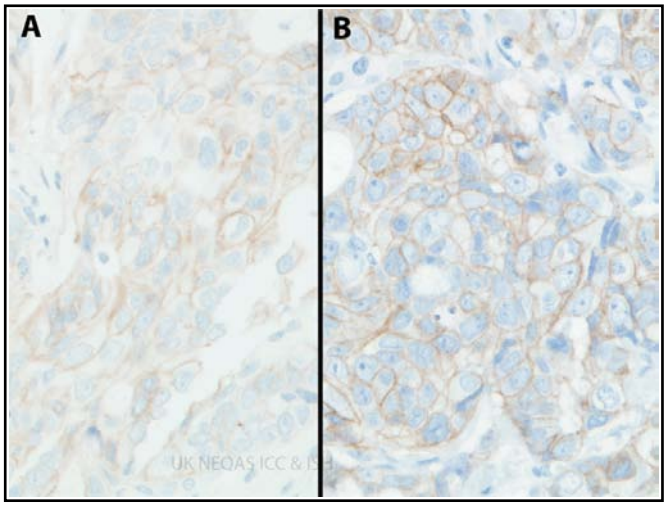


Fig 2. (A, B) Two good examples of HER2 2+ staining on the NEQAS distributed gastric sample 'B', both showing complete membrane staining of weak to moderate intensity. (A) Stained with the Dako HercepTest and (B) stained with the Ventana 4B5 Pathway. CC1 mild retrieval.

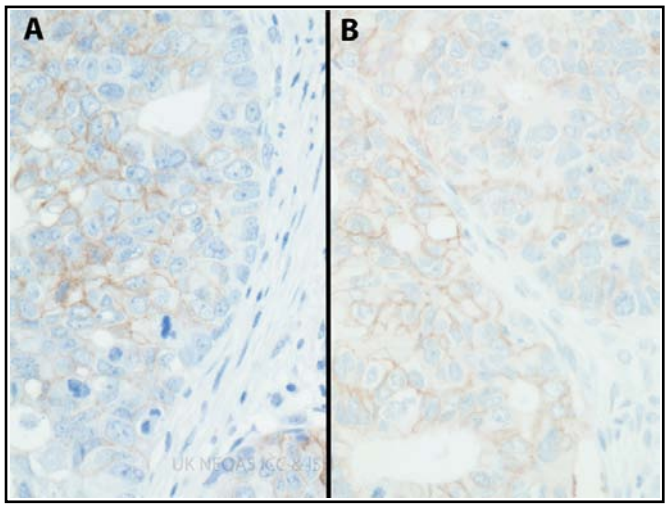


Fig 3. (A, B) Two good examples demonstrating HER2 2+ protein expression on the NEQAS distributed gastric sample 'C'. As expected, the tumour shows weak to moderate complete membranous staining. (A) Stained with the Dako HercepTest and (B) stained on the Ventana platform with the 4B5 Pathway.

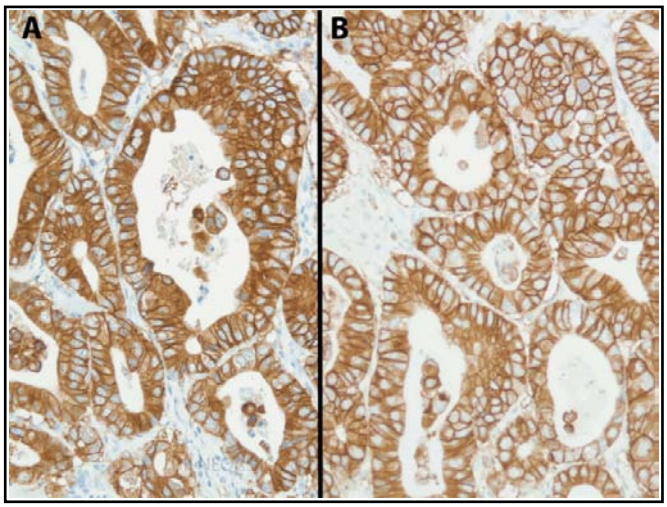


Fig 4. (A, B) Two good examples of HER2 3+ staining on the NEQAS distributed gastric tumour sample 'D'. Both images show strong complete membrane staining, which is much more intense than that seen with the 2+ samples shown in Figs 2 & 3. (A) Stained with the Dako HercepTest and (B) stained with the Ventana 4B5 Pathway on the Benchmark XT.

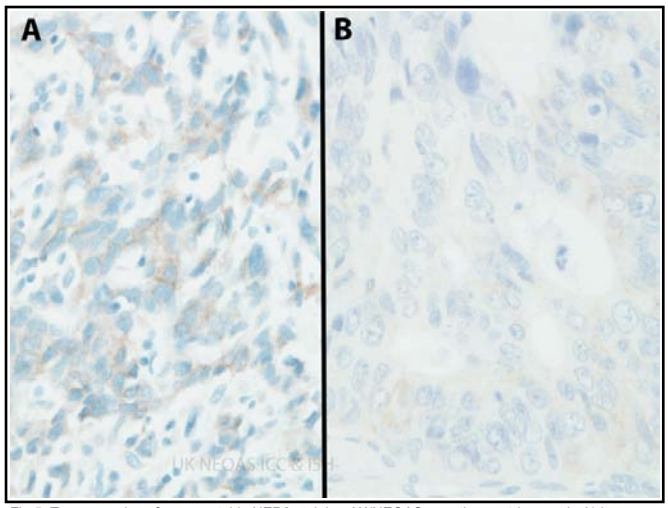


Fig 5. Two examples of unacceptable HER2 staining: 'A'(NEQAS negative gastric sample A) is showing inappropriate non-specific staining. 'B' (NEQAS 2+ gastric sample B) is much weaker than expected and appears to be negative; at most of 1+ HER2 expression level.

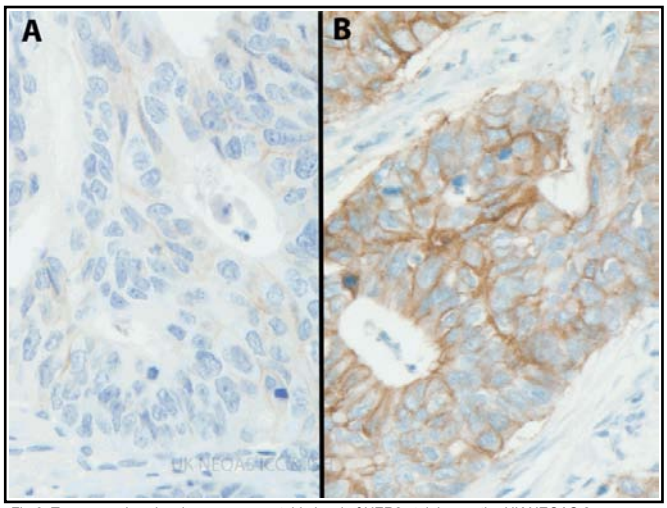
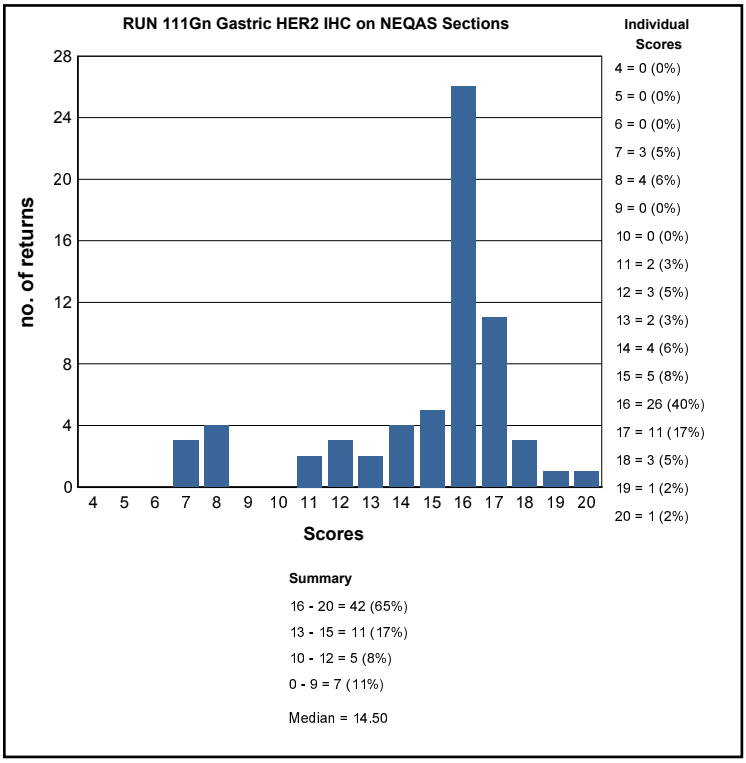
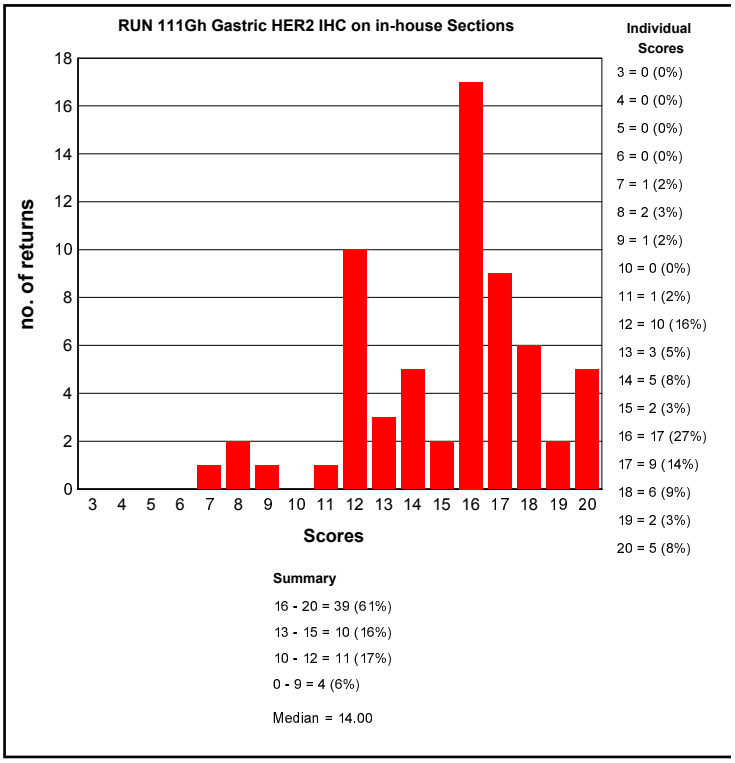


Fig 6. Two examples showing an unacceptable level of HER2 staining on the UK NEQAS 2+ gastric sample C: In 'A' the staining is too weak and more representative of 1+ staining. In 'B' the staining is excessive and more representative of 3+ staining. Both stained on the Ventana platform with the 4B5 antibody: Example'A' with 8 minutes antibody incubation time, and

GRAPHICAL REPRESENTATION OF PASS RATES





ANTIBODIES AND OTHER TECHNICAL PARAMETERS EMPLOYED BY PARTICIPANTS IN THE GASTRIC HER2 ICC MODULE

The following tables record the number of participants (N) using each primary antibody and the percentage (%) of these participants achieving acceptable staining (Score >12/20) on UK NEQAS sections.

Gastric HER2 ICC Run: 111

Primary Antibody	N	%
Dako Link HercepTest SK001 (poly)	6	100
Labvision / Neomarkers RM-9103 (SP3)	1	100
Leica Oracle HER2 Bond IHC (CB11)	1	0
Leica Oracle HER2 Bond IHC (CB11)	1	0
Ventana Confirm 790-4493 (4B5)	14	64
Ventana Confirm 790/800-2996 (4B5)	2	100
Ventana Pathway 790-100 (4B5)	5	60
Ventana Pathway 790-2991 (4B5)	33	94

Gastric HER2 ICC Run: 111

Automation	N	%
Dako Autostainer Link 48	4	100
Dako Autostainer Plus Link	3	100
Leica Bond Max	1	0
Leica Bond-III	1	0
Ventana Benchmark GX	4	75
Ventana Benchmark ULTRA	21	71
Ventana Benchmark XT	29	93

Gastric HER2 ICC Run: 111

Heat Mediated Retrieval	N	%
Dako PTLink	6	100
Leica ER1 25 mins	2	0
Ventana CC1 16mins	1	100
Ventana CC1 20mins	2	50
Ventana CC1 24mins	1	100
Ventana CC1 32mins	5	60
Ventana CC1 36mins	11	64
Ventana CC1 52mins	1	100
Ventana CC1 56mins	1	100
Ventana CC1 64mins	3	100
Ventana CC1 mild	23	91
Ventana CC1 standard	6	100

Gastric HER2 ICC Run: 111

Detection	N	%
AS PER KIT	7	71
Dako EnVision FLEX (K8000/10)	1	100
Dako HerCep Test Autor (SK001)	4	100
Leica Bond Polymer Refine (DS9800)	1	0
Ventana iView system (760-091)	2	100
Ventana OptiView Kit (760-700)	2	100
Ventana UltraView Kit (760-500)	44	82

Gastric HER2 ICC Run: 111

Enzyme Retrieval	N	%
NOT APPLICABLE	28	82
Ventana Protease	1	100

Gastric HER2 ICC Run: 111

Chromogen	N	%
AS PER KIT	9	89
DAKO DAB+	1	100
Dako FLEX DAB	3	100
Leica Bond Polymer Refine kit (DS9800)	2	50
Ventana DAB	2	100
Ventana iview	1	100
Ventana Ultraview DAB	45	80

BEST METHODS

A selection from just a few of the best methods employed by participants

Gastric HER2 IHC - Method 1

Participant scored 18/20 (UK NEQAS Slide) and 17/20 (In House slide) using this method.

Primary Antibody:	Ventana Pathway 790-2991 (4B5) , 32 Mins Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana iView Kit
Main Buffer:	AS PER KIT
HMAR:	Ventana CC1 mild
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	Ventana iView system (760-091) Prediluted

Gastric HER2 IHC - Method 2

Participant scored 20/20 (UK NEQAS Slide) and 16/20 (In House slide) using this method.

Primary Antibody:	Ventana Pathway 790-2991 (4B5) , 16 Mins
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.5
HMAR:	Ventana CC1 mild
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB, Time 1: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins

Gastric HER2 IHC - Method 3

Participant scored 19/20 (UK NEQAS Slide) and 16/20 (In House slide) using this method.

Primary Antibody:	Ventana Pathway 790-2991 (4B5) , 16 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 36mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

Gastric HER2 IHC - Method 4

Participant scored 16/20 (UK NEQAS Slide) and 20/20 (In House slide) using this method.

Primary Antibody:	Dako Link HercepTest SK001 (poly)
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB
Detection:	Dako HerCep Test Autor (SK001)

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	Gold Standard	Second Antibody
Antigens Assessed:	Cyclin D1	BCL6
Tissue Sections circulated:	Reactive Tonsil and Mantle Cell Lymphoma	Reactive Tonsil and Follicular Cell Lymphoma
Number of Registered Participants:	222	
Number of Participants this Run	209 (94%)	

Introduction

Gold Standard: Cyclin D1

Cyclin D1 is a 36KDa protein encoded by the CCND1 (bcl-1) gene. It is part of the cyclin family, and functions as a regulator of Cyclin-dependent kinases (CDKs) CDK4 and CDK6., whose activity is required for cell cycle G1/S transition. Mutations, amplification and overexpression of the cyclin D1 gene alters cell cycle progression, and are seen frequently in a variety of tumours, including breast carcinomas, head and neck squamous cell carcinoma and oesophageal cancers. Cyclin D1 is also expressed in some lymphoid neoplasms, and immunohistochemical staining of Cyclin D1 is particularly useful to diagnose mantle cell lymphoma (MCL) and to distinguish this from chronic lymphocytic leukaemia, small lymphocytic lymphomas and other non-Hodgkin lymphomas (Chan et al., Swerdlow et al.). This diagnosis is a very important one to make due to the tumour's unresponsiveness to treatment and it's prognosis.

Features of Optimal Immunostaining (Figs 1, 2, 4 & 6):

- Moderate to strong staining in the squamous epithelium of the tonsil.
- Nuclear staining in the majority of tumour cells of the MCL.
- Characteristically the staining will feature a mixture of strongly, moderately and weakly stained cells.
- Minimal background staining.

Features of Sub-optimal Immunostaining (Figs 3 & 5):

- Very weak staining of the tonsil squamous epithelial cells or in the tumour nuclei of the MCL.
- Uneven staining.
- Diffuse or predominantly cytoplasmic staining.
- Non-specific or background staining.

References:

1. Chan JKC. Expert opinion: Immunostaining for cyclin D1 and the diagnosis of mantle cell lymphoma: is there a reliable method? Histopathol1999; 34:7-20.
2. Swerdlow SH, Williams ME. From centrocytic to mantle cell lymphoma: A clinicopathologic and molecular review or 3 decades. Hum pathol 2002; 33:7-20.

Second Antigen: BCL-6

BCL-6 (B-Cell CLL/Lymphoma 6, zinc finger protein 51) is a 95 kDa zinc-finger transcription factor. It is required for germinal centre formation and is involved in the differentiation of normal germinal centre B-cells. BCL-6 acts a sequence-specific repressor of transcription and is a suppressor of p53 expression (Phan and Dalla-Favera). As an immunohistochemical marker, BCL-6 is used alongside a panel of other lymphoma markers to determine lymphomas of B-cell origin: it is most commonly detected in germinal centre neoplasms such as follicular lymphoma, diffuse large B-cell lymphoma, Burkitt's lymphoma and lymphocyte predominant Hodgkin's lymphoma (Cattoretti et al., Skinnider et al., Wlodarska et al., Raible et al.) BCL-6 expression is absent in acute lymphatic leukaemia and mantle cell lymphoma.

Features of Optimal Immunostaining (Figs 7, 8 & 11)

- Moderate to strong nuclear staining of all germinal centre B-cells in the distributed tonsil and lymph node (follicular lymphoma).
- Clean background with no non-specific staining

Features of Sub-optimal immunostaining (Figs 9, 10 & 11)

- Weak, uneven, or partially missing staining of relevant cells
- Poor/diffuse nuclear localisation.
- High background or non-specific staining of cell types not expected to stain.

References:

1. RT Phan and R Dalla-Favera. The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells,. Nature 2004; 432: 635-639.
2. G Cattoretti et al. BCL-6 protein is expressed in germinal-centre cells. Blood. 1995;86: 45-53.
3. BF Skinnider et al. BCL-6 and BCL-2 protein expression in diffuse large B-cell lymphoma and follicular lymphoma: correlation with 3q27 and 18q21 chromosomal abnormalities. Hum Pathol. 1999; 30: 803-808.
4. I Wlodarska et al. Frequent occurrence of BCL6 rearrangements in nodular l lymphocyte predominance Hodgkin lymphoma but not in classical Hodgkin lymphoma. Blood Jan 15; 101(2): 706-10.

Assessment Summary:

After carrying out an internal audit of participants scores for the **Cyclin D1** assessment, we noted a higher than expected 'unacceptable' rate for the NEQAS distributed Cyclin D1 'L' slides. Even though the NEQAS samples were tested prior to distribution, it was only after assessing that the team agreed that there may have been some fixation issues with one of the NEQAS distributed samples, which had an adverse effect on samples stained by labs predominantly using the Leica Bond automated platforms. A new set of Cyclin D1 slides were therefore distributed to all participating labs to stain for a re-assessment. There was a great improvement in the staining on the newly distributed slides, particularly with the Leica Bond users, however, the overall acceptable pass rate was still lower than the previous assessment: 68% received an acceptable pass, compared to 85% in the previous run (110). A further 19% received a borderline pass and 27 labs (13%) failed the assessment. The reason for failure was mostly due to very weak staining, which was mostly noted in the UK NEQAS MCL tumour, but also observed in the tonsil. Many borderline scores showed weak or background staining, but were still considered diagnostic. The participants' in house tissue scored slightly higher than the NEQAS distributed samples. Again, weak staining was the main reason for failure on the in-house tissue, but also many sections were of poor tissue quality. Most labs provided either tonsil or a MCL tumour as their in house control, but it was good to see that many labs are now using both a tonsil and MCL multi-tissue control. Similarly to the previous Run (110) the most popular clone was the SP4, and in particular the Labvision (Thermo Sci) SP4 antibody, which showed an acceptable pass rate of 60%. The Ventana rabbit monoclonal antibody SP4-R was also very popular, used by 41 participants and showed an acceptable pass rate of 95%. It was noted that even on the

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second set of UK NEQAS distributed slides, the Leica P2D11F11 clone showed very weak staining on all the commercially available platforms. This has also been an observation also noted on previous Cyclin D1 assessments.

The secondary antibody, **BCL-6,** showed higher pass rates than the Cyclin D1 assessment with 79% receiving an acceptable pass and a further 12% achieving a borderline. 18 labs (9%) failed the assessment, and again the main reason for failure or a borderline pass was due to weak staining on both the UK NEQAS distributed tonsil and FCL. Several slides showed excessive background staining (example illustrated in Fig 10). The participants' in-house controls showed slightly higher pass rates than the NEQAS distributed slides: 81% received an acceptable pass, a further 16% achieved a borderline score, and only 5 labs (2%) failed the assessment. The main reasons for failure on the in-house tissue was due to excessive inappropriate background or non-specific staining. Borderline scores were mostly due to weak staining. The Dako PG-B6p clone was the most popular choice of antibody, which showed an acceptable pass rate of 77%. The Leica/ Novocastra LN22 and the Ventana 1GI191E/A8 clones were

Selected Images showing Optimal and Sub-optimal Immunostaining

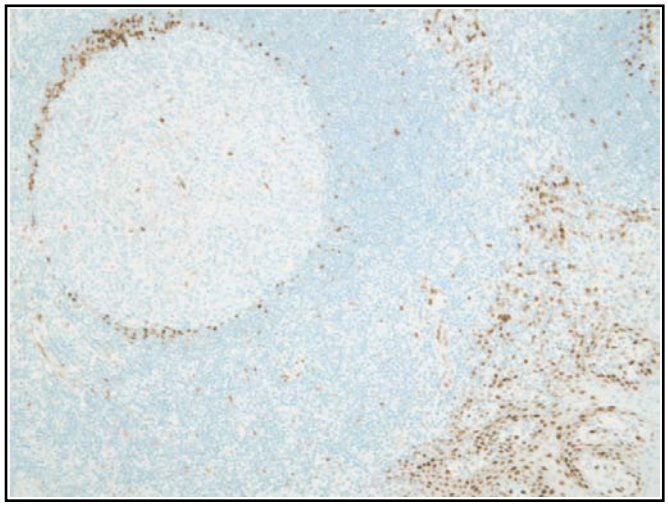


Fig 1. Optimal demonstration of Cyclin D1 in the UK NEQAS reactive tonsil section. The squamous epithelial cells show moderate to strong nuclear staining and there are a few endothelial cells staining in the germinal centre. Section stained with the LabVision SP4 antibody, 1:50 on the Ventana ULTRA CC1 retrieval for 64 minutes.

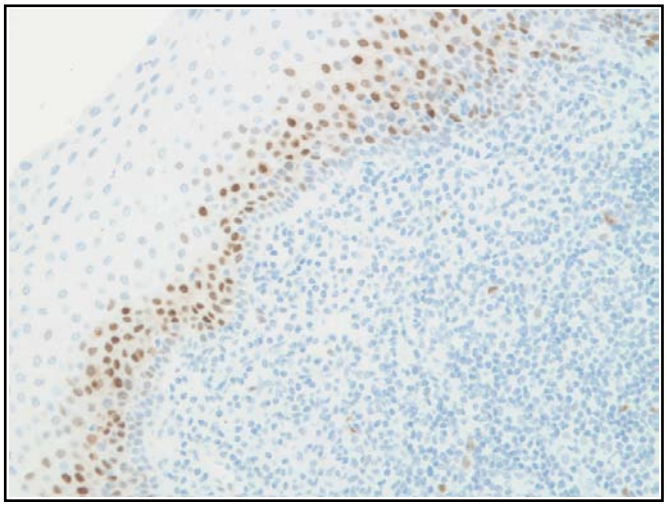


Fig 2. Good demonstration of Cyclin D1 on the UK NEQAS reactive tonsil section, showing moderate to strong nuclear staining of the suprabasal squamous epithelial cells. Section stained with the Dako EP12 RTU antibody, on Dako Autostainer 48 with pretreatment in the PT link.

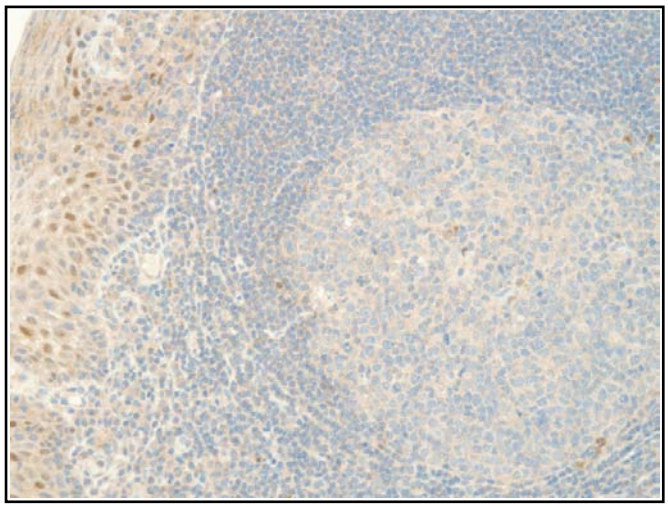


Fig 3. Suboptimal staining of Cyclin D1 on the UK NEQAS distributed tonsil section. The demonstration is weak with background staining. The Dako DCS-6 antibody was used, 1:100, on the Leica Bond III ER2 pre-treatment for 20 minutes.

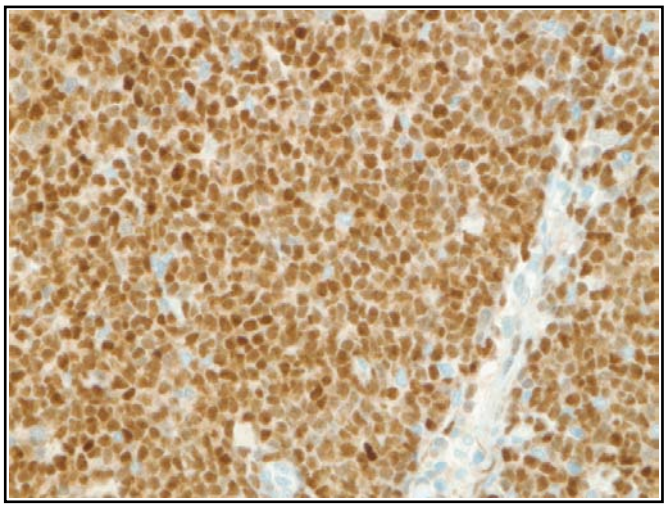


Fig 4. Optimal staining for Cyclin D1 on the UK NEQAS distributed mantel cell lymphoma (MCL): Virtually all of the tumour cells show strong nuclear staining. Stained with the Ventana SP4-R antibody on the Benchmark ULTRA with CC1 retrieval for 64 minutes.

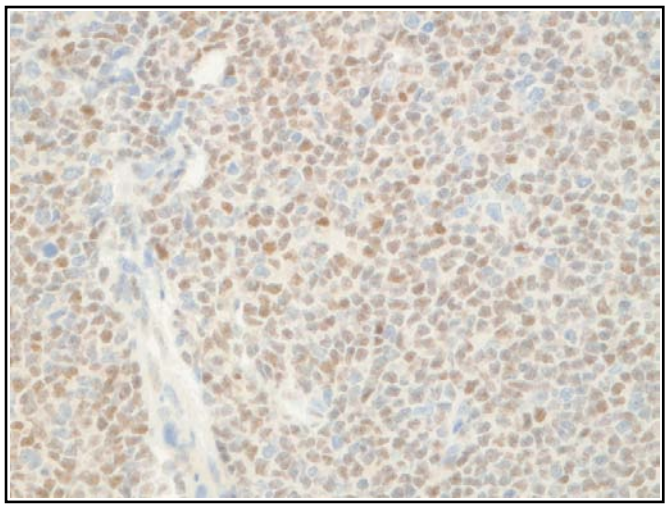


Fig 5. Sub-optimal demonstration of Cyclin D1 on the UK NEQAS distributed mantel cell lymphoma. Tumour cells are demonstrated but the intensity of staining is much weaker than expected (compare to Fig 4). Weaker staining is most likely due to lack of antigen retrieval. Stained with the Leica P2D11F11 antibody on the BondMax and no pre-treatment.

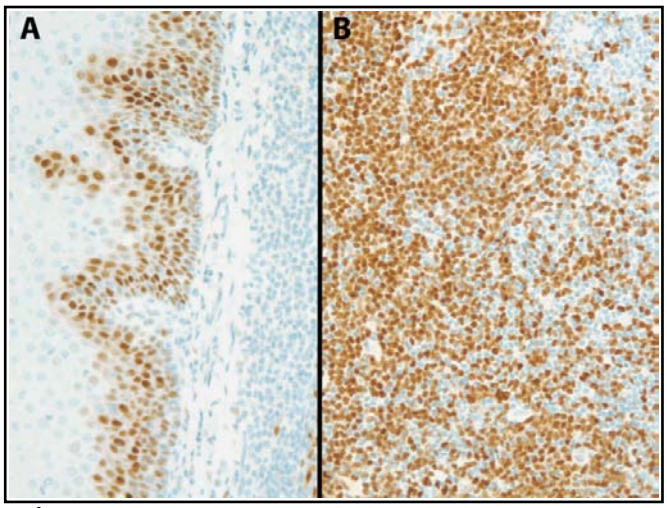


Fig 6. Good example of an in house control stained with Cyclin D1. The composite control contained both tonsil (A) and MCL (B) tissue and both sections stained optimally. Section stained with the Ventana SP4-R pre-diluted antibody on the Benchmark ULTRA, CC1 retrieval for 56 minutes.

Selected Images showing Optimal and Sub-optimal Immunostaining

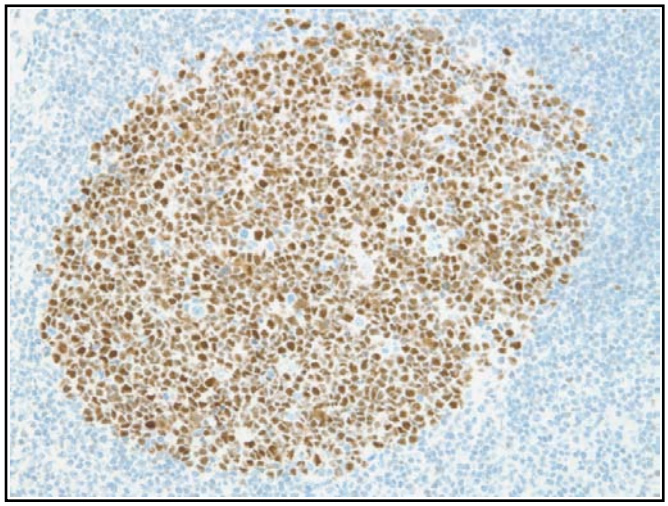


Fig 7. Optimal demonstration of BCL6 in the UK NEQAS distributed reactive tonsil. The section shows strong, distinct nuclear staining in virtually all the germinal centre B-cells and no background staining. Section stained with the Dako PG-B6p RTU antibody on the Dako Omnis with antigen retrieval for 30 minutes and FLEX detection.

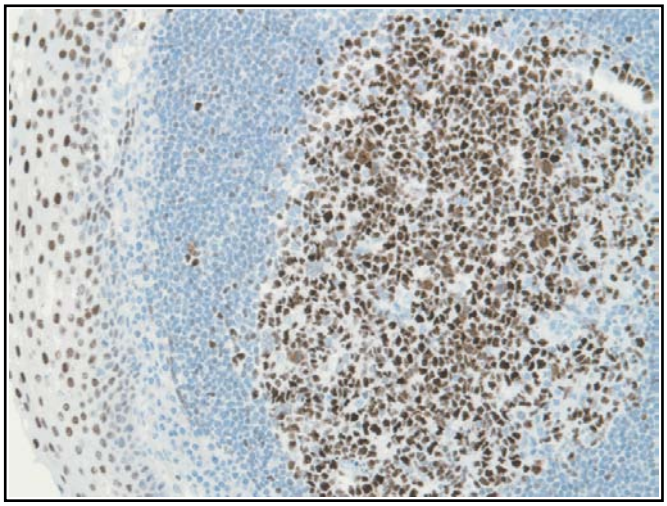


Fig 8. Good demonstration of BCL6 on the UK NEQAS distributed reactive tonsil. The section shows strong and distinct nuclear staining of the germinal centre B-cells, while the squamous epithelial cells show weak to moderate staining. Stained with the Dako PG-B6p antibody, 1:25, on the Dako Autostainer with retrieval in the PT link for 20 minutes, high pH buffer.

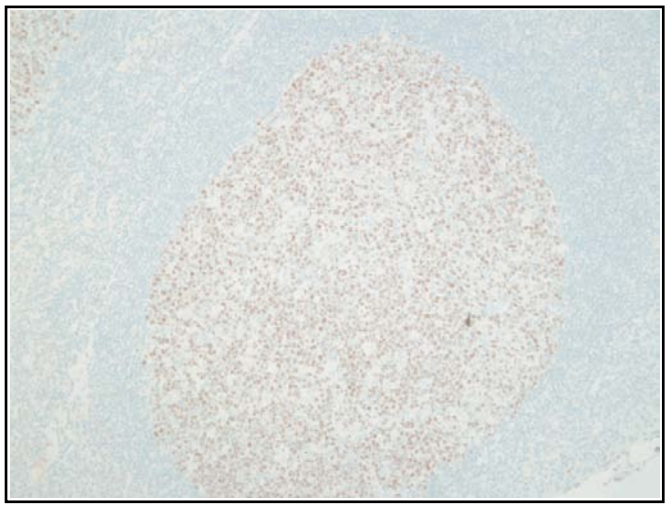


Fig 9. Poor demonstration of BCL6 in the UK NEQAS distributed reactive tonsil. Although the majority of germinal centre cells expected to stain are demonstrated, the staining is too weak (compare to Figs 7&8). Section stained with the Cell Marque G1191E/A8 antibody on the Leica BondMax with ER1 retrieval for 10 minutes.

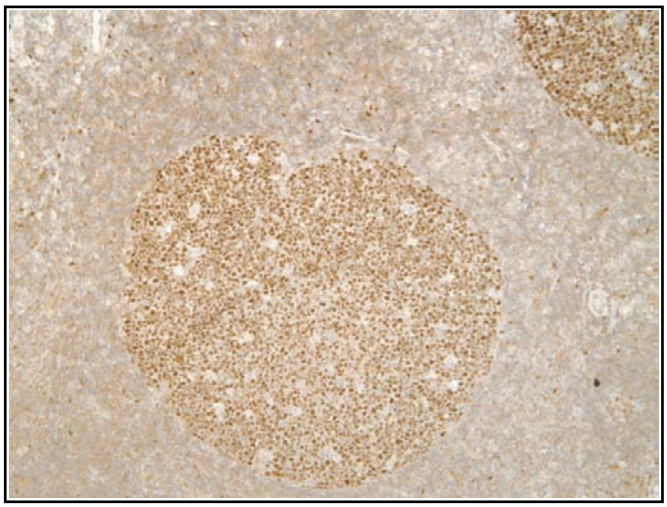


Fig 10. Sub-optimal demonstration of BCL6 on the UK NEQAS distributed reactive tonsil, showing excessive background staining. Stained with the Cell Marque G1191E/A8 antibody, 1:2000, on the Dako Autostainer and antigen retrieval for 26 minutes in the Labvision PT module (citrate buffer).

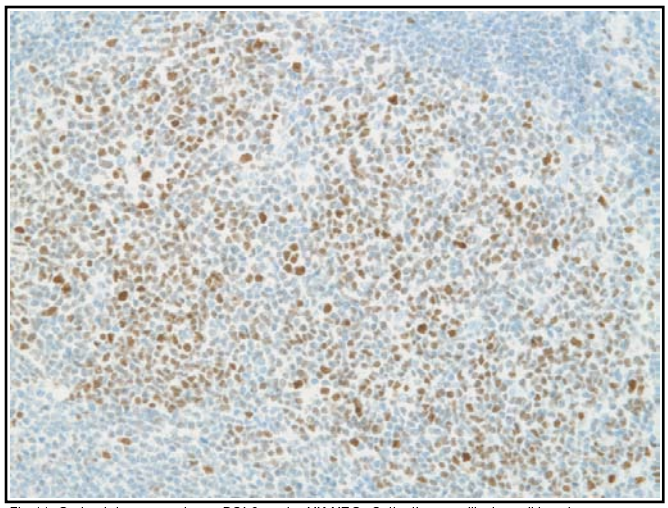


Fig 11. Optimal demonstration of BCL6 on the UK NEQAS distributed follicular cell lymphoma tissue. Most of the neoplastic cells show moderate to strong nuclear staining, while the background remains clean. Same protocol as Fig 7.

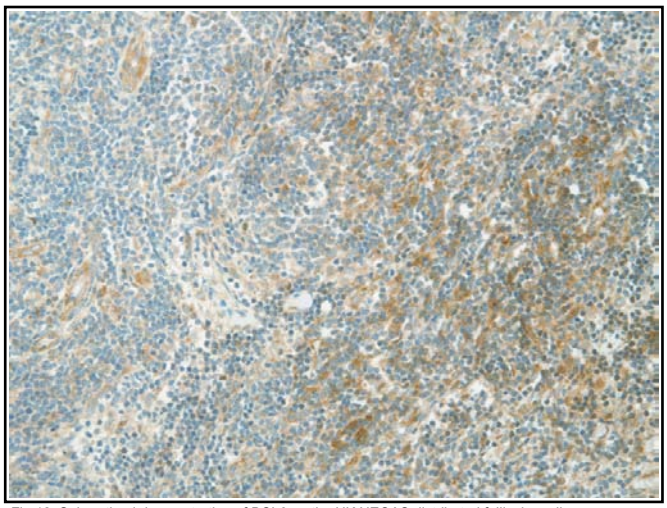
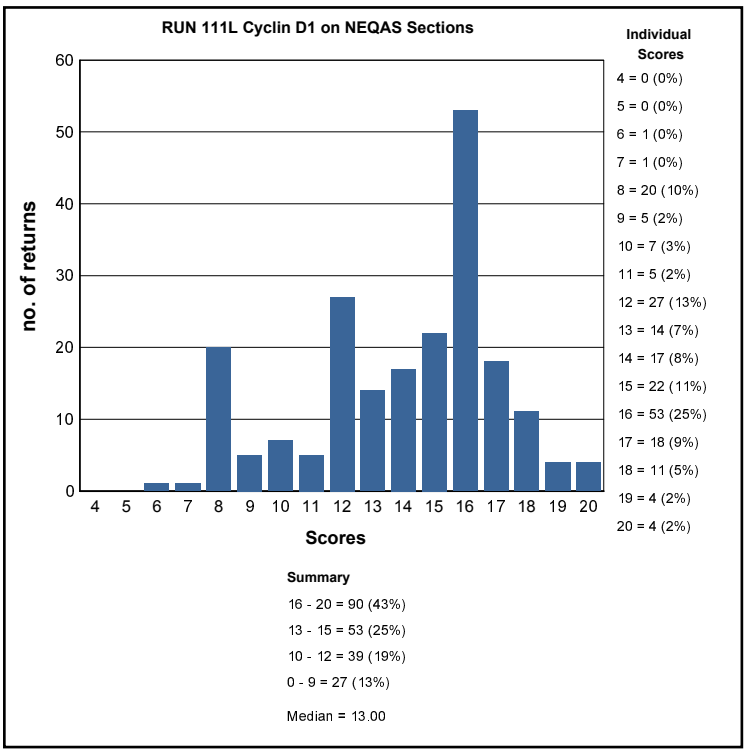
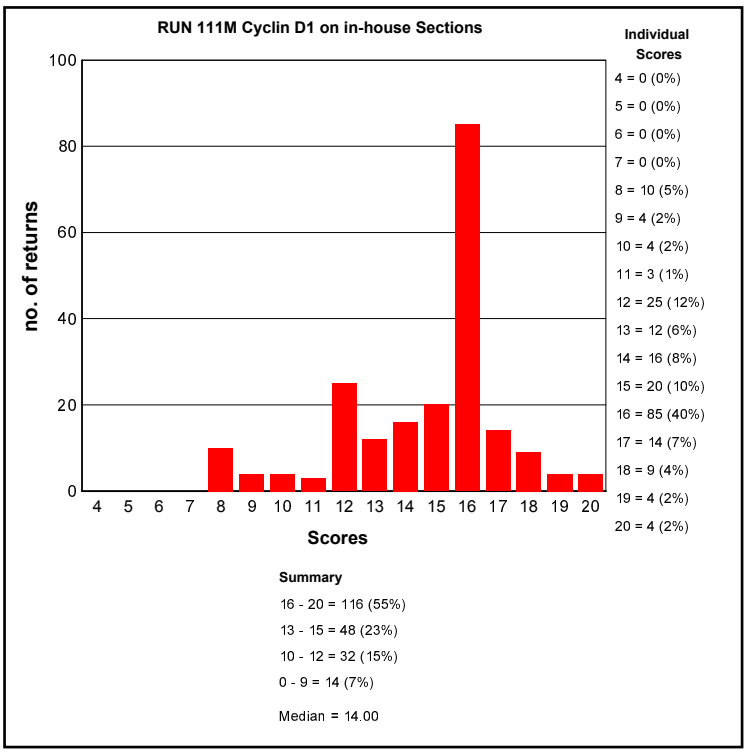
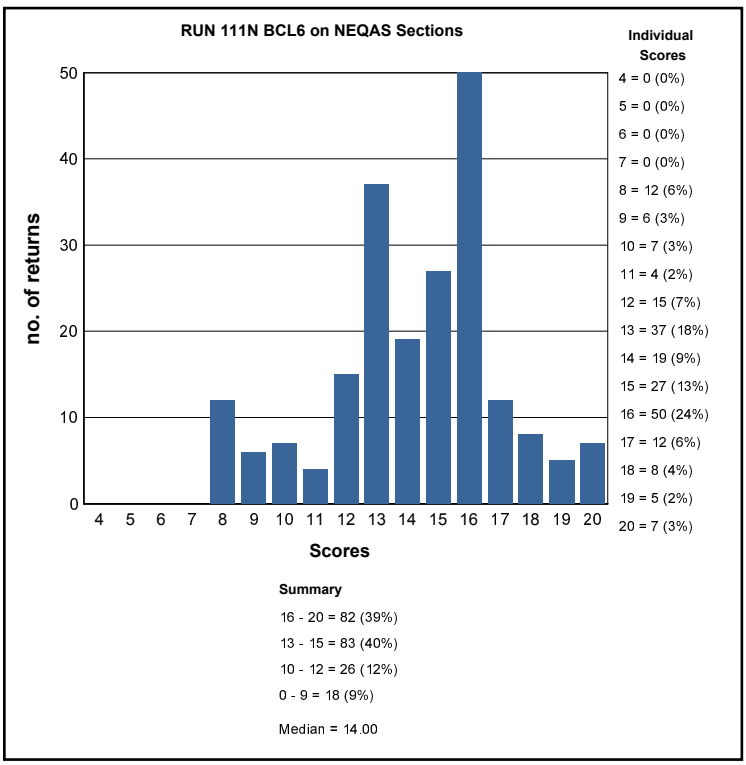


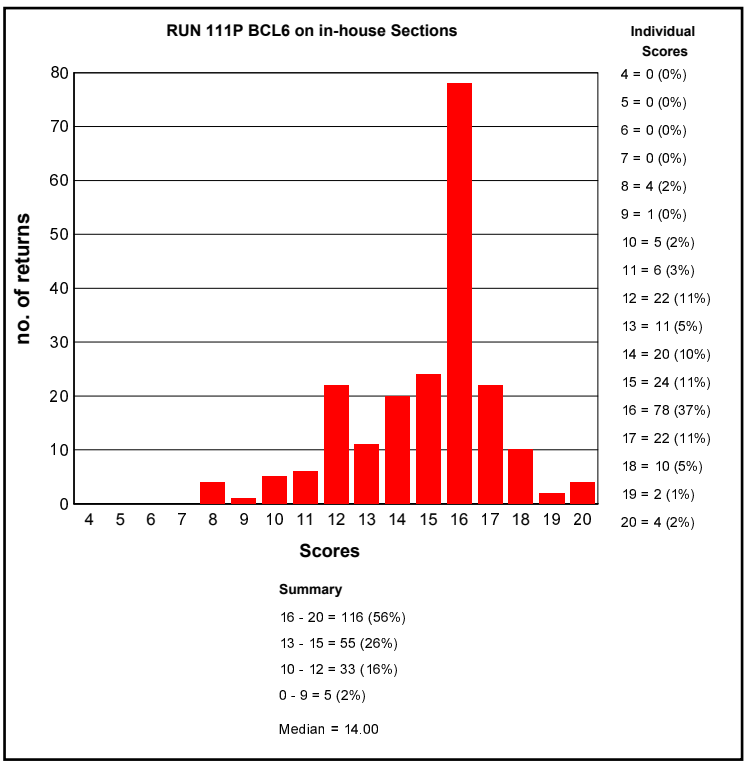
Fig 12. Sub-optimal demonstration of BCL6 on the UK NEQAS distributed follicular cell lymphoma. The sections shows inappropriate non-specific staining, making it difficult to determine the true specific staining of B-cells. Same protocol as Fig 9.

GRAPHICAL REPRESENTATION OF PASS RATES









ANTIBODIES AND OTHER TECHNICAL PARAMETERS EMPLOYED BY PARTICIPANTS IN THE LYMPHOMA MODULE

The following tables record the number of participants (N) using each primary antibody and the percentage (%) of these participants achieving acceptable staining (scores 12/20) on UK NEQAS sections.

Lymphoma Run: 111

Primary Antibody : Cyclin D1

Antibody Details	N	%
AbCam SP4 16663	1	100
Cell Marque (RBT14) CRC011	1	100
Cell Marque (SP4) 241-R	10	50
Dako (DCS-6) M7155	1	0
Dako (EP12) M3642	17	59
Dako (SP4) M3635	2	50
Dako FLEX RTU (SP4) IR152	1	100
Dako RTU (EP12) IR083/IS083	18	89
Lab Vision/Thermo Sci MS 210P (DCS-6)	1	0
LabVision/Thermo Sci (SP4) RM-9104	63	60
Leica/Novo (P2D11F11) NCL-Cyclin D1-GM-CE	7	0
Leica/Novo RTU (P2D11F11) NCL-RTU-CyclinD1	2	0
Menapath (SP4) MP-307	2	100
NeoMarkers/Thermo Sci (EPR2241IHC) RM-2113	10	60
Neomarkers/Thermo Sci MS 210 PO (DC5-6)	2	50
Other	13	54
Vector rbm VP-RM03 (SP4)	5	60
Ventana (SP4-R) 790-4508	41	95
Ventana 250-2723 (P2D11F11)	1	100
Ventana rbm 760-4282 (SP4)	5	100

Lymphoma Run: 111

Primary Antibody : BCL6

Antibody Details	N	%
Cell marque 227M-94/95/96/97/98	14	71
Dako BCL6 M7211 (PG-B6p)	59	64
Dako RTU FLEX Link IR625 (PG-B6p)	9	67
Dako RTU Omnis (PG-B6p) GA625	3	100
Labvision Bcl-6 Ab-2 (BL6,02) mm	1	100
Leica RTU PA0204 (LN22)	14	64
Novocastra NCL-BCL-6 (P1F6) mm	4	100
Novocastra NCL-L-BCL-6-564 (LN22) mm	50	96
Other	4	50
Ventana 760-4241 (1GI191E/A8)	43	91

Lymphoma Run: 111

Heat Mediated Retrieval	BCL6 N	BCL6 %	Cyclin D1 N	Cyclin D1 %
_Ventana Benk ULTRA CC1 (Stan.)	1	100	0	0
Dako Omnis	5	100	4	75
Dako Pascal	1	100	1	0
Dako PTLink	28	64	25	76
Lab vision PT Module	2	50	2	0
Leica ER1 10 mins	1	0	0	0
Leica ER1 20 mins	1	100	1	100
Leica ER1 30 mins	1	0	1	0
Leica ER1 40 mins	0	0	1	0
Leica ER2 10 mins	1	0	1	100
Leica ER2 20 mins	42	76	27	37
Leica ER2 30 mins	22	82	27	41
Leica ER2 40 mins	5	80	10	40
Microwave	1	100	2	50
None	0	0	1	100
Other	0	0	1	0
Pressure Cooker	4	100	2	50
Steamer	1	100	1	0
Ventana CC1 20mins	0	0	1	100
Ventana CC1 24mins	1	100	0	0
Ventana CC1 32mins	8	100	10	90
Ventana CC1 40mins	3	67	7	100
Ventana CC1 48mins	3	100	2	100
Ventana CC1 52mins	2	100	0	0
Ventana CC1 56mins	4	75	3	100
Ventana CC1 64mins	28	93	25	92
Ventana CC1 72mins	2	100	0	0
Ventana CC1 76mins	3	67	3	100
Ventana CC1 80mins	1	100	0	0
Ventana CC1 88mins	1	100	0	0
Ventana CC1 8mins	0	0	1	100
Ventana CC1 92mins	3	67	3	33
Ventana CC1 extended	6	67	4	50
Ventana CC1 mild	3	67	10	100
Ventana CC1 standard	18	72	25	84
Ventana CC2 48mins	1	100	0	0
Ventana CC2 64mins	0	0	2	100
Ventana CC2 standard	1	100	0	0
Water bath 95-98 OC	0	0	2	100

Lymphoma Run: 111

Enzyme Mediated Retrieval	BCL6 N	BCL6 %	Cyclin D1 N	Cyclin D1 %
AS PER KIT	1	100	2	50
NOT APPLICABLE	86	85	101	65
Other	0	0	1	100

Lymphoma Run: 111	BCL6		Cyclin D1	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	15	73	15	67
BioGenex SS Polymer (QD 420-YIKE)	1	100	1	100
Dako EnVision FLEX (K8000/10)	2	50	6	83
Dako EnVision FLEX+ (K8002/12)	15	80	15	67
Dako Envision HRP/DAB (K5007)	2	100	4	50
Dako Envision+ HRP mouse K4004/5/6/7	3	33	0	0
Dako Envision+ HRP rabbit K4008/9/10/11	0	0	2	100
LabVision UltraVision LP HRP (TL 125 HLJ)	1	100	0	0
Leica Bond Polymer Define (DS9713)	1	0	1	0
Leica Bond Polymer Refine (DS9800)	64	73	61	39
MenaPath X-Cell Plus (MP-XCP)	1	100	0	0
None	1	100	1	100
NOT APPLICABLE	1	100	3	67
Other	9	89	7	43
Ventana iView system (760-091)	2	100	4	75
Ventana OptiView Kit (760-700)	37	95	34	100
Ventana UltraView Kit (760-500)	46	76	53	81

Lymphoma Run: 111	BCL6		Cyclin D1	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	1	100	1	100
Dako Autostainer Link 48	26	65	22	77
Dako Autostainer plus	3	67	3	33
Dako Autostainer Plus Link	3	67	3	100
Dako Omnis	4	100	4	75
LabVision Autostainer	2	100	4	25
Leica Bond Max	45	64	32	28
Leica Bond X	0	0	1	0
Leica Bond-III	33	91	38	50
Menarini - Intellipath FLX	1	100	0	0
None (Manual)	3	100	2	0
Other	0	0	1	100
Ventana Benchmark GX	5	80	3	67
Ventana Benchmark ULTRA	52	90	59	93
Ventana Benchmark XT	30	73	35	80

Lymphoma Run: 111	BCL6		Cyclin D1	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	27	78	25	76
BioGenex Liquid DAB (HK153-5K)	1	100	1	100
DAKO DAB+	1	0	2	50
Dako EnVision Plus kits	1	100	3	100
Dako FLEX DAB	24	75	21	76
Dako REAL EnVision K5007 DAB	3	100	4	50
Leica Bond Polymer Refine kit (DS9800)	66	76	62	39
menapath xcell kit DAB (MP-860)	1	100	0	0
NOT APPLICABLE	0	0	2	50
Other	12	83	13	77
Sigma DAB (D5905)	1	100	1	0
Ventana DAB	19	100	20	95
Ventana iview	2	100	1	100
Ventana Ultraview DAB	49	76	54	81
Vision BioSystems Bond X DAB	1	0	0	0

BEST METHODS - Gold Standard Antibody

A selection from just a few of the best methods employed by participants

Cyclin D1 - Method 1

Participant scored 20/20 (UK NEQAS Slide) and 18/20 (In House slide) using this method.

Primary Antibody: AbCam SP4 16663 , 32 Mins, 37 ºC Dilution 1: 100
Automation: Ventana Benchmark ULTRA
Method: Ventana UltraView DAB
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 64mins
EAR: NOT APPLICABLE
Chromogen: Ventana Ultraview DAB, 37 ºC., Time 1: 12 Mins, Time 2: 12 Mins
Detection: Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC Prediluted

Cyclin D1 - Method 2

Participant scored 17/20 (UK NEQAS Slide) and 19/20 (In House slide) using this method.

Primary Antibody: Dako (EP12) M3642 , 40 Mins, 21 ºC Dilution 1: 100
Automation: Dako Autostainer Link 48
Method: Dako FLEX kit
Main Buffer: Dako FLEX wash buffer, PH: 7.6
HMAR: Dako PTLink, Buffer: HIGH PH TARGET RETRIEVAL SOLUTION, PH: 9
EAR:
Chromogen: Dako FLEX DAB, 21 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection: Dako Envision+ HRP rabbit K4008/9/10/11 , 15 Mins, 21 ºC Prediluted

Cyclin D1 - Method 3

Participant scored 20/20 (UK NEQAS Slide) and 16/20 (In House slide) using this method.

Primary Antibody:	Ventana (SP4-R) 790-4508
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

Cyclin D1 - Method 4

Participant scored 18/20 (UK NEQAS Slide) and 16/20 (In House slide) using this method.

Primary Antibody:	LabVision/Thermo Sci (SP4) RM-9104 , 16 Mins, 37 ºC Dilution 1: 50
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	Ventana OptiView Kit (760-700)

BEST METHODS - Secondary Antibody

A selection from just a few of the best methods employed by participants

BCL6 - Method 1

Participant scored 20/20 (UK NEQAS Slide) and 17/20 (In House slide) using this method.

Primary Antibody:	Novocastra NCL-L-BCL-6-564 (LN22) mm , 15 Mins, RT ºC Dilution 1: 1/40
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), Time 1: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, RT ºC Prediluted

BCL6 - Method 2

Participant scored 20/20 (UK NEQAS Slide) and 20/20 (In House slide) using this method.

Primary Antibody:	Dako BCL6 M7211 (PG-B6p) , 30 Mins, 23 ºC Dilution 1: 25
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: Target retrieval solution High pH, PH: 9
EAR:	
Chromogen:	Dako EnVision Plus kits, 23 ºC., Time 1: 10 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 15 Mins, 23 ºC Prediluted

BCL6 - Method 3

Participant scored 20/20 (UK NEQAS Slide) and 19/20 (In House slide) using this method.

Primary Antibody:	Dako BCL6 M7211 (PG-B6p) , 15 Mins, r/t ºC Dilution 1: 20
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 30 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

BCL6 - Method 4

Participant scored 20/20 (UK NEQAS Slide) and 18/20 (In House slide) using this method.

Primary Antibody:	Novocastra NCL-L-BCL-6-564 (LN22) mm , 32 Mins, 36 ºC Dilution 1: 100
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.4
HMAR:	Ventana CC1 32mins
EAR:	NOT APPLICABLE
Chromogen:	Other, 37 ºC., Time 1: 8 Mins, Time 2: 4 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 37 ºC Prediluted

Neil Bilbe

	Gold Standard	Second Antibody
Antigens Assessed:	Synaptophysin	GFAP
Tissue Sections circulated:	Cerebellum and GBM (oligo)	Glioblastoma and brain cortex.
Number of Registered Participants:	63	
Number of Participants this Run	60 (95%)	

Introduction

Gold Standard: Synaptophysin

Synaptophysin is a 38kDa integral synaptic vesicle glycoprotein with four transmembrane domains. It is present in neuroendocrine cells and in virtually all neurons in the brain and spinal cord involved in synaptic transmission. Synaptophysin is also detected in the epithelium of the choroid plexus, adrenal cortical cells, goblet cells and Paneth cells (Wiedenmann et al). The antibody is a useful marker of a wide spectrum of neuroendocrine tumours, including neuroblastomas, ganglio-neuroblastomas, ganglioneuromas, gangliogliomas, pheochromocytomas, and paragangliomas (Gould et al). Neuroendocrine neoplasms of epithelial type are also detected, including pancreatic islet-cell neoplasms, medullary thyroid carcinomas, pituitary and parathyroid adenomas, bronchopulmonary and gastrointestinal tract carcinoids. Synatposhysin also stains positive for adrenal cortical adenomas.

Features of Optimal Immunostaining:

- Intense, punctate staining around the neuron perikarya.
- Intense staining in normal cerebellum.
- Clean background.

Features of Sub-optimal Immunostaining:

- Weak or uneven staining.
- Poor localisation of antigen
- Excessive background staining, or non-specific staining of cell types or components not expected to stain.
- Tissue destruction due to harsh heat induced antigen retrieval.

References:

1. Wiedenmann B, et al. Synaptophysin: A marker protein for neuroendocrine cells and neoplasms. Proc Natl Acad Sci 1986; 83:3500-4.
2. Gould VE et al. Synaptophysin expression in neuroendocrine neoplasms as determined by immunocytochemistry. Am J Pathol 1987; 126:243-57.
3. Kwon SE, Chapman ER. Synaptophysin regulates the kinetics of synaptic vesicle endocytosis in central neurons. Neuron 2011:70:847-85.

Second Antigen: GFAP

Glial Fibrillary Acidic Protein (GFAP) is a 50kDa intermediate filament protein that is expressed by numerous cell types of the central nervous system (CNS) including astrocytes and ependymal cells but not in glial cells (Jacque *et al*). Functionally, GFAP is thought to provide structural stability and shape to astrocytic processes (Eng *et* al). Outside the CNS, GFAP may be demonstrated in Schwann cells, enteric glia cells, salivary gland neoplasms, metastasising renal carcinomas, papillary meningiomas and breast myoepithelial cells. GFAP is also known to be an important protein involved in repair after CNS injury caused by trauma, genetic disorders or chemicals, where it is upregulated and forms a glial scar. Conversely, there is a progressive loss of GFAP production with increasing astrocyte malignancy, hence, fewer tumour cells stain positive for GFAP in malignant astrocytomas than in less malignant astrocytomas and normal brain. GFAP is also important in the identification of many other glial tumours and the presence of gliosis: Glioblastoma and oligodendroglioma are usually positive for GFAP, whereas ganglioglioma, primitive neuroectodermal tumours and plexus carcinoma show varying levels of GFAP staining. GFAP is also expressed in Scwannomas, neurofibromas, chondromas and pleomorphic adenomas (Viale *et al*).

Features of Optimal Immunostaining:

- Intense, specific staining in the perikarya of tumour cells.
- Clean background with no non-specific staining.

Features of Sub-optimal Immunostaining:

- Weak or uneven staining of glioma cells.
- Diffuse, poorly-localised staining.
- Excessive background or non-specific specific staining.

References:

1. **Jacque CM, et al.** Determination of glial fibrilary acidic protein (GFAP) in human brain tumours. 1978; J Neuro Sci 35 (1): 147-55.
2. **Eng LF, et al.** Glial fibrillary acidic protein:GFAP-thrity-one years 1969-2000. Neurochem Res 2000;25:1439-51.
3. **Viale G, et al.** Glial fibrillary acidic protein immunoreactivity in normal and diseased human breast. Virchows Arch A Pathol Anat 1991; 418: 339-48.

Assessment Summary:

There were 63 active labs; three EU labs did not submit at all, and another had subsequently withdrawn from the scheme. This left a total of 240 slides for assessment, 60 for each antigen: **G, H, J, and K.** So effectively all participants submitted all their NEQAS and in-house sections. It appears that this has increased from previous runs. Whether this is a result of labs having to place their in-house section onto the Control/Patient slides is difficult to gauge. Of the 120 in-house samples submitted (**H & K)** six (5%) were on a separate slide, received from 4 individual labs. These were due to section lifting, the size of section and basically forgetting to do so.

Of the 240 sections assessed 91.3% (219) passed, 6.7% (16) were borderline, and 2.1% (5) failed: ≤ 9/20. Two were on the NEQAS Gold (Synaptophysin), one on the 2nd antigen (GFAP), and two on the in-house 2nd antigen **(K)**. There were no borderline or failed in-house synaptophysin slides **(H).**

The two Golds **(G)** slides both failed due to very weak or absence of staining. The 1° antibodies employed were; a Dako M0776, SY38 clone (total 4 users), diluted 1:300, with ER2 for 20 min on a Bond Max; and a Novacastra NCL-L-SYNAP-299 (total 10 users), 27G12 clone, diluted 1:100, with CC1 for 32 mins on a Ventana Benchmark XT*

The GFAP slide **(J)** used a Sigma monoclonal GA5 clone (1 other user); the stated dilution was 1:15000, stained for 92 mins, with a CC1 for 8 mins, (see figure 11). This participant also failed the Synaptophysin* assessment, albeit using a more common antibody, employed by 17% of labs, in contrast to the sigma GFAP primary used by only two labs (3%).

In-house control material:

Both the failed in-house GFAP slides **(K)** were on a piece of appendix, but the samples used were of poor quality and considered unsuitable as a choice of control material.

58% of participants submitted a sample of neurological origin, or considered to be an appropriate choice, for a synaptophysin control **(H)**. Conversely only 3 labs did not send in a neurological sample for the GFAP **(J)**; two were appendixes (failed), and one was a section of eye globe (borderline), the rest (95%) were mostly cortex or cerebellum.

Selected Images showing Optimal and Sub-optimal Immunostaining

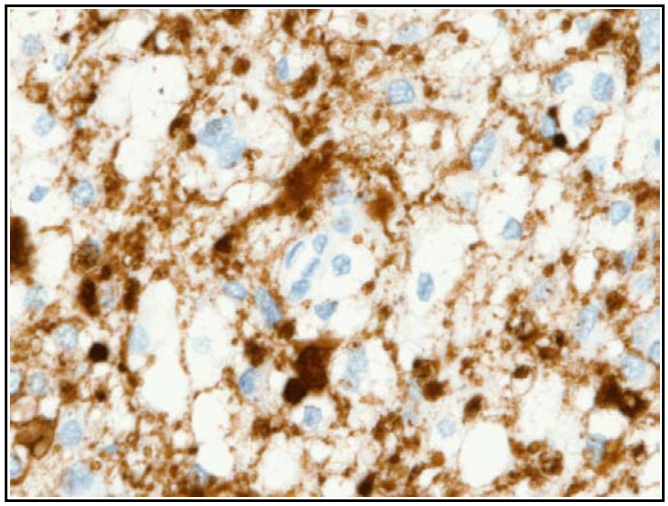


Fig 1. Excellent demonstration of synaptophysin on the NEQAS tumour sample. Glioma cells are nicely contrasted, the counterstain intensity is optimal, and the background is clean. Dako antibody, clone SY38, 1:50, 20 mins, with a low pH Dako Omnis RT, and the RTU Dako OMNIS Flex for 10 mins.

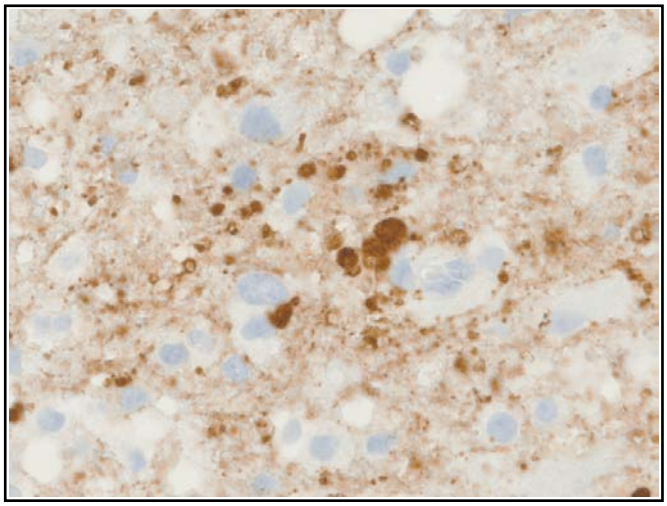


Fig 2. Sub-optimal synaptophysin demonstration on NEQAS sample. The tumour is weakly stained, and is patchy. This slide was assessed as borderline. Dako Clone DAK-SYNAP, prediluted, 20 mins, a Dako PT Link 20 mins, with the RTU Dako EnVision FLEX+ for 20 mins, on a Dako Autostainer Link 48.

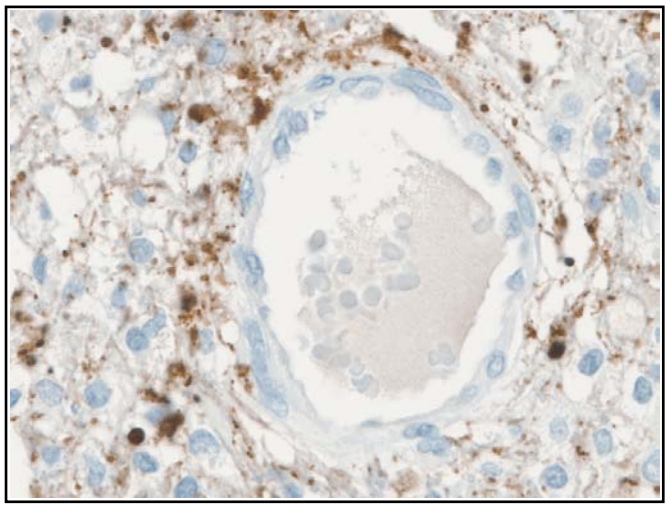


Fig 3. Sub-optimal synaptophysin staining, some tumour cells are either weakly stained or not demonstrated at all. One would expect to see more glioma cells around the vessel. Novocastra NCL-SYNAP-299 (27G12), 1:50, with a Ventana CC1 mild, the Ventana UltraView Kit, on the Ventana Benchmark XT.

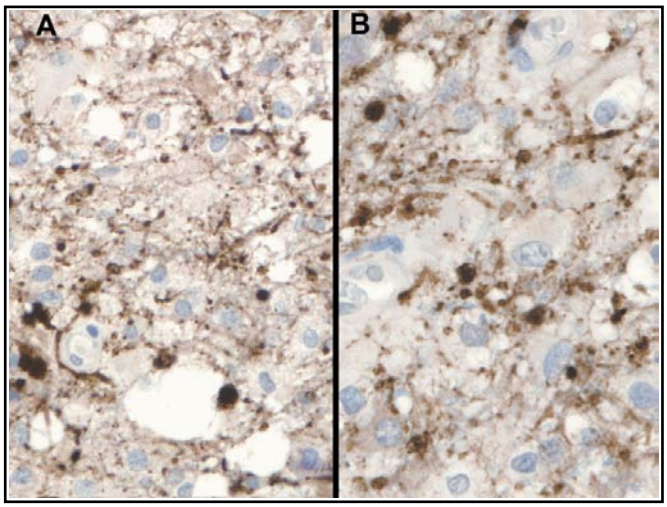


Fig 4. Good staining of the NEQAS oligodendroglioma sample, but unfortunately there is some background staining, shown at low (A) and high (B) power. Overall this was assessed as a low pass. Dako polyclonal, 1:200, for 15 mins; no RT; Leica Bond Polymer Refine RTU, 8 min; on a Leica Bond-III.

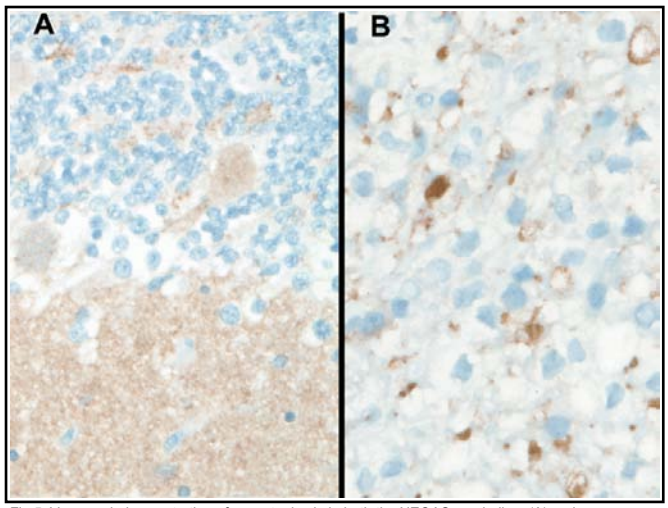


Fig 5. Very weak demonstration of synaptophysin in both the NEQAS cerebellum (A) and tumour (B), This slide received a low borderline score. RTU Ventana CONFIRM SP-11, 32 mins, with Ventana CC1 for 24 mins, using a Ventana Optiview kit and the Ventana Benchmark ULTRA.

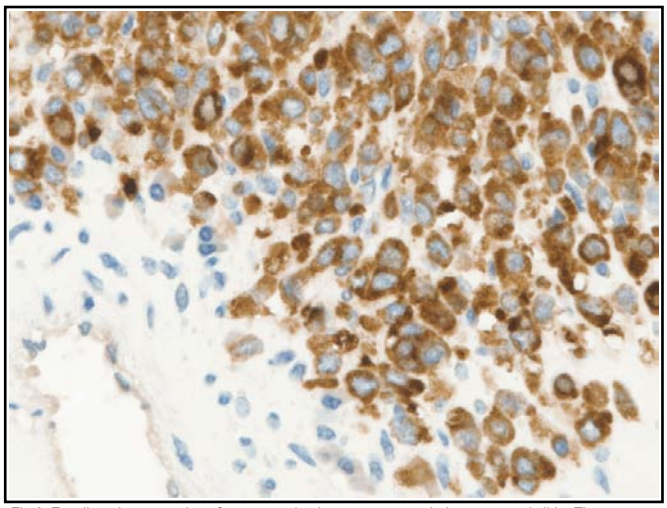


Fig 6. Excellent demonstration of a neuroendocrine tumour on an in-house control slide. The negative stroma and positive tumour cells are nicely contrasted. Novocastra synaptophysin, clone 27G12, 1:10, 15 mins; Leica ER1 20 mins; Leica Bond Polymer Refine, 8 mins; on a Leica Bond-III.

Selected Images showing Optimal and Sub-optimal Immunostaining

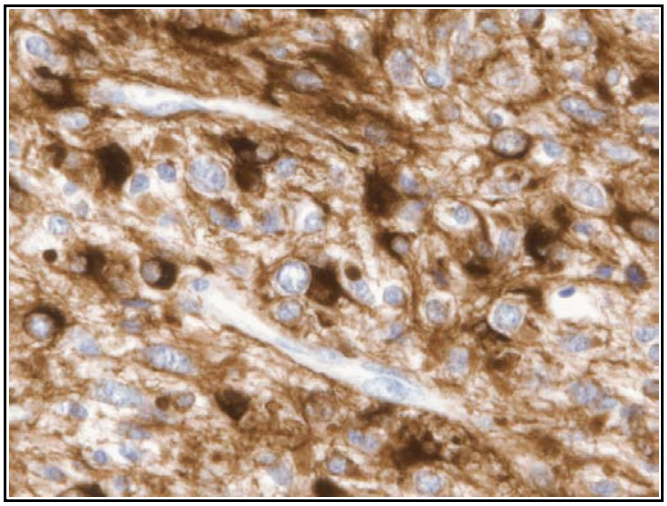


Fig 7. Optimal demonstration of GFAP on the NEQAS tumour section. The counterstain is of the correct intensity which aids the contrast. Dako polyclonal, 1:500; no RT; with the Leica Bond Polymer Refine (DS9800); on the Leica Bond Max.

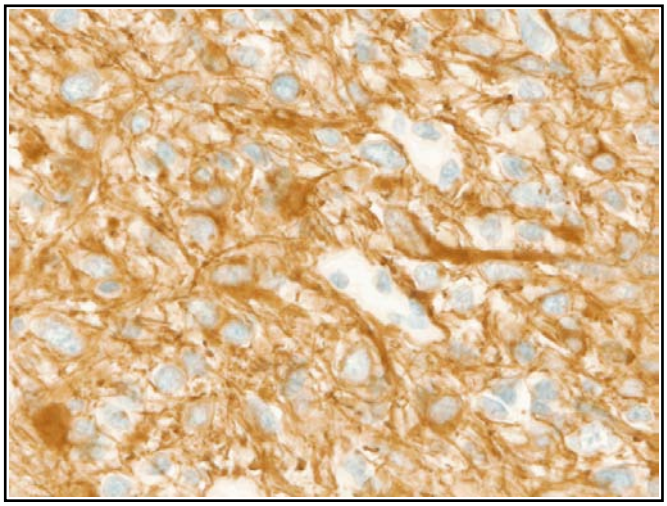


Fig 8. Sub-optimal GFAP demonstration on the NEQAS tumour tissue. Although still adequate for diagnosis, the staining is weak (see fig 7) and bland. Dako Z0334 polyclonal, 1:3000, 32 mins; hot Ventana CC1 for 64 mins; RTU Ventana UltraView Kit, 12 mins; on the Ventana Benchmark ULTRA.

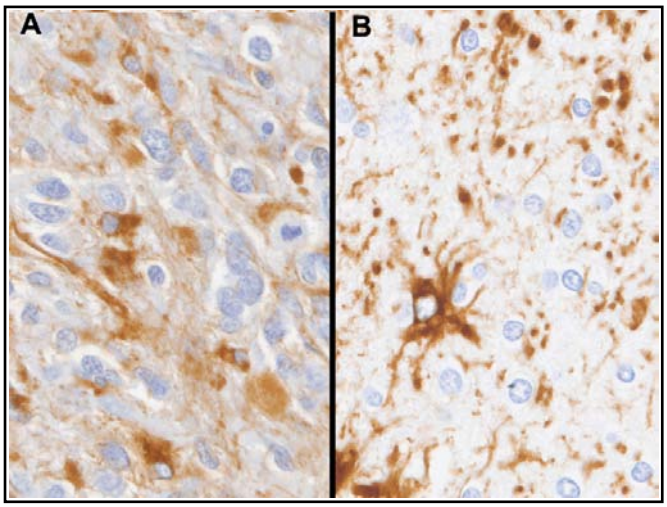


Fig 9. Weak GFAP staining on the NEQAS tumour (A) and brain (B) sections. This slide was assessed as borderline for diagnostic purposes. Dako polyclonal (as fig 7, 8) but no dilution given; no RT; using a Dako EnVision FLEX+ (K8002/12) kit, on the Dako Autostainer Link 48.

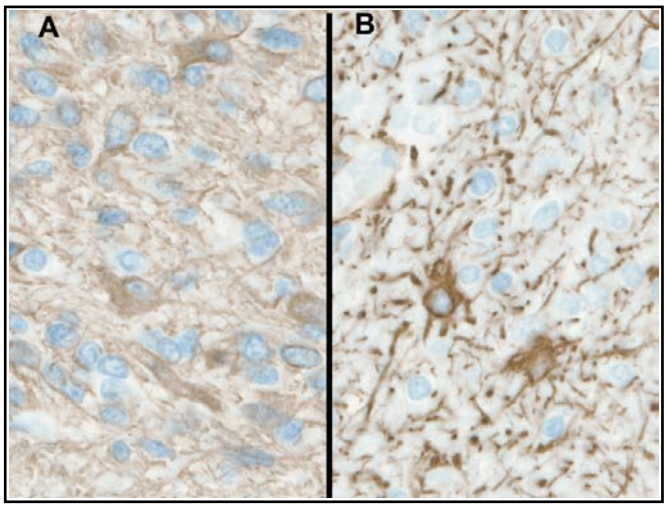


Fig 10. Interesting GFAP result on the NEQAS tumour (A) and brain (B) sections. Although adequately stained, this participant employed a monoclonal primary: Dako M0761 (6F2), 1:400, 20 mins; Dako PTLink; Dako EnVision FLEX kit, 20 mins; with a Dako FLEX DAB chromogen, giving this grey colouration.

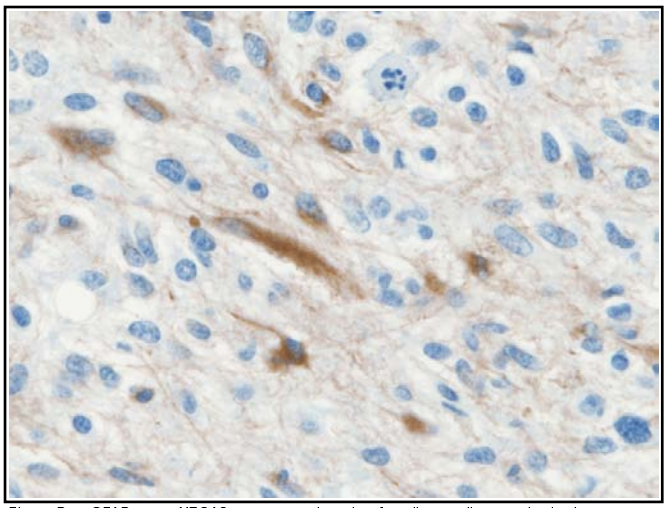


Fig 11. Poor GFAP on the NEQAS tumour sample; only a few glioma cells are stained, with the majority negative. This failed the assessment. Sigma G3895 (GA5) monoclonal, stated dilution 1:15000, for 92 mins; UltraView Kit; Ventana CC1 8 mins; on a Ventana Benchmark XT.

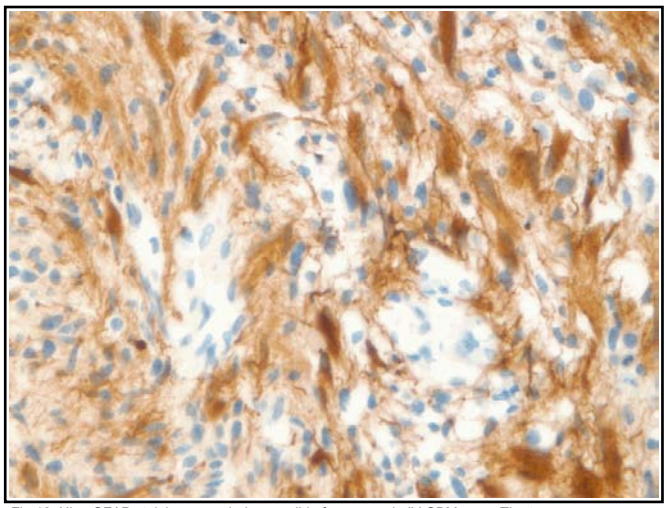
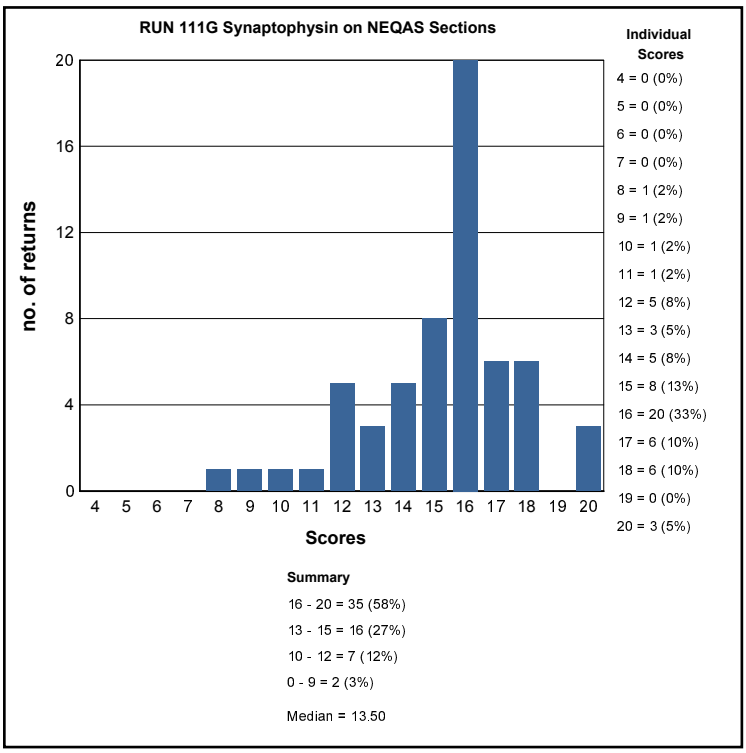
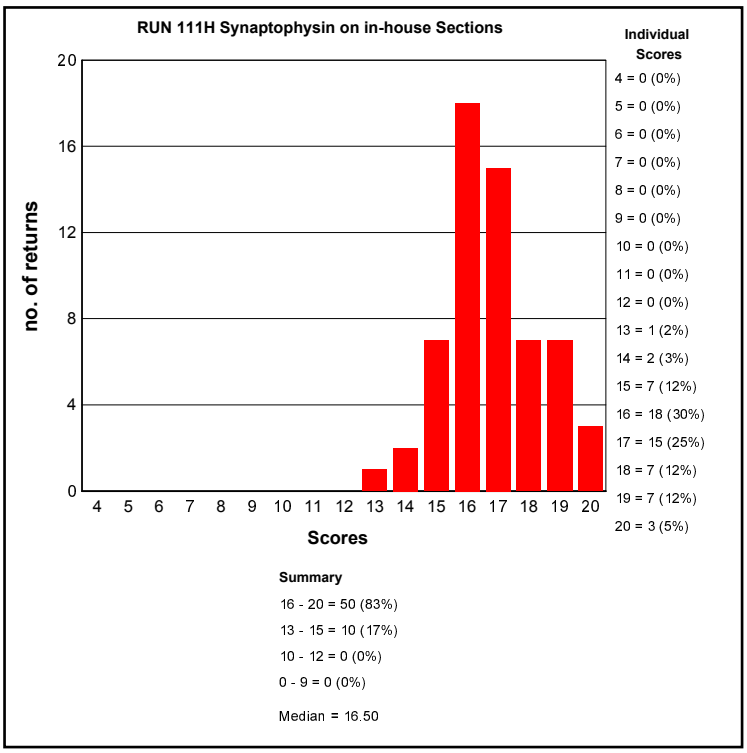
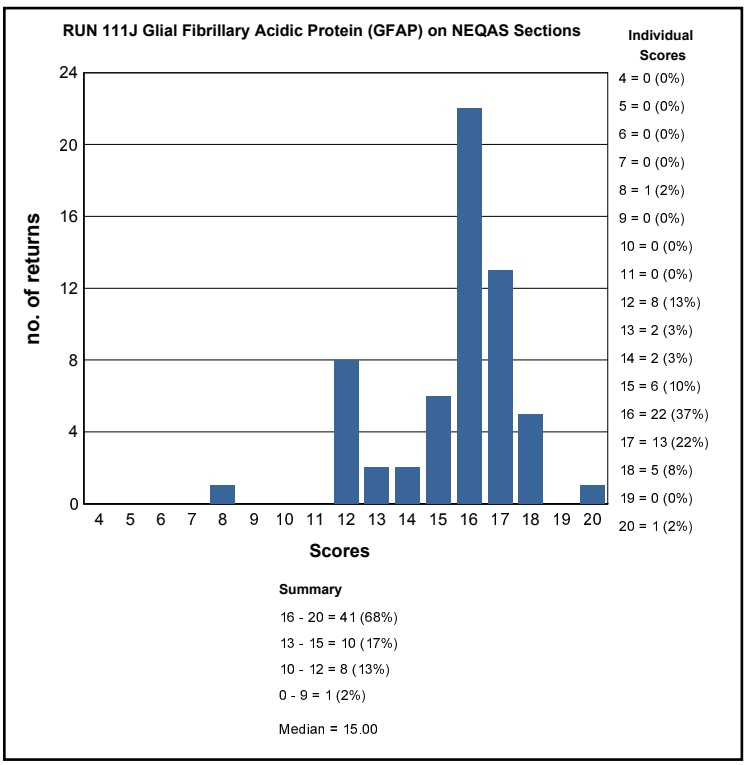


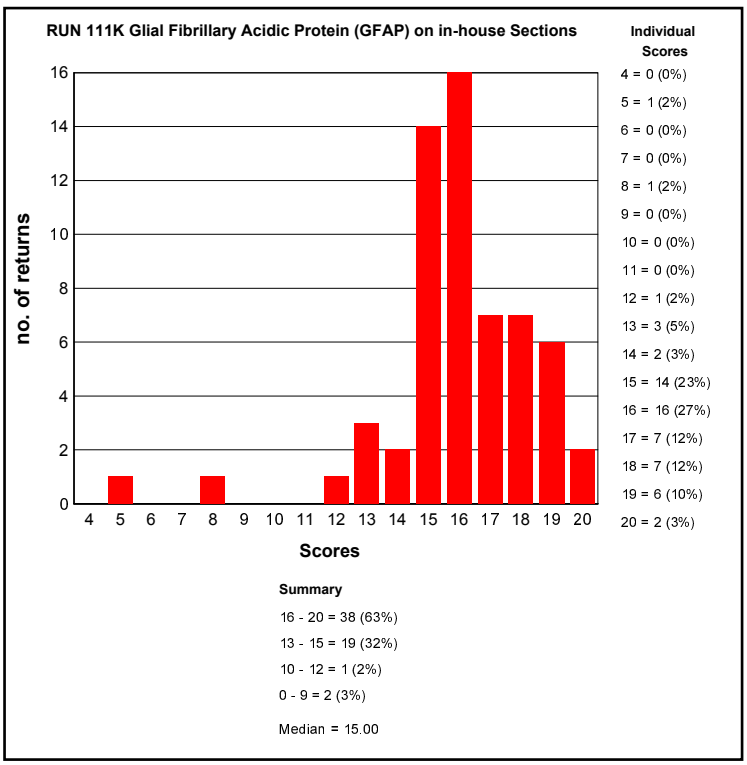
Fig 12. Nice GFAP staining on an in-house slide from a grade IV GBM case. The tumour stands out beautifully, against a clean background. Dako polyclonal, 1:1000; Leica ER1 10 mins; Leica BondMAx Refine KIT; using a Leica Bond Max.

GRAPHICAL REPRESENTATION OF PASS RATES









ANTIBODIES AND OTHER TECHNICAL PARAMETERS EMPLOYED BY PARTICIPANTS IN THE NEUROPATHOLOGY MODULE

The following tables record the number of participants (N) using each primary antibody and the percentage (%) of these participants achieving acceptable staining (scores 12/20) on UK NEQAS sections.

Neuropathology Run: 111

Primary Antibody : Synaptophysin

Antibody Details	N	%
BioGenex AM 6 (clone SNP)	2	100
Biogenex MU 363 UC	1	100
Dako A0010 (polyclonal)	1	100
DAKO FLEX IR776 (SY38)	3	100
Dako M0776 (clone SY38)	4	50
Novocastra Bond RTU PA0299 (rb poly)	2	100
Novocastra NCL-L-SYNAP-299 (27G12)	10	80
Novocastra NCL-SYNAP-299 (27G12)	10	80
Novocastra TRU-SYNAP-299 (27G12)	1	100
Other	14	93
Ventana 760-4595 (MRQ-40)	4	100
Ventana CONFIRM 790-4407 (SP-11)	8	75

Neuropathology Run: 111

Primary Antibody : Glial Fibrillary Acidic Protein (GFAP)

Antibody Details	N	%
Cell Marque (EP672Y) 258R	1	100
Dako IR524 (R Poly)	3	100
Dako M0761 (6F2)	12	75
Dako Z0334 (R Poly)	29	83
Immunon 490740RB	1	100
Novocastra NCL-GFAP-GA5 (GA5)	2	100
Novocastra PA0026 RTU (GA5)	3	100
Other	1	100
Sigma G3895 (GA5)	2	50
Ventana 760-4345 (EP672Y)	6	100

Neuropathology Run: 111

Heat Mediated Retrieval	Glial Fibrillary Acidic Protein (GFAP)		Synaptophysin	
	N	%	N	%
_Leica BondMax ER2	0	0	1	100
Biocare Decloaking Chamber	1	0	1	100
Dako Omnis	1	100	1	100
Dako PTLink	9	89	12	83
Leica ER1 10 mins	2	100	0	0
Leica ER1 20 mins	3	100	3	100
Leica ER1 30 mins	2	100	5	80
Leica ER2 20 mins	5	100	9	78
Leica ER2 30 mins	0	0	1	100
Microwave	2	100	0	0
None	12	83	1	100
Other	1	0	1	100
Ventana CC1 16mins	1	100	0	0
Ventana CC1 20mins	1	100	0	0
Ventana CC1 24mins	0	0	2	0
Ventana CC1 32mins	1	100	3	67
Ventana CC1 36mins	1	100	0	0
Ventana CC1 40mins	1	100	0	0
Ventana CC1 48mins	1	100	1	100
Ventana CC1 52mins	0	0	2	100
Ventana CC1 56mins	0	0	2	100
Ventana CC1 64mins	2	100	6	100
Ventana CC1 8mins	3	33	0	0
Ventana CC1 mild	4	75	3	67
Ventana CC1 standard	4	100	6	100

Neuropathology Run: 111

Enzyme Mediated Retrieval	Glial Fibrillary Acidic Protein (GFAP)		Synaptophysin	
	N	%	N	%
Dako Proteinase K (S3020)	1	100	0	0
NOT APPLICABLE	29	79	36	86
Other	1	100	0	0
VBS Bond Enzyme 1	5	80	0	0
Ventana Protease 1 (760-2018)	3	100	0	0

Neuropathology Run: 111	Glial Fibrillary Acidic Protein (GFAP)		Synaptophysin	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	6	100	8	100
Biocare polymer (M4U534)	1	0	1	100
Dako EnVision FLEX (K8000/10)	2	50	2	100
Dako EnVision FLEX+ (K8002/12)	5	80	5	80
Dako Envision HRP/DAB (K5007)	1	100	1	100
Leica Bond Polymer Refine (DS9800)	19	89	18	83
None	0	0	1	0
Other	1	100	1	100
Vector Elite ABC Kit (PK-7200)	1	100	0	0
Ventana OptiView Kit (760-700)	6	83	9	78
Ventana UltraView Kit (760-500)	18	83	14	86

Neuropathology Run: 111	Glial Fibrillary Acidic Protein (GFAP)		Synaptophysin	
Automation	**N**	**%**	**N**	**%**
Dako Autostainer Link 48	9	89	12	83
Dako Autostainer Plus Link	2	50	0	0
Dako Omnis	1	100	1	100
Leica Bond Max	8	88	7	86
Leica Bond X	1	100	0	0
Leica Bond-III	11	91	13	85
Menarini - Intellipath FLX	1	0	1	100
None (Manual)	2	100	1	100
Ventana Benchmark ULTRA	15	87	15	87
Ventana Benchmark XT	10	80	10	80

Neuropathology Run: 111	Glial Fibrillary Acidic Protein (GFAP)		Synaptophysin	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	9	89	13	92
Dako DAB+ REAL Detection (K5001)	1	100	0	0
Dako EnVision Plus kits	2	100	1	100
Dako FLEX DAB	9	78	7	71
Dako REAL EnVision K5007 DAB	0	0	1	100
Leica Bond Polymer Refine kit (DS9800)	19	89	18	83
Other	3	67	3	67
Ventana DAB	2	100	3	100
Ventana Ultraview DAB	15	80	14	86

BEST METHODS - Gold Standard Antibody

A selection from just a few of the best methods employed by participants

Synaptophysin - Method 1

Participant scored 20/20 (UK NEQAS Slide) and 20/20 (In House slide) using this method.

Primary Antibody: Dako M0776 (clone SY38) , 20 Mins Dilution 1: 50
Automation: Dako Omnis
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako Omnis, Buffer: Dako low pH TRS
EAR:
Chromogen: Dako FLEX DAB, Time 1: 5 Mins, Time 2: 5 Mins
Detection: Other , 10 Mins

Synaptophysin - Method 2

Participant scored 17/20 (UK NEQAS Slide) and 20/20 (In House slide) using this method.

Primary Antibody: Novocastra NCL-L-SYNAP-299 (27G12) , 15 Mins, 24 ºC Dilution 1: 10
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER1 20 mins
EAR:
Chromogen: Leica Bond Polymer Refine kit (DS9800)
Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins, 24 ºC

Synaptophysin - Method 3

Participant scored 18/20 (UK NEQAS Slide) and 20/20 (In House slide) using this method.

Primary Antibody:	DAKO FLEX IR776 (SY38)
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	AS PER KIT
HMAR:	Dako PTLink
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	AS PER KIT

Synaptophysin - Method 4

Participant scored 16/20 (UK NEQAS Slide) and 19/20 (In House slide) using this method.

Primary Antibody:	Ventana CONFIRM 790-4407 (SP-11) , 24 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 48mins, Buffer: CC1
EAR:	NOT APPLICABLE
Chromogen:	Other, 37 ºC., Time 1: 8 Mins, Time 2: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 37 ºC Prediluted

BEST METHODS - Secondary Antibody

A selection from just a few of the best methods employed by participants

Glial Fibrillary Acidic Protein (GFAP) - Method 1

Participant scored 17/20 (UK NEQAS Slide) and 18/20 (In House slide) using this method.

Primary Antibody:	Dako Z0334 (R Poly) , 20 Mins, 20 ºC Dilution 1: 2000
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	None
EAR:	Dako Proteinase K (S3020)
Chromogen:	Dako EnVision Plus kits
Detection:	AS PER KIT

Glial Fibrillary Acidic Protein (GFAP) - Method 2

Participant scored 18/20 (UK NEQAS Slide) and 18/20 (In House slide) using this method.

Primary Antibody:	Dako M0761 (6F2) , 15 Mins, 24 ºC Dilution 1: 500
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER1 20 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, 24 ºC

Glial Fibrillary Acidic Protein (GFAP) - Method 3

Participant scored 18/20 (UK NEQAS Slide) and 16/20 (In House slide) using this method.

Primary Antibody:	Dako Z0334 (R Poly) , 24 Mins Dilution 1: 5000
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 mild
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

Glial Fibrillary Acidic Protein (GFAP) - Method 4

Participant scored 17/20 (UK NEQAS Slide) and 19/20 (In House slide) using this method.

Primary Antibody:	Novocastra NCL-GFAP-GA5 (GA5) , 15 Mins, RT ºC Dilution 1: 1000
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, RT ºC Prediluted

Neil Bilbe and Irena Srebotnik Kirbis

	Gold Standard	Second Antibody
Antigens Assessed:	Melanoma markers	CD3
Sample circulated; cytospins and cell block sections:	Human melanoma, cervical cancer, and bladder tumour cell lines, plus an effusion (CS), FNA and RBCs (CB).	Effusion, human cell lines B & T lymphocytes, carcinoma cells.
Number of Registered Participants:	82: 57 cell block (69.5%) and 25 cytospin (30.5%)	
Number of Participants this Run	81 (99%)	

Introduction

Gold Standard: Melanoma markers

S100 is a multigene family of low molecular weight proteins and is demonstrated in some Langerhans' cells and melanocytes of the skin, interdigitating reticulum cells in lymph nodes, medullary epithelial reticular cells in the thymus, chondrocytes in cartilagenous tissue, adipocytes in some, but not other biopsies, myoepithelial cells in salivary glands and breast, folliculostellate cells of the pituitary gland, and Schwann cells and glial cells of nervous tissue. Weak labelling is found in epithelial cells of the mammary and sweat glands. It stains the majority of malignant melanomas.

Normal adult tissues that exhibit positive staining with anti-melanosome **HMB45,** include melanocytes (fetal and subset melanocytes containing immature melanosomes), retinal pigment epithelia (prenatal and infantile). Positive results aid in the classification of melanomas and melanocytic lesions and also aid in distinguishing metastatic amelanotic melanomas from other poorly differentiated tumours of uncertain origin. Anti-melanosome HMB45 stains most melanomas (excluding desmoplastic), melanocytic atypical hyperplasia, melanocytic neuroectoderm of infancy, angiomyolipoma, and various naevi are also stained by anti-melanoma, HMB45.

The antibody **Melan-A** labels melanocytes and is a useful tool for the identification of melanomas, and if melanoma is ruled out, for adrenocortical carcinomas. Melan-A, isolated as a melanoma-specific antigen, is a transmembrane protein composed of 118 amino acids with uncertain function. Melan-A is expressed in skin, retina and the majority of cultured melanocytes and melanomas, whereas a vast variety of other tissues and cancers do not express Melan-A.

Features of Optimal Immunostaining:

- Intense cytoplasmic staining of tumour cells.
- Clean background.
- No non-specific staining of other cell types
- Adequate nuclear counterstain.

Features of Sub-optimal Immunostaining:

- Weak, uneven or no staining of tumour cells
- Excessive background staining.
- Non-specific staining of cell types or components not expected to stain.
- Excessive or very weak nuclear counterstain.

References

1. Liu K, Dodge R, Glasgow BJ, Layfield LJ. Fine-needle aspiration: Comparison of smear, cytospin, and cell block preparations in diagnostic and cost effectiveness. Diagn Cytopathol. 1998;19:70–4.
2. Dalquen P, Sauter G, Epper R, Kleiber B, Feichter G, Gudat F. Immunocytochemistry in diagnostic cytology. Recent Results Cancer Res. 1993;133:47–80
3. Leung SW, Bedard YC. Immunocytochemical staining on ThinPrep processed smears. Mod Pathol 1996; 9(3): 304–6.
4. Beaty M, Fetsch PA, Wilder AM, Marincola FM, Abati A. Effusion cytology of malignant melanoma. Cancer (Cancer Cytopathol) 1997; 81(1): 57–63.

Second Antigen: CD3

CD3 antigen is a complex of closely related polypeptide chains found on the surface membrane of T-lymphocytes in close physical association with the T-cell receptor (TCR). CD3 appears in the cytoplasm early in T-cell maturation (Wood et al), and then becomes membrane-associated later in cell maturation. Staining is therefore confined to the cytoplasm and/or cell membrane. The most common use of the CD3 antibody is in lymphoma diagnosis as it is present in the majority of mature T-cell neoplasms (90%), although occasionally tumours lose the antigen during the neoplstic process. It is not found in B-cell or non-lymphoid malignancies. There is several polyclonal and monoclonal antibodies now available for formalin fixed paraffin wax-embedded (FFPE) sections. However, the range of antibodies for cytology preparations is much wider, although several antibodies work well on both FFPE and cytology preps.

Features of Optimal Immunostaining:

- Strong, crisp and well-localised staining in the cytoplasm and cell membranes of T-cells.
- Clean background with no staining of the carcinoma cells within the cytological effusion.
- Adequate counter-stain.

Features of Sub-optimal immunostaining:

- Weak, uneven, or less T-cells staining than expected .
- Diffuse staining in the membranes.
- High background or non-specific staining of cell types not expected to stain, including the carcinoma cells within the cytology effusion.
- Excessive or very weak counterstain

References

1. Joel F, Leong W-M, Leong S-Y. Essential markers in malignant lymphoma: A dioagnositc approach. J Histotech 2002; 25(4): 215-227.
2. Wood Km, Pallesen g, Ralfkiaer E, et al. Heterogeneity of CD3 antigen expression in T-cell lymphoma. Hisopthol 1993;22:311-317.

References (cell blocks in cytology)

1. Nithyananda A. Nathan; Cell Block Cytology; Improved Preparation and Its Efficacy in Diagnostic Cytology Am J Clin Pathol 2000;114:599-606

Assessment Summary:

82 active labs were registered for this module; only one lab did not submit any slides at all. Two labs did not submit an in-house melanoma control **(S)**, and there was a slide missing for each of the CD3 antigens **(T & U)**. This resulted in a total of 320 slides for assessment.

The overall number of passed slides was 282 (88.1%), those scored as borderline 28 (8.8%), and a total of 10 slides failed the assessment (3.1%):

	Pass	Bord	Fail	No of slides		Pass	Bord	Fail
R	73	6	2	81	R	90%	7%	2%
S	67	11	1	79	S	85%	14%	1%
T	72	4	4	80	T	90%	5%	5%
U	70	7	3	80	U	88%	9%	4%
Total	282	28	10	320	Average	88.1%	8.8%	3.1%

Samples sent to the participants, both originate from the same supplier . Cytospins are prepared and provided directly by this laboratory. In addition, a single FFPE cell block for each antigen is sent to UK NEQAS ICC & ISH. These blocks are then sub-divided into 2 or 3 smaller blocks before sectioning, and validated prior to dispatch. Samples are stained with *all* appropriate markers and any additional antigens: CK or CD20

Examples of the validation samples sent with the slides:

Melanoma (HMB45, Melan A, or S100)

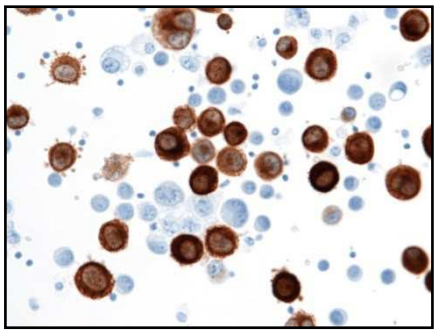


Fig 1: Melan A on cytospin prepared from 111R

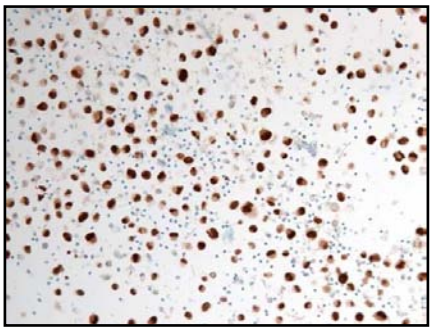


Fig 2: S-100 on cell block prepared from 111R

CD3

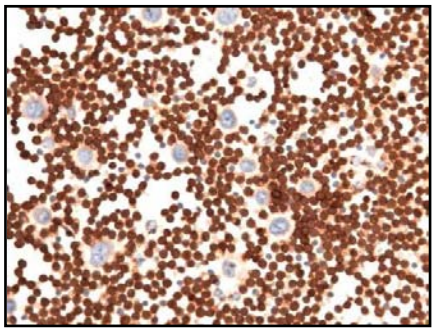


Fig 3: CD3 on cytospin prepared from 111T

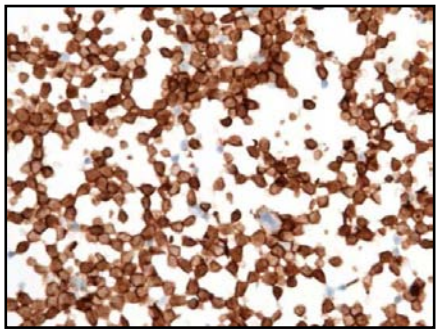


Fig 4: CD3 on cell block prepared from 111T

Assessment outcomes :

There were 10 failed slides from 7 individual laboratories; three participants failed both their NEQAS and in-house CD3 assessment.

Melanoma (R & S)

This was the slightly better of the two antigens, with only two failed NEQAS slides **(R)** and a single in-house control slide **(S)**. The two failed slides both employed an S100 antibody*, one on a CB: Dako poly, 1:2000, with CC1 on the Benchmark Ultra (see fig 4); the second on a CS: Leica Bond RTU, no retrieval, on the Bond Max. The assessors commented that the Dako CB slide had weak staining, with non-specific demonstration of some components, allied with some background as well. The cells on the CS slide had been completely destroyed, although there was no mention of any associated pre-treatment(s).

The single in-house failure **(S)** was a scrappy piece of skin, weakly stained, using a Ventana prediluted, Melan A, MART-1, 1° antibody, with CC1 on the Benchmark Ultra.

* **N.B. for Run 112 we are requesting S100 only**

CD3 (T & U)

N.B. One participant stated that they rarely, if ever, used CD3, even though this was in their repertoire. They had requested a cytospin preparation, and stained it with a Novocastra primary, PS1 clone, diluted 1:200, without retrieval (normal for a cytospin sample) on a Bond III. They had asked in their data entry for some protocol advice. This lab failed both slides.

Interestingly, two other labs also using this antibody, both achieved reasonable results (16/20 each), but both were on FFPE cell block sections, with CC1 retrieval, diluted 1:50, as per the supplier's datasheet. Feedback was sent to the participant as requested. Without changing to cell blocks in the future or introducing a new antibody supplier it is difficult to see how, or if, significant improvement can be made.

The other two participants who failed both CD3 slides, used: 1) Dako rbpoly, 1:50, with ER2, on a Bond III (CB) (see fig 9), and 2) Novocastra (LN10), 1:200, without retrieval, again on a Bond III (CS). The staining on the CB sample (1) was diffuse, with inappropriate staining and excessive background; but on the CS (2) staining tended to be weak and uneven rather than non-specific. The in-house samples for these 3 labs were: a cell block from a pleural effusion, a smear also from an effusion, and lastly a thin prep from a lymph node sample.

The remaining lab that failed just their NEQAS CD3 slide **(T)** also used the LN10 clone but on a CB, 1:100, with ER2, on a Bond Max. In this case some of the tumour cells had stained and there was evidence of excessive pre-treatment.

Numerically, the number of failed cell block and cytospin NEQAS slides was equal; 3 each. Percentage-wise though, this translates to 3% and 6% respectively, due to the request levels for each sample.

In-house controls sample type summary:

	Mel (S)	%	CD3 (U)	%	Average
FFPE	49	62.0%	44	55.0%	**58.5%**
CB	14	17.7%	17	21.3%	**19.5%**
CS	13	16.5%	15	18.8%	**17.7%**
Smear	1	1.3%	3	3.8%	**2.6%**
Thin prep	2	2.5%	1	1.3%	**1.9%**
Totals	**79**	**100.0%**	**80**	**100.0%**	**100.0%**

In comparison to the previous run for Melanoma markers (110), the FFPE numbers have risen 6%, cell block samples have dropped by approximately 5%, and there has been minor changes to the other sample types.

Selected Images showing Optimal and Sub-optimal Immunostaining

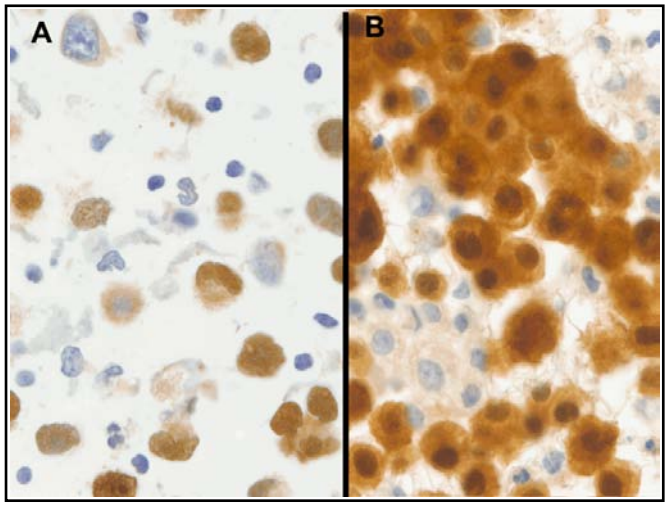


Fig 1. Good demonstration of melanoma on the NEQAS Cell Block (A) and an in-house cell block preparation of a clot from a pleural effusion (B), albeit there is slight background staining. Novocastra RTU S100, for 10 mins, Leica ER1 10 mins, on the Leica Bond-III and the Leica Bond Polymer Refine.

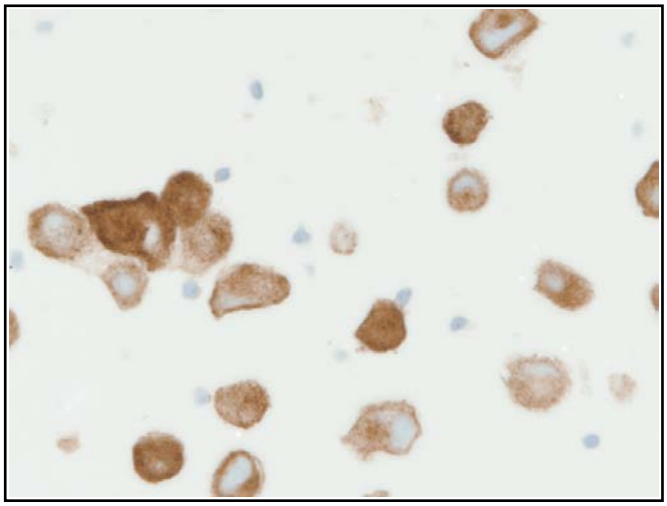


Fig 2. Sub-optimal demonstration on the NEQAS CB section; tumour staining is weak as is the counterstain and some cells are not adequately stained. Novocastra Melan A, 1:20, 48 mins, Ventana CC1 standard, on the Ventana Benchmark XT, detection layer of Ventana UltraView Kit (760-500).

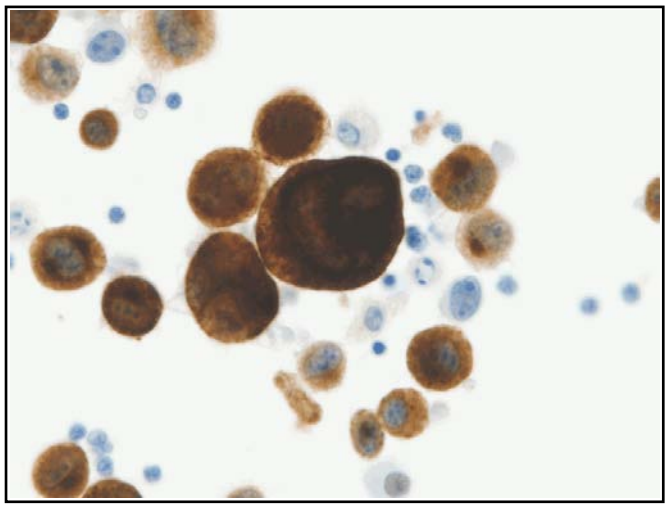


Fig 3. Optimal demonstration of melanoma cells on the NEQAS cytospin (CS). Non-tumour cells are completely unstained and the background is clean. Novocastra Melan A, 1:50, no RT, on Leica Bond-III, with the Leica Bond Polymer Refine kit. No times were given.

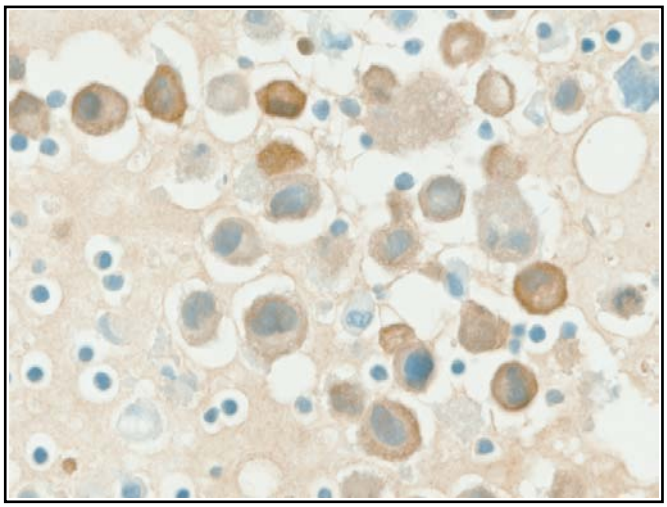


Fig 4. Poor demonstration of melanoma on the NEQAS CB section. The background matrix is all staining, and it was difficult to accurately diagnose on this slide and therefore failed the assessment. Dako poly S100, 1:2000, 32 mins, with Ventana CC1 64mins, on Ventana Benchmark ULTRA and RTU UltraView Kit.

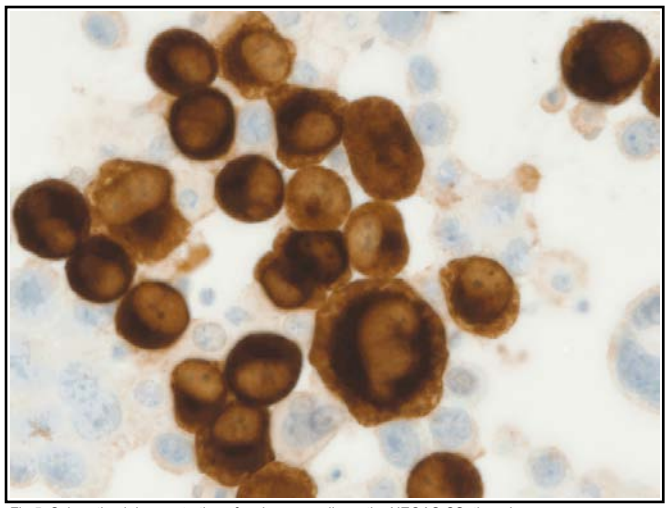


Fig 5. Sub-optimal demonstration of melanoma cells on the NEQAS CS; there is some non-specific staining, although this is adequate for diagnostic purposes. RTU Dako FLEX Melan A, 20 mins, have stated that Dako Omnis RT was employed, and Omnis platform, along with the pre-diluted Flex kit for 20 mins.

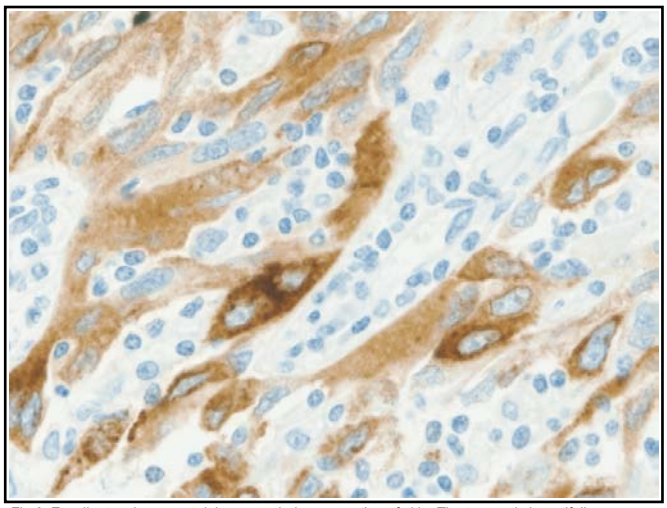


Fig 6. Excellent melanoma staining on an in-house section of skin. The tumour is beautifully demonstrated, counterstain is optimal, and background is clean. Dako monoclonal Melan A, 1:200, 30 mins, Dako PT Link high pH, on Dako Autostainer, with Dako EnVision FLEX+ for 20 mins.

Selected Images showing Optimal and Sub-optimal Immunostaining

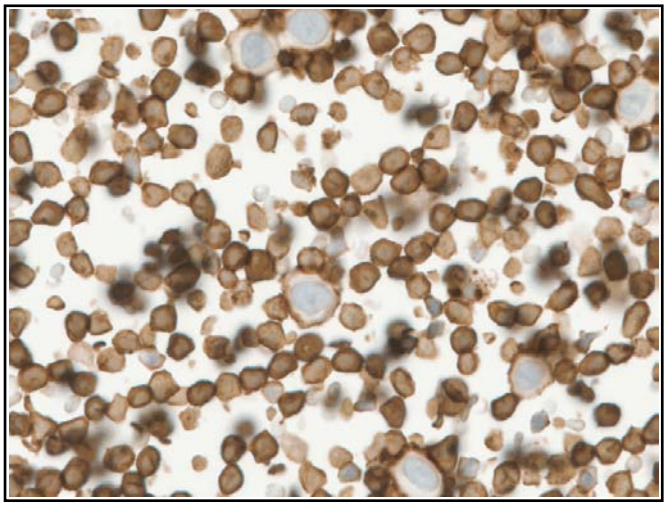


Fig 7. Sub-optimal CD3 demonstration of T-cells on NEQAS CB section. Staining is not crisp, counterstain is weak, and there is some non-specific staining. Novocastra RTU (LN10), Leica ER2 20 mins, on the Leica Bond-III, with the Leica Bond Polymer Refine. No times were given.

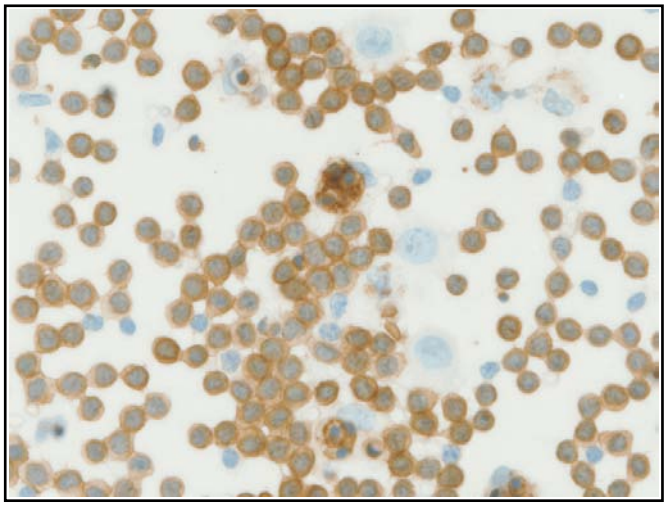


Fig 8. Sub-optimal CD3 demonstration on NEQAS CS sample; staining is slightly weak, and there is a hint of inappropriate staining, but assessed to be still of good quality (compare fig 10). Novocastra CD3 LN10, 1:50, 32 mins, no RT, on Ventana Benchmark ULTRA, with RTU Ventana UltraView Kit, 8 mins at 37 C.

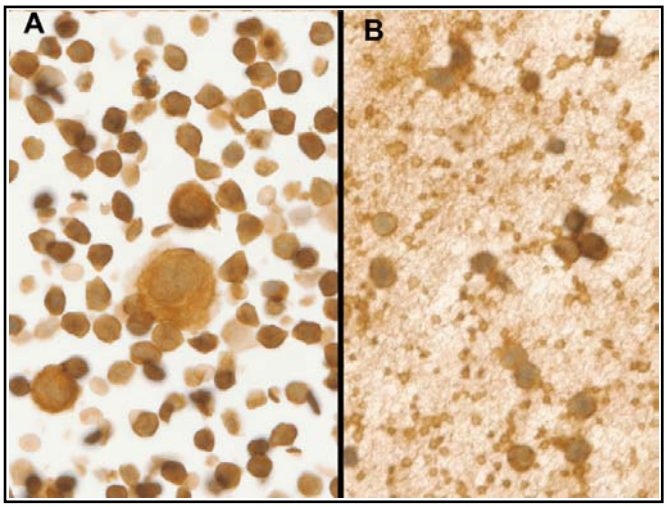


Fig 9. Poor demonstration of CD3 on both the NEQAS (A) and in-house pleural effusion (B) CB sections. All cells are stained in both preparations, and both these slides failed the assessment. Dako poly, 1:50, 15 mins, with Leica ER2 20 mins, on Leica Bond-III, and the Leica Bond Polymer Refine kit.

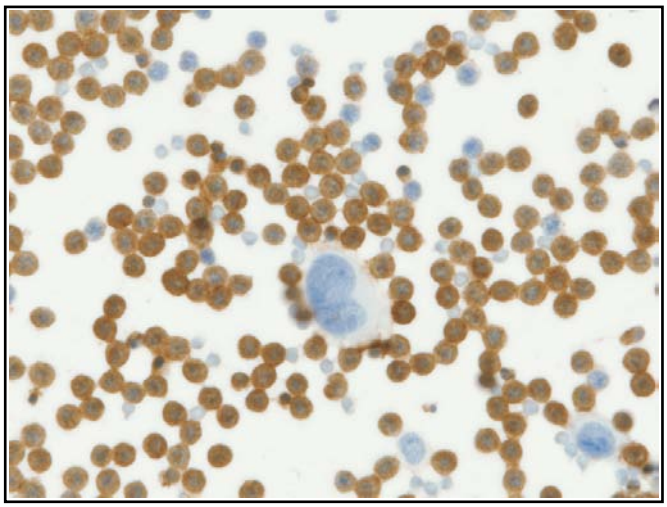


Fig 10. Optimal demonstration of CD3 in a NEQAS CS sample. All tumour cells are negative and the background is clean. Novocastra NCL-CD3 (UCHT1), 1:50, no RT, on Leica Bond-III, and Leica Bond Polymer Refine kit.

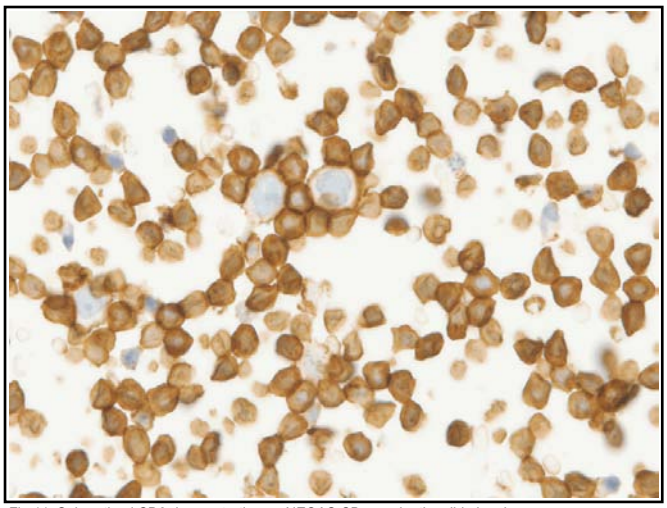


Fig 11. Sub-optimal CD3 demonstration on NEQAS CB sample; the slide has been over retrieved, and there is some evidence of cell damage; assessed as borderline. Novocastra NCL-L-CD3-565 (LN10), 32 mins, Ventana CC1 64mins, on the Ventana Benchmark ULTRA.

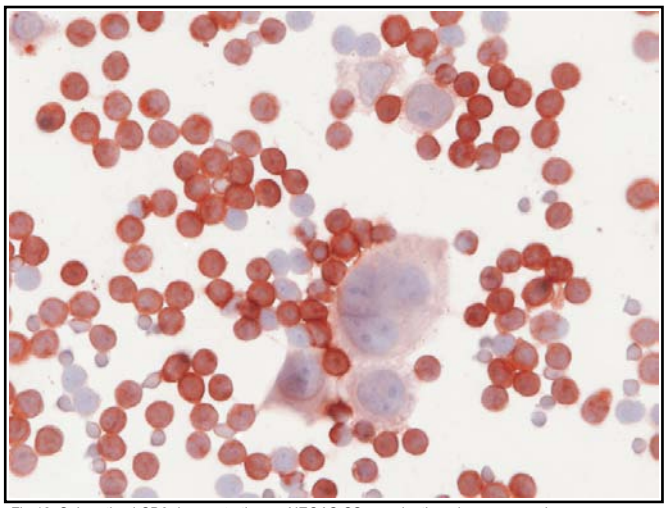
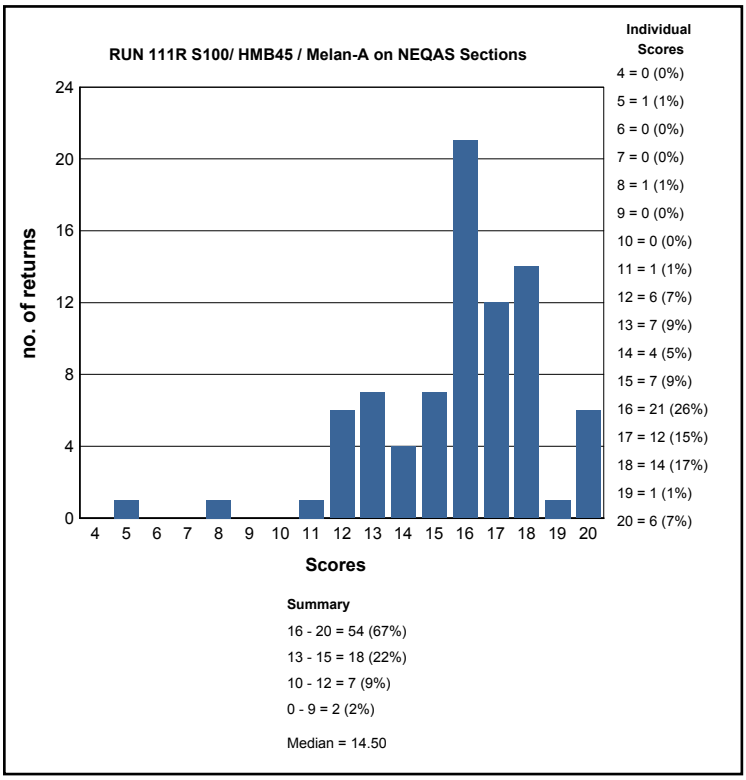
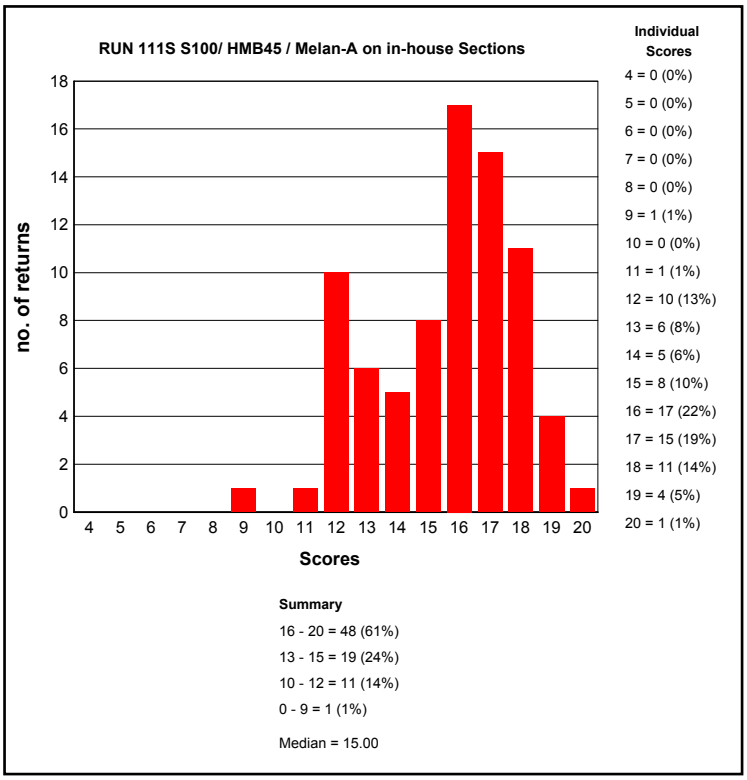
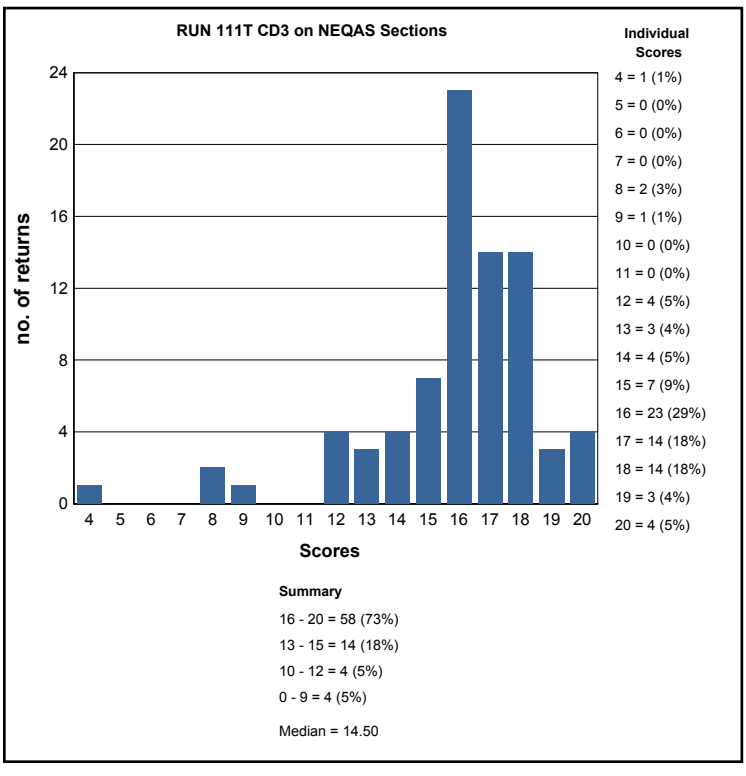


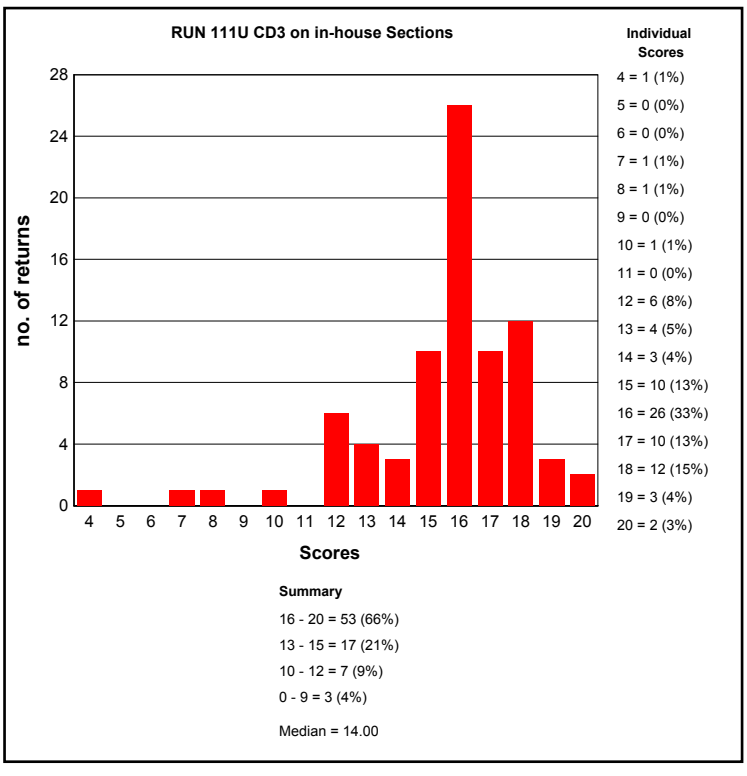
Fig 12. Sub-optimal CD3 demonstration on NEQAS CS sample; there is pronounced non-specific staining. Dako A0452 (rbpoly), 1:20, 30 mins, no RT, Leica Bond Max, using a red chromogen with the Leica Bond Polymer Refine kit (DS9800), 8 mins, at 20 C.

GRAPHICAL REPRESENTATION OF PASS RATES









ANTIBODIES AND OTHER TECHNICAL PARAMETERS EMPLOYED BY PARTICIPANTS IN THE CYTOLOGY MODULE

The following tables record the number of participants (N) using each primary antibody and the percentage (%) of these participants achieving acceptable staining (a score ≥12/20) on UK NEQAS sections.

Cytology Run: 111

Primary Antibody : S100/ HMB45 / Melan-A

Antibody Details	N	%
Biogenex Melan A (MART-1) (A103) AM361/MU361	2	100
Cell Marque CMA710 (HMB45)	1	100
Dako FLEX RTU Melan A (A103) IR633	6	83
Dako M0634 (HMB45)	9	100
Dako M7196 (A103 Melan A)	17	94
Dako U7025 (clone HMB45)	1	100
Dako Z0311 (S100)	3	33
Novocastra/Leica NCL-HMB45 (HMB45)	1	100
Novocastra/Leica NCL-MELAN A (Melan A)	15	87
Novocastra/Leica NCL-RTU-MelanA(A103) PA0233	2	100
Novocastra/Leica NCL-S100p (S100)	1	100
Other	10	80
Ventana Melan A (MART-1) 790-2990	13	92

Cytology Run: 111

Primary Antibody : CD3

Antibody Details	N	%
Dako A0452 (rbpoly)	6	67
Dako IR053 RTU FLEX Link (polyclonal)	6	100
Dako M7254 (F7.2.38)	3	100
LabVision/Neomarkers RM 9107S (SP7)	5	80
Leica/Novocastra NCL-CD3 (UCHT1)	5	100
Leica/Novocastra NCL-CD3-PS1 (PS1)	2	50
Leica/Novocastra NCL-L-CD3-565 (LN10)	25	88
Leica/Novocastra NCL-L-CD3-PS1 (PS1)	1	100
Leica/Novocastra PA0553 RTU (LN10)	3	100
Leica/Novocastra RTU-CD3-PS1 RTU (PS1)	1	100
Other	7	86
Vector VPC316 (PS1)	2	100
Ventana 790 2921 (PS1)	2	100
Ventana 790-4341 (2GV6)	12	100

Cytology Run: 111

Primary Antibody : S100/ HMB45 / Melan-A

Antigen Retrieval	N	%
YES	35	43
NO	46	57
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	35	
Not Specified	0	

Cytology Run: 111

Primary Antibody : CD3

Antigen Retrieval	N	%
YES	37	46
NO	44	54
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	37	
Not Specified	0	

Cytology Run: 111

Heat Mediated Retrieval

Cytology Run: 111

Heat Mediated Retrieval

Cytology Run: 111

Enzyme Mediated Retrieval

Cytology Run: 111

Enzyme Mediated Retrieval

Cytology Run: 111

Detection	CD3		S100/ HMB45 / Melan-A	
	N	%	N	%
AS PER KIT	4	75	6	100
Dako Duet St.ABC (K0492)	1	100	0	0
Dako EnVision FLEX (K8000/10)	1	100	0	0
Dako EnVision FLEX+ (K8002/12)	7	100	9	89
Dako Envision HRP/DAB (K5007)	2	100	2	100
Dako Envision+ HRP rabbit K4008/9/10/11	1	0	0	0
Leica Bond Polymer Refine (DS9800)	19	74	21	95
None	2	100	4	75
NOT APPLICABLE	1	100	0	0
Other	4	100	7	71
Ventana iView system (760-091)	2	100	4	100
Ventana OptiView Kit (760-700)	11	100	10	100
Ventana UltraView Kit (760-500)	21	90	17	76

Cytology Run: 111

Automation	CD3		S100/ HMB45 /	
	N	%	N	%
Dako Autostainer	2	100	1	100
Dako Autostainer Link 48	9	100	12	100
Dako Autostainer plus	1	100	0	0
Dako Autostainer Plus Link	2	100	1	100
Dako Omnis	1	100	3	33
Leica Bond Max	14	79	11	91
Leica Bond-III	12	75	13	100
Ventana Benchmark GX	3	100	3	67
Ventana Benchmark ULTRA	21	90	22	82
Ventana Benchmark XT	15	93	15	93

Cytology Run: 111

Chromogen	CD3		S100/ HMB45 /	
	N	%	N	%
AS PER KIT	9	100	9	78
Dako DAB Liquid (K3465)	1	100	0	0
DAKO DAB+	1	100	1	0
Dako FLEX DAB	10	100	12	92
LabVision DAB	0	0	1	100
Leica Bond Polymer Refine kit (DS9800)	22	73	20	95
Other	5	100	7	100
Ventana DAB	8	100	7	100
Ventana Enhanced Alk. Phos. Red Detection Kit	0	0	1	0
Ventana iview	2	100	4	100
Ventana Ultraview DAB	22	86	19	84

BEST METHODS - Gold Standard Antibody

A selection from just a few of the best methods employed by participants

S100/ HMB45 / Melan-A - Method 1

Participant scored 20/20 (UK NEQAS Slide) and 18/20 (In House slide) using this method.

Primary Antibody:	Dako M0634 (HMB45) , 40 Mins, 37 ºC Dilution 1: 20
Automation:	Ventana Benchmark XT
Method:	Ventana iView Kit
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	None
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB
Detection:	Ventana iView system (760-091)

S100/ HMB45 / Melan-A - Method 2

Participant scored 19/20 (UK NEQAS Slide) and 18/20 (In House slide) using this method.

Primary Antibody:	Dako FLEX RTU Melan A (A103) IR633
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	AS PER KIT

S100/ HMB45 / Melan-A - Method 3

Participant scored 20/20 (UK NEQAS Slide) and 17/20 (In House slide) using this method.

Primary Antibody:	Novocastra/Leica NCL-MELAN A (Melan A) , 30 Mins, RT ºC Dilution 1: 1:100
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Other
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Other

S100/ HMB45 / Melan-A - Method 4

Participant scored 17/20 (UK NEQAS Slide) and 17/20 (In House slide) using this method.

Primary Antibody:	Cell Marque CMA710 (HMB45) , 19 Mins Dilution 1: 100
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 36mins
EAR:	
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana OptiView Kit (760-700)

BEST METHODS - Secondary Antibody

A selection from just a few of the best methods employed by participants

CD3 - Method 1

Participant scored 20/20 (UK NEQAS Slide) and 20/20 (In House slide) using this method.

Primary Antibody:	Leica/Novocastra NCL-CD3 (UCHT1) , 20 Mins Dilution 1: 100
Automation:	Leica Bond-III
Method:	AS PER KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER1 40 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800), Time 1: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins

CD3 - Method 2

Participant scored 19/20 (UK NEQAS Slide) and 18/20 (In House slide) using this method.

Primary Antibody:	Dako IR053 RTU FLEX Link (polyclonal) , 20 Mins, 21 ºC Prediluted
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	AS PER KIT
HMAR:	Dako PTLink, Buffer: Dako High pH TRS
EAR:	
Chromogen:	AS PER KIT, 21 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	, 20 Mins, 21 ºC Prediluted

CD3 - Method 3

Participant scored 18/20 (UK NEQAS Slide) and 18/20 (In House slide) using this method.

Primary Antibody:	Vector VPC316 (PS1)
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: dako high ph, PH: 9.5
EAR:	
Chromogen:	Dako FLEX DAB, 21 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako Envision HRP/DAB (K5007) , 20 Mins, 21 ºC Prediluted

CD3 - Method 4

Participant scored 18/20 (UK NEQAS Slide) and 19/20 (In House slide) using this method.

Primary Antibody:	Other , 20 Mins, 37 ºC Dilution 1: 500
Automation:	Ventana Benchmark GX
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.4
HMAR:	
EAR:	
Chromogen:	AS PER KIT
Detection:	None

The Alimentary Tract Module: GIST Run 111

Suzanne Parry

	First Antibody	Second Antibody
Antigens Assessed:	CD117	CD34
Tissue Sections circulated:	GIST, Appendix & Desmoid	GIST, Appendix & Desmoid
Number of Registered Participants:	116	
Number of Participants this Run	114 (98%)	

Introduction

Gold Standard: CD117

CD117 (c-Kit) is a transmembrane tyrosine kinase growth factor receptor involved in cell differentiation. It is expressed by melanocytes, mast cells and interstitial cells of Cajal, and is also expressed in gastrointestinal stromal tumours (GIST). Until recently, patients with GIST faced a limited choice of treatment regimes, which included radiotherapy and surgery with limited success. However, Glivec (Imatinib), which was originally developed for chronic myeloid leukaemia (CML), has proved effective in the targeted treatment of GISTs by directly inhibiting the action of CD117.

Features of Optimal Immunostaining: **(See Figs 1, 2, & 6)**

- Good localisation of CD117 to mast cells in the appendix and desmoid sections (Fig 1 desmoid not shown)
- Good localisation of CD117 to interstitial cells of Cajal (Fig 6B)
- Good localisation of CD117 to cells of the GIST (Fig 2)
- No staining of the desmoid tumour

Features of Sub-optimal Immunostaining: **(See Figs 3, 4 & 5)**

- Weak and/or patchy staining of the tumour cells of the GIST (Fig 4)
- Little or no staining of the mast cells
- Excessive background or non specific staining (Figs 3 & 5)
- Staining of the desmoid tumour

Second Antibody: CD34

CD34 is a transmembrane glycoprotein, which is expressed on immature hematopoietic stem/progenitor cells, capillary endothelial cells and embryonic fibroblasts. It can also be found in splenic marginal zones, dendritic interstitial cells around vessels, nerves, hair follicles, muscle cells and sweat glands in various tissues. CD34 labels capillaries in most tissues but may be absent in large veins and arteries, and is negative in the sinus endothelium of placenta and spleen. CD34 is an excellent indicator of vascular differentiation, regardless of the tumour grade. Prior to the discovery of CD117, CD34 positivity was the best available indicator for a GIST diagnosis, however, it is not a very specific marker for this use. Overall, about 60-70% of GISTs are positive for CD34, although this varies by tumour location: CD34 expression is highest in gastric GISTs (85%), but is only seen in about 50% of small intestinal GISTs (Hasegawa et al; Rudolph et al.). Of the less common locations, CD34 is expressed in 100% of GISTs of the oesophagus, 65% colonic, 96% of rectal GISTs, and 65% of GISTs in non-GI locations, such as the mesentery and omentum (Miettinen et al). In summary, although GISTs often stain for CD34, this staining is not exclusive to GISTs, for example, competing diagnoses, such as schwannoma and solitary fibrous tumours, may also stain for CD34.

Features of Optimal Immunostaining: (See Figs 7, 9 11A & 12)

- Strong staining of the endothelial cells in the blood vessels and lymphatic vessels throughout the appendix (Fig 7)
- Good localisation of CD34 to the interstitial cells of Cajal in the appendix
- A strong, distinct membranous reaction of virtually all the neoplastic cells of the gastrointestinal stromal tumour (Figs 8 & 12A)
- Minimal background staining (Figs 7, 8, 11A &12)

Features of Sub-optimal Immunostaining: (See Figs 9, 10 &11B)

- Weak or negative staining of the endothelial cells and other elements in the appendix and desmoid (Figs 9 & 11B)
- Non-specific nuclear staining (possibly caused by over pre-treatment)
- Weak and/or patchy staining of the tumour cells of the GIST (Fig 10)
- Excessive background / non specific staining
- Staining of the tumour cells in the desmoid tumour

Tissue Distribution and Assessment Procedure

Composite slides comprising of a GIST, appendix and desmoid tumour were distributed to all participants for them to stain with the requested antibodies (CD117 and CD34) using their routine protocol. The circulated tissue was sequential sections from the same tissue block used for both the requested antibodies.
Assessment was carried out around a multi-header microscope with each slide being assessed by 4 independent assessors and a microscope lead. All 3 samples on the slide were viewed, and then a final overall score out of 5 was given by each individual assessor. These four scores were then combined to give an overall score out of 20.

References

1. Cordless et al., Biology of Gastrointestinal Stromal Tumours. J Clin Oncol 2004, 22(18): 3813-3825.
2. Blay et al., Consensus meeting for the management of gastrointestinal stromal tumors. Report of the GIST Consensus Conference of 20-21 March 2004, under the auspices of ESMO. Ann Oncol 2005 6: 566-578.
3. Parfitt JR, Rodriguez-Justo M, Feakins R, Novelli MR (2008) Gastrointestinal Kaposi's sarcoma: CD117 expression and the potential for misdiagnosis as gastrointestinal stromal tumour. Histopathology 2008, 52: 816-23.
4. Hasegawa T et al, Gastrointestinal stromal tumors: consistent CD117 immunostaining for diagnosis, and prognostic classification based on tumor size & MIB-1 grade. Hum Pathol. 2002 Jun;33(6):669-76.
5. Rudolph et al, Gastrointestinal mesenchymal tumors - immunophenotypic classification and survival analysis. 2002 Sep;441(3):238-48. Epub 2002 Jul 6.
6. Miettinen et al, Immunohistochemical spectrum of GISTs at different sites and their differential diagnosis with a reference to CD117 (KIT). Mod Pathol. 2000 Oct;13(10):1134-42.

Assessment Summary:

Results from the **CD117** assessment showed acceptable pass, borderline pass and fail rates of 78%, 17% and 5% respectively, which are very similar figures to the previous assessment (run 110). The main reasons for the sub-optimal marks were due to very weak staining of the GIST or non-specific and background staining, predominantly in the desmoid tumour. Inappropriate antibody dilution factor and antigen retrieval were the main causes of these issues. The most popular CD117 antibody choice still remains the Dako polyclonal, used by 83% of participants in this assessment run, and showed an acceptable pass rate of 79%.

The Alimentary Tract Module: GIST Run 111

Suzanne Parry

The second antibody assessed for the Alimentary Tract module for Run 111 was **CD34**. The overall results from the assessment showed very good pass rates: 96% of labs achieved an acceptable level of staining, and 4% (N=4) received a borderline pass of 12/20. It was pleasing to see that no labs failed the assessment. The borderline labs were all marked down for weak staining. However, 3 of these borderline labs received an acceptable level of staining on their in-house tissue. Only 2 of these borderline labs provided their full methodology details, but it was clear that these 2 labs are using a good protocol that should also work on the UK NEQAS material. The team felt that maybe the slides had not been stained soon on arrival, which may explain the weaker staining on the NEQAS sample compared to the in-house control which would have been freshly cut onto the same slides as the NEQAS tissue. Again, the most popular CD34 antibody was the Dako QBend 10 clone. This was used by 48% of participating labs, and showed an overall acceptable pass rate of 84%. The Leica/Novocastra QBend 10 was also popular; used by 28% of labs and showed an excellent pass rate of 100%. The Ventana QBend 10 clone, used by 21 labs (18%), also showed an acceptable pass rate of 100%. It was noted that both the Dako and Leica QBend 10 antibodies produced good staining on all of the commercial platforms, such as the Leica Bond, Dako autostainer, Labvision autostainer, and Ventana Benchmark and ULTRA machines. The Ventana QBend 10 antibody was only used by Ventana platform users as recommended by the supplier.

UK NEQAS ICC recommends that labs use a normal control, such as an appendix or normal bowel epithelium, along with a known positive GIST for their in-house controls. Over the last year of Alimentary Tract assessments there has certainly been an increase in labs submitting composite controls including both normal and positive tumour tissue.

Similarly to a patient clinical sample, wherever possible it is important to chose the appropriate tissue block for IHC testing to not only include the tumour, but hopefully have some normal epithelium, or with the lab control, to include another tissue to act as a normal control.

Selected Images showing Optimal and Sub-optimal Immunostaining

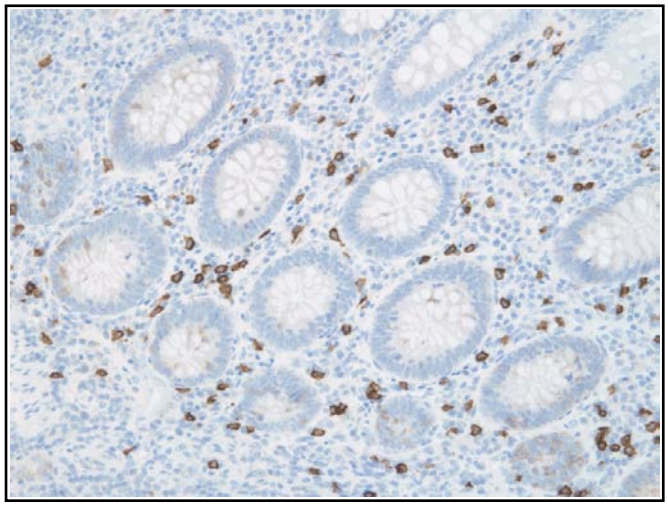


Fig 1. Good demonstration of CD117 in the UK NEQAS ICC distributed appendix. Staining in the mast cells is well localised, while the background remains clean. Section stained with the Dako polyclonal antibody, 1:300, PT Link retreieval with high pH buffer on the Dako Autostainer and FLEX detection.

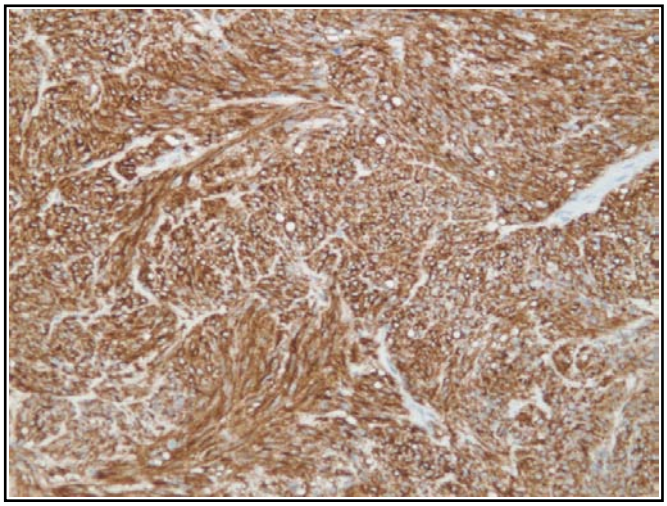


Fig 2. Optimal demonstration of CD117 in the UK NEQAS distributed GIST section. All the neoplastic cells show strong and well-localised predominantly membranous and cytoplasmic staining, while the vessels remain unstained. Stained with the Dako polyclonal antibody, 1:100, on the Leica Bond III, ER2 retrieval for 20 minutes.

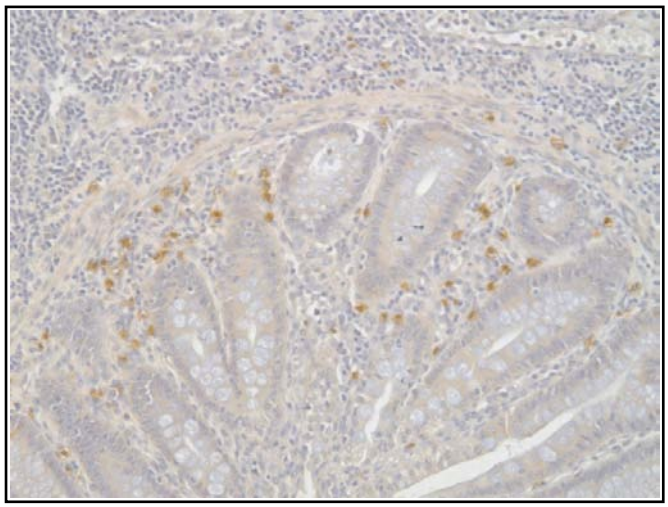


Fig 3. Sub-optimal demonstration of CD117 in the UK NEQAS appendix. Although the mast cells are staining as expected, there is also non-specific background staining. The GIST and desmoid sections on the same slide also showed excessive background staining. Stained with the Dako polyclonal antibody, 1:75, with no pre-treatment on the Dako Autostainer.

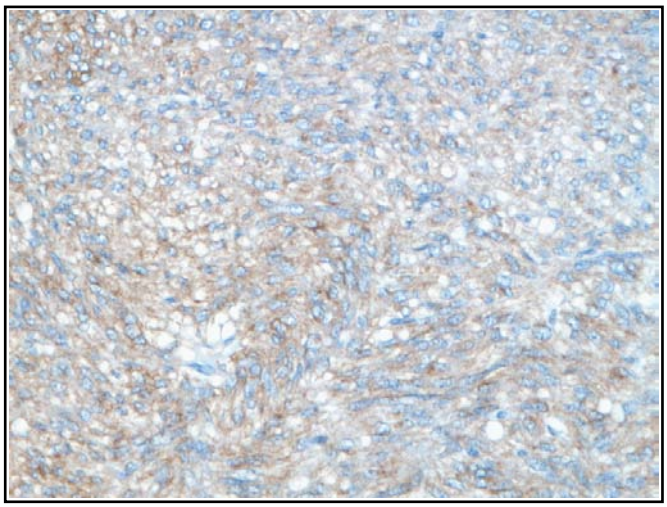


Fig 4. Poor demonstration of CD117 in the UK NEQAS distributed GIST section: The neoplastic cells only show weak and diffuse staining (compare with Fig 2). Section stained with the Dako polyclonal antibody, 1:100, on the Ventana Benchmark XT and no antigen retrieval.

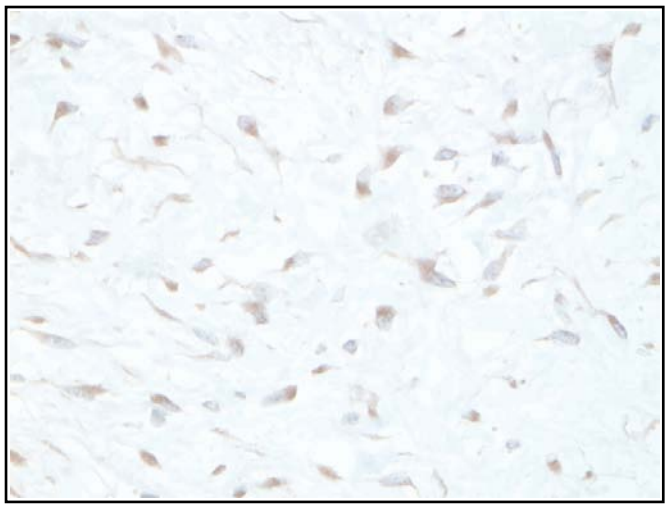


Fig 5. Poor demonstration of CD117 in the UK NEQAS distributed desmoid tumour. Although the mast cells stained as expected, the section also showed inappropriate non-specific staining. The Dako polyclonal antibody was used at a dilution of 1:50 on the Ventana Benchmark XT with CC1 mild retrieval.

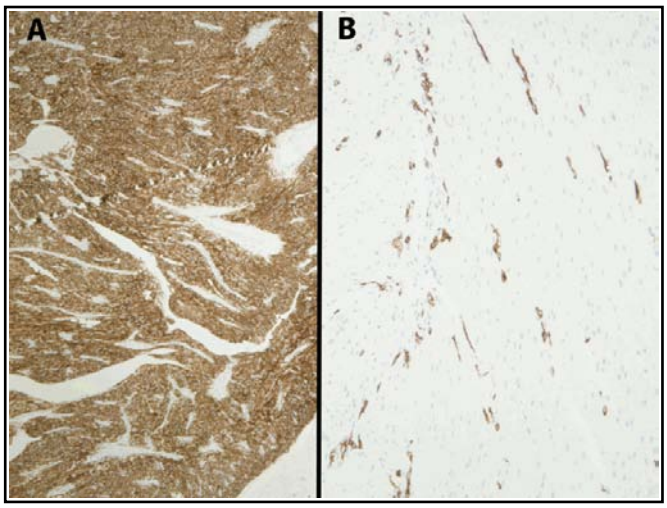


Fig 6. Good example of an in house control stained with CD117. The GIST (A) and the cells of Cajal in the adjacent tissue (B) are optimally demonstrated. Same protocol as Fig 2.

Selected Images showing Optimal and Sub-optimal Immunostaining

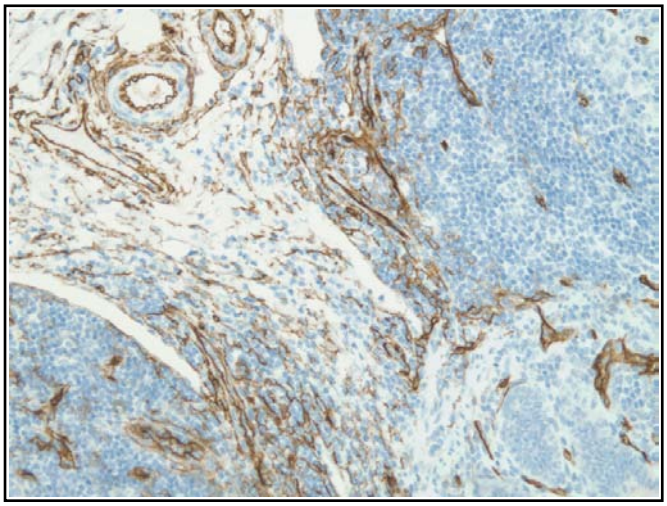


Fig 7. Optimal demonstration of CD34 in the UK NEQAS distributed appendix section. The example shows strong distinct staining of the vessels and endothelial cells while the background remains clean. Section stained with the Dako QBend 10 antibody, 1:100, on the Dako Autostainer and pre-treated in the PT Link with high pH buffer.

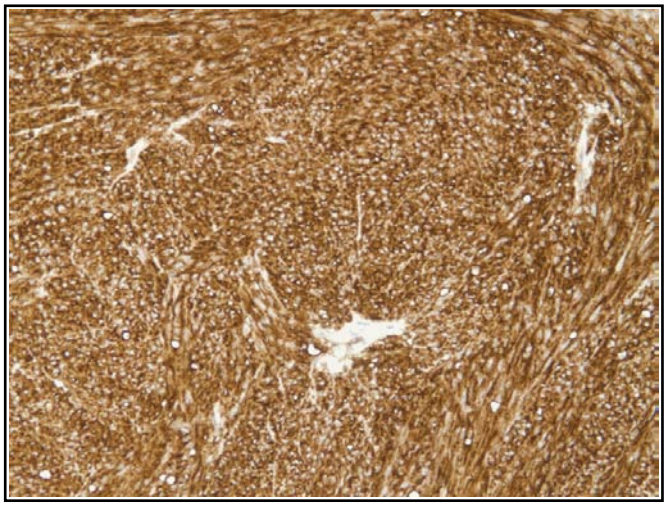


Fig 8. Good demonstration of CD34 in the UK NEQAS distributed GIST. The example shows intense, well localised membranous and cytoplasmic staining. Section stained with the Leica QBend 10 antibody, 1:200, on the Bond III with ER2 retrieval for 20 minutes.

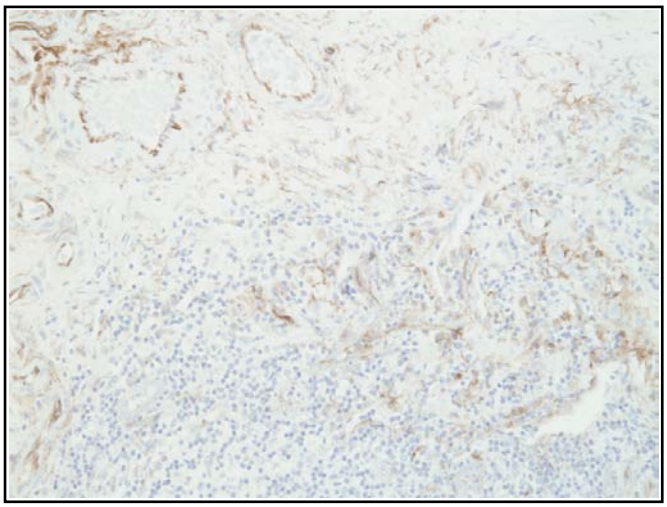


Fig 9. Insufficient staining of CD34 in the UK NEQAS distributed appendix. Although the vessels are demonstrated, the staining is weak and diffuse (compare to Fig 7).

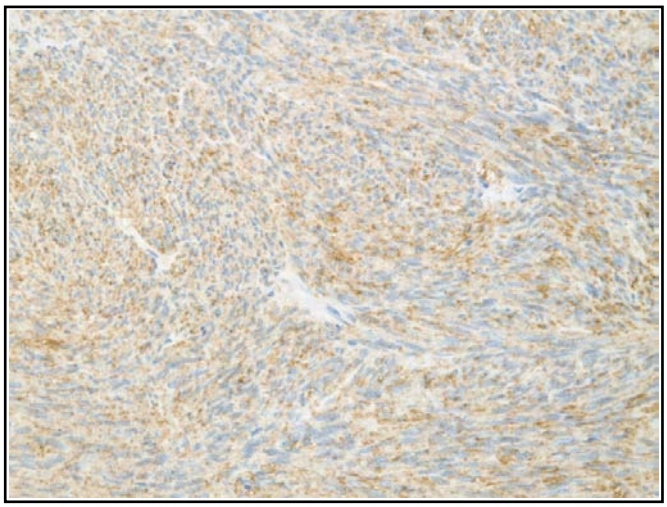


Fig 10. Sub-optimal staining for CD34 in the UK NEQAS GIST section. Tumour cells are demonstrated but the staining is weaker than expected (compare to Fig 8). A combination of antibody dilution (too dilute) and too short antigen retrieval are the likely reason for the weaker than expected staining: Dako QBend 10 antibody, 1:300, Leica ER2 retrieval for 10 minutes on

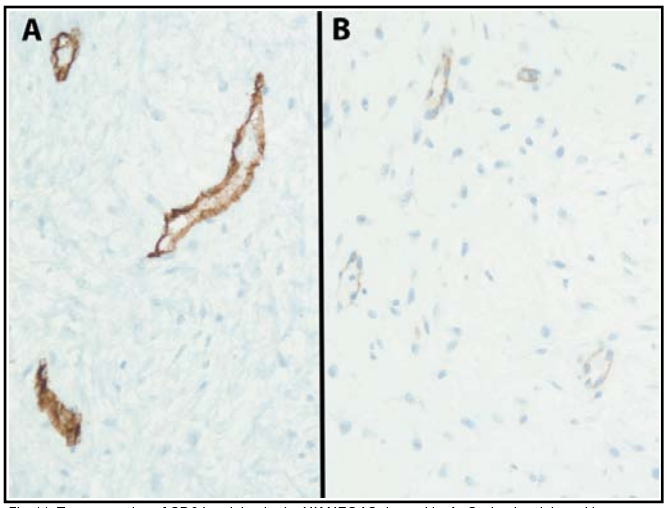


Fig 11. Two examples of CD34 staining in the UK NEQAS desmoid. (A) Optimal staining with strong staining in the endothelial cells and small vessels, whith minimal background. (B) Very weak staining. (A) stained with the Ventana QBend 10 pre-diluted antibody on the Benchmark. (B) Stained with the Dako QBend 10 antibody on the Leica Bond III without pre-treatment.

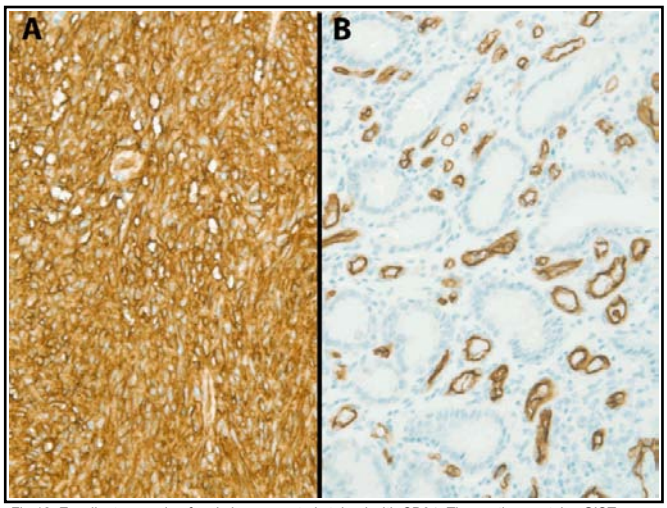
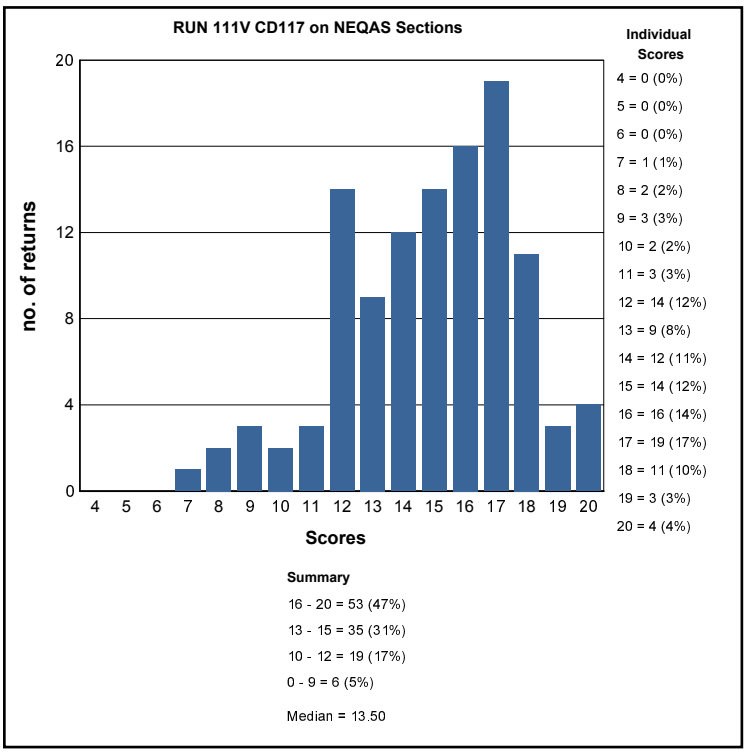
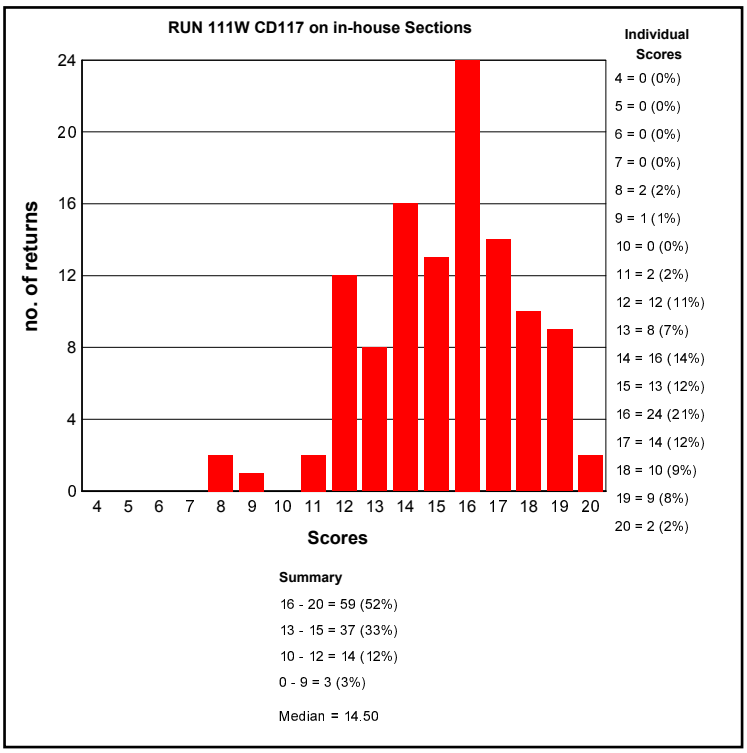
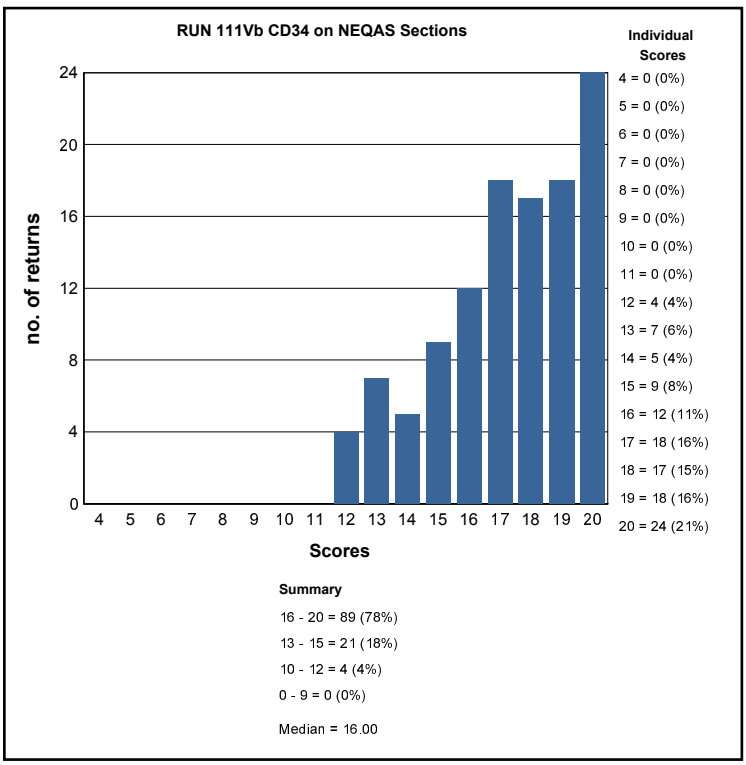


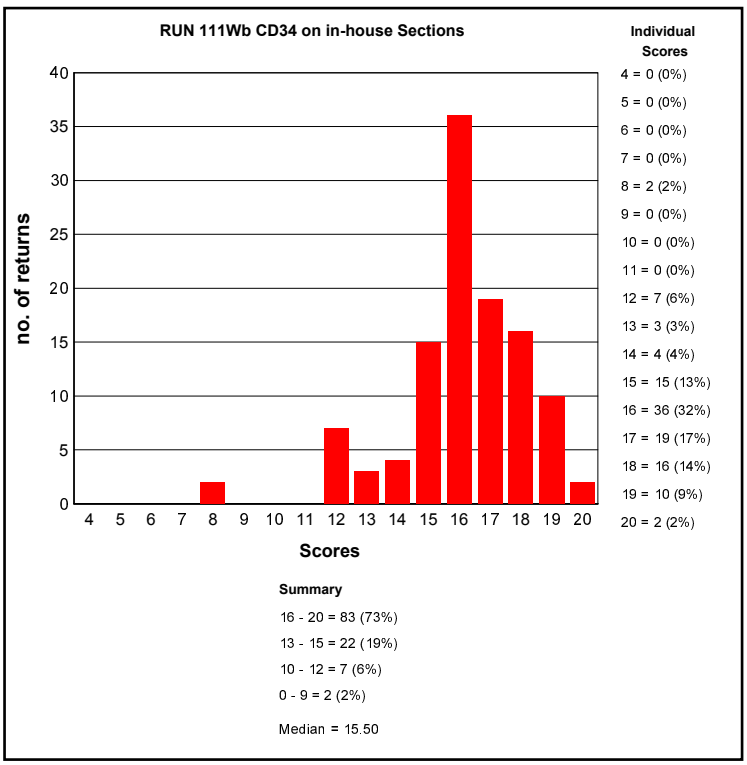
Fig 12. Excellent example of an in house control stained with CD34. The section contains GIST (A) and placenta (B) tissues; both showing strong crisp positive staining while the background remains clean. Section stained with the Leica QBend 10 antibody, 1:200, on the Ventana ULTRA with CC1 mild antigen retrieval and Optiview detection kit.

GRAPHICAL REPRESENTATION OF PASS RATES









ANTIBODIES AND OTHER TECHNICAL PARAMETERS EMPLOYED BY PARTICIPANTS IN THE ALIMENTARY TRACT PATHOLOGY MODULE

The following tables record the number of participants (N) using each primary antibody and the percentage (%) of these participants achieving acceptable staining (scores 12/20) on UK NEQAS sections.

Alimentary Tract Pathology Run: 111

Primary Antibody : CD117

Antibody Details	N	%
Cell Marque 117R/S-xx (YR145)	4	100
Dako A4502 (rb poly)	95	79
Epitomics AC-0029 (EP10)	1	100
Leica/Novocastra NCL-CD117 (T595)	1	100
Other	1	100
Spring Bioscience M3264 (SP26)	1	0
Ventana 790-2951 (9.7)	10	60

Alimentary Tract Pathology Run: 111

Primary Antibody : CD34

Antibody Details	N	%
Dako IR/IS632 RTU (QBend10)	4	75
Dako M7165 (QBend10)	48	96
Leica NCL-END (QBend10)	14	100
Leica PA0212 (QBend10)	9	100
Leica NCL-E-ND (QBend10)	7	100
Leica RTU-END (QBend10)	2	100
Other	5	100
Serotec MCA 547 (QBend10)	2	50
Vector VP C345 (QBend10)	1	100
Ventana 790-2927 (QBend10)	21	100

Alimentary Tract Pathology Run: 111

Heat Mediated Retrieval	CD117 N	CD117 %	CD34 N	CD34 %
Dako Omnis	0	0	1	100
Dako PTLink	15	100	14	93
Lab vision PT Module	1	100	1	100
Leica ER1 10 mins	1	100	1	100
Leica ER1 20 mins	5	60	9	89
Leica ER1 30 mins	3	100	4	100
Leica ER2 10 mins	1	100	2	100
Leica ER2 20 mins	18	83	21	100
Leica ER2 30 mins	9	100	2	100
None	3	33	3	100
Pressure Cooker	1	100	2	100
Pressure Cooker in Microwave Oven	0	0	1	100
Ventana CC1 16mins	1	100	3	100
Ventana CC1 20mins	0	0	1	100
Ventana CC1 32mins	4	25	7	100
Ventana CC1 36mins	7	71	7	71
Ventana CC1 48mins	3	100	0	0
Ventana CC1 52mins	2	100	1	100
Ventana CC1 56mins	4	75	1	100
Ventana CC1 64mins	9	67	5	100
Ventana CC1 88mins	1	100	0	0
Ventana CC1 8mins	1	0	5	100
Ventana CC1 extended	0	0	1	100
Ventana CC1 mild	9	44	12	100
Ventana CC1 standard	15	80	6	100

Alimentary Tract Pathology Run: 111

Enzyme Mediated Retrieval	CD117 N	CD117 %	CD34 N	CD34 %
AS PER KIT	0	0	5	100
NOT APPLICABLE	73	75	47	96
VBS Bond Enzyme 1	0	0	1	100
Ventana Protease 1 (760-2018)	0	0	1	100

Alimentary Tract Pathology Run: 111	CD117		CD34	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	7	86	9	100
BioGenex SS Polymer (QD 420-YIKE)	0	0	1	100
BioGenex SS Polymer (QD 430-XAKE)	1	100	0	0
Dako EnVision FLEX (K8000/10)	1	100	1	100
Dako EnVision FLEX+ (K8002/12)	7	100	7	86
Dako Envision HRP/DAB (K5007)	1	0	2	100
Dako Envision+ HRP mouse K4004/5/6/7	0	0	1	100
Dako Envision+ HRP rabbit K4008/9/10/11	1	100	0	0
Leica Bond Polymer Refine (DS9800)	35	86	33	97
None	1	100	1	100
NOT APPLICABLE	1	100	1	100
Other	4	100	3	100
Ventana iView system (760-091)	2	50	1	100
Ventana OptiView Kit (760-700)	17	65	13	100
Ventana UltraView Kit (760-500)	34	68	36	94

Alimentary Tract Pathology Run: 111	CD117		CD34	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	1	100	1	100
Dako Autostainer Link 48	13	100	12	92
Dako Autostainer plus	2	50	1	100
Dako Autostainer Plus Link	2	100	1	100
Dako Omnis	0	0	1	100
Leica Bond Max	13	100	15	100
Leica Bond-III	25	80	24	96
Other	0	0	1	100
Ventana Benchmark GX	2	100	2	100
Ventana Benchmark ULTRA	29	69	28	93
Ventana Benchmark XT	26	62	22	100

Alimentary Tract Pathology Run: 111	CD117		CD34	
Chromogen	**N**	**%**	**N**	**%**
A. Menarini Liquid Stable DAB kit	1	100	0	0
AS PER KIT	17	82	16	100
BioGenex Liquid DAB (HK153-5K)	0	0	1	100
Dako DAB+ REAL Detection (K5001)	0	0	1	0
Dako EnVision Plus kits	2	100	3	100
Dako FLEX DAB	9	100	6	100
Dako REAL EnVision K5007 DAB	1	0	2	100
Leica Bond Polymer Refine kit (DS9800)	33	85	34	97
Other	4	75	4	100
Ventana DAB	7	57	6	100
Ventana iview	2	50	1	100
Ventana Ultraview DAB	37	70	38	95

BEST METHODS - Gold Standard Antibody

A selection from just a few of the best methods employed by participants

CD117 - Method 1

Participant scored 20/20 (UK NEQAS Slide) and 19/20 (In House slide) using this method.

Primary Antibody: Dako A4502 (rb poly) , 20 Mins Dilution 1: 100
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 20 mins
EAR: NOT APPLICABLE
Chromogen: Leica Bond Polymer Refine kit (DS9800), Time 1: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins

CD117 - Method 2

Participant scored 18/20 (UK NEQAS Slide) and 19/20 (In House slide) using this method.

Primary Antibody: Dako A4502 (rb poly) , 32 Mins Dilution 1: 100
Automation: Ventana Benchmark ULTRA
Method: Ventana UltraView DAB
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 64mins, Buffer: cc1, PH: 8
EAR: NOT APPLICABLE
Chromogen: Ventana Ultraview DAB, rt ºC., Time 1: 5 Mins
Detection: Ventana UltraView Kit (760-500) , 8 Mins Prediluted

CD117 - Method 3

Participant scored 17/20 (UK NEQAS Slide) and 19/20 (In House slide) using this method.

Primary Antibody:	Ventana 790-2951 (9.7) , 20 Mins, 37 ºC
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana OptiView Kit (760-700)

CD117 - Method 4

Participant scored 19/20 (UK NEQAS Slide) and 19/20 (In House slide) using this method.

Primary Antibody:	Dako A4502 (rb poly) , 25 Mins, 21 ºC Dilution 1: 100
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: high flex solution
EAR:	
Chromogen:	Dako FLEX DAB, 21 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako Envision+ HRP rabbit K4008/9/10/11 , 25 Mins, 21 ºC Prediluted

BEST METHODS - Secondary Antibody

A selection from just a few of the best methods employed by participants

CD34 - Method 1

Participant scored 20/20 (UK NEQAS Slide) and 19/20 (In House slide) using this method.

Primary Antibody:	Dako M7165 (QBend10) , 20 Mins, 20 ºC Dilution 1: 50
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako Wash Buffer (S3006)
HMAR:	Dako PTLink
EAR:	NOT APPLICABLE
Chromogen:	Dako EnVision Plus kits
Detection:	Dako EnVision FLEX+ (K8002/12)

CD34 - Method 2

Participant scored 20/20 (UK NEQAS Slide) and 19/20 (In House slide) using this method.

Primary Antibody:	Leica NCL-END (QBend10) , 15 Mins Dilution 1: 100
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

CD34 - Method 3

Participant scored 20/20 (UK NEQAS Slide) and 18/20 (In House slide) using this method.

Primary Antibody:	Ventana 790-2927 (QBend10) , 32 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.8
HMAR:	Ventana CC1 64mins, Buffer: CC1, PH: 8.5
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB, 37 ºC., Time 1: 8 Mins, Time 2: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins Prediluted

CD34 - Method 4

Participant scored 20/20 (UK NEQAS Slide) and 20/20 (In House slide) using this method.

Primary Antibody:	Dako M7165 (QBend10) , 60 Mins, 24 ºC Dilution 1: 40
Automation:	
Method:	Envision
Main Buffer:	TBS + Tween, PH: 7.4
HMAR:	Pressure Cooker, Buffer: Citrate buffer, PH: 6
EAR:	
Chromogen:	Dako REAL EnVision K5007 DAB, 24 ºC., Time 1: 10 Mins
Detection:	Dako Envision HRP/DAB (K5007) , 30 Mins, 24 ºC Prediluted

Lynch Syndrome/HNPCC Run 111

Keith Miller and Suzanne Parry

	Gold Standard	Second Antibody
Antigens Assessed:	MSH2	MSH6
Tissue Sections circulated:	Normal Appendix & Colonic Tumours	Normal Appendix, & Colonic Tumours
Number of Registered Participants:	82	
Number of Participants This Run:	75 (91%)	

General Introduction

In Lynch/ Hereditary Non-Polyposis Colorectal Cancer (HNPCC) syndrome, tumours that have lost mismatch repair (MMR) function, immunohistochemistry (IHC) can be used to show abnormal MMR protein expression. Lynch/HNPCC syndrome sufferers inherit one germline mutant MMR allele and one normal, wild-type MMR allele. During tumour formation, the normal allele is inactivated by mutation or loss or other mechanism, thus leaving no expression from functional alleles. MMR protein abnormal expression in Lynch/HNPCC tumours may thus be detected by IHC in two patterns:

(i) Complete loss of expression (when there is no expression of that MMR protein or only expression of a truncated or mutant protein to which the antibody does not bind) or

(ii) Patchy/weak expression (if the mutation generates a prematurely truncated but variably stable protein, or a protein with alterations to the epitope recognised by the antibody, e.g. missense mutation).

It is important to realise, however, that a small proportion, perhaps 5-20% of Lynch/HNPCC-related tumours do not exhibit any abnormality on analysis by IHC even though they have lost MMR function, as manifested by microsatellite instability (MSI) and this may be due to mutations that functionally inactivate the MMR protein but allow its expression as a stable protein with nuclear localisation and intact epitope.

Mismatch Repair Markers

Where possible, IHC should be carried out using an automated staining machine. Manual IHC is prone to significant variation, both within and between batches. Whereas automated immunostaining generates more consistent and reproducible staining patterns. There are 4 MMR antibodies available to aid in the differential identification of colorectal cancers. These are MLH1, MSH2, MSH6 & PMS2, and the use of all 4 antibodies is recommended rather than just using the 2 markers (MLH1 & MSH2). There is a heterodimeric association of proteins, such that a loss of MLH1 expression is almost always accompanied by the loss of PMS2 expression, and a loss of MSH2 is almost always accompanied with the loss of MSH6. Therefore, the expression changes to the heterodimeric binding partners of these proteins acts as a very useful confirmatory finding. The antigenic epitopes for the four anti-MMR antibodies are particularly fixation-sensitive, so immunostaining patterns should only be assessed in well-fixed regions of the tissue section, for example where the adjacent normal epithelium or the intra- or peri-tumoural lymphoid cells or stromal cells, such as fibroblasts, show clear, strong nuclear immunopositivity for all four of the MMR proteins. Any poorly fixed parts of the tumour should not be used to confirm a diagnosis.

Clinical Interpretation & Reporting Guidelines

We recommend reporting MMR IHC findings in tumours as either:

a) Normal: Demonstrating strong immunopositivity of the tumour cell nuclei for all four MMR proteins, similar in staining intensity to that of the adjacent normal epithelium or intra-tumoural lymphoid cells or stromal cells.

b) Negative: Showing complete loss of staining of one or more MMR proteins, relative to the strongly intensive immunopositivity of the adjacent normal epithelium or intra-tumoural lymphoid cells or stromal cells.

c) Patchy/weak: Staining intensity of one or two of the MMR proteins is either weak or patchy in the tumour cell nuclei, compared to adjacent normal epithelium or intra-tumoural lymphoid cells or stromal cells, that show strong nuclear positivity in a well fixed region of the tumour. This patchy/weak staining may sometimes be accompanied by staining in the cytoplasm rather than the nucleus, whereas, the adjacent normal epithelium or intra-tumoural activated lymphocytes or stromal cells show the usual pattern of strong nuclear immunopositivity for the MMR protein(s). This suggests that destabilisation of the MMR protein complexes has occurred and the proteins are no longer bound to the nuclear DNA.

Clinical Reporting

Reports should conclude with a statement that evidence of abnormal MMR expression by IHC has, or has not, been found. If abnormal MMR expression has been found the comment is made that this is compatible with, but not necessarily diagnostic of Lynch/HNPCC syndrome, and that, if not already organised, referral of the individual and their family should be made to clinical genetic services.

If abnormal MMR expression has not been found, the covering statement is made that MSI studies should be carried out to include or exclude MMR functional abnormality in the tumour (because of the proportion of tumours with MSI that do not exhibit an abnormality of MMR protein expression).

Because abnormality of MMR protein expression is evidence in itself of loss of MMR function in that tumour, a comment may also be made that MSI studies are not needed.

A small minority of tumours may show unusual combinations of abnormal MMR protein expression, e.g. loss of both MLH1 and MSH2, or three out of the four MMR proteins. MSH2, or three out of the four MMR proteins. It is suggested that the IHC be repeated and then a clinico-pathological review of the case be carried out as necessary, to decide whether to proceed with testing of other tumours from the same individual or family, or proceeding directly to mutation detection studies.

Further Discussion on MMR proteins

Abnormality of MMR protein expression is not in itself necessarily diagnostic of Lynch /HNPCC syndrome, as around 15% of sporadic colon cancers show loss of MLH1 staining due to acquired promoter methylation, and some sporadic colorectal tumours will also lose MSH2, MSH6 or PMS2. While there is evidence that abnormal MMR expression in rectal cancers is more predictive of Lynch syndrome, it is by no means an absolute. Where available and appropriate, it can be suggested that testing for the *BRAF* V600E mutation is a way of distinguishing a proportion of MLH1-negative tumours as sporadic. It is important to appreciate the full interpretation of the tumour, where the intensity of immunopositivity in tumour

cell nuclei can be directly compared with the strong nuclear staining intensity in well fixed adjacent normal epithelium or lymphoid cells or stromal cells. Artefactually, distorted staining patterns due to poor fixation (affecting both stromal cells as well as tumour cells) in central parts of the tumour are irrelevant. It should be borne in mind that geneticists may use the immunohistochemical expression pattern of MMR proteins to interpret mutations of uncertain significance, and that both false -negative and false-positive staining (or interpretation) may potentially cause misinterpretation of DNA sequence findings. This could result in a mutation being interpreted as pathogenic when it is, in fact, neutral in effect, and thus result in inappropriate predictive genetic testing being offered to the family. Hence, care is needed in both staining and interpretation.

Assessment Procedure:

Composite slides were distributed to all participants for them to stain with **MSH2** (1st antibody) and **MSH6** (2nd antibody) using their routine protocols. The sections comprised of normal appendix and two colonic tumours (one with loss of MMR protein and one with no loss of MMR). Assessment of the stained slides was carried out around a multi-header microscope, with each slide being assessed by 4 independent assessors and a microscope lead. All samples on the slide were viewed, and then a final overall score out of 5 was given by each individual assessor. These four scores were then combined to give an overall score out of 20.

Features of Optimal Immunostaining: (Figs 1, 2, 3, 6, 7, 8 & 9)

Appendix: (Figs 1, 7 & 8)

Strong nuclear staining of the epithelium at the base and lower third-half of the crypts with fading of nuclear staining intensity in the middle and upper thirds of the crypts, to negative or weak staining of epithelial nuclei at the luminal surface.

- Strong staining of lymphoid follicles.

Tumour without loss of MMR protein: (Figs 2, 6A & 9)

Strong nuclear staining in the tumour cells.

- Strong nuclear staining in the lymphocytes and stromal cells.

Tumour with loss of MMR protein: (Figs 3 & 6B)

- Strong nuclear staining in the lymphocytes and stromal cells.
- No staining in the tumour cells.

Features of Sub-optimal Immunostaining: (Figs 4, 5, 10, 11 &12)

Appendix: (Fig 4)

- Weak, uneven, partially missing staining of relevant cells.
- Excessive background or non-specific specific staining.

Tumour without loss of MMR protein: (Figs 5 & 10)

- Weak or no staining in the tumour cells.
- Weak or no staining in the lymphocytes and stromal cells.
- Excessive background or non-specific staining.

Tumour with loss of MMR protein: (Figs 11 & 12)

- Weak or no staining in the lymphocytes and stromal cells.
- Excessive background or non-specific staining.
- False positive staining in the tumour cells.

Assessment Summary:

The pass rates for the **MSH2** assessment on the UK NEQAS sections was slightly higher than the previous time this antibody was assessed (Run 109), with 67% of labs achieving an acceptable pass, and a further 25% of participants receiving a borderline score (10-12/20). Only 6 labs (8%) failed the assessment, which again showed an improvement from the previous MSH2 run when 14 labs failed the assessment. There was a very similar correlation between the staining on the NEQAS sections and the participants' in-house controls: The in-house sections showed an acceptable pass rate of 71%, borderline passes of 22% and failure of 7% (N=5). Similarly to previous runs, the main reason for a failed or borderline score was mostly due to weak staining. The Ventana G219-1129 antibody clone was used by most labs in this assessment, and showed a pass rate of 86%. The Dako FE11 clone is also becoming very popular; now used by 12 labs with a pass rate of 83% in the current run.

The **MSH6** assessment showed a very similar pass rate to that of the MSH2 with 68% of labs receiving an acceptable pass on the UK NEQAS material, and a further 21% achieving a borderline. 9 labs (12%) failed the assessment , and again this was mostly due to weak staining. However, 3 labs failed due to inappropriate nuclear staining in the tumour known to be negative. Excessive antigen retrieval was most likely the cause or this. Labs that received a borderline score were also mostly marked down due to weak staining, while other labs showed excessive and background staining. The Dako EP49 antibody was the most popular antibody used in this assessment by 30 labs, and showed a pass rate of 85%. The Ventana 44 clone was also popular; used by 20 labs and with a pass rate of 75%.

In-House Control Tissue Recommendations

UK NEQAS ICC recommends that labs use both a normal control, such as an appendix or normal colonic epithelium, along with a colonic tumour of known mismatch marker expression. Better still would be to include both a positive and negative tumour alongside some normal epithelial control material. The appendix or normal colonic epithelium are ideal controls to demonstrate the intensity of nuclear staining, and to highlight the gradation of intensity of epithelial cell staining down the crypts. An in-house colonic tumour of known expression will highlight any weak staining (in a known positive tumour) or any false positive staining (in a known negative tumour). Furthermore, the tumours should show staining in the stromal cells and lymphocytes which act as an internal normal control.

Over the latest assessments it has been noted that most labs are now using composite in-house control tissue (as described above); mostly using tissue microarray (TMA) cores or small punches: These are ideal in clinical practice as they are small enough to enable the control and test sample to be placed on the same slide enabling labs to place control sections on all test samples for better internal quality control.

References

1. Vasen HF, Möslein G, Alonso A et al.,(2007) Guidelines for the clinical management of Lynch syndrome (hereditary non-polyposis cancer). J Med Genet. 44 6):353-62. Free download from: http://www. jmg.bmj.com/cgi/content/full/44/6/353.
2. Dr Philippa Brice. Biomarkers in familial colorectal cancer screening. Expert workshop, 14th February 2006. Public Health Genetics Unit, Cambridge, UK. Free download from: http://www.phgfoundation.org/file/2743/.
3. Arends MJ, Frayling I. Mismatch Repair Deficiency in Hereditary and Sporadic Colorectal Cancer. In: "The Effective Management of Colorectal Cancer" (4th Edition), UK Key Advances in Clinical Practice series. Eds: Cunningham D, Topham C, & Miles A. ISBN 1-903044-43- X. 2005. Chapter 2, pp25-40.
4. Frayling IM, Happerfield L, Mattocks C, Oakhill K, Arends MJ. Application of Molecular Diagnostics to Hereditary Nonpolyposis Colorectal Cancer (chapter 32). In: "Molecular Diagnostics, For the Clinical Laboratorian" (2nd Edition). Eds: Coleman WB & Tsongalis GJ. Humana Press Inc., NJ. 2005. ISBN: 1-59259-928-1, ISBN13: 978-1-58829-356-5; ISBN10: 1-58829-356-4. pp 375 – 392.
5. Poulogiannis G, Frayling IM, Arends MJ. DNA Mismatch Repair Deficiency in Sporadic Colorectal Cancer and Lynch Syndrome. *Histopathology* 2010; 56: 167 - 179.a.
6. Vasen F, et al. Revised guidelines for the clinical management of Lynch syndrome (HNPCC): recommendations by a group of European experts. Gut 2013;62: 812-823.

Selected Images showing Optimal and Sub-optimal Immunostaining

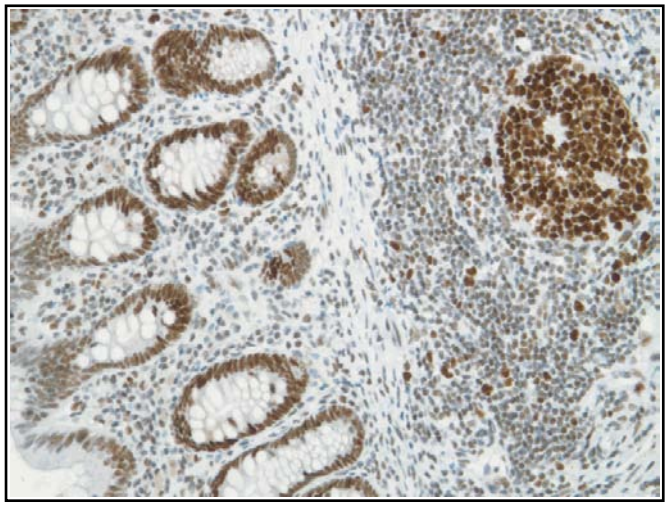


Fig 1. Optimal demonstration of MSH2 in the UK NEQAS distributed appendix, showing strong staining of the epithelial and stromal cells, with fading of staining intensity towards the luminal surface of the epithelial crypts. Stained with the Ventana pre-diluted G219-1129 antibody on the Benchmark XT, CC1 retrieval for 40 minutes and OptiView detection.

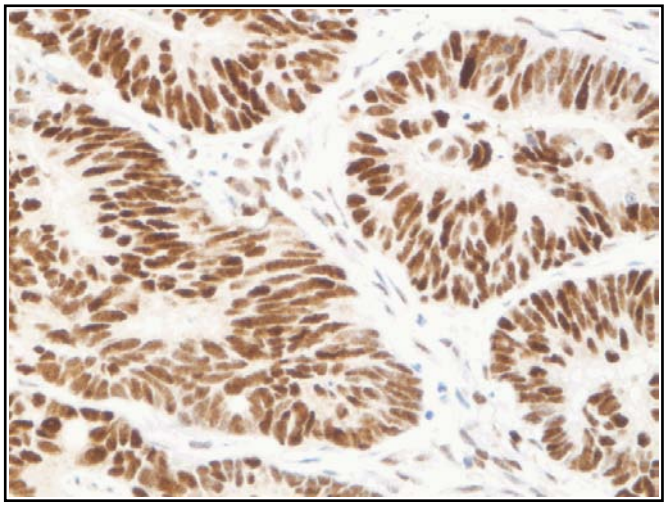


Fig 2. Good demonstration of MSH2 in the UK NEQAS positive colonic tumour: The section shows strong nuclear staining of virtually all the tumour cells and the intratumoural lymphocytes and stromal cells. Section stained with the Novocastra 25D12 antibody, 1:25, on the Leica Bond III with ER2 antigen retrieval for 20 minutes.

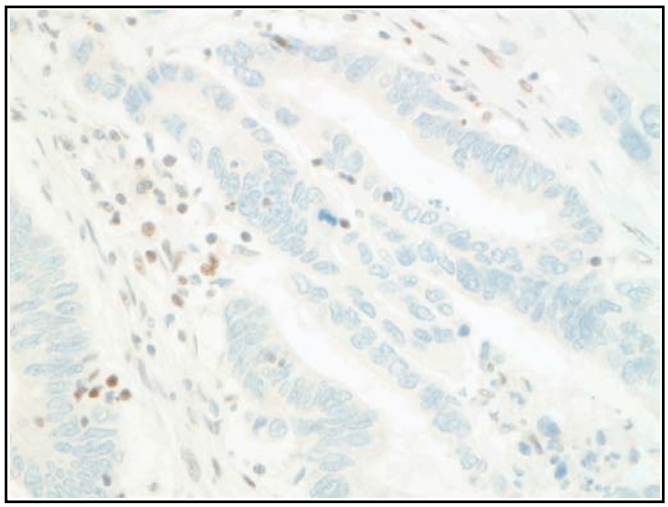


Fig 3. Acceptable staining of MSH2 on the UK NEQAS distributed MSH2 negative colonic tumour: Only the intratumoral lymphocytes and stromal cells are staining positive. Section stained with the Ventana G219-1129 prediluted clone on the Ventana ULTRA platform with CC1 48 minutes antigen retrieval and OptiView detection kit.

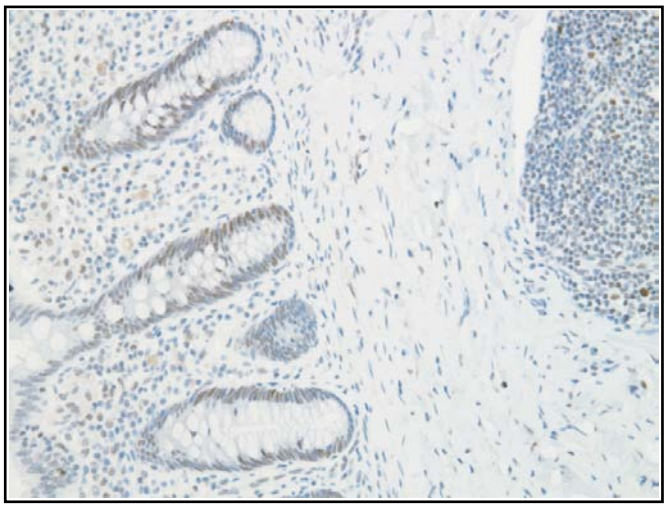


Fig 4. Weak demonstration of MSH2 on the UK NEQAS distributed appendix section (compare to Fig 1). Section stained with the Leica 25D12 antibody, 1:30, on the Leica Bond III with ER1 antigen retrieval for 30 minutes.

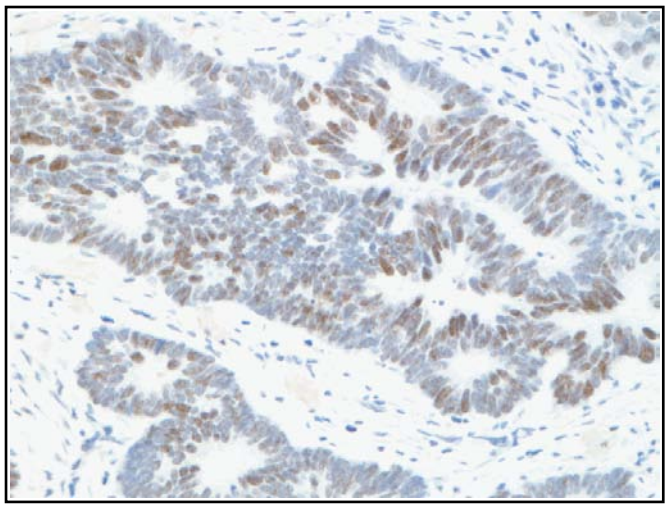


Fig 5. Sub-optimal demonstration of MSH2 on the UK NEQAS distributed MSH2 positive colonic tumour (compare with fig 2). The staining is weak and with less tumour cells staining than expected.

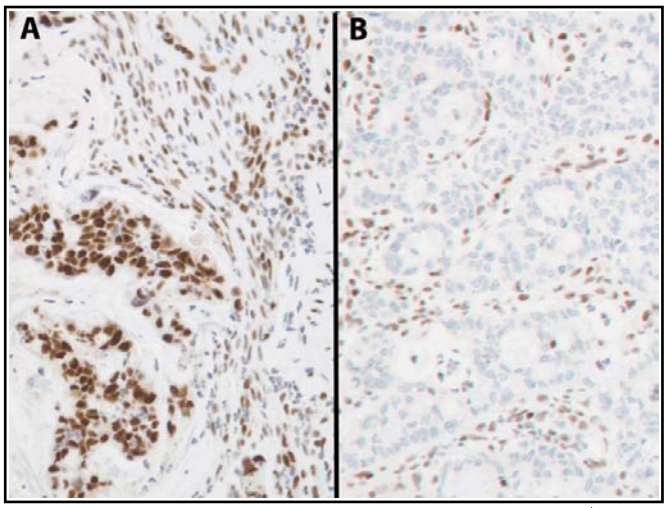


Fig 6. Good example and staining of an in-house control for MSH2: The section includes (A) positive and (B) negative expressing tumours. Both tumours also demonstrate distinct staining of the intratumoral lymphocytes and stromal cells. Stained with the Cell Marque G219-1129 antibody, 1:200, on the Ventana Benchmark XT, CC1 standard and OptiView detection.

Selected Images showing Optimal and Sub-optimal Immunostaining

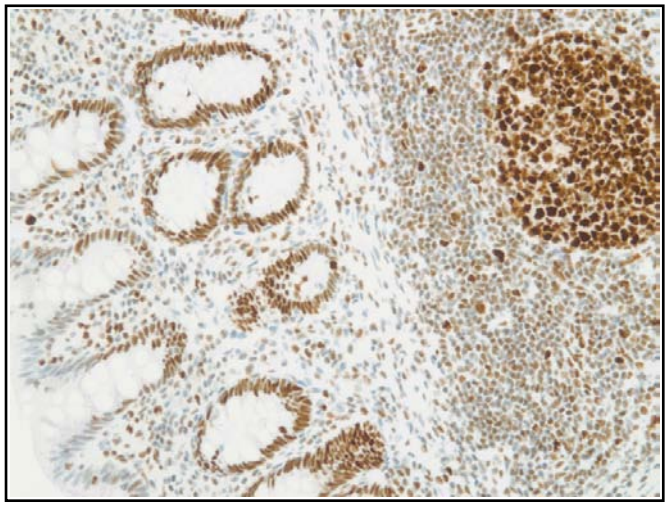


Fig 7. Optimal demonstration of MSH6 in the UK NEQAS distributed appendix. Strong MSH6 staining of the epithelial and stromal cells, with fading of staining intensity towards the luminal surface of the epithelial crypts. Stained with the Dako RTU EP49 antibody on the Dako Autostainer and pre-treatment in the PT Link.

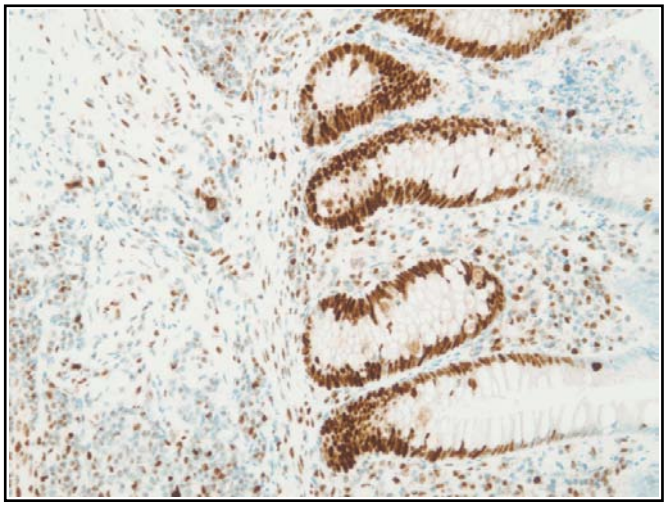


Fig 8. Good demonstration of MSH6 in the UK NEQAS distributed appendix, showing strong to moderate nuclear staining in the basal and lower half of the epithelial crypts, which fades towards the luminal surface. Section stained with the Ventana pre-diluted 44 antibody, on the ULTRA Autostainer with CC2 pre-treatment for 92 minutes and OptiView detection.

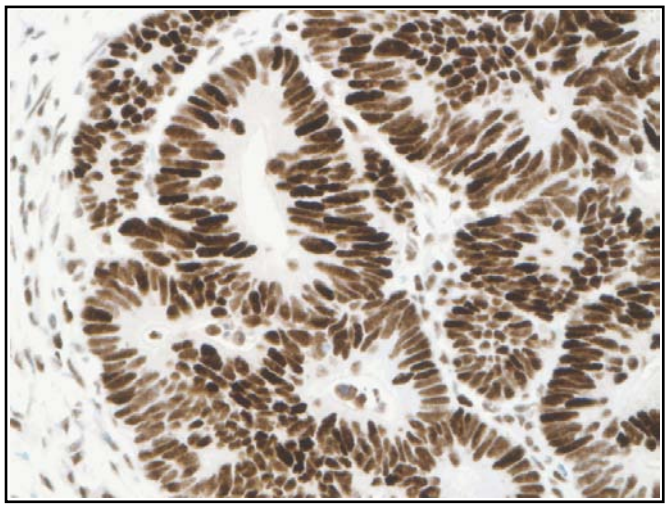


Fig 9. Optimal demonstration Of MSH6 in the UK NEQAS tumour with normal expression of MSH6. The section shows strong nuclear staining of virtually all neoplastic cells, with distinct staining in the intratumoral lymphocytes and stromal cells. Section stained with the Dako RTU EP49 antibody on the Leica Bond III, ER2 antigen retrieval for 30 minutes.

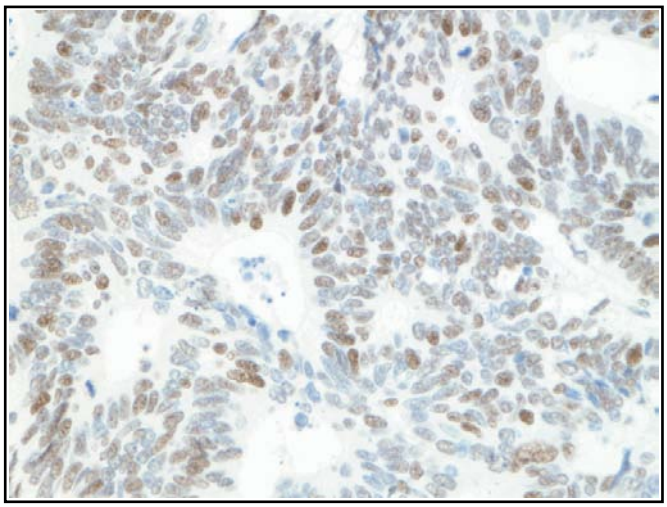


Fig 10. Sub-optimal staining of MSH6 on the UK NEQAS distributed positive colonic tumour (compare with Fig 9). Proportion & intensity of tumour cells is lower than expected with lack of staining of surrounding lymphocytes & stromal cells. Stained using BD Pharmingen 44/MSH6 clone, 1:1500 dilution on the Leica Bond, ER2 for 20 minutes.

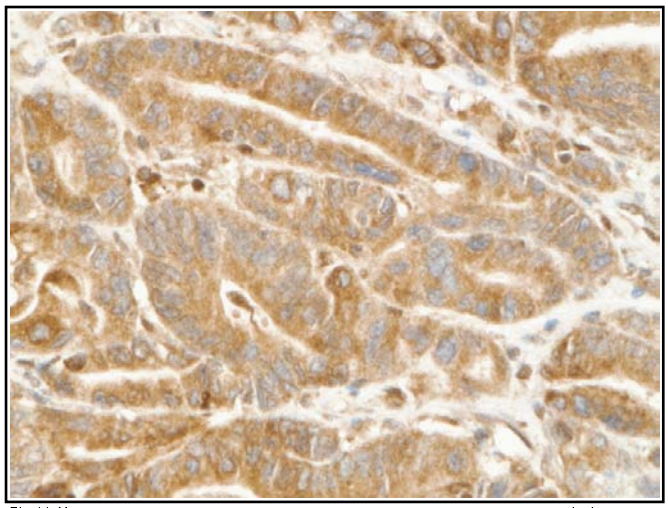


Fig 11. Unacceptable demonstration of MSH6 on the UK NEQAS distributed negative colonic tumour. As expected the tumour nuclei are negative, but section shows excessive non-specific cytoplasmic staining. Stained with the BD Pharmingen 44 clone (1:50), on the Dako Autostainer with Labvison PT Module pretreatment.

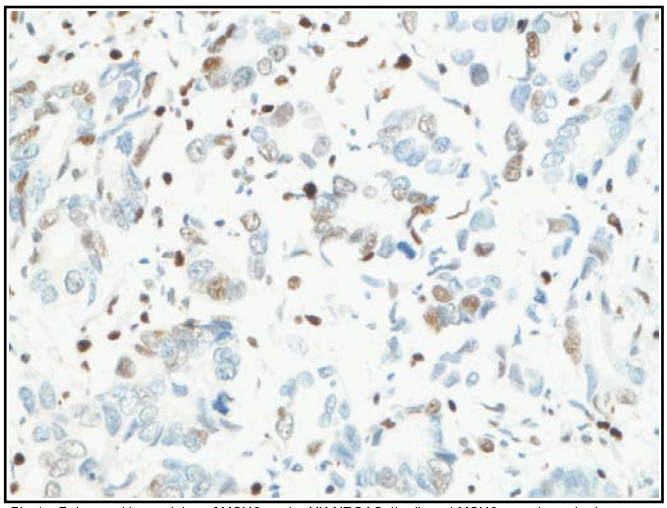
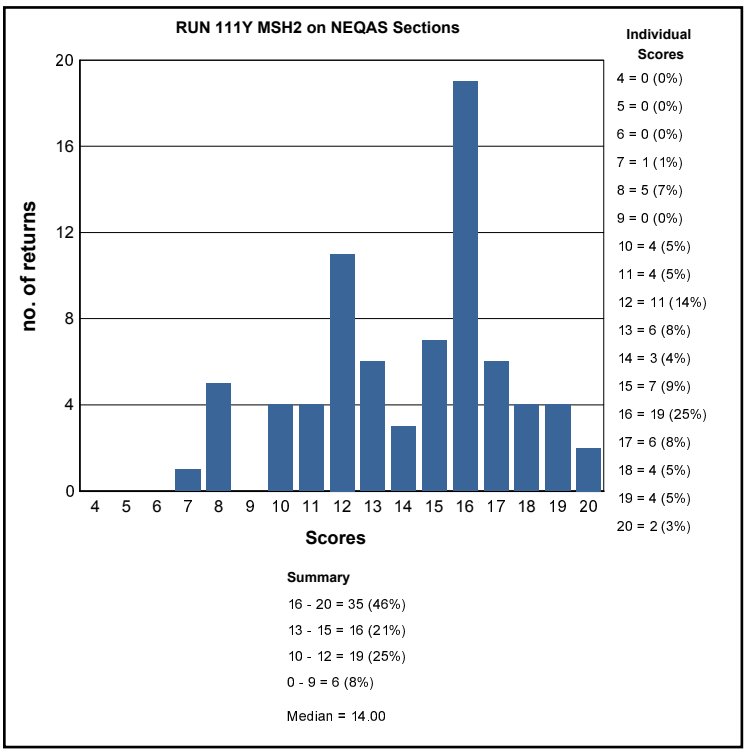
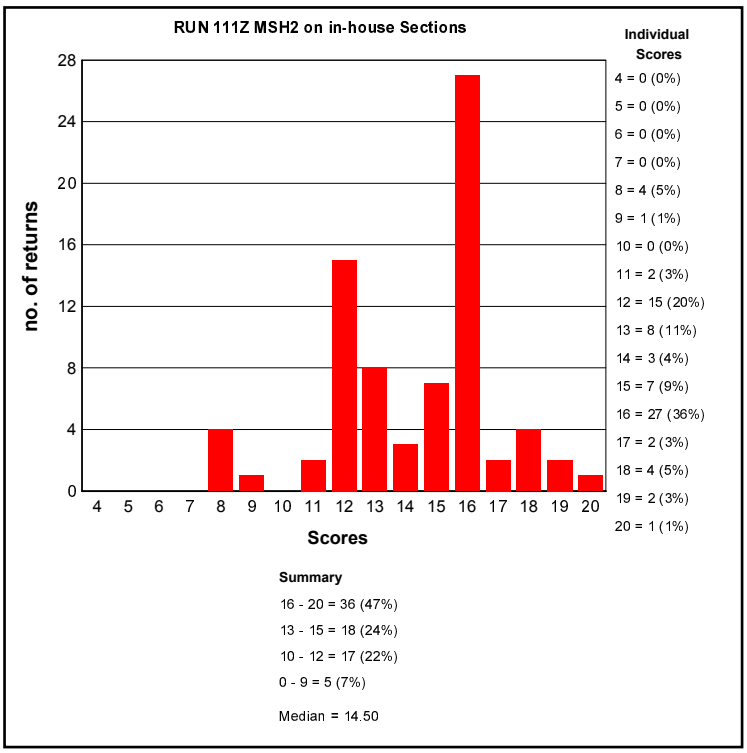
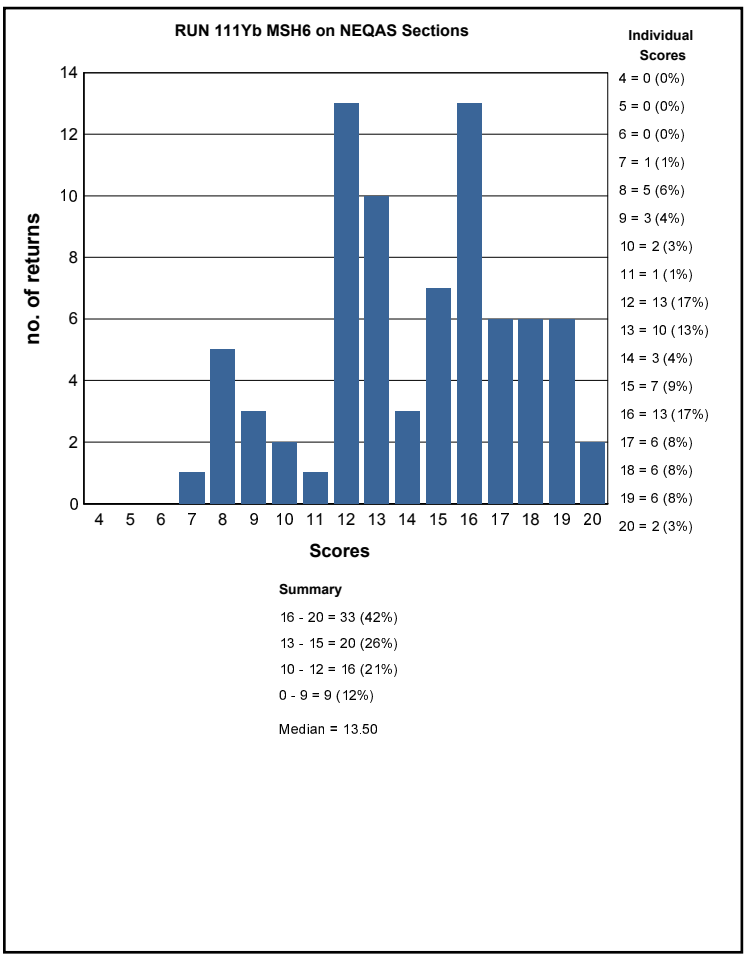


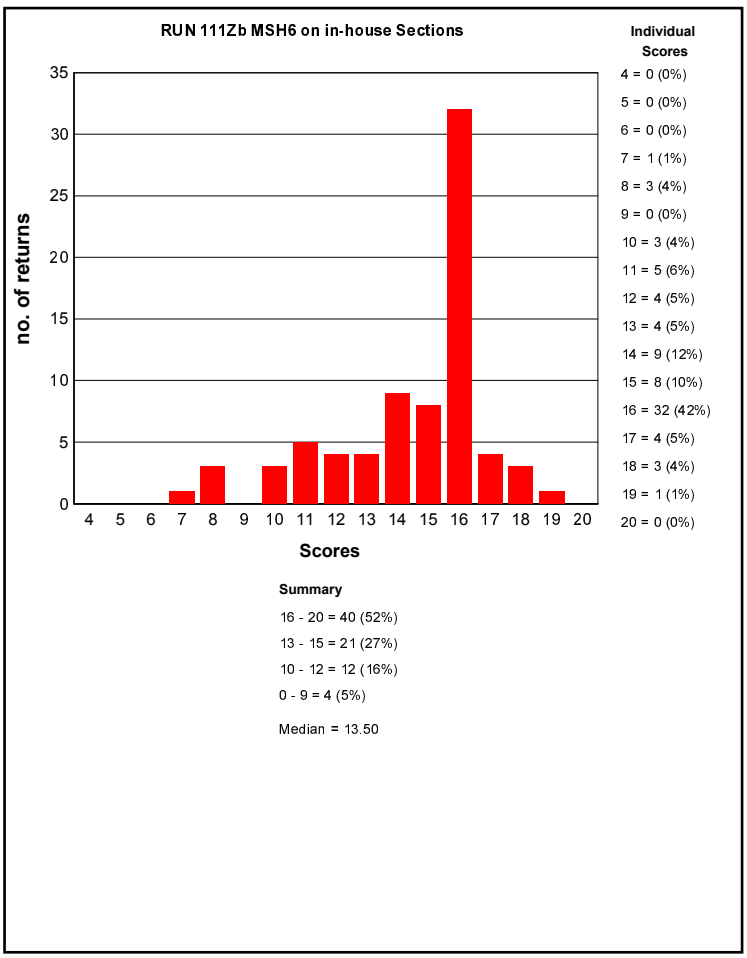
Fig 12. False positive staining of MSH6 on the UK NEQAS distributed MSH6 negative colonic tumour. The inappropriate staining is possibly due to excessive antigen retrieval. Section stained with the Dako EP49 clone, 1:50, on the Dako Autostainer plus using Dako PT Link retrieval for 60 mins in high pH buffer solution.

GRAPHICAL REPRESENTATION OF PASS RATES









ANTIBODIES AND OTHER TECHNICAL PARAMETERS EMPLOYED BY PARTICIPANTS IN THE HNPCC MODULE

The following tables record the number of participants (N) using each primary antibody and the percentage (%) of these participants achieving acceptable staining (scores 12/20) on UK NEQAS sections.

HNPCC Run: 111

Primary Antibody : MSH2

Antibody Details	N	%
BD Bio/Pharmingen 556349 (G219-1129)	5	40
Calbiochem NA26/T (GB12)	1	0
Calbiochem NA27/T (FE11)	1	0
Cell Marque CMAx/Cx (G219-1129)	6	50
Dako FLEX RTU IR085 (FE11)	12	83
Dako M3639 (FE11)	4	100
Epitomics AC-0211 RED2	1	100
Invitrogen 33-7900 (FE11)	2	100
Leica/Novocastra Bond RTU PA0048 (25D12)	2	0
Leica/Novocastra NCL-MSH2 (25D12)	10	30
Other	2	50
Ventana 760-4265 (G219-1129)	29	86

Primary Antibody : MSH6

Antibody Details	N	%
A. Menarini MP-265-CMK/PM	1	0
Abcam AB92471 (EPR3945)	2	50
BD T. Labs/BioSci/Pharmingen 610918 (44)	7	71
BD T. Labs/BioSci/Pharmingen 610919 (44/MSH6)	5	20
Cell Marque 287M-14/15/16 (44)	1	0
Cell Marque 287R-24/25/26 (SP93)	2	100
Cell Marque 287R-27/28 RTU (SP93)	1	0
Dako FLEX RTU IR086 (EP49)	11	91
Dako M3646 (EP49)	19	79
Epitomics AC-0047 (EP49)	2	100
Leica/Novocastra Bond RTU PA0597 (PU29)	1	0
Leica/Novocastra NCL-L-MSH6 (PU29)	2	50
Other	3	0
Ventana CONFIRM 790-4455 (44)	20	75

HNPCC Run: 111

Heat Mediated Retrieval	MSH2 N	MSH2 %	MSH6 N	MSH6 %
Biocare Decloaking Chamber	1	100	1	100
Dako PTLink	9	100	9	89
Lab vision PT Module	1	100	1	0
Leica ER1 20 mins	1	100	0	0
Leica ER1 30 mins	1	0	2	50
Leica ER1 40 mins	0	0	1	0
Leica ER2 20 mins	11	55	12	67
Leica ER2 30 mins	7	29	6	67
Leica ER2 40 mins	6	33	6	50
Steamer	1	0	1	0
Ventana CC1 24mins	0	0	1	0
Ventana CC1 32mins	10	60	3	33
Ventana CC1 36mins	1	100	0	0
Ventana CC1 40mins	1	100	2	100
Ventana CC1 44mins	1	100	0	0
Ventana CC1 48mins	4	100	3	100
Ventana CC1 56mins	2	50	3	33
Ventana CC1 64mins	11	91	10	80
Ventana CC1 72mins	0	0	3	67
Ventana CC1 80mins	0	0	1	100
Ventana CC1 88mins	1	100	2	100
Ventana CC1 8mins	0	0	1	100
Ventana CC1 92mins	1	100	1	100
Ventana CC1 extended	1	0	0	0
Ventana CC1 mild	2	50	1	0
Ventana CC1 standard	2	100	4	50
Ventana CC2 48mins	1	0	0	0
Ventana CC2 64mins	1	0	0	0
Ventana CC2 92mins	0	0	3	100

HNPCC Run: 111

Enzyme Mediated Retrieval	MSH2 N	MSH2 %	MSH6 N	MSH6 %
AS PER KIT	2	100	2	50
NOT APPLICABLE	36	64	35	66

HNPCC Run: 111	MSH2		MSH6	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	5	60	5	60
Biocare polymer (M4U534)	1	100	1	100
Dako EnVision FLEX (K8000/10)	1	0	2	50
Dako EnVision FLEX+ (K8002/12)	6	100	3	100
Leica Bond Polymer Refine (DS9800)	24	42	25	60
None	0	0	2	100
Other	2	100	2	0
Ventana OptiView Kit (760-700)	31	84	27	78
Ventana UltraView Kit (760-500)	6	50	9	56

HNPCC Run: 111	MSH2		MSH6	
Automation	**N**	**%**	**N**	**%**
Dako Autostainer Link 48	7	100	6	100
Dako Autostainer plus	2	100	2	0
Dako Autostainer Plus Link	1	100	2	100
LabVision Autostainer	1	0	1	0
Leica Bond Max	10	40	9	56
Leica Bond-III	16	44	18	61
Menarini - Intellipath FLX	1	100	1	100
Ventana Benchmark GX	1	0	1	0
Ventana Benchmark ULTRA	22	77	21	76
Ventana Benchmark XT	16	75	15	73

HNPCC Run: 111	MSH2		MSH6	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	19	79	18	61
Dako EnVision Plus kits	2	100	2	100
Dako FLEX DAB	5	80	5	80
Leica Bond Polymer Refine kit (DS9800)	24	38	24	67
Other	7	100	7	57
Ventana DAB	12	67	9	89
Ventana Ultraview DAB	8	75	12	58

BEST METHODS - Gold Standard Antibody

A selection from just a few of the best methods employed by participants

MSH2 - Method 1

Participant scored 19/20 (UK NEQAS Slide) and 16/20 (In House slide) using this method.

Primary Antibody: Leica/Novocastra NCL-MSH2 (25D12) , 20 Mins Dilution 1: 25
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 20 mins
EAR: NOT APPLICABLE
Chromogen: Leica Bond Polymer Refine kit (DS9800), Time 1: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins

MSH2 - Method 2

Participant scored 16/20 (UK NEQAS Slide) and 18/20 (In House slide) using this method.

Primary Antibody: Dako M3639 (FE11) Dilution 1: 75
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 40 mins
EAR: NOT APPLICABLE
Chromogen: Leica Bond Polymer Refine kit (DS9800)
Detection: Leica Bond Polymer Refine (DS9800)

MSH2 - Method 3

Participant scored 19/20 (UK NEQAS Slide) and 16/20 (In House slide) using this method.

Primary Antibody:	Ventana 760-4265 (G219-1129) , 16 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana apk Wash (250-042)
HMAR:	Ventana CC1 standard
EAR:	
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700)

MSH2 - Method 4

Participant scored 19/20 (UK NEQAS Slide) and 19/20 (In House slide) using this method.

Primary Antibody:	Dako FLEX RTU IR085 (FE11) , 20 Mins, 21 ºC Prediluted
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	AS PER KIT
HMAR:	Dako PTLink, Buffer: Dakp High pH TRS
EAR:	
Chromogen:	AS PER KIT, 21 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	AS PER KIT , 20 Mins Prediluted

BEST METHODS - Secondary Antibody

A selection from just a few of the best methods employed by participants

MSH6 - Method 1

Participant scored 19/20 (UK NEQAS Slide) and 17/20 (In House slide) using this method.

Primary Antibody:	Dako M3646 (EP49) , 25 Mins, 21 ºC Dilution 1: 25
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER1 30 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

MSH6 - Method 2

Participant scored 20/20 (UK NEQAS Slide) and 16/20 (In House slide) using this method.

Primary Antibody:	Dako FLEX RTU IR086 (EP49) , 20 Mins, 20 ºC Prediluted
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink
EAR:	NOT APPLICABLE
Chromogen:	Dako EnVision Plus kits
Detection:	Dako EnVision FLEX+ (K8002/12)

MSH6 - Method 3

Participant scored 20/20 (UK NEQAS Slide) and 19/20 (In House slide) using this method.

Primary Antibody:	Dako M3646 (EP49)
Automation:	Dako Autostainer Plus Link
Method:	AS PER KIT
Main Buffer:	AS PER KIT
HMAR:	Dako PTLink
EAR:	AS PER KIT
Chromogen:	AS PER KIT
Detection:	AS PER KIT

MSH6 - Method 4

Participant scored 19/20 (UK NEQAS Slide) and 16/20 (In House slide) using this method.

Primary Antibody:	Dako FLEX RTU IR086 (EP49)
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 56mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700)

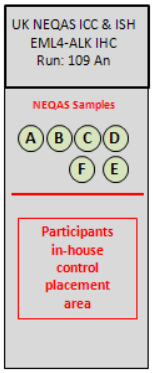
ALK (NSCLC) IHC Module Run 111

Merdol Ibrahim, Suzanne Parry, Dawn Wilkinson, Neil Bilbe, David Allen, Steven Forrest, Perry Maxwell, Tony O'Grady, Jane Starczynski, Phillipe Taniere, John Gosney, Keith Kerr, Erik Thunnissen & Keith Miller

Sample and Slide Distribution

Antibody assessed	ALK
Samples Circulated	Composite slide (see table below)
Number Participants	42

Distributed slide layout and Table 1 below illustrate the positioning of the distributed samples along with their pre-



Sample code	Sample	FISH status (Vysis)	IHC status (Roche D5F3)
A	Appendix	-ve	+ve for ganglion cells & axons
B	Normal lung	-ve	-ve
C	NSCLC adenocarcinoma	+ve (inversion + deletion)	+ve
D	NSCLC adenocarcinoma	-ve	-ve
E	Cell line: 100% adenocarcinoma	-ve	-ve
F	Cell line: 50% isogenic + 50% adenocarcinoma	+ve (inversion)	Approx. 50% +ve & 50% -ve

Introduction

Non-small cell lung cancer (NSCLC) accounts for approximately 80%[1] of lung cancers, with a 5 year survival rate of 17%[1,2]. Existing therapies have limited benefit due to their non-specific targeted approach. However, with the possibility of identifying underlying molecular or mutational changes certain sub-groups of patients may be eligible candidates for more precise targeted therapies, and thus, have a greater chance of survival. A therapeutic drug currently showing good clinical response is Crizotinib[3,4], intended for patients showing anaplastic lymphoma kinase (ALK) rearrangements.

One of the fusion partners of ALK has shown to be echinoderm microtubule associated protein-like 4 (EML4)[5] found in 3-6.7%[6-10] of patients. The EML4-ALK rearrangement was initially assessed using fluorescence in situ hybridisation (FISH). The FISH technique involves the identification of genetic abnormalities using break-apart FISH probes, to identify whether there are genomic rearrangements or deletions between the EML4-ALK fusion pair[3,4]. Brightfield immunohistochemistry (IHC) has also enhanced the possibility of detecting ALK rearrangements[11]. However, IHC for ALK rearrangements can also be challenging due to the relatively low ALK protein concentrations, and therefore requiring more sensitive and/or enhanced detection systems. A more recent publication by Savic and colleagues[12] indicates that certain IHC antibodies perform equally well but still recommend a combined use with FISH testing

Update to the Assessment Criteria

After the initial pre-pilot assessment (Run 108), the assessment procedure has been further updated to include:

Same slide NEQAS and In-house controls

The UK NEQAS ICC & ISH distributed samples were placed and orientated as shown in in the 'Distributed slide layout and Table1. The microscope slide also had an area for participants to cut and place their appropriate in-house control material alongside the NEQAS sample. This set-up made sure that both the NEQAS and in-house controls had the same methodology applied to all samples. The request to include in-house samples also provided information on the selection and type of ALK in-house control that participants are using (see results below).

Interpretation criteria incorporating staining intensity

During the pre-pilot assessment the scoring criteria for ALK IHC employed a simple '+ve'/'-ve' (positive/negative) interpretation for each of the NEQAS distributed samples. This has now been updated for the IHC positive samples to also include feedback on the intensity of the observed staining including 3+ (high), 2+ (medium) and 1+ (low). Assessors were therefore asked to score each of the ALK IHC positive samples as: +ve (3+) OR +ve (2+) OR +ve (1+). This allowed more informative feedback on the intensity of staining on the ALK positive samples but also reflected the different scoring system employed by participants themselves (see Table 2)

Assessment process

- An assessment panel/team consisted of 4 assessors and a microscope driver (lead).
- Each of the two assessment teams scored all the samples independently, providing interpretation of the samples along with comments highlighting if there were interpretation and technical issues.
- Each of the 4 assessors within each team scored the slides with an overall possible score out of 5, with marks summed together to give a final score out of a maximum of 20:

Table 2: Assessment interpretation

Score	Interpretation
16-20/20:	**Excellent:** Samples of very good staining quality and show the expected level of staining
13-15/20:	**Acceptable:** Some slight technical issues noted by some of the assessors, but overall the staining is suitable for interpretation
10-12/20:	**Borderline:** Overall the staining is clinically relevant but technical improvements can be made.
4-9/20:	**Unacceptable:** Overall the samples are of unacceptable quality for clinical interpretation and technical improvements need to be made. Marks may have been deducted due to: - False positive/negative ALK IHC membrane staining - Excessive cytoplasmic staining - Non-specific staining etc
Marks may also be deducted for reasons which will be shown on individual participant reports such as: Poor quality/choice of in-house control tissue, poor/inadequate fixation, damaged cell morphology (over retrieval) etc.	

- During this assessments all participants slides were scored twice to make sure the panel were consistent in their scoring.

Results

Features of Acceptable Staining

- ALK IHC positive tumour samples (sample C) (Fig. 2A & B) should demonstrate granular cytoplasmic staining in the majority of the tumour nuclei.
- ALK IHC positive cell line samples (sample F) (Fig. 2D & E) should demonstrate granular cytoplasmic staining in approximately 50% of the sample preparation. ALK IHC negative cell lines (sample E) (Fig 2G) should show no staining for ALK IHC
- ALK IHC negative tumour sample (sample D) (Fig. 2G) should show no ALK IHC staining
- Normal lung (sample B) (Fig 2H) should generally be negative although there may be some staining within macrophages
- Appendix (sample A) (Fig 2I) should show staining in ganglion cells and axions

Features of Unacceptable Staining

- False negative or absence of ALK staining where tumour cells should be staining positive
- Non-specific / Excessive Tyramide staining (Fig 3 B & C)
- Absence of staining in appendix

Pass Rates & Methodologies

Figure 1A, shows the overall pass rates on the NEQAS distributed samples. Overall there was 86% (n=36) of participants who achieved an acceptable/excellent result, with 10% (n=4) achieving borderline acceptability and 5% (n=2) who's samples were found to be unacceptable.

The reasons for the borderline and unacceptable results were due to staining being too weak (sensitivity down), false positive staining or excessive tyramide staining.

A breakdown of the submitted methods (table 1) showed the use of 3 different antibody clones (D5F3, ALK1 and 5A4) used either as part of an assay (Roche diagnostics) or lab devised methods:

- The Roche D5F3 (Tables 1 & 2) was the main antibody used accounting for 72% (n=28) of users, with 89% demonstrating either an acceptable or excellent staining quality on the NEQAS distributed samples. All those that submitted the D5F3 on the Ventana platform appeared to use the recommended protocol with the enhanced OptiView detection system. The main reason for the borderline and unacceptable results was due to either non-specific tyramide staining, which in one instance (Fig 3B) was soo stronger that the assessors interpreted the sample as being positive for ALK.
- The Leica Biosystems 5A4 clone (Tables 1 & 2) was used by 7 participants in either as a concentrate (n=6) or prediluted (n=1). The concentrate 5A4 clone was used on numerous staining platforms (Table 2) and had a an overall pass rate of 50% (n=3). In previous assessments the 5A4 clone used in conjunction with the Ventana platform and Optiview detection showed staining comparable to the Ventana ALK assay; Again this was observed during this assessment but unfortunately a second participant only achieved a borderline result using this methods, due to the lower sensitivity staining. This may have been due to the participant not using the tyramide amplification step but we had insufficient protocol data to forma full conclusion
- The cell signalling D5F3 clone (Table 1 and 2) was used by 2 participants and had a 100% pass rate
- The Dako ALK1 (Table 1 & 2) (not recommended by Dako for NSCLC) was used only by a single participant who's staining was deemed to acceptable, although the staining intensity was lower than that seen by the Optiview users.

Scoring Systems Employed by Participants

Of the 38 participants who submitted scoring methodology data, 82% (n=31) used a simple +ve/-ve scoring methods with 18% (n=7) using an intensity based scoring method (3+,2+,1+ and Neg.).

In-house Controls

The scoring of in-house controls is not solely based on the quality of ALK staining but the choice and suitability of the controls in gauging the sensitivity and specificity of ALK in the lung setting. Assessors also look at the quality and preservation of the submitted samples.

Forty out of forty two (95%) participants submitted in-house controls alongside the NEQAS distributed sample (Fig. 1B), and of these participants 37% (n=15) were assed as having submitted an acceptable in-house control, 59% (n=24) showing borderline acceptability and 5% (n=2) unacceptable in-house controls.

As indicated in the table below participants not submitting an appropriate control or solely a single section are scored a maximum 'borderline' score (10-12/20).

Submission on in-house controls for assessments

- Participants in-house control tissue should consist of composite control as outlined above
- Participants submitting solely a single section e.g. a single positive/negative lung control, appendix, lymphoma etc will be scored a maximum 'borderline' score (10-12/20).

Tissue types submitted

Table 4 shows the tissue types submitted.

- 43% submitted only a single in-house control, which was deemed to be unacceptable so a maximum borderline score (12/20) was given. 60% of these participants submitted a single tonsil section, with a further 27% submitting a single ALK positive lung tumour
- 28% submitted at least 2 controls with over 50% including both an ALK positive and negative control
- 31% submitted at least three or more in-house controls with a variety of tissue types

Lymphoma control

Lymphoma as a control in the lung setting for ALK IHC is not recommended and gives a false security of the sensitivity of the test, as illustrated in Figs 5E and F, where the Dako ALK1 clone was used. In this example, even though the lymphoma sample shows the expected level of staining (Fig 5F), the NEQAS distributed NSCLC sample (Fig 5E) showed false negative IHC staining.

Appendix control

Appendix appears to be useful to gauge the sensitivity of ALK IHC, but is not recommended as a 'single tissue control' and

where used should be used alongside a multi-tissue control (see below)

Recommended ALK IHC controls

The UK NEQAS ICC & ISH ALK IHC assessment panel recommends that the ideal control should consist of:

a. NSCLC ALK IHC positive tumour: Gauges sensitivity
b. NSCLC ALK IHC negative tumour: Gauges specificity
c. -Appendix may also be used alongside the lung tumour controls (see Fig 6): Gauges both sensitivity and specificity

- Commercially available control material (e.g. cell lines, xenografts etc) showing at least positive and negative ALK IHC expression may also be an acceptable alternative.
- In all cases the control material should initially be validated using FISH.
- It is also recommended that control material should be cut and placed alongside the clinical sample being tested.

Acknowledgements

We are grateful for Novartis Pharmaceuticals UK Limited for providing an educational grant which was used to help setup the NSCLC ALK EQA module. Novartis are not privy to any data/results until they are publically available.

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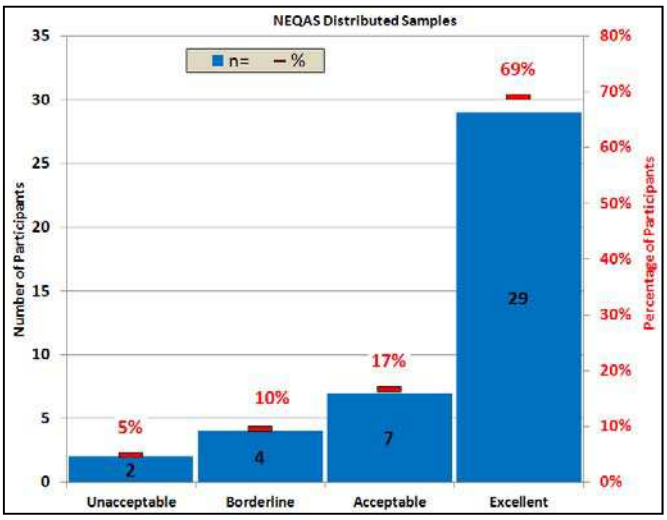
Fig1. Pass Rates: Below graphs show (A) NEQAS and (B) Participant in-house pass rates

A. NEQAS Sample Pass Rates



B. IN-HOUSE Sample Pass Rates

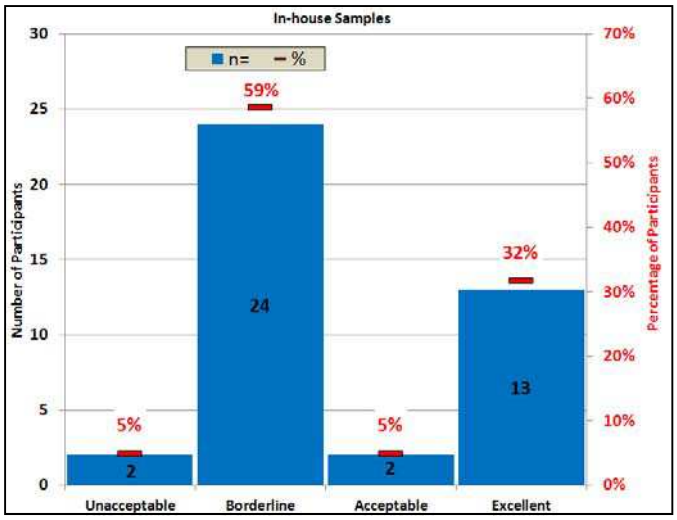


Table 1: Antibodies submitted and respective pass rates (n=39)

Antibody	n=	% of total methods submitted	Excellent	Acceptable	Borderline	Unacceptable
Cell Signalling Tech. D5F3	2	5%	2 (100%)	-	-	-
Dako M7195 (ALK1)	1	3%	-	1 (100%)	-	-
Genemed (D5F3)	1	3%	1 (100%)	-	-	-
Novocastra NCL-ALK (5A4)	6	15%	2 (33%)	1 (17%)	2 (33%)	1 (17%)
Novocastra PA0306 (5A4)	1	3%	-	1 (100%)	-	-
Ventana/Roche (D5F3)	28	72%	21 (75%)	4 (14%)	2 (7%)	1 (4%)

Table 2: Pass rates showing associated antibody, automated platform and detection systems employed

Primary antibody	Automation Instrument	Detection kit	Excellent	Acceptable	Borderline	Unacceptable
Cell Signalling Tech. D5F3	LabVision Autostainer	Dako Envision HRP/DAB (K5007)	1 (100%)	-	-	-
	Ventana Benchmark XT	Ventana OptiView Kit (760-700)	1 (100%)	-	-	-
Dako M7195 (ALK1)	Dako Autostainer Link 48	DAKO Envision FLEX+ mouse Linker	-	1 (100%)	-	-
Genemed (D5F3)	Ventana Benchmark XT	Ventana OptiView Kit (760-700)	1 (100%)	-	-	-
Novocastra NCL-ALK (5A4)	Dako Autostainer Link 48	Dako EnVision FLEX+ (K8002/12)	-	1 (100%)	-	-
		DAKO Envision FLEX+ mouse Linker	1 (100%)	-	-	-
	Leica Bond-III	Leica Bond Polymer Refine (DS9800)	-	-	1 (50%)	1 (50%)
	Ventana Benchmark ULTRA	Ventana OptiView Kit (760-700)	-	-	1 (100%)	-
	Ventana Benchmark XT	Ventana OptiView Kit (760-700)	1 (100%)	-	-	-
Novocastra PA0306 (5A4)	Leica Bond Max	Leica Bond Polymer Refine (DS9800)	-	1 (100%)	-	-
Ventana/Roche (D5F3)	Ventana Benchmark	Ventana OptiView Kit (760-700)	3 (100%)	-	-	-
	Ventana Benchmark ULTRA	Ventana OptiView Kit (760-700)	5 (100%)	-	-	-
	Ventana Benchmark XT	Ventana OptiView Kit (760-700)	13 (65%)	4 (20%)	2 (10%)	1 (5%)

Table 3: Scoring systems used for respective antibody clones

		Scoring Methods Used	
Antibody (clone)	**n =**	**+ve / -ve**	**3+,2+,1+, neg.**
Ventana/Roche (D5F3)	27	89%	11%
Novocastra NCL-ALK (5A4)	6	67%	33%
Cell Signalling Tech. D5F3	2	-	100%
Dako M7195 (ALK1)	1	100%	-
Genemed (D5F3)	1	100%	-
Novocastra PA0306 (5A4)	1	100%	-
	Overall	**82%**	**18%**

Table 4: Controls and tissue types submitted

no. in-house controls submitted per participant	no. participants	% Participants	Tissue types submitted
0	0	0%	-
1	17	43%	60%: Appendix 27%: NSCLC (+ve) 13%: Lymphoma (+ve) 13%: NSCLC cell block (+ve)
2	11	28%	55%: NSCLC (+ve) + NSCLC (-ve) 9%: NSCLC cell line (+ve) + NSCLC cell line (-ve) 9%: Appendix (+ve) + Other (+ve) 9%: Other (+ve) + Other (-ve) 9%: NSCLC (+ve) + Lymphoma 9%: NSCLC (+ve) + Other (-ve)
3	7	18%	29%: NSCLC (+ve) + Lymphoma (-ve) + Other (-ve) 14%: 2 x NSCLC (+ve) + Lymphoma (+ve) 14%: NSCLC (+ve) + Appendix (+ve) + Other (-ve) 14%: 2 x ALCL (+ve) + Appendix (+ve) 14%: NSCLC (+ve) + NSCLC (-ve) + Appendix (+ve) 14%: 2 x NSCLC (+ve) + SCLC (-ve)
>3	5	13%	-

Figure 2

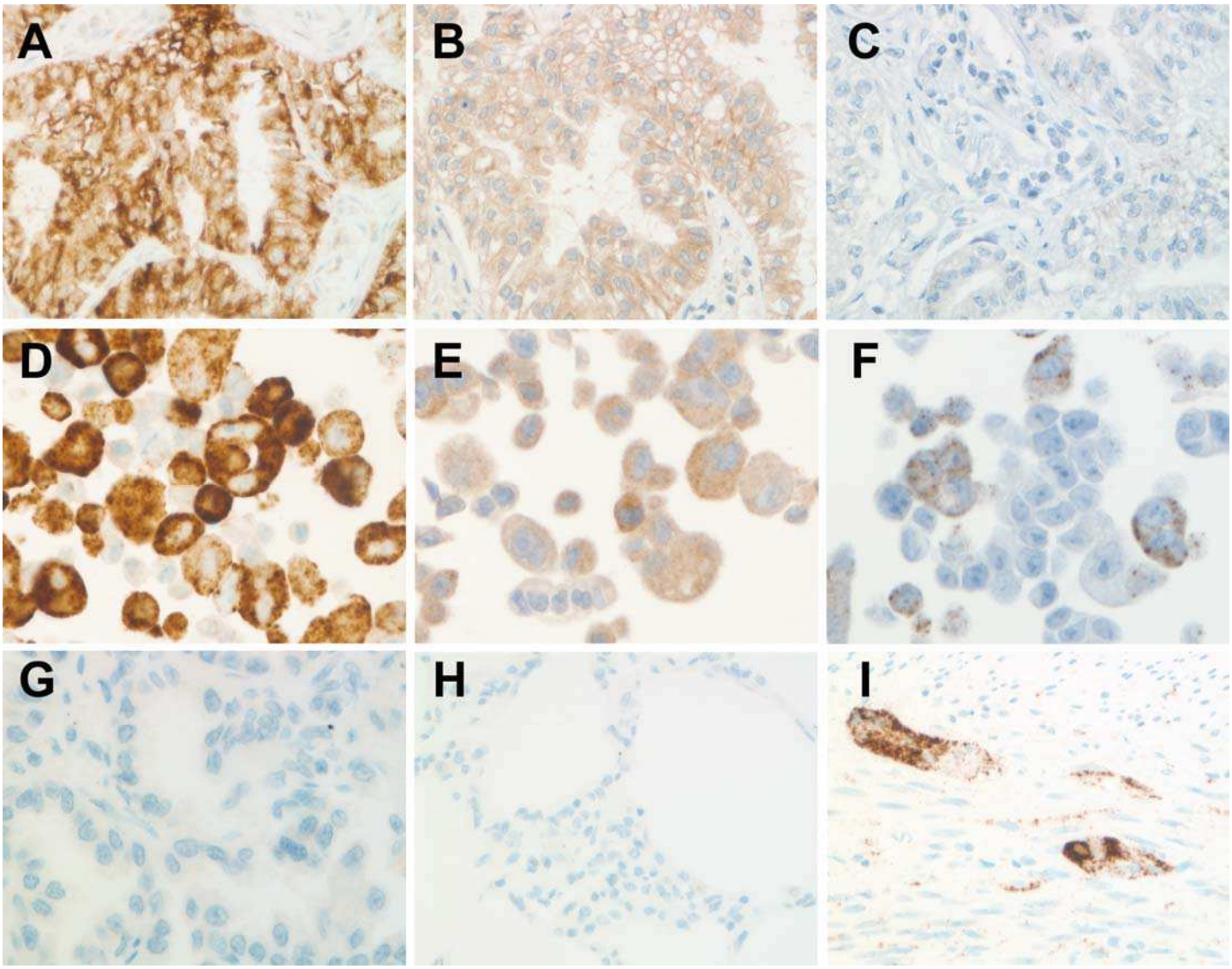


Fig 2
(A,C) NEQAS distributed ALK positive tumour (sample 'C'). (A & B) Good demonstration of ALK showing the acceptable levels of staining. (A) stained on the Ventana Benchmark XT using the D5F3 assay and OptiView detection (B) Stained using Novocastra 5A4 on a Bond III with the Leica Bond Polymer refine detection. (C) Unacceptable ALK staining, leading to a false-negative result. This sample was also stained using Novocastra 5A4 on a Bond III with the Leica Bond Polymer refine detection. The false-negative staining could have been due to a failed test rather than an issue with the methodology itself.

(D,F) NEQAS distributed ALK positive cell line (Sample 'F'). (D & E) Acceptable levels of staining. (D) stained on the Ventana Benchmark XT using the D5F3 assay with OptiView detection (E) Stained using Novocastra 5A4 on a Bond III with the Leica Bond Polymer refine detection. (F) Staining is much weaker than expected (compare to D & F) with many of the expected ALK positive cells not staining.

(G) Acceptable demonstration of ALK in the NEQAS distributed negative tumour (Sample 'D'). Stained using the same methods as in Figure A.

(H) Acceptable demonstration of ALK in the NEQAS distributed normal lung (Sample 'B'). Stained using the same methods as in Figure A.

(I) NEQAS distributed appendix tissue (Sample 'A'), showing very nice demonstration of the ganglion cells and axons. Stained using the Ventana Benchmark XT.

Figure 3

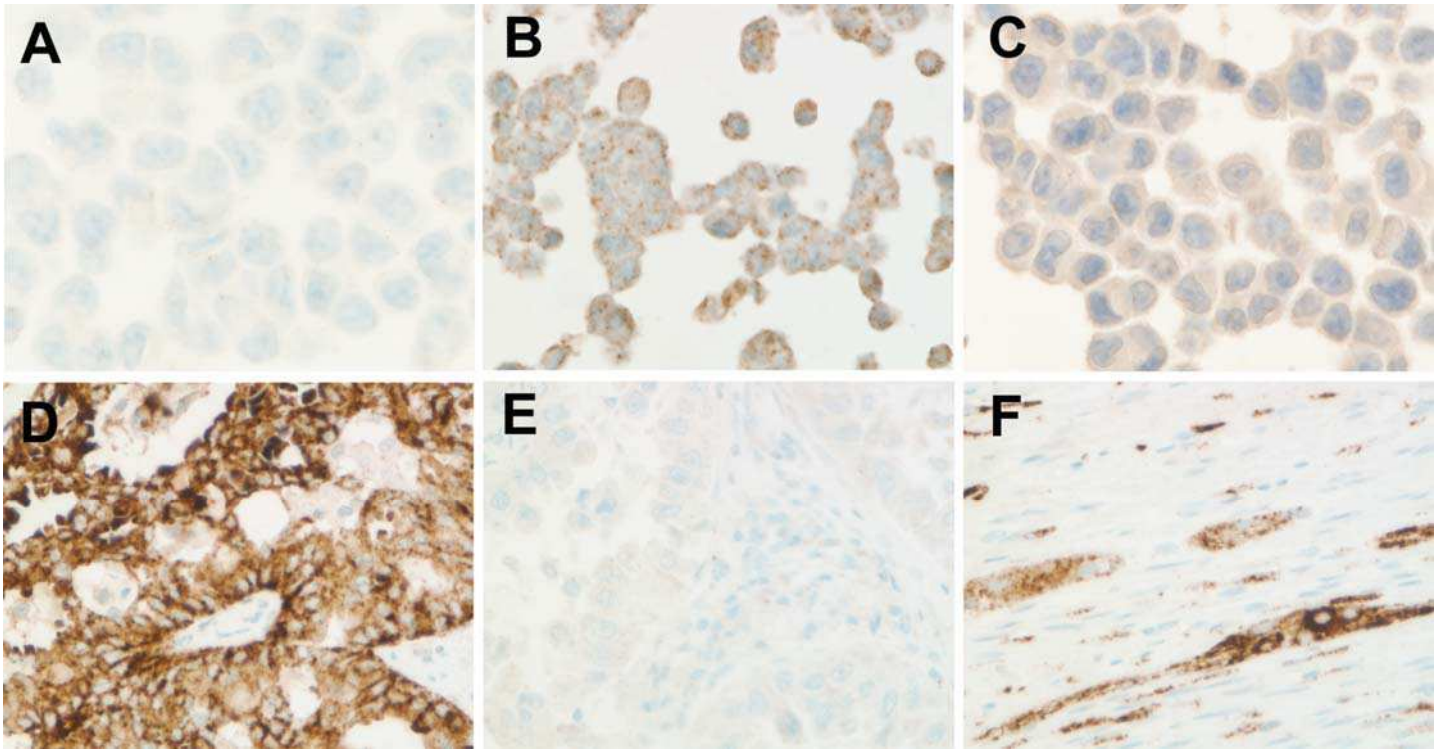


Fig 3
(A) Good demonstration of ALK staining in the NEQAS distributed negative cell line (Sample 'E'). Stained on the Ventana Benchmark XT using the D5F3 assay using OptiView detection

(B) Excessive tyramide deposits in the NEQAS distributed negative cell line (Sample 'E'; compare with fig 2A). Stained using the Ventana Benchmark XT with the D5F3 assay and OptiView detection. From observations at NEQAS excessive tyramide deposits may be associated with excessive baking of slides.

(C) Excessive background in the NEQAS distributed negative cell line (Sample 'E'; compare with fig 2A). Stained using Novocastra 5A4 on a Bond III with the Leica Bond Polymer refine detection.

(D-F) Good in-house control submission showing (A) ALK positive NSCLC tumour (B) ALK negative NSCLC and (F) ALK positive appendix control. Stained on the Ventana Benchmark XT using the D5F3 assay and OptiView detection

The HER2 ISH Module - Interpretive Run 40

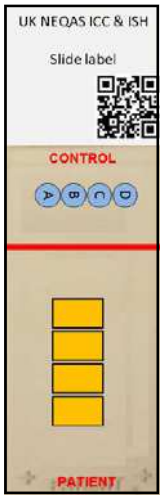
Merdol Ibrahim and Suzanne Parry

Gene Assessed:	HER2
Sections Circulated:	Composite slide consisting of 4 breast carcinoma sections (see table below)
Number of Registered Participants:	173
Number of Participants This Run	142 (82%)

Tissue Expression levels: The summary box below gives details of the HER2 phenotype and genotype of the tissues circulated for this assessment, which comprised of formalin fixed and paraffin processed sections from a composite block of four human breast carcinomas.

Sample	HER2 status by IHC	HER2 status by FISH
A	**2+**	Amplified
B	**2+**	Non-Amplified
C*	**2+**	Non-amplified
D	**2+**	Non-Amplified

Tissue Section Positioning: Tissue sections were positioned on microscope slides as illustrated in the



NEQAS samples

Area to place in-house samples

Introduction

Breast cancer HER2 status should be determined in all newly diagnosed and recurrent breast cancers. In-situ hybridization (ISH), using either fluorescent (FISH)[4] or brightfield chromogenic methods[5] are now recognised techniques to determine the amplification status of breast cancers in either 2+ IHC equivocal cases or as an upfront method. The chromogenic assays (**CISH**) are now used as abundantly as **FISH.** Initially only the single HER2 probe methods were used, and several of these are still available, such as the Ventana Silver ISH (SISH)[3], however, over the past few years the dual probe (ratio) based techniques have become the preferred methods of choice.

For UK laboratories who are accredited to carry out diagnostic HER2 ISH testing, participation in an external quality assessment (such as the UK NEQAS ICC & ISH) is a requirement, and therefore poor performance is monitored on a regular basis to enhance confidence in HER2 ISH testing. It should also be emphasised that the scheme remains very much open to non-UK laboratories as an aid to monitoring and improving performance.

Recommendations

There are several recent recommendations regarding HER2 ISH testing including those by CAP (USA) and UK[6,7]. It is advisable that these guidelines are followed and processes of introducing and quality controlling clinical HER2 ISH testing are properly validated prior to their introduction into the laboratory.

Updated Assessment Procedure

The assessment of slides now utilises a statistical method in order to provide more concise information with regards to inter-observer variability in enumerating HER2 copy, chromosome 17 and overall ratios (See table: **Statistical Approach to the Scoring System**).

Assessment Results

All participants who took part in the current ISH module assessment (Run 40) are using a dual probe (ratio) method. 54% of labs (N=71) are using the fluorescence ISH technique, ad 46% (N=61) are using chromogenic ISH techniques.

Overall the acceptable pass rate for all participants was 71% (≥30/36), and a further 24% received a borderline pass (24-29/36). 7 labs (5%) failed the assessment (scores of <24/36). FISH users alone showed an acceptable pass rate of 82%, and the labs using chromogenic methods showed an acceptable pass rate of 68%.

Frequency Histograms

Frequency histograms of the assessment results are also provided showing the distribution of HER2 copy and Cen17 counts and ratio calculations. Participants are encouraged to use the provided frequency histograms to gauge their performance and variability in counts for each of the distributed samples.

HER2 ISH Method and Probe Enumeration

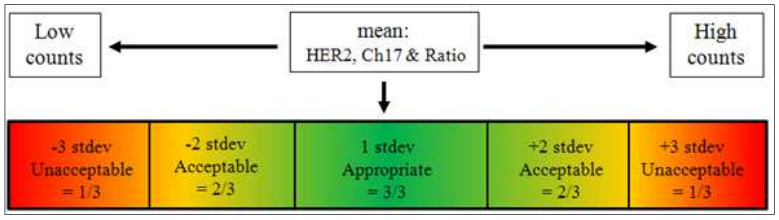
The table on the next page (table 1) shows the various ISH methods employed along with mean HER2, Cen17 counts and ratio calculations.

References

1. Walker RA, Bartlett JM, Dowsett M, Ellis IO, Hanby AM, Jasani B, Miller K. Pinder SE. (2008) HER2 testing in the UK: further update recommendations. J Clin Pathol;61818-824.
2. Bartlett JM, Starczynski J, Atkey N, Kay E, O'Grady A, Gandy M, Ibrahim M, Jasani B, Ellis IO, Pinder SE, Walker RA. (2011) HER2 testing in the UK: recommendations for breast and gastric in-situ hybridisation methods. J Clin Pathol; 64: 649-653.
3. Moore, D. S. and McCabe, G. P. Introduction to the Practice of Statistics, 4th ed. New York: W. H. Freeman, 2002.

Table: Statistical Approach to the Interpretive ISH Scoring System

1. All participant data is initially evaluated to exclude '**outliers**'. An outlier is defined as those scores that are 1.5 times the interquartile range (IQR) outside of the lower and upper quartiles.
2. A mean score is then generated for the submitted HER2 copy, chromosome 17 and ratios.
3. Counts for HER2 copy, Cen17 and ratio are scored individually such that results within +/- 1 standard deviation (stdev) of the mean are score 3/3, +/- 2 stdev = 2/3 and ≥ +/- 3 stdev = 1/3. A non submission is scored 0/3.



4. For each of the samples there is therefore a possible score out of 9 (those using a ratio method) and 3 (those using a single copy method). As NEQAS ICC & ISH distributes 4 samples there is a possible score out of 36 (those using a ratio method) and 12 (those using a single copy method). (score = 3).

Dual Probe: Ratio Scoring Method

- Marks are awarded for each criteria (HER2 copy, Cen17and Ratio) with a possible score out of 9 for each sample (A,B,C & D). For the 4 samples, the marks are added together to give a possible score out of 36.
- Marks are awarded for each criteria (HER2 copy, Cen17& Ratio) by comparing your score to standard deviation (stdev) from the mean score, such that: (a) 3/3 = within 1 stdev from the mean, (b) 2/3 = within 2 stdev from the mean (c) 1/3 = > 3 stdev from the mean.
- Score interpretation: 36/36 = Excellent ; 30-35/36 = Acceptable ; 24-29/36 = Borderline ; <24/36 = Unacceptable

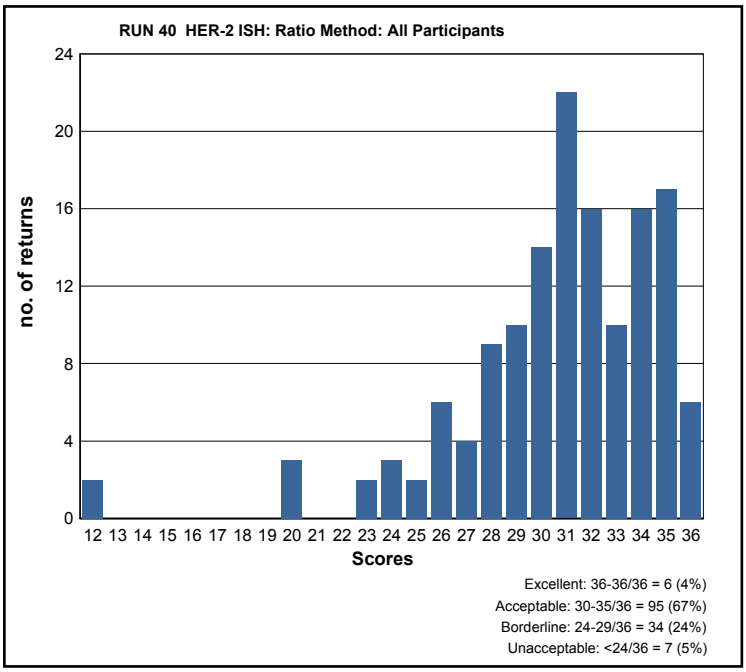
Single Probe: HER2 Copy Scoring Method

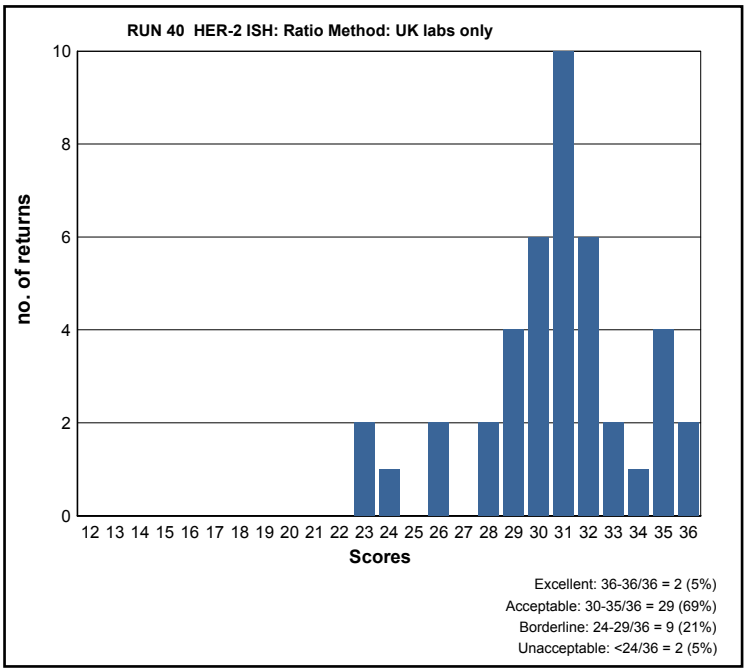
- Marks are awarded for HER2 copy counts, with a possible score out of 3 for each samples (A,B,C & D). For the 4 samples, the marks are summed together to give a possible score out of 12.
- Marks are awarded for HER2 copy counts by comparing your counts with the standard deviation (stdev) from the mean score, such that: (a) 3/3 = within 1 stdev from the mean, (b) 2/3 = within 2 stdev from the mean (c) 1/3 = > 3 stdev from the mean.
- Score interpretation: 12/12 = Excellent ; 10-11/12 = Acceptable ; 8-9/12 = Borderline ; <8/12 = Unacceptable

Table 1: Methods and mean counts/ratios (n/a= not applicable ; n/d = not done):

		Sample A: mean (stdev)			Sample B: mean (stdev)			Sample C: mean (stdev)			Sample D: mean (stdev)		
METHOD	n=	Copy No	Cen17	Ratio	Copy No	Cen17	Ratio	Copy No	Cen17	Ratio	Copy No	Cen17	Ratio
Ratio: Dako DuoCISH	1	5.3 (n/a)	1.9 (n/a)	2.78 (n/a)	2.33 (n/a)	2.45 (n/a)	0.95 (n/a)	2.04 (n/a)	1.86 (n/a)	1.09 (n/a)	2.72 (n/a)	2.38 (n/a)	1.14 (n/a)
Ratio: Dako IQFISH pharmDX	9	6.41 (0.99)	2.77 (0.48)	2.33 (0.22)	2.5 (0.33)	2.46 (0.41)	1.02 (0.13)	2.31 (0.53)	1.95 (0.29)	1.18 (0.15)	2.91 (0.24)	2.07 (0.39)	1.44 (0.22)
Ratio: Dako Pharm Dx	4	5.7 (0.44)	2.23 (0.39)	2.57 (0.23)	2.75 (0.18)	2.64 (0.22)	1.05 (0.09)	2.98 (1.07)	2.16 (0.31)	1.38 (0.28)	3.16 (0.25)	2.21 (0.35)	1.42 (0.22)
Ratio: Kreatech Probes	4	5.94 (0.57)	2.48 (0.59)	2.62 (1.07)	2.43 (0.04)	2.36 (0.21)	1.04 (0.08)	2.07 (0.11)	1.65 (0.05)	1.28 (0.12)	2.81 (0.23)	2.13 (0.41)	1.34 (0.2)
Ratio: Leica HER2 FISH TA9217	9	6.04 (0.31)	2.1 (0.38)	2.94 (0.47)	2.7 (0.28)	2.41 (0.27)	1.12 (0.12)	2.58 (0.52)	2.01 (0.51)	1.3 (0.21)	3.17 (1.34)	2.2 (0.98)	1.47 (0.54)
Ratio: Other - CISH	1	5.25 (n/a)	1.8 (n/a)	2.92 (n/a)	2.45 (n/a)	2.55 (n/a)	0.96 (n/a)	2.3 (n/a)	1.8 (n/a)	1.28 (n/a)	2.98 (n/a)	1.75 (n/a)	1.7 (n/a)
Ratio: Other - FISH	2	6.65 (n/a)	2.18 (n/a)	3.94 (n/a)	2.12 (n/a)	1.9 (n/a)	1.2 (n/a)	1.86 (n/a)	1.51 (n/a)	1.24 (n/a)	3.12 (n/a)	2.1 (n/a)	1.54 (n/a)
Ratio: Pathvysion Vysis Kit	48	5.95 (0.91)	2.49 (0.58)	2.52 (0.75)	2.49 (0.48)	2.5 (0.4)	1.02 (0.24)	2.22 (0.54)	1.94 (0.39)	1.15 (0.13)	2.78 (0.52)	2.19 (0.34)	1.28 (0.19)
Ratio: Ventana BDISH 800-098/505	7	5.77 (n/a)	2.18 (n/a)	2.66 (n/a)	2.48 (n/a)	2.34 (n/a)	1.01 (n/a)	3.49 (n/a)	1.94 (n/a)	1.83 (n/a)	3.09 (n/a)	2.2 (n/a)	1.41 (n/a)
Ratio: Ventana DDISH (780/800-4422)	41	6.07 (1.95)	2.01 (0.62)	2.93 (0.87)	2.68 (0.81)	2.12 (1.01)	1.24 (0.37)	2.28 (0.77)	1.69 (0.51)	1.26 (0.31)	3.16 (0.91)	1.81 (0.53)	1.69 (0.46)
Ratio: Ventana Inform Silver ISH	8	5.28 (1.57)	1.92 (0.38)	2.79 (0.53)	2.59 (0.49)	2.24 (0.31)	1.17 (0.17)	2.16 (0.37)	1.68 (0.27)	1.3 (0.19)	3.08 (0.66)	1.86 (0.27)	1.66 (0.34)
Ratio: Zytovision ZytoDot 2C	3	5.41 (n/a)	1.65 (n/a)	3.44 (n/a)	2.83 (n/a)	1.97 (n/a)	1.39 (n/a)	2.07 (n/a)	1.61 (n/a)	1.3 (n/a)	2.81 (n/a)	1.57 (n/a)	1.89 (n/a)
Ratio: Zytovision ZytoLight	5	6.14 (1.06)	2.31 (0.1)	2.71 (0.46)	2.7 (0.72)	2.69 (0.56)	1 (0.05)	2.06 (0.03)	1.72 (0.04)	1.2 (0.04)	2.99 (0.33)	2.17 (0.15)	1.39 (0.21)

GRAPHICAL REPRESENTATION OF PASS RATES (ratio methods only)





METHODS USED and PASS RATES

The following tables record the number of participants (N) using each method, antibody and the percentage (%) of these participants achieving acceptable interpretation. For those using a dual probe (ratio) method the pass rate is ≥30/36 and for those using a single HER2 copy method the pass rate is ≥10/12

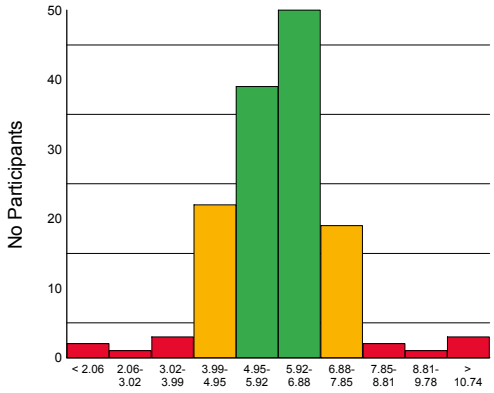
Ratio Method	N	% Pass (score ≥ 30/36)
Ratio: Dako DuoCISH	1	100%
Ratio: Dako IQFISH pharmDX	9	78%
Ratio: Dako Pharm Dx	4	75%
Ratio: Kreatech Probes	4	100%
Ratio: Leica HER2 FISH TA9217	9	89%
Ratio: Other - CISH	1	100%
Ratio: Other - FISH	2	50%
Ratio: Pathvysion Vysis Kit	48	79%
Ratio: Ventana BDISH 800-098/505	7	43%
Ratio: Ventana DDISH (780/800-4422)	41	56%
Ratio: Ventana Inform Silver ISH	8	75%
Ratio: Zytovision ZytoDot 2C	3	33%
Ratio: Zytovision ZytoLight	5	100%

Copy Method (Shown Only When Applicable)	N	% Pass (score ≥ 10/12)

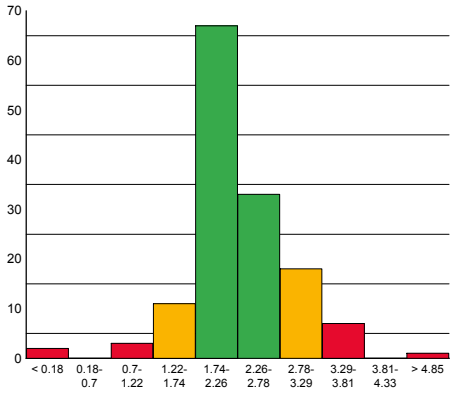
FREQUENCY HISTOGRAMS

The below histograms show distribution of HER2 copy, Chr17 and Ratio results for this assessment

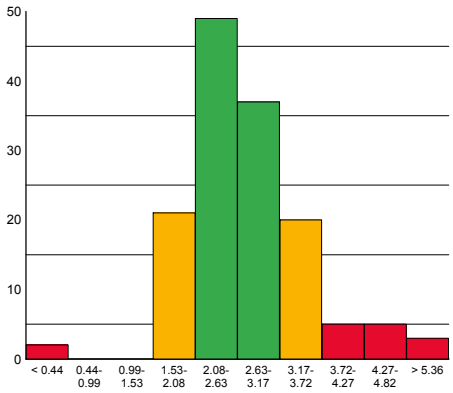
Green = mean +/- 1 Stdev Amber = mean +/- 2 Stdev Red = mean ≥ +/- 3 stdev



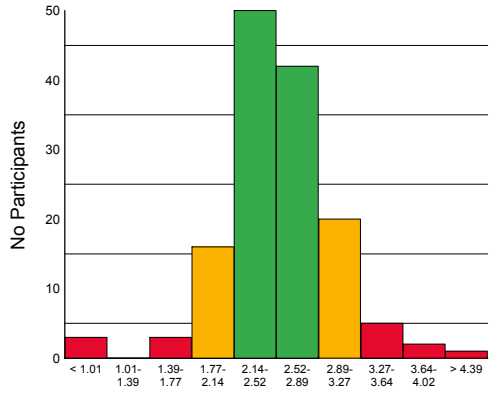
Sample A: Av. HER2 copy



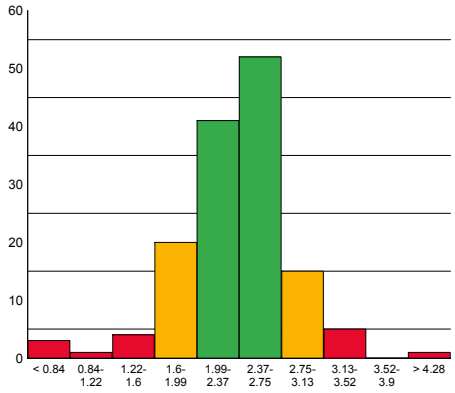
Sample A: Av. Chr17



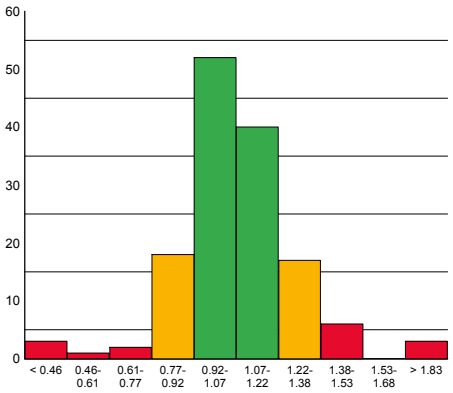
Sample A: Ratio



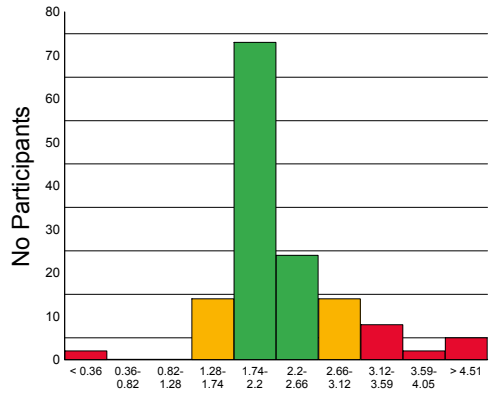
Sample B: Av. HER2 copy



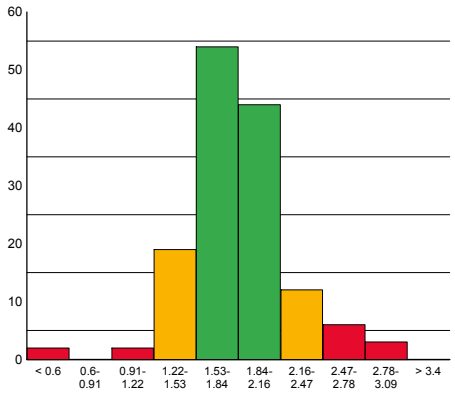
Sample B: Av. Chr17



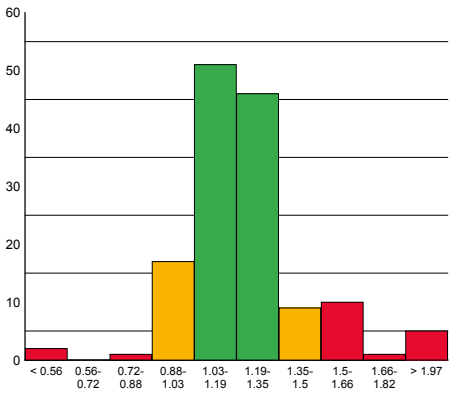
Sample B: Ratio



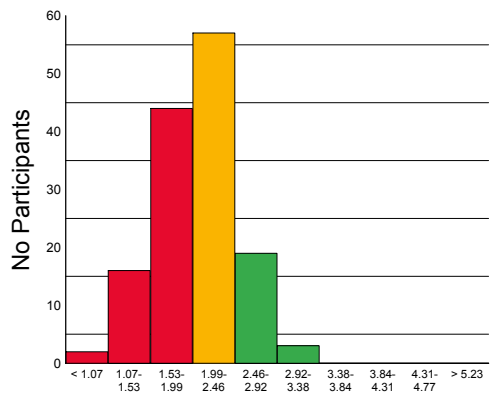
Sample C: Av. HER2 copy



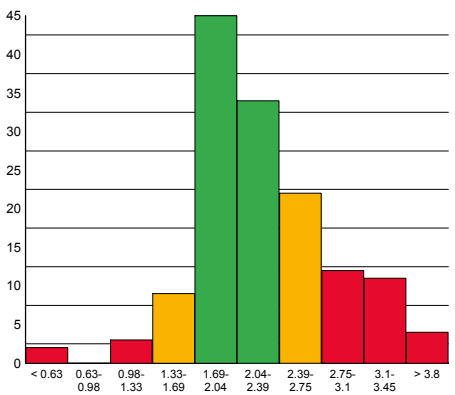
Sample C: Av. Chr17



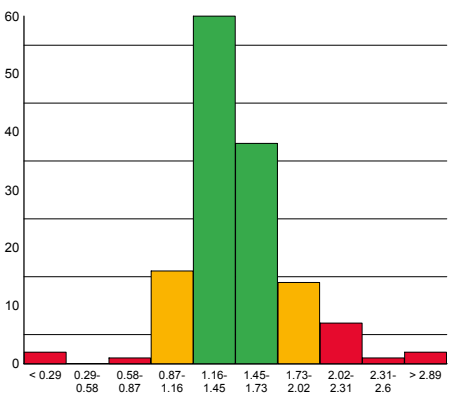
Sample C: Ratio



Sample D: Av. HER2 copy



Sample D: Av. Chr17



Sample D: Ratio

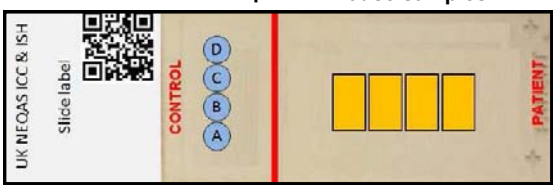
The HER2 ISH Module - Technical (Pilot) Run 40

Merdol Ibrahim and Suzanne Parry

Gene Assessed:	HER2
Sections circulated:	Composite slide consisting of 4 breast carcinoma sections (see table below)
Number of Registered Participants:	173
Number of Participants Taking Part this Run	132 (76%) (71 Fluorescent and 61 Chromogenic)

The Summary Box below gives details of the HER2 phenotype and genotype of the tissue sections circulated for this assessment, which comprised of formalin fixed and paraffin processed sections from a composite block of four human breast carcinomas

Sample	HER2 status by IHC	HER2 status by FISH
A	**2+**	Amplified
B	**2+**	Non-Amplified
C*	**2+**	Non-amplified
D	**2+**	Non-Amplified



Tissue Section Positioning: Tissue sections were positioned on microscope slides as illustrated in the image on the left (same sample as the interpretive ISH). The area below the RED line is for participants to place their in-house samples

Assessment Procedure

Chromogen ISH (CISH / SISH / BDISH / DDISH etc.) was assessed around a multi-header microscope with each slide being reviewed by 4 independent assessors, each providing scores out of 5 for the slide as a whole (not the individual tissue sections). The scores are added together to give an overall score out of 20 along with any assessor feedback comments.

Fluorescent ISH (FISH) was assessed by a team of assessors who viewed the slides together on a large HD screen which was linked to the fluorescent microscope. Each assessor provided their opinion and feedback, and then an overall pass rate comment was given.

A summary of the assessment scoring criteria and it's interpretation is shown in the table on the next page
Each of the UK NEQAS tissue sections (A-D) (as well as in-house samples where submitted). These were then individually assessed for the quality of ISH staining. Assessors **do not count t**he HER2/Cen17 signals, but the accuracy of signal enumeration is assessed in the 'interpretive' section of the HER2 ISH module.

Important: If two separate slides are submitted with separate Cen17 and HER2 copy numbers (e.g. Ventana/Roche SISH), then the assessors regard this as a single result, and as such, combined comments are placed in a single results column.
If cores from a combination of the 2 distributed slides have been used to carry out the counts for the interpretive assessment, then it is important that participants indicate on the slide and the online methodology form as to which cores were used, as these will be the cores reviewed in the technical part of the assessment.

In-house 'example/s' are requested for this module and participants will be required to place them on the distributed NEQAS slides (as shown in the image above).

Results Summary

CISH Results

Selected images Figs 1-6 show examples of the acceptable and unacceptable levels of staining for the different methods.

The overall results from the CISH technical assessment saw a similar pass rate from the previous Run (39): 54% of labs received an acceptable pass and a further 21% achieved a borderline pass. However, 15 labs (25%) failed the assessment, which was mostly due to weak Cen 17 signals, and several labs also showed excessive signals obscuring the nuclei and signals outside of the nuclei. Both of these are features we have seen in previous assessment runs during the UK NEQAS assessment year. Labs that received borderline passes were mostly marked down due to weak Cen 17 signals, but these were still deemed readable. Again some slides showed dust-like silver chromogen signals, but unless obscuring the specific HER2 signals the slides was still considered readable and therefore did not fail, and were given a borderline pass. These observational results refer mainly to the Ventana BDISH or DDISH methods, which were used by most labs (77%) who submitted brightfield ISH slides for this technical assessment.

The Dako DuoCISH method was used by a few labs, but the data has only collected the results from 1 lab as the other participants using this kit did not submit their methodology details. However, these slides were recognised as being stained with the Dako DuoCISH kit by the assessment team: With this kit the HER2 signals are red and the Cen17 signals are blue. Characteristically, this assay also shows a lot of red blush in the cytoplasm. Data from the 1 lab shown in the report graphs shows that this lab achieved a borderline level of staining. Marks were lost due to weak probe signals. The Zytovision ZytoDot 2C method was used by 3 labs, but unfortunately all 3 labs failed the assessment due to unreadable

Table below shows a summary of the assessment scoring criteria and interpretation.

Scoring Interpretation	
Acceptable Individual Assessor 4-5/5 or Overall score ≥13/20	The NEQAS distributed samples and/or in-house tissue show a good/very good standard of staining and are suitable for interpretive analyses.
Borderline Individual Assessor 3/5 or Overall Score = 10-12/20	The NEQAS distributed samples and/or in-house tissue is borderline interpretable with 2 out of the 4 assessors indicating that improvements can be made with respect for example: Excessive / Weak Nuclear staining Level of probe hybridisation Excessive background staining **Also see assessor comments on your report**
Unacceptable Individual Assessor 1-2/5 or Overall score ≤9/20 =	The NEQAS distributed samples and/or in-house tissue is unacceptable for interpretation with at least 3 out of the 4 assessors indicating that there are hybridisation issues, which could be due: Excessive / Weak Nuclear staining Poor probe hybridisation Missing Her2 copy no. / CEN 17 Excessive background staining **Also see assessor comments on your report**
Score = 0	Slide Not submitted for assessment

HER2 signals and weak or Cen 17 signals. The ZytoDot 2C CISH assay is recognised as having green HER2 signals and red Cen17 signals.

FISH Results

Images of acceptable and unacceptable levels of staining are illustrated in figures 7-12.

Overall the FISH results showed better pass rates than the previous assessment run (39): 75% of labs received an acceptable pass, compared to 62% in run 39, and a further 6% achieved a borderline. However, there was still a fail rate of 20%, but again this was an improvement on the previous run where 25% of labs failed the assessment. Similarly to the CISH assessment, weak or no signals was the reason for failure or borderline marks. The team does still wonder if signal fading may be lost during transit. However, most slides arrive to the assessment with very strong signals and no sign of fading, including slides from overseas countries, including Australia and the United Arab Emirates. The UK NEQAS ISH scheme does provide recommendations to try and prevent any loss of signal (see later paragraph on Recommendations for Returning FISH slides).

The Pathvysion Vysis kit still remains the most popular FISH method. In the current run (40) this assay was used by 58% of labs, and showed an acceptable pass rate of 61%, which was much higher than the previous assessment where only 32% of labs received an acceptable pass. A further 7% of labs using the Vysis kit received a borderline for the current run. The Dako IQFISH and the Leica FISH kits were the next popular choices of kits, and both showed excellent pass rates of 100% (N=10 and N=9 respectfully). A variety of other kits were used, including the Dako Pharm Dx, Kreatech Probes and the Zytovision ZytoLight, all of which performed well, although the numbers of users of these kits is very low.

Validating ISH

It is important to emphasise that when introducing ISH into the laboratory or when changing methodology, e.g. from FISH to CISH, the new technique must be fully validated and staff must undergo extra training to gain expertise with the new method employed.

The UK NEQAS data indicates that there is a definite move towards the use of the Ventana DDISH method and laboratories introducing either this method or another ISH method should make sure that:

- Staff 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% is recommended for both diagnostic results (amplified and non-amplified status) and
- numerical results (for both HER2 and Cen17).
- Clinical and UK NEQAS samples should be assessed for quality of hybridisation prior to interpretation. You should make sure that the probe signals are easily discernible, normal cells show the expected HER2 and/or Cen17 signal, and there is minimal background and leaching of the signal.
- Further validation guidelines can be found in: Bartlett et al., (2011) HER2 testing in the UK: Recommendations for Breast and Gastric In-situ Hybridisation Methods. J Clin Pathol.64(8):649-653.

Important: Laboratories experiencing any staining problems should contact the relevant company for further support.

Recommendations for Returning FISH Slides for NEQAS Assessments

a. Sections should be mounted using a fluorescence antifade mounting media, which prevents loss/quenching of fluorescence e.g. Vectashield (Vector labs), Dako Fluorescence Mounting Medium (Dako), Fluoromount (SIGMA/Aldrich, VWR). Note: The Abbott Vysis kit already contains DAPI in phenylenediamine dihydrochloride, which is an anti fading reagent, but we have found that some laboratories also sued the above mentioned mounting media.
b. Seal the coverslip edges onto the slide using clear varnish or another sealing agent, which will stop the slide from drying out.
c. Send back FISH slides as soon as you have finished your own interpretation.
d. There is no need to send back slides packed in ice/dry ice. Please return in the slide mailer that is provided.

How to Use this Report to Compare NEQAS Cell Line 'Technical' & 'Interpretive' Results:

Once you have your results for both the 'Interpretive' and 'Technical' HER2 ISH modules, use both results to troubleshoot whether there are interpretive and/or technical issues with your ISH methodology.
The table below shows a brief troubleshooting guide, which we hope will assistant you further.
UK NEQAS ICC & ISH recommends that laboratories should continually monitor their performance to make sure that both technical and interpretive results are frequently audited.

Interpretive & Technical Troubleshooting Guide		
Interpretive Result	**Technical Result (pre-pilot module)**	**Overall Feedback**
Appropriate or Acceptable	Acceptable	**Acceptable** The NEQAS samples show a good standard of staining **and** have been interpreted correctly
Unacceptable	Acceptable	**Unacceptable** The NEQAS samples show a good standard of staining **BUT** there appears to be issues with interpretation i.e. HER2 copy number and/or Cen17 overestimated/underestimated. **Recommend that scoring/counting criteria is reviewed**
Appropriate or Acceptable	Borderline	**Borderline Acceptable** The NEQAS samples are of borderline acceptability for staining quality. **Recommend that technical method (kit/assay) is further optimised.**
Unacceptable	Borderline	**Unacceptable** The technical staining can be improved as this may be effecting interpretation. **Recommend that technical method (kit/assay) is optimised (or where relevant a standardised kit is used as per instructions) as interpretation may be affected by the borderline quality of staining.**
Appropriate or Acceptable	Unacceptable	**Unacceptable** The NEQAS samples are unacceptable for interpretation and caution should be taken when interpreting from these slides. **Recommend that technical method (kit/assay) is optimised (or where relevant a standardised kit is used as per instructions) as interpretation, although appears correct, may lead to incorrect interpretation of clinical cases.**
Unacceptable	Unacceptable	**Unacceptable** The NEQAS samples are unacceptable for technical staining and interpretation. **Reporting from such cases is very likely to lead to incorrect interpretation of clinical cases.** **If there is persistent underperformance:** • **seek assistance from kit/assay manufacturer** • **seek assistance from UK NEQAS or colleagues** • **re-validate protocol (retrospectively and prospectively)** • **review scoring criteria** • **send clinical cases to a reference centre to confirm your results**

Selected Images showing Optimal and Sub-optimal Immunostaining

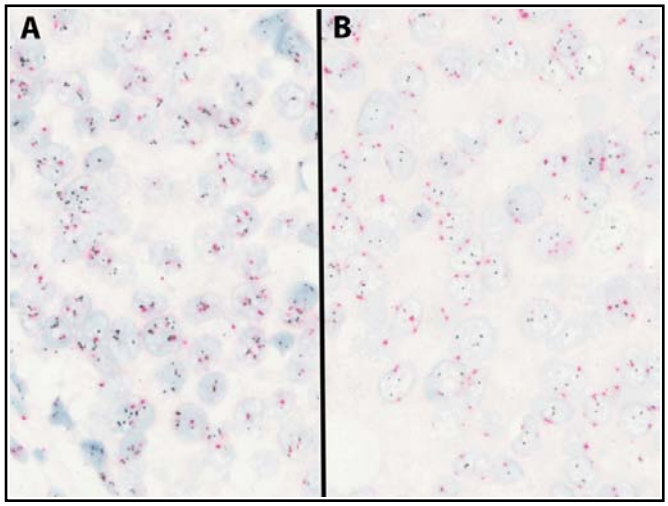


Fig 1. Acceptable Ventana DDISH staining in the UK NEQAS distributed samples: Both (A) amplified and (B) non-amplified cases show distinct HER2 (black) and Chr17 signals (red) with the expected level of copies per cell.

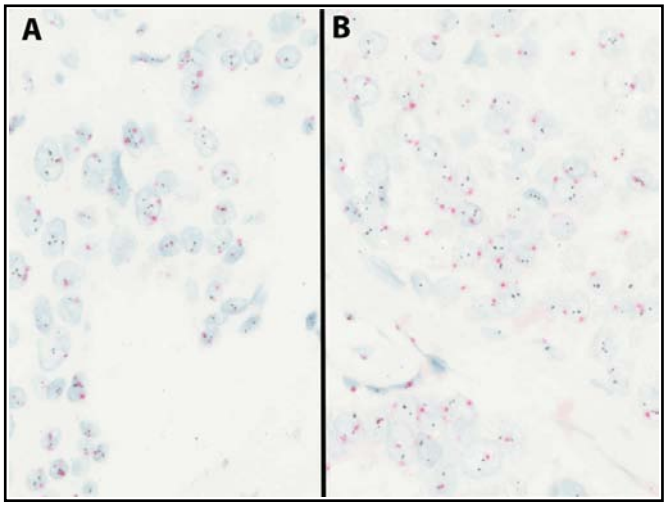


Fig 2. Acceptable Ventana DDISH in the UK NEQAS (A) sample 'C' and (b) sample 'D'. Both samples are non-amplified, showing the expected level of copies per nuclei. Both the HER2 (black) and Chr17 (red) signals are strong and clear.

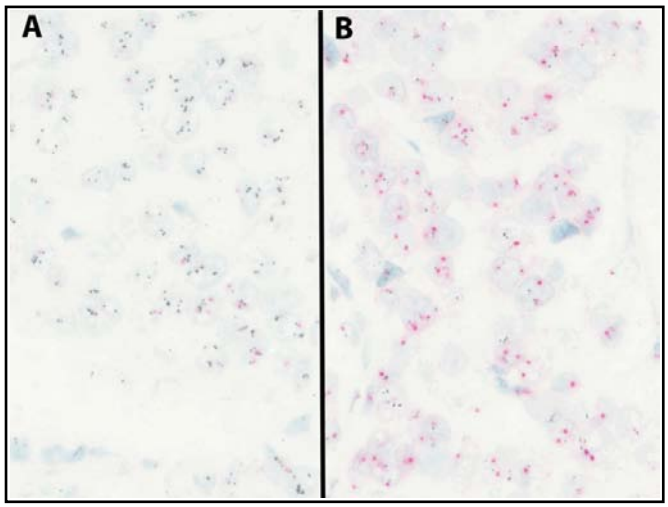


Fig 3. Two examples of slides that received a fail for the UK NEQAS technical assessment. The examples are the amplified sample 'A'. (A) The Chr17 signals are weak and uneven. (B) The HER2 signal are very low (compare to Fig 1A). Both slides stained with Ventana DDISH.

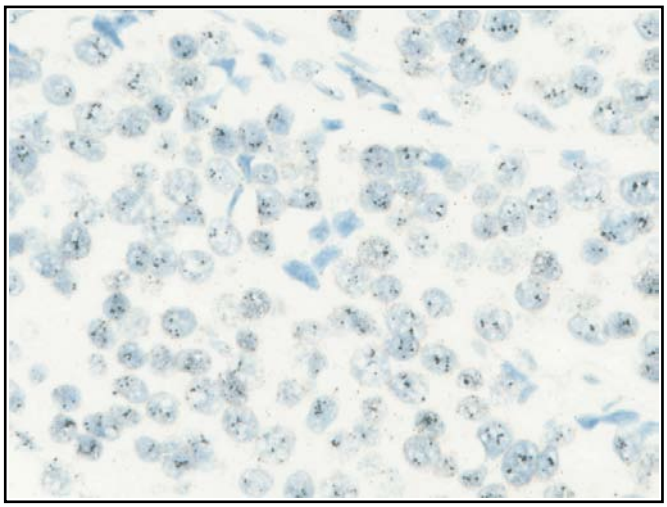


Fig 4. Unacceptable level of staining in the UK NEQAS sample 'A', showing excessive silver chromogen deposit. The single probe Ventana CISH kit was used.

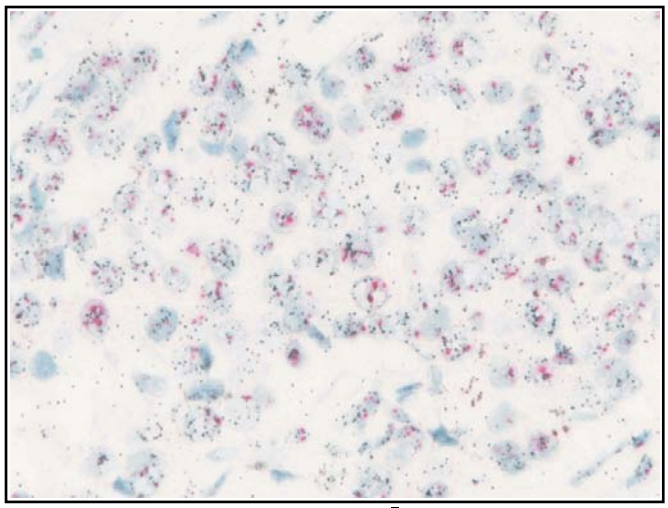


Fig 5. Unacceptable staining in the UK NEQAS 'sample 'A'. The example shows excessive signals with many outside of the nuclei. The slide is therefore unreadable. However, the participant did attempt to make a count on this section. Stained with the Ventana DDISH kit.

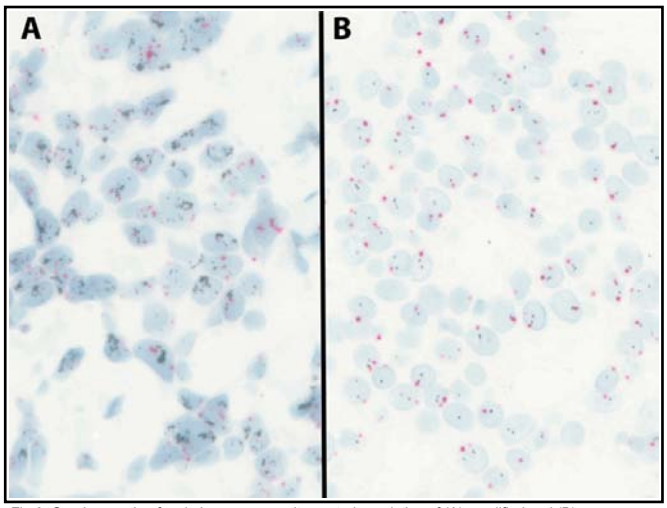


Fig 6. Good example of an in house composite control consisting of (A) amplified and (B) non-amplified tissue samples. Both cases show strong, clear and distinct HER2 signals (black) and Chr17 signals (red).

Selected Images showing Optimal and Sub-optimal Immunostaining

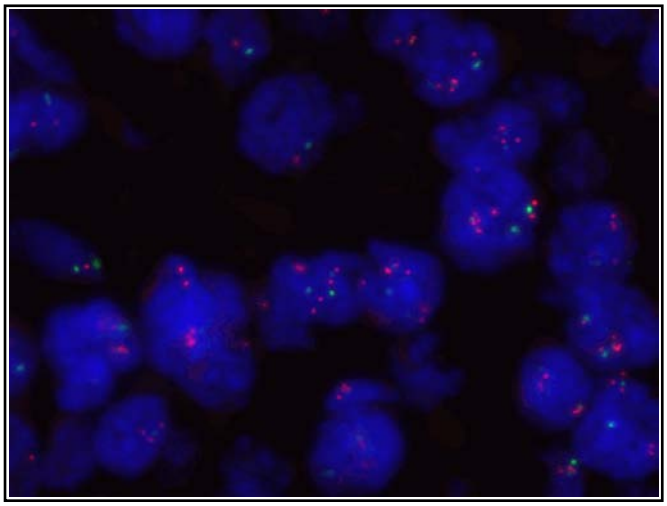


Fig 7. Acceptable FISH examples from UK NEQAS distributed 'amplified' sample 'A', stained using Leica HER2 FISH TA9217. The nuclei demonstrate distinct HER2 signals (red) and Chr17 signals (green).

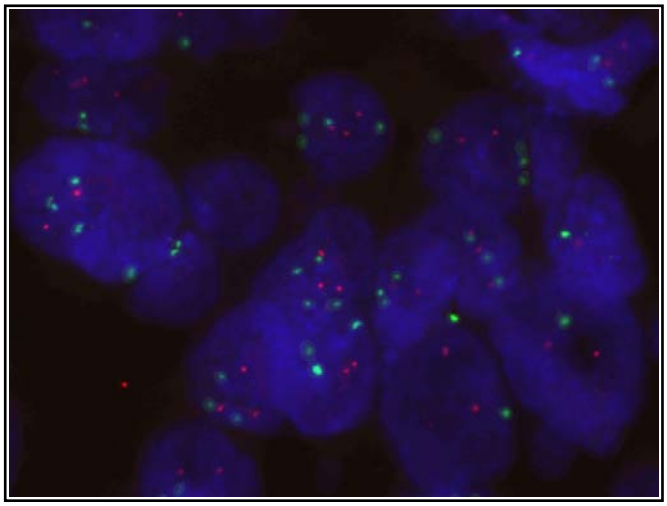


Fig 8. Acceptable FISH examples from UK NEQAS distributed 'non-amplified' sample 'B', stained using the Pathvysion Vysis Kit with distinct HER2 signals (red) and Chr17 signals (green).

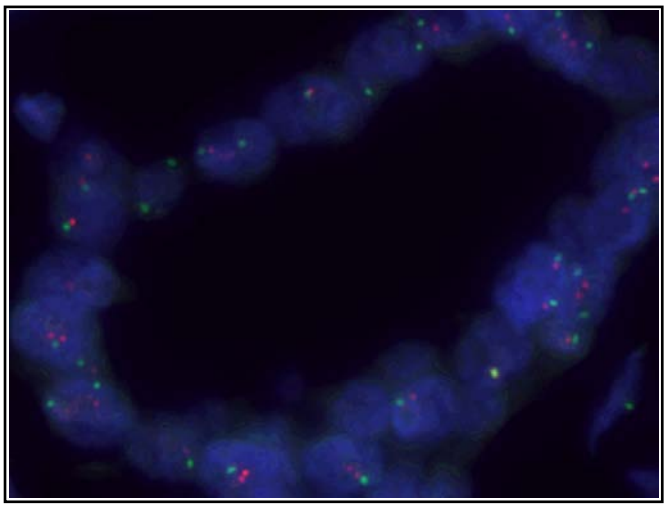


Fig 9. Acceptable FISH examples from UK NEQAS distributed 'non-amplified' sample 'C', stained using the Dako IQFISH PharmDX with distinct HER2 signals (red) and Chr17 signals (green).

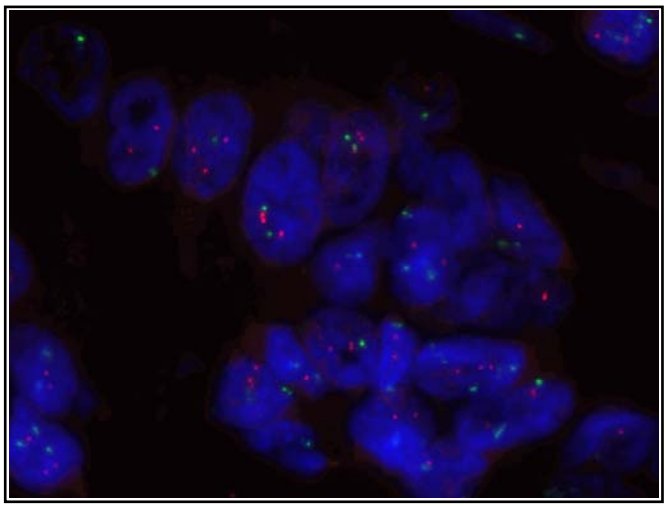


Fig 10. Acceptable FISH examples from UK NEQAS distributed 'non-amplified' sample 'D', stained using the Leica HER2 FISH TA9217 with distinct HER2 signals (red) and Chr17 signals (green).

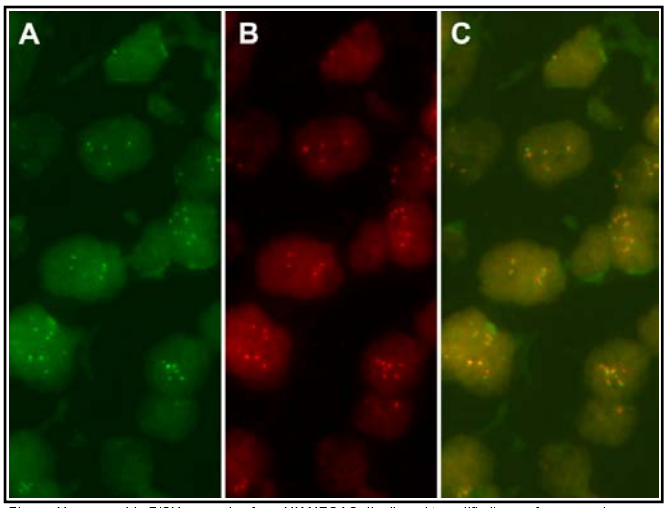


Fig 11. Unacceptable FISH examples from UK NEQAS distributed 'amplified' case from sample 'A' both stained using the Pathvysion Vysis Kit. (A) Non-specific Chr17 signal (B) Neither HER2 or Chr17 signals were observed.

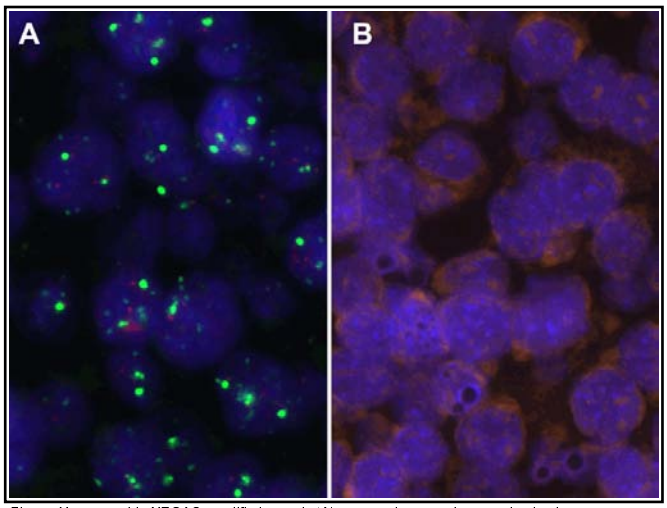
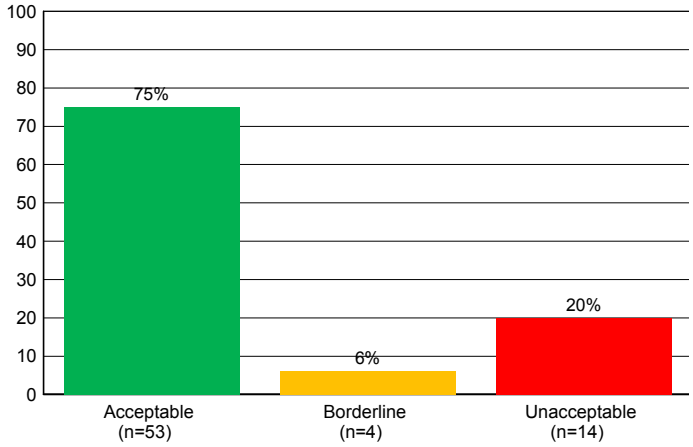


Fig 12. Unacceptable NEQAS amplified sample 'A' purported to have been stained using the Vysis kit, but appears to show break-apart probes (A) Chr17 (B) HER2 and (C) combined image.

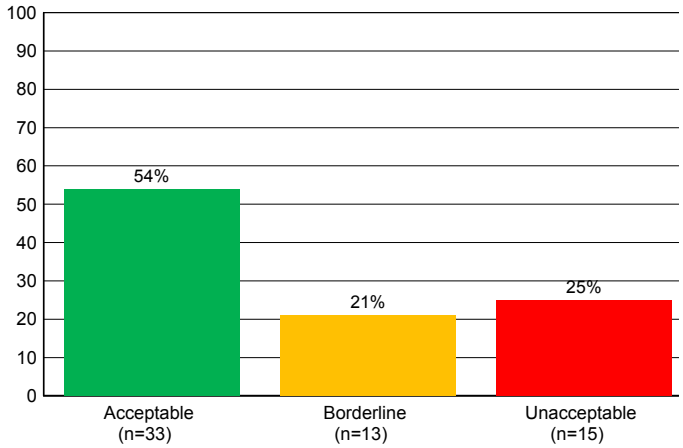
Technical ISH: Pass Rates and Methods

Overall Pass Rates

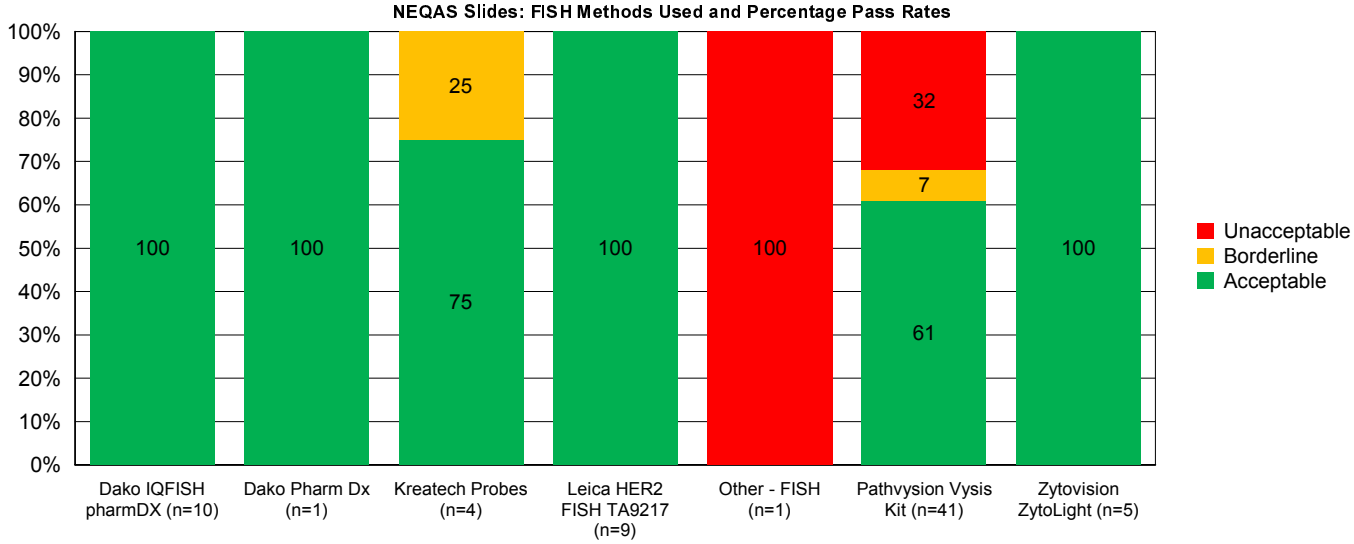
FISH NEQAS slide (n=71)



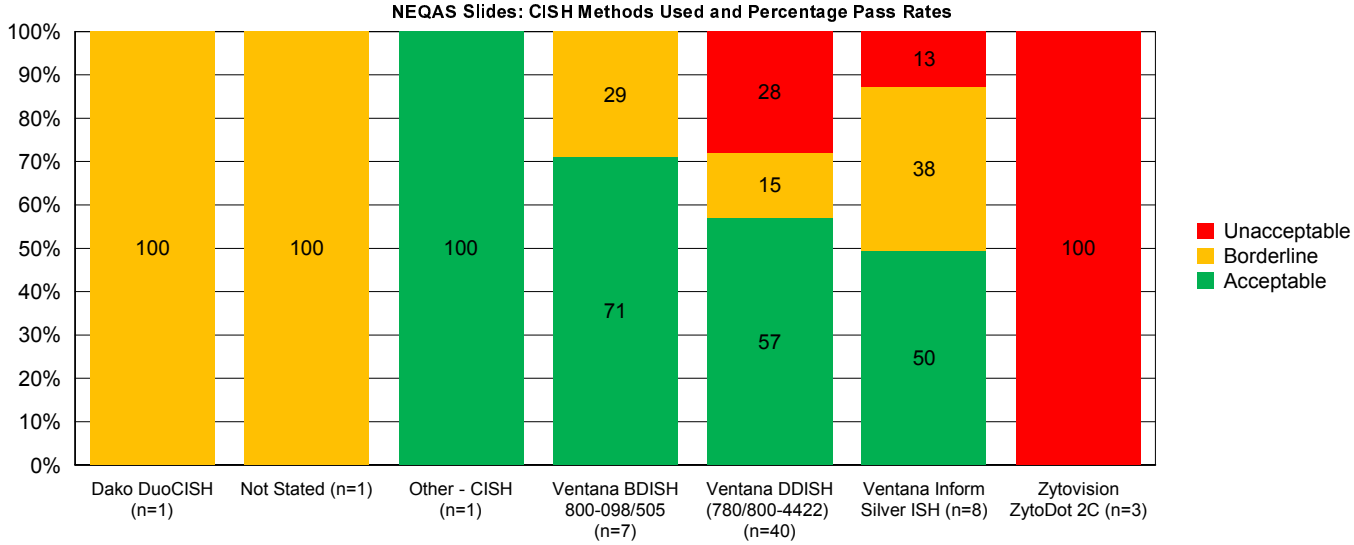
CISH NEQAS Slide (n=61)



NEQAS Slides: FISH Methods Used and Percentage Pass Rates



NEQAS Slides: CISH Methods Used and Percentage Pass Rates



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