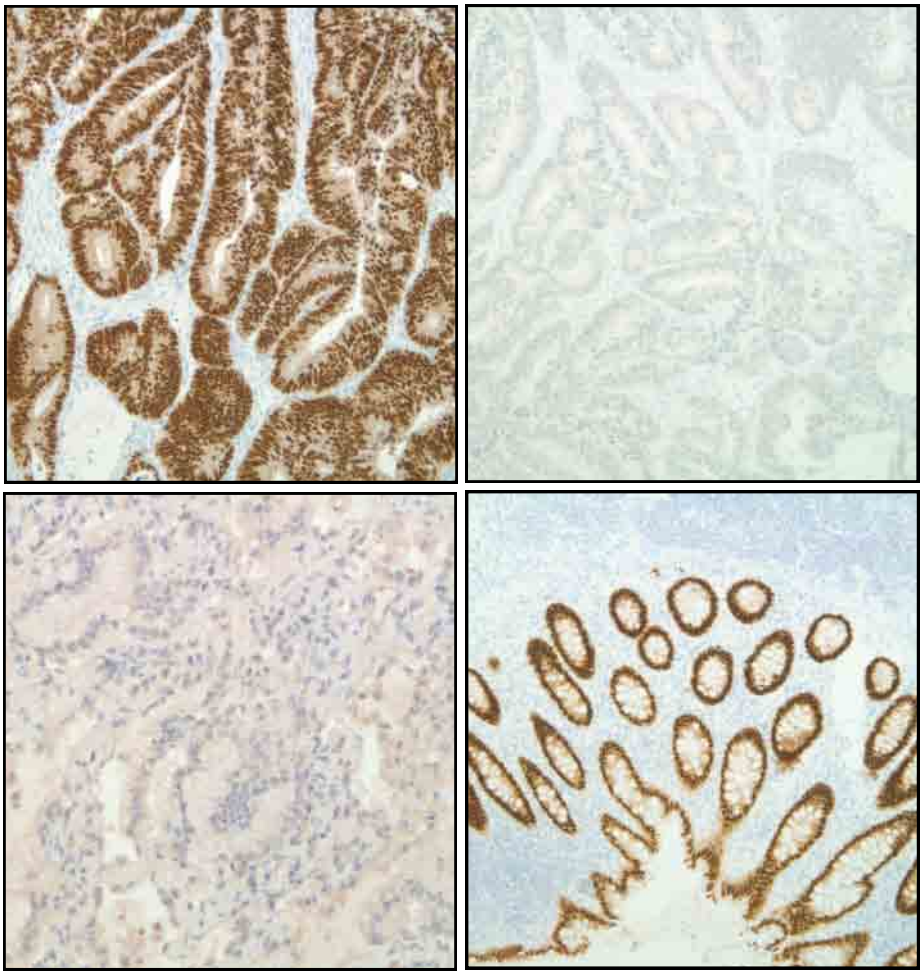
UK NEQAS
International Quality Expertise

Immunocytochemistry

Improving Immunocytochemistry for Over 25 Years

Results - Summary Graphs - Pass Rates
Best Methods - Selected Images

Assessment Dates: 23rd June — 15th July 2016



Cover Photo: Taken from the : General Module 2nd Antibody (C & D).

Top Left: Optimal CDX2 demonstration on NEQAS colon sample (C)
Top Right: Poor demonstration of CDX2 on NEQAS colon tissue (C)
Bottom Left: Sub-optimal, non-specific CDX2 staining on NEQAS lung section (C)
Bottom Right: Excellent in-house demonstration of CDX2 on a piece of appendix (D)

Articles / Reports

Immunocytochemistry Modules

In situ Hybridisation Modules

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- Slide Adhesion Study

General Information

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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 this assessment.

United Kingdom
Mr C Abbott, Bath
Mr D Allen, London
Prof M Arends, Edinburgh
Mr N Bilbe, London
Ms Y Bissett, Kilmarnock
Mr D Blythe, Leeds
Ms A Brown, London
Ms A Clayton, Preston
Dr C Cardozo, Preston
Mr A Dodson, London
Mr R Fincham, Cambridge
Mr D Fish, Warwick
Mrs S Forrest, Liverpool
Mr S Forrest, Liverpool
Dr I Frayling, Cardiff
Ms J Freeman, London
Mr J Gregory, Birmingham
Dr N Guppy, London
Ms L Happerfield, Cambridge
Ms J Hogarth, Newcastle
Dr R Hunt, Stockport
Dr M Ibrahim, London
Ms S Jordan, London
Ms L Kane, Glasgow
Mr J Linares, London
Dr B Mahler Araujo, Cambridge
Dr P Maxwell, Belfast
Mr K Miller, London
Ms A Newman, London
Dr D Pandit, Preston
Ms S Parry, London
Dr M Pitt, Cambridge
Dr E Provenzano, Cambridge
Ms F Rae, Edinburgh
Mr G Rock, Birmingham
Mr J Ronan, Nottingham
Dr J Starczynski, Birmingham
Dr P Taniere, Birmingham
MS C Thomas, Preston
Dr P Wencyk, Nottingham
Mrs D Wilkinson, London
Ms H White, Maidstone
Mrs J Williams, Portsmouth

Germany
Dr I Nagelmeier, Kassel

Iran
Dr T Khan, Tehran

Ireland
Dr T O'Grady, Dublin
Dr K McAllister, Dublin

Netherlands
Prof E Thunnissen, Amsterdam

Portugal
Mr J Matos, Lisbon
Dr A Ferro, Lisbon
Mr R Roque, Lisbon

Slovenia
Ms I Kirbis, Ljubljana.

Journal layout and design prepared by UK NEQAS ICC & ISH

ISH Best Practices

A Tissue/Slides

1 Pre-analytics:
Verify sample collection conditions (6-48 hours in NBF)

2 Water Quality:
Ensure water quality is as per CAP guidelines

3 Pre-processing/Sectioning:
- Ensure tissue fits into cassette
- Cut 4 um thick sections

4 Slides:
- Use Superfrost Plus slides or equivalent charged
- Do not 'double dip' slides

B Pre-run

1 Labels:
Centre label with no overhang

2 Dispensers:
- inspect all tips and remove plugs
- prime liquid meniscus to end of dispenser

3 Slide Placement:
Placed towards operator

4 Protocol:
- Ensure protocol optimisation
- Start with package insert protocol

5 Instrument Priming:
Prime instrument at least once before run0

C Post-run

1 Post-Processing Slides:
- Wash slides in soapy DI water
- If using red detection, dry slides by baking at 60°C for 60 min
- Dip in clean xylene (< 30 sec), then permanently mount

2 Bulk Preparation:
Rinse with DI water

3 Decontaminate Carboys:
Decontaminate 20 L bulk carboys every quarter per operator's manual

4 Instrument:
- Decontaminate instrument per operator's manual quarterly
- Level 1/Preventative Maintenance
- Ensure L1/PM are performed according to recommended schedule

For online training on our Dual ISH Assay, please visit **www.her2dualish.com**

For a full description of ISH Best practices, please contact your local Product Specialist.

www.roche.com **www.ventana.com**

UK NEQAS ICC & ISH User Satisfaction Survey: 2016

Seema Dhanjal, Neil Bilbe and Keith Miller

Overview

At June 2016, approximately **560** active participants were registered with UK NEQAS ICC & ISH. Excluding any central contacts or agents, and new laboratories recently joining the scheme, a total of **520** labs were emailed, with a link to the online survey. The survey was open for 4 weeks – the closing date was chosen as 28th June 2016 to allow time for review prior to the 2016 AMR.

Regional breakdown (520 Labs):

UK & Eire: 216 Labs – 42%
Rest of World: 304 Labs – 58%

At the start of the survey, there were 3 mandatory fields to complete:

- Participant Code
- Region (UK & Eire or ROW)
- Modules participated in LAST YEAR (Runs: 110 – 113)

There were 30 questions/sections. Respondents were asked to give a rating of **Very Satisfied, Satisfied, Neutral, Dissatisfied, or Very Dissatisfied** to the first 16 questions; Q.17 asked for the reason for participation in UK NEQAS ICC & ISH, Q.18 how likely they are to continue to use our service; Q.19 to 24, required a **Yes or No** type response.

Q. 25 asked participants to rate the quality of the EQA material by sample type: a) tissue sections and cell lines, b) cell blocks and cytospins (cytology module only)

Q. 26 asked if a change in methods based on EQA results had been made

Q. 27 asked whether any reassessments requests had been made.

The final three questions (Q. 28, 29, 30) asked for the rating of our service; firstly by level of satisfaction, secondly, with an overall score out of 10, and thirdly by individual modules subscribed to, which was new to this year.

At the end of each section, or following some individual questions, there were comments sections allowing participants to express their views, or make any comments they felt would be useful to UK NEQAS ICC & ISH management.

Return details

Over 150 responses were received; any duplicate, incomplete and unusable, or multiple entries were removed, leaving a total of 144 replies for analysis.

The overall response rate to the survey was therefore approximately 28%, an increase from the 19% last year.

79 UK & Eire labs responded out of a total of 216 labs registered in the UK & Eire (42%) which represents **37%** of all registered UK & Eire labs.

65 Non-UK & Eire (OS) replied out of a total of 304 registered with the scheme (58%), which represents **21%** of all registered OS labs.

Overall analysis of satisfaction

Results from the response to Q. 28 relating to overall satisfaction levels.

- Very Satisfied (44) 30.8%
- Satisfied (93) 65%
- Neutral (6) 4.2%
- Very/Dissatisfied 0%

Effectively, over 95% of users were either Very Satisfied or Satisfied with the service; 4.2% responded to being Neutral. There were *no* Dissatisfied responses.

Response by region:

2016	UK (N=79)	OS (N=64)
Very Satisfied	26 (32.9%)	18 (28.1%)
Satisfied	50 (63.3%)	43 (67.2%)
Neutral	3 (3.8%)	3 (4.7%)
Very/Dissatisfied	0 (0%)	0 (0%)

Comparisons with data from 2015 Survey

2015	UK (N=45)	OS (N=59)
Very Satisfied	18 (40%)	21 (35.5%)
Satisfied	26 (57.7%)	34 (57.6%)
Neutral	1 (2.2%)	4 (6.7%)
Very/Dissatisfied	0 (0%)	0 (0%)

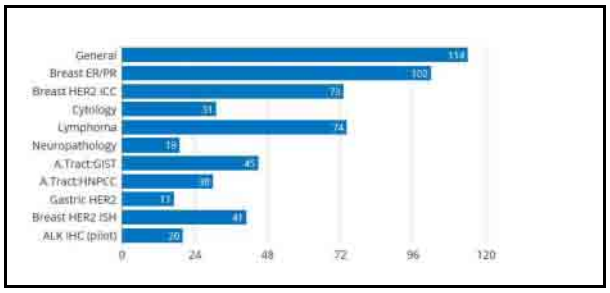
Responses	2015 (%)	2016 (%)
Very Satisfied	37.5	30.8
Satisfied	57.6	65
All other responses	4.8	4.2

The overall levels of satisfaction remain *relatively* unchanged, but there has been a decrease in the number of Very Satisfied (↓ 6.7%) responses, off-set by an increase in Satisfied responses (↑ 7.4%). There was a corresponding drop (minor) in the number of Neutral responses ↓ 0.6.

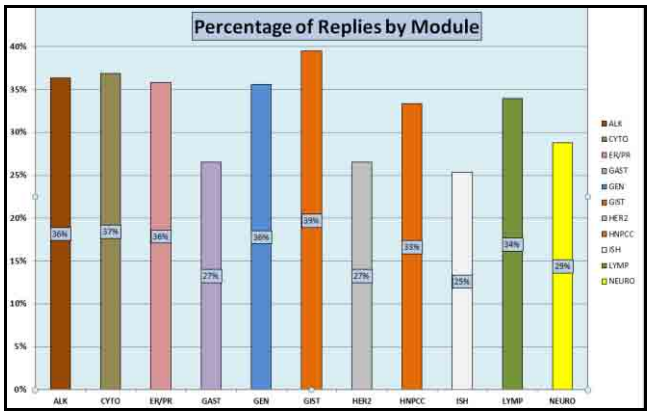
There appears to have been a universal drop of 6-7% in the levels of Very Satisfied responses from all regions.

Some module related data

The average number of modules that all registered labs participate in is just over 3. The number for those actually responding is 3.9: The combined number of modules for all labs responding was 566 (143 replies), the number of labs for each module can be seen in the table below:



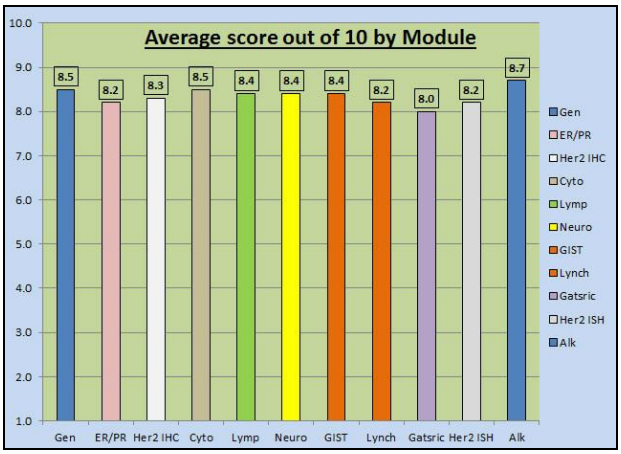
The actual percentage of labs registered for each module who responded was:



On average 33% of labs registered for a particular module responded to the survey, with a median of 34%. Lowest response 25% (Her2 ISH), to the highest 39% (GIST).

Overall rating of UK NEQAS ICC & ISH out of 10 (Q. 29) and by individual module (Q. 30)

The overall average score (Q.29) was 8.2, (8.3 last year), this was unrelated to any specific modules.



The average the scores from all the *individual* modules (Q. 30) is 8.3, same as the overall score from last year.

The lowest was 8.0 (Gastric Her2) and the highest of 8.7 (ALK).

In a change to last year, when we didn't ask for the scores of individual modules in the survey, but cross tabulated from the data, the range of scores this year was 0.7 (0.3 last year).

The biggest *increases* in scores were for the ALK (0.6), GIST (0.3) and General (0.2); the Gastric Her2, Breast Her2 ISH both *decreased* by 0.2. The remaining modules either remained the same (Lymphoma and Neuropathology) or changed +/- by 0.1 (ER/PR, Her2 IHC, Cytology, and Lynch).

All these changes are minor and essentially ratings for the scheme and modules are consistent year on year. The most noticeable change was for the ALK IHC module which has progressed from a pre-pilot, pilot, to a live module over the last two years, which may explain the increase in satisfaction compared to other modules.

Replies to individual questions

The response to individual questions was not mandatory, therefore the number of participants leaving feedback varied from question to question, and topic to topic.

Using the same method employed for previous surveys, the percentage of non-satisfied responses derived from totalling up all the relevant individual questions (Q. 1- 16) is 3.2% a slight decrease (3.5%), but in line with previous years.

Levels of dissatisfaction 2016

The main findings and from this year's survey were:

- Turnaround times: same as last year at 8.5%
- Assessor comments: decreased to 11.3% from 14.5%
- Web based format of results: decreased from 6% to 1.4%
- Time to stain samples: increased from 1.7% to 6.3%
- This year there was a decreased level of satisfaction for the 'Number of Meetings' of 7.7% up from 3% last year.
- All questions relating to communication: cover letters, technical help, communication with the scheme, website; have all shown a raised level of satisfaction with the average dissatisfied responses dropping from 3.4% to 1.5%.

Quality of material provided by UK NEQAS

We asked the participants to give us feedback on the quality of the EQA material/samples provided. This was not module specific, although cell lines are only used on a few modules (ALK and Breast Her2) and the two questions relating to cytology material (cytospins and cell blocks) were only visible to those who selected Cytology at the beginning of the survey.

	FFPE (n=141)	Cell Lines (n=93)	Cytospins (n=23)	Cell Blocks (n=22)
Excellent	7.1%	15.1%	4.3%	9.1%
Very Good	41.1%	44.1%	65.2%	59.1%
Good/Acceptable	46.8%	36.6%	30.4%	31.8%
Poor	5.0%	3.2%	0.0%	0.0%
Very Poor	0.0%	1.1%	0.0%	0.0%

The main observation was the better overall results for the cytology material against the FFPE and cell lines, and the noticeable absence of any poor or very poor responses for the cytospins or cell blocks.

Comments, suggestions, and feedback about the service

Comments were returned throughout the survey; these were collated and categorized into 10 main areas:

- Sample and results section (26 comments)
- Participant feedback and communication section (18)
- UK NEQAS ICC & ISH meetings section (11)
- Complaints about the service (6)
- Treatment of in-house samples and UK NEQAS ICC & ISH samples (16)
- Assessment of in-house controls (11)
- Quality of the UK NEQAS ICC & ISH EQA material (24)
- Use of EQA results to improve in-house staining (54)
- Reassessment requests (8)
- General comments and feedback about the service (24)

It is not possible to list all the comments; many were not relevant to the section or questions they appeared in. Several participants made the same or a similar comment in several sections.

In total of **200** comments were received, a scheme reply and comments on some of these are given further down.

The main themes are summarised below:

- Not enough time between results and next run to tweak protocols
- Assessor comments are contradictory, probably due to stock comments – more free text would be better
- Scores of $^{4}/_{5}$ do not have comments
- More meetings (2) and locations outside of London
- Still some problems with section adhesion
- For cytology we often run tissue sections
- In-house section results are important as we work up protocols based on these rather than NEQAS
- More thought about feedback on in-house slides as the material available to labs differs from site to site
- Sections have been arriving scratched or damaged
- Many labs use their results to optimise methods
- Two new antibodies every run to increase panels
- Specialised modules for less common markers
- NEQAS sections are too close to the Red line on the slides
- *Plus lots of complimentary comments about the scheme*

Summary

This year's response level of 28% was encouraging, after the poor response from last year's survey of around 20%, albeit this required at least one reminder.

Levels of participant satisfaction remain high at c. 95%, similar to 2015, but the actual level of Very Satisfied respondents has dropped back to 30%, the first decrease for 3 years, and down from 37.5% last year. The difference is consistent between both OS and UK participants with both showing a shift from Very Satisfied to Satisfied of 6-7%.

The results for the scores out of 10 are still of the same magnitude, with an 8.2 average, even with scores being given for individual modules, as against one single overall score last year. In fact most participants appeared to give the same score for all modules participated in and there was little evidence of any differential marking by the respondents.

The E-Journal showed a slightly decreased satisfaction level at 85% although surprisingly some participants returned a response of 'Not Applicable'

The overall levels of participants returning a rating on the quality of the UK NEQAS ICC & ISH sample quality, was again very good:

- 141 Formalin fixed paraffin sections (FFPE)
- 93 Cell lines (CL)
- 23 Cytospins (CS)
- 22 Cell block sections (CB)

The sample quality rating shows a steady decline in improvement; the paraffin sections (FFPE) had a rating of Poor (5%); the cell lines had a rating of 3.2% for Poor, but more disappointingly a Very Poor rating of 1.1%. No modules sent FFPE samples received a Very Poor rating.

Cross tabulation shows a wide range of results across the modules. Some had no poor responses (ALK, HNPCC), the rest ranged from 2% (ISH) to 11% (Neuropathology) with a median of 5%.

None of the cytological samples had any poor ratings.

The overall Excellent rating across all samples was 9%, down from 11% last year.

UK v OS levels of dissatisfaction

Five areas where dissatisfaction has continuously been high were used to gauge whether there were any discernible regional (UK/Eire v ROW) differences:

Turnaround times: 14% v 2%

Assessor comments: 13% v 8%

Web based format of results: 2% v 0%

Time given to stain/return samples: 6% v 8%

Number of meetings: 7% v 10%

The main findings, when comparing the regional responses is that the UK & Eire labs appear to be less satisfied than last year, whereas the Rest of the World are much more satisfied. Indeed, for the ROW there are some very noticeable changes from previous years with significant drops in the dissatisfied rates.

It is difficult to know why this has happened, perhaps the expectations and requirements (e.g. UKAS) of the UK/Eire labs influences this more so than for overseas participants.

UK NEQAS ICC & ISH response to some areas of dissatisfaction, and participants' comments.

1. *Not enough time between runs to tweak results*

As the number of modules and the complexity of the scheme have grown, the interval between completion of a run and initiation of the next has understandably diminished. The information required to complete the run is not just reports and scores, there is much more which is produced (images, best methods, graphs) and required (validation and run reviews).

2. *Assessor comments can be contradictory, probably due to stock comments – more free text would be better*

The assessor scoring sheets have been modified over the last few years which has allowed not only more 'stock' comments, but also greatly expanded the free text comments areas. It would not be possible to limit reports solely to free text comments due to the time this would require for the sessions.

3. *Still problems with section adhesion*

We are constantly monitoring section adhesion problems, by way of participant feedback, communications, and comments, plus any issues that we find during the actual assessments. In addition to this we are still testing various slide types to try and decide which would be the best overall choice, but are aware that no one slide would suffice for all modules, platforms and protocols.

4. *Specialised modules less common markers*

This is a common theme, due to the scheme not able to request all markers that laboratories stock, and subsequent issues during accreditation visits (UKAS). The problem is that in order for EQA to be worthwhile, as wide a range of users and protocols are required, and setting up new modules for 10 – 20 labs would also mean that economically it would not be viable for either NEQAS or the participants.

A more realistic approach would be to have small networks of labs, which could carry out their own lab-to-lab QA, or for there to be a subscription based referral/feedback service provided by the scheme.

5. *In-house sections and feedback due to variations from site to site*

This topic has been the subject of much discussion. Giving feedback on samples where there is often little or no details about either the sample type (tissue, tumour) or preparation (fixation, processing) can lead to guesswork, which is not in the remit of EQA schemes. The material sent out by NEQAS has documented details and reference standards to consult and compare against. It is also evident that labs use controls in different ways; some using the same multi-block approach for all or the majority of markers, others trying to tailor the control to the 'case or antigen'. Therefore it is difficult to know or assume how labs interpret their or use in-house control results (60% of labs said that generally they do not change their protocols as a result of EQA results).

UK NEQAS ICC & ISH staff and management would like to thanks all the users and participants for their time and comments whilst completing this survey. If you require any further information, or wish to contact the office concerning this article, please write, call, or email: info@ukneqasiccish.org

UK NEQAS ICC & ISH Participants Meeting 2016

UK NEQAS ICC & ISH Participant Meeting September 9th

UK NEQAS ICC & ISH welcomed around 60 delegates from the UK and Eire, from 50 individual laboratories, to its Participant Meeting held at the Grange Holborn Hotel, in Central London.

Six external speakers, in addition to UK NEQAS ICC & ISH staff and associates, contributed to a full day's programme. The meeting was complimented by the presence of 5 trade stands, who provided much appreciated sponsorship for the meeting.

The day was chaired by **Keith Miller**, UK NEQAS ICC & ISH Scheme Director. Keith started by reminding the delegates of the recent departure of Merdol Ibrahim as Scheme Manager.

A range of disease entities were covered, alongside several scheme related talks.

The first presentation was a joint effort given by consultant genetic pathologist **Dr Ian Frayling** from Cardiff, and consultant histopathologist **Professor Mark Arends** from Edinburgh. Their talk covered the developments in DNA Mismatch Repair (MMR), with a comprehensive review of Lynch and related syndromes, including the aetiology, diagnosis, and management; plus a thorough overview of the MMR immunohistochemical demonstration, interpretation and quality assessment issues relating to the disease.

Professor John Gosney, of Royal Liverpool UH, gave the next presentation on: The detection and interpretation of PD-L1 in NSCLC. Starting with the biology, and then covering the histological appearance of the tumour, particularly in relation to the associated immune response, he then progressed to PD-L1 expression and prognosis. He rounded off his talk by covering the diagnostic requirements and pitfalls, and finally posing the question: 'is PD-L1 the best biomarker?'

Seema Dhanjal, Quality Manager, presented the findings from the latest User Survey sent out to participants in June. 29% of labs submitted, up from 20% in 2015. Overall levels of satisfaction remain unchanged at 95% although there was a swing of 6-7% from Very Satisfied to Satisfied responses. As in previous years there were no dissatisfied replies. Seema then compared 2016 to 2015 for a number of topics and then showed the results for individual modules, and quality of material, before summarizing the individual comments received, of around 200.

Dr Andrew Wotherspoon, consultant histopathologist at Royal Marsden Hospital concluded the morning session with a presentation: The role of IHC in the evaluation of lymphoid proliferations. He started by emphasizing the importance of fixation, processing and sectioning; then highlighting the lengthy classification. The roles, uses and pitfalls of immunocytochemistry were covered, along with the diagnostic pathways employed. The importance of QC and experience were stressed, and not to forget that the H&E is still the most important stain in histopathology.

An excellent hot buffet lunch in the Constellation restaurant followed, before reconvening for the afternoon sessions.

Andrew Dodson, from ICR, and Deputy Director of UK NEQAS ICC & ISH started proceedings with an overview of our parent body, UK NEQAS; its structure, personnel and wide ranging roles and how it regulates the numerous EQA schemes which come under its control. The organisation has various working groups and committees, with the Executive probably being the most important and influential. Cellular Pathology has two representatives on this, including Andrew himself.

Barbara De la Salle, of UK NEQAS Haematology, spoke on PREPQ End to End Quality, a system designed to collect errors from diagnostic laboratories in line with ISO: 15189 and the Pathology QA Review of 2014. A Working Group was set-up as a pan-UK NEQAS and disciplinary process. A number of design models were considered in order to collate and collect the errors. The number of participants (voluntary) has risen from an initial 10-20 to 250+ currently. Problems with data formats, IT, and staff and time issues all added to the challenges. Extending this to Cellular Pathology labs is being considered.

Two presentations from UK NEQAS ICC & ISH support scientists, **Dawn Wilkinson** and **Neil Bilbe** followed.

Dawn gave an update of recent scheme news and changes, starting with our move to Finsbury Business Centre in January, the appointment of Seema Dhanjal as Quality Manager also in January, which was quickly followed by our first UKAS assessment under ISO: 17043 in February, not to mention the 6 month surveillance, and Extension To Scope visits a few days before the meeting. Single slide and section adhesion issues, and a round up of some module changes (status, poor performance, and proposed additions) concluded the talk.

Neil covered the subject 'How do we choose what markers to request?' which outlined the processes involved in deciding the antibodies to be requested each EQA year, primarily through the Antibody Repertoire survey, collating the levels of markers for each module, applying our criteria for Golds (90% +) and 2nd antigens (75% upwards). Possible markers, new and current, are then passed to our suppliers, who will see if both suitable and sufficient cases and material can be prepared. The issues of Ethics (PM, HTA, RC Path) and ensuring suitably experienced assessors are available to assess new markers were also stated.

Suzanne Parry, currently Acting Scheme Manager, gave us an update of the ALK IHC module. She started with the background involved in setting up the module from pre-pilot (2014) to live (2016). From an initial survey response of 100, around 30-50 labs have participated in the module since. Antibody clones, NEQAS samples (both cell lines and tissue), in-house control tissues used, validation procedures, and methodologies were all covered. The most widely used and best performing clone is still the Roche D5F3. The EQA results show the use of multiple methods and some potential pitfalls. A FISH module is to be introduced sometime in the future.

Dr Naomi Guppy, Research Manager in UCL-AD was the last speaker of the day, her topic was IHC and ISH in the diagnosis of Infectious Agents. The biology, life cycles, clinical presentations as well as the histological picture of several infectious diseases were covered, starting with HPV, the herpes virus family: CMV in particular; then the organisms Helicobacter pylori and Treponema pallidum were discussed. Treatment, management, and the various techniques available were shown, along with their diagnostic uses and pitfalls.

This completed the programme of formal presentations.

Keith Miller, rounded things up by thanking all the speakers for their time and contribution to the day; the trade for their support; the UK NEQAS team for the organisation and assistance throughout the meeting; and the delegates for coming, all of which contributed to a successful meeting. He wished everyone a safe journey home.

Barbara De la Salle presenting

An attentive audience

The presentation pdfs can be viewed and downloaded from the scheme's website: www.uknegasiccish.org/downloads/

Slide Adhesion Study – The type of slide you use is an important factor for optimal immunohistochemistry

Dawn Wilkinson

Introduction

In May 2014, we piloted '**single slide assessment'** beginning with the hormone receptor module. This was successful so we decided to roll it out to all modules in May 2015.

The main reason why we did this was to comply with ISO 17043 standards: To ensure the same protocol is applied to both the 'NEQAS' and 'in-house' samples (stained at the same time, same type of slide, same batch, same run and on the same position within the machine). Other reasons were to reduce laboratory EQA reagent costs, decrease turnaround times for reports and assessments, to manage the increase in workload, and an easier comparison of the NEQAS and IN-HOUSE samples by assessing sections in parallel. This helped to increase consistency and reproducibility by our assessors and to provide greater feedback.

The pilot single slide assessment revealed no indication of adhesion issues. The slides were later reviewed again, and it was confirmed that there had been no adhesion issues in the pilot single slide assessment .

Single Slide Survey

Once we had rolled out the single slide assessment to all modules, the majority of participants were satisfied with the single slide approach, however some laboratories notified the scheme that they were having adhesive issues with their in-house controls not staying on the slide.

UK NEQAS ICC and ISH, decided to investigate further. We sent out a slide survey to evaluate why some participants 'in-house' controls were not adhering onto NEQAS slides and to see if we could pinpoint a single common denominator as to why some laboratories were having issues.

We asked a number of questions, including: How long after receipt of the NEQAS slides do you handle them? Draining time? Drying time and temperature? Which slides do you use for IHC? (full presentation can be found on our website: www.ukneqasiccish.org

The overall conclusion from the survey was that there was no single common denominator identified. The survey highlighted a number of different variables within the pre-analytical process. We recognise that the pre-analytical factors such as fixation, processing are important to produce optimal IHC as well as standardised staining methods. However, the type of slide and how the slide is treated and baked once the sections are cut varied immensely from laboratory to laboratory even before the 'analytical 'staining stage.

UK NEQAS ICC & ISH R&D

UK NEQAS ICC and ISH decided to explore the adhesion issue further as there was no single answer as to why some laboratories had adhesion issues and others did not.

We went back to basics and examined our slides. It is important to UK NEQAS ICC & ISH to ensure our participants are provided with high quality material. Therefore the fact that a small percentage of laboratories were having adhesion issues required NEQAS to probe further and find a solution.

We began by scrutinizing the 'positively charged slide' (covalent coupling of amino groups to slide surfaces). The advent of the adhesive slide has definitely helped in improving tissue retention, however lifting can still occur. The best prevention is properly fixed and processed tissue, however the type of slide you use is of equal importance.

A failed slide, due to lifting and tissue loss, not only leads to loss of time, money and effort on the part of the laboratory, but more importantly can lead to a longer diagnostic turnaround time or misdiagnosis, which leads to an adverse effect on patient care.

UK NEQAS ICC & ISH discovered that **not** all 'positively charged' slides are created equal. Traditionally adhesive slides have been **'hydrophobic'** in nature, however manufacturers have been quietly introducing '**hydrophilic'** slides behind the scenes. There are also performance differences dependent on the quality of the raw materials of the glass and/or the consistency with which the coating is applied.

Two important areas in IHC is tissue retention and reagent dispersal. **'Hydrophilic** slides' allow better tissue retention and reagent dispersal which in turn would prevent lifting during staining and, therefore resolving the implications in patient care mentioned previously, and also a benefit of reducing laboratory reagent costs due to less reagent use. We decided to investigate this further . How would you know if the slides you are using are **'hydrophobic** 'or '**hydrophilic**' in nature?

Slide Wettability

Slide Wettability is one of the most important features of adhesion slides. Uniform wettability of a microscope slide allows unbiased observations using a microscope and therefore is an important property.

A surface is said to be wetted if a liquid spreads over the surface evenly without the formation of droplets. When the liquid is water and it spreads over the surface without the formation of droplets, the surface is said to be hydrophilic.

Wettability can be detected by the drop contact angle (Figs1 & 2). The smaller contact angles indicate greater hydrophilicity and larger contact angles indicate greater hydrophobicity. Lower contact angles encourage reagent dispersal. (Fig 3) Hydrophilic slides have significantly smaller contact angles. (Figs1-3 Heras et al: Hydrophilic Plus Slides: A Surface -Modified Slide for Improved Tissue Adhesion. D.Siena: (2014): The Impact of Microscope Slides on Immunohistochemistry).

Fig 1

Fig 2

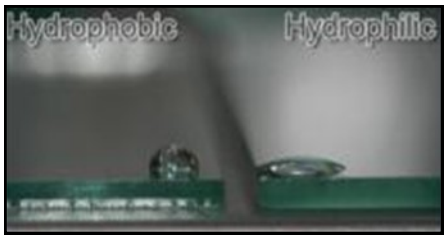
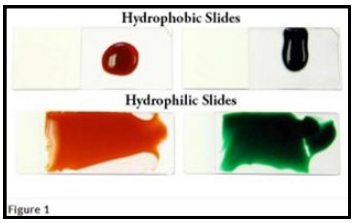


Fig 3



A simple test for uniform wettability: The Dip Test

To test for uniform wettability simply dip the slide into water, the slide should remain wet with a nice even layer of water on the face of the slide. If the water beads and runs off a slide, it indicates poor wettability which is a sign of poor surface quality and corrosion has already started. For a video demonstration visit http://www.youtube.com/user/TheDURANGroup#p/a .

A point to consider is that adhesive slides start to degrade the minute the box is opened. Environmental factors (heat, moisture, time) can also effect fluidics (uniform wettability) UK NEQAS ICC and ISH always ensure the slides we use are well within the expiry date and we always request that you stain your slides as soon as possible on receipt into the laboratory.

Hydrophobic vs Hydrophilic Slides

How do you know if the slides you are using are 'Hydrophobic' or 'Hydrophilic'? There are simple tests you can carry out to see if your slide is on the hydrophobic or hydrophilic spectrum.

Slide Adhesion Study – The type of slide you use is an important factor for optimal immunohistochemistry

Dawn Wilkinson

UK NEQAS ICC AND ISH IN-HOUSE EXPERIMENTS

We tested 11 different adhesive slides from different companies/ suppliers. We also included a non-adhesive slide.

Exp 1: Wettability: Water drop test: 200 ul of distilled water were dropped onto slides from an equal distance. (Fig 5). Given a score 1-3. Water spreads or pools on the slide depending on whether the slide is hydrophilic/hydrophobic

1-Hydrophilic
2-Middle of spectrum
3-Hydrophobic

Exp 2: Dip test: Uniform wettability is an important indicator of the surface quality of a slide. A good quality slide should exhibit uniform wettability. The cheaper slide surfaces can contain defects, dirt or glass corrosion, which result in poor wettability. To test for uniform wettability simply dip the slide into water, the slide should remain wet with a nice even layer of water on the face of the slide. If the water beads and runs off a slide, it indicates poor wettability which is a sign of poor surface quality and the corrosion is already started. Given a score:

1-Uniform wettability
2-Some beading occurring
3-Pooling of water (hydrophobic) major beading

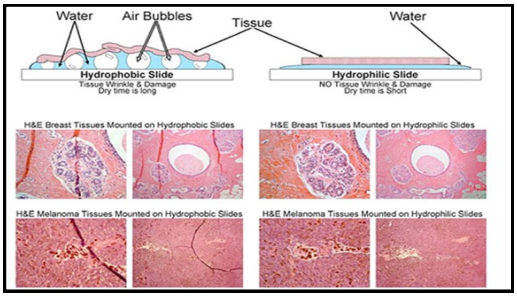
Exp 3: "Movability" of section: To test if the paraffin section is moveable once picked up from the water bath onto the slide. The more hydrophilic a slide is the easier it will be to move the section until the water has dissipated and the section has touched the surface of the slide. Given a score:

1- Moves easily
2-Moves
3-No movement

Exp 4: Draining/drying time: Hydrophobic slides take longer to drain due to trapped water compared to hydrophilic slides which drain very quickly.

1-Up to 5 mins
2-10 mins
3-15 mins
4-20+ mins

Slides that displayed a tendency to be hydrophobic experienced tissue wrinkling and damage upon retrieval by heat-based methods due to hydrophobic slides trapping water (Fig 4-Heras et al).



Exp 5: Double Dipping NEQAS prepared slides from run 111, were allowed to come up to room temperature and then the support scientist cut 3 um sections onto the NEQAS slide as a participant would and within the same time frame as the participant. The slides were drained and baked following UK NEQAS recommendations.

Please see picture of stained slides below. All slides were stained with ER or HER2. The sections were microscopically examined for tissue loss or lifting on the NEQAS slide. No tissue loss or lifting was noted after following the UK NEQAS ICC & ISH recommended draining and baking protocols.

Result table:

Slide	Slide type	Exp 1	Exp 2	Exp 3	Exp 4
A	Superfrost plus yellow	2	2	2	3
B	Superfrost plus thermo	2	2	1	2
C	TOMO (IN DATE)	1	1	1	1
D	Leica Bond Plus	2	2	1	2
E	Marienfeld R (HistoBond R)	2	2	3	4
F	Surgipath Xtra	3	3	3	4
G	Colourfrost plus	3	3	3	4
H	Starfrost	3	3	2	3
I	Uncoated slide superfrost	1	2	1	2
J	TOMO out of date	2	2	2	1
K	Marienfeld S (Histobond S)	3	3	3	4
L	Dako (not in picture)	1	1	1	1

Results

The slides that performed well and showed the best slide surface qualities and wettability on the hydrophilic spectrum from our in-house experiments were the TOMO and DAKO 'positively charged slides'.

The Leica Bond plus and Superfrost plus performed well, and exhibit a more hydrophilic slide. The CellPath HistoBond 'R' are the current NEQAS slides and exhibited 'hydrophobic' qualities and therefore was on the more hydrophobic spectrum, which could explain the adhesion issue. However The NEQAS slides weren't the most hydrophobic slides, with Surgipath Xtra and Colourfrost Extra exhibiting the most hydrophobic qualities of the slides tested.

We asked participants what slides they used in their current practice in our survey. The 3 most popular slides on the responses we received were Thermo Superfrost (35.7%), Leica Bond Plus(13.9%) and Surgipath Xtra (10.3%). 26% of participants did not state which slides they used routinely. The choice of slide used within your laboratory, will be dependent on factors such as which IHC platform you use and cost.

Slide Adhesion Study – The type of slide you use is an important factor for optimal immunohistochemistry

Dawn Wilkinson

Audit of slides

We decided to compare our original chosen slide with a more hydrophilic slide from the same company and manufacturer and a known hydrophilic slide 'TOMO' from a different company.

We sent 3-4 different slides out to laboratories who offered to participate in this trial. We asked them to cut their in house control onto the slides provided and to drain and bake the slides using their routine method and send them back to UK NEQAS ICC & ISH. The team routinely carried out an assessment giving the scores out of 20, and noting any lifting for both the NEQAS sections and the 'in-house' samples.

Results of Audit

The overall result of the audit showed that TOMO a type of ' hydrophilic' slide performed better than our current slides and the more hydrophilic slide from our supplier. There was still some lifting, we think this may be how different laboratories drain and dry their slides before staining. We recommend that you follow UK NEQAS ICC & ISH recommendations for treatment of slides prior to staining. We also looked at the TOMO slides sent unbaked prior to sending to participants might be a concern. However, the baked slides had slightly poorer results compared to the unbaked slides.

Result Table

Type of Slide	Same scores as Histobond 'R'(original)	Poorer scores than original	Better scores than original	Lifting
Histobond 'R' (NEQAS)				21%
Histobond 'S' (NEQAS)	44%	17%	39%	21%
TOMO unbaked (NEQAS)	19%	6%	75%	16%
TOMO 'baked'(NEQAS)	19%	6%	75%	5%
Histobond 'R'				42%
Histobond 'S' (in-house)	58%	26%	16%	32%
TOMO unbaked (in-house)	59%	18%	23%	21%
TOMO 'baked'(in-house)	54%	23%	23%	21%

Discussion

NEQAS ICC and ISH want to provide the best possible service to our participants. Although most participants had no adhesion issues, a minority of our participants were having difficulty with adhesion. UK NEQAS ICC & ISH carried out research of our current slides. We did not want to change the slides without carrying out a thorough investigation, as we distribute between 6000-7000 slides per run. No one slide fits all. The current UK NEQAS ICC & ISH slides were in between the most hydrophilic type slides, such as DAKO or TOMO (Matasumi) slides and the most hydrophobic slides X-tra, Thermo shandon, Starfrost.

The NEQAS slide was on the more hydrophobic spectrum compared to the TOMO or DAKO slides which were the most hydrophilic out of all the slides tested. NEQAS ICC and ISH cannot advocate one slide over another however, from the results of our research and participants feedback we have decided to look into changing over to a more hydrophilic slide, this should ensure better adhesion of the participants sample and better wettability, which is an important factor in the advancement of IHC automation and the different methods used within the IHC platforms to ensure good reagent spread.

The solution drop test was carried out before environmental stress to the slides (humidity, heat, staining). Some 'positively charged' slides withstand environmental stress better than others. (Recommended slide storage and handling - Ventana, Roche) It is thought that environmental stress can effect the properties of the glass/coating.

Some interesting points were noted during our R&D and going back to basics of the simple slide as something we handle every day. As simple as they seem, determining and choosing the correct slide for a particular process, is a good starting point to provide optimal IHC. It is also important to ensure standardisation of storage of slides, draining, drying, and baking of your 'positively charged 'slides'.

Storage-According to manufacturers' instructions: storage conditions can considerably affect the quality and performance of the microscope slide. Microscope slides should not be stored on floors, and should be kept away from doors, heating and air conditioning ducts to prevent and minimise temperature and humidity changes (constant room temperature). Slides should not be stored with reagents/solvents together as the slide surface can turn hydrophobic. Environmental factors may affect fluidics(uniform reagent coverage across the surface of positively charged slides) and can therefore affect staining.

Rotation-Slides should be rotated. Rotation is the first line of defence against temperature and humidity changes resulting in moisture contamination (soda lime glass is subject to an aging process). Once a box of adhesive slides are opened they can begin to corrode. The chance of moisture contamination increases with the age of the product. Minimise the amount of time slides are stored outside of packaging, limit exposure to room humidity, minimise plastic exposure (recommended by Ventana). Test your slides with the simple dip test, to see if the surface of your slide has corroded.

Adhesion issues-If you are having adhesion problems substitute tap water for distilled water in your water bath (especially in hard water areas). Drain your slides appropriately before putting in the oven, if water is trapped underneath the wax, it will not matter the length of time in the oven, the section will still fall off. Hydrophilic slides have 2-3 times the number of binding sites (higher spatial density of amino groups) than hydrophobic slides, therefore better tissue adherence and the lower the contact angle the better the reagent spread. It has been noted that there can be a trend towards stronger IHC staining in the hydrophilic slides. The uniform wetting property of the hydrophilic microscope slide, rapid and even spreading of reagents over the entire working area of the slide is essential for uniform results and the uniform distribution of reagents during IHC staining may reduce slide to slide variability (Heras.A et al). A hydrophilic slide drains quicker and therefore saving time.

Hydrophobicity-It is important to be aware that some IHC stainers use hydrocarbon oils to dewax slide or as a liquid coverslip this may increase the hydrophobicity nature of the slide, if already using a more hydrophobic slide. Hydrophobic behaviour is generally observed by surfaces with critical surface tensions less than 35 dynes/cm. At first, the decrease in critical surface tension is associated with oleophilic behaviour, i.e., the wetting of surfaces with hydrocarbon oils. As the critical surface tensions decrease below 20 dynes/cm, the surfaces resist wetting by hydrocarbon oils and are considered oleophobic as well as hydrophobic, therefore may prevent even spreading of reagents over the entire working area of the slide.

Manufacturer/Supplier/Printing onto adhesive slides– UK NEQAS ICC & ISH have slides customised. A point for us to take into consideration is the raw glass specifics, it is of importance to be aware that the raw glass is a purchased part and the raw glass for each product may be treated differently in the hands of the supplier. The glass can arrive in large sheets from the manufacturer, to prevent the sheets sticking together , some slide manufacturers spray a thin coating of oil between the sheets of glass. This prevents moisture penetrating between the sheets. If this oil is not removed prior to high temperature 'baking on' of the ink on the slides it can cause the surface of the glass to remain hydrophobic. It can also cause the cell coating to adhere unevenly resulting in fluorescent particles to adhere to the surface and poor wettability. Once the oil is baked on, it is very difficult to remove. Eliminating the oil by using a detergent prior to inking, will eliminate the oil and may also contribute to a more hydrophilic slide. (Sarkissan M et Al).

Conclusion

After our investigation UK NEQAS ICC and ISH have decided to move over to a more 'hydrophilic' microscope slide that performs well and can withstand potential environmental stress such as time, heat and humidity, to suit the unique technical needs of UK NEQAS ICC and ISH.

The scheme is looking into the different 'hydrophilic' slides on the market and researching cost, quality of the surface of these slides, and customisation to suit UK NEQAS ICC and ISH requirements.

We all take the simple slide for granted as we use it everyday, however it is important to remember that the choice of slide you use when carrying out IHC, is as vital as any other step in the staining process to provide optimal IHC staining.

The General Pathology Module Run 114

Julie Williams and Dawn Wilkinson

	Gold Standard	Second Antibody
Antigens Assessed:	Ki-67	CDX2
Tissue Sections circulated:	Breast carcinoma x 2 and normal tonsil.	Normal appendix, adenocarcinoma of lung and colon.
Number of Registered Participants:	310	
Number of Participants this Run	298 (96%)	

Introduction

Gold Standard: Ki-67

Ki-67 is a 345-395 kDa nuclear protein involved in the maintenance and up regulation during the cell division cycle. Ki-67 is present in the cell nuclei in all actively proliferating cells during late G1, S, M and G2 stages of the cell cycle and mitosis, but is not expressed in cells during the resting phase G0 (non-cycling cells). The Ki-67 antigen is used to measure the growth fraction of a given cell population (Ki-67 labelling index), i.e. the percentage of cells staining positive for Ki-67. The Ki-67 labelling index (LI) is used to assess the course of cancer in various solid tumours: It is known to correlate with tumour grade, survival and recurrence. For example, in the lymphoma setting the percentage nuclear staining with Ki-67 is used as a prognostic marker: Tumour grade is closely associated with the percentage of Ki-67 stained nuclei in non-Hodgkin's lymphoma (Brown et al, Hall et al.); with <20% Ki-67 expression seen in low grade lymphomas and >20% Ki-67 expression associated with high grade lymphomas. Furthermore, low grade lymphomas with a labelling index >5% have a worse prognosis than those with an index of <5%. In glial tumours the Ki-67 proliferative index can also be used to aid differentiation between benign (LI <10%) and malignant (LI >10%) lesions. Low grade astrocytomas have a labelling index of 0-5%, while higher grade anaplastic and glioblastomas usually have an index >10% (Torp). In the breast cancer setting, the Ki-67 proliferative index is used as both a predictive and prognostic marker (Dowsett et al).

Features of Optimal Immunostaining:
Tonsil: (Fig 1 & 2)
- Intense and well-localised nuclear staining of 80-90% of the germinal centre B-cells
- Intense staining of the basal epithelial cells
- Clean background with no non-specific staining

Breast Tumour A: (Fig 4 & 5)
- Well-localised nuclear staining in approx. 40% of tumour cells, with varying intensity of expression
- Clean background with no non-specific staining in the stroma or cell types not expected to stain

Breast Tumour B: (Fig 4 & 5)
- Intense and well-localised nuclear staining in approx. 80-90 % of tumour cells, with varying intensity of expression.
- Clean background with no non-specific staining in the stroma or cell types not expected to stain

Features of Sub-Optimal Immunostaining: (Fig 3 & 6)
- Weak, uneven, partially missing staining of relevant cells
- Fewer tumour cells staining than expected in the breast

References:
1. M Dowsett, et **al.** Assessment of Ki-67 in Breast cancer: Recommendations from the International Ki-67 in Breast Cancer Working Group. JNCI 2011 103
2. Kontzoglou K, Palla G, et al. (2013). Correlation between Ki67 and breast cancer prognosis." Oncology 84(4): 219-225.
3. Polley MY, Leung SC, et al. International Ki67 in Breast Cancer Working Group of the Breast International and G. North American Breast Cancer (2013). "An international Ki67 reproducibility study." J Natl Cancer Inst 105(24): 1897-1906.

Second Antigen: CDX2

Homeobox CDX2 is a protein that in humans is encoded by the CDX2 gene. This is expressed uniformly and strongly in the nuclei of all intestinal epithelial cells (from duodenum to rectum), and is involved in the regulation of both cell proliferation and differentiation of these cells.
CDX2 antibodies are useful in identifying malignant cells from primary and metastatic tumours of the gastrointestinal (GI) tract. Other tumours occasionally expressing CDX2 are large cell pulmonary adenocarcinoma, prostate adenocarcinoma and yolk sac tumour.
Despite CDX2 being an important and relatively sensitive and specific marker for intestinal adenocarcinomas, it is important to use the marker within a panel .

Features of Optimal Immunostaining:
Appendix: (Fig 7 &12)
- Strong uniform distinct staining in virtually all epithelial cells
- No background staining

Colon Adenocarcinoma: (Fig 8)
- Strong uniform distinct staining in virtually all epithelial cells
- No background staining

Features of suboptimal Immunostaining: (Fig 9,10 &11)
- Weak, uneven or no staining
- Diffuse staining
- Excessive background staining
- Non-specific diffuse inappropriate staining in the lung adenocarcinoma

References:
1. Borrisholt M. et al. Demonstration of CDX2 is highly antibody dependent.-Appl.Immunohistochem.Mol.Morphol.2013;1:64-72
2. Barbareschi M,Murer B, Colby TV, Chilosi M, Macri E,Loda M, et al. CDX-homeobox gene expression is a reliable marker of colorectal adenocarcinoma metastases to the lungs.Am J Surg Pathol 2003;27:141-9
3.Werling RW, Yaziji H, Bacchi CE, Gown AM. CDX2, a highly sensitive and specific marker of adenocarcinomas of intestinal origin: an immunohistochemical survey of 476 primary and metastatic carcinomas. Am J Surg Pathol 003;27:303-10.

Assessment Summary

Ki67

298 laboratories submitted their slides for the Ki67 assessment and 295 labs submitted their in-house control sections for this run. The results show a decrease of 4% in acceptable results from the last time the Ki67 was carried out (Run 105) as shown in the table below:

Ki-67 Pass Rates :		
	Run 105	**Run 114**
Acceptable	84% (N=292)	80% (N=240)
Borderline	13% (N=44)	12% (N=36)
Unacceptable	3% (N=10)	7%(N=22)

Julie Williams and Dawn Wilkinson

The reason for failure was either due to weak/very weak staining or a lower percentage of cells staining than expected. As an example, the Dako MIB-1 clone was used by a lab at a dilution of 1:400, which is too dilute , and may explain why the staining for this participant was weak. Dako recommend the antibody to be used at a dilution of between 1:75-1:150. The most popular clone was then Dako (M7240) MIB-1 clone, used by 156 participants with a pass rate of 70%. Another popular choice was the Ventana (790-429-86) 30-9 clone, used by 60% of participants, and shoed an acceptable pass rate of 97%. Compared to Run 105, when the Ki-67 was last assessed, there was an increase in the users of the Leica (PA0230) RTU K2 clone. This showed an acceptable pass rate of 91%.

The overall pass rates of the in-house controls was higher than that on the NEQAS material, with 84% of participants achieving an acceptable pass, compared to 80% on the NEQAS material. A possible explanation for this is because many labs are using appendix on its own as their in house control. Appendix on it's own does not always identify when the assay may be slightly sub-optimal. NEQAS therefore recommends a composite control with tumours of known expression in addition to a normal appendix control to help gauge the sensitivity of the assay.

CDX2

The CDX2 antibody was chosen as the 2nd marker. This was the first time that UK NEQAS IHC & ISH has assessed for CDX2, and most labs did stock this antibody and therefore submitted slides for the assessment. However, 29 labs (10%) of those that submitted slides stained with the alternative antibody of CK7 as they did not stock the antibody.

Similarly to the Ki-67 assessment, the pass rates for CDX2 were very good. These are summarised in the table below:

CDX2 Pass Rates Run 114:	
Acceptable	95% (N=236)
Borderline	3% (N=8)
Unacceptable	2% (N=5)

All the slides that failed the assessment showed very weak or little demonstration of CDX2 and were therefore considered unacceptable. The in-house results showed similar pass rates to the NEQAS distributed slides, with 96% receiving an acceptable pass, a further 2% achieved a borderline and 3 labs (1%) failed on their in-house material. Again, weak or little staining was the reason for poor marks.

Of the 29 labs that submitted the alternative antibody CK7, 28 labs (98%) received acceptable passes, and 1 lab received a borderline score due to weak staining. All 29 labs received acceptable passes on their in-house material.

For the CDX2 assessment the EPR2764Y clone from Ventana was the most popular choice of antibody. This was used by 167 labs and showed a pass rate of 95%. The Ventana D5/16B4 antibody was also a popular choice, used by 73 laboratories, and showed an acceptable pass rate of 100%. The Dako DAK-CDX2 clone (IS/IR080) was used by 52 participants, and this showed an acceptable pass rate of 100%. For the participants' in-house controls a range of tissues were used, including appendix, colon, pancreas and large bowel; with many labs using a multi-sample control with several of these tissues incorporated.

Selected Images showing Optimal and Sub-optimal Immunostaining

Fig 1. Optimal demonstration of Ki-67 in the UK NEQAS distributed tonsil section. Strong nuclear staining is shown in 80-90% of germinal centre B-cells, while the background remains clean. Stained with the Dako MIB1 antibody (1:200), on the Dako Autostainer using PT link high pH buffer retrieval.

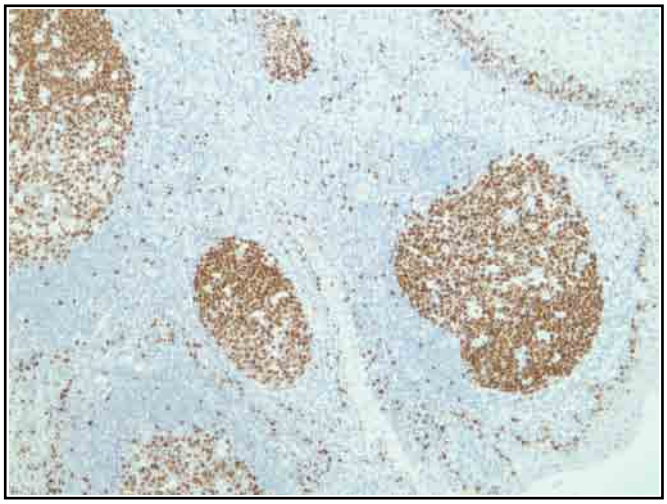


Fig 2. Optimal demonstration of Ki-67 in the UK NEQAS distributed tonsil section. Even at low power it is clear to see the strong nuclear staining in approximately 90% of the tumour cells and in the basal squamous epithelial cells. Stained manually using the Leica MM1 clone, waterbath high pH antigen retrieval and Dako Envision detection.

Fig 3. Unacceptable staining of Ki-67 in the UK NEQAS tonsil section (compare to Figs 1&2). Staining is not only weak, but the percentage of nuclei staining is lower than expected. This is most likely due to the antibody being too dilute. Stained with the Dako MIB1 antibody (1:400), on the Leica BondMax with ER2 retrieval for 20 mins.

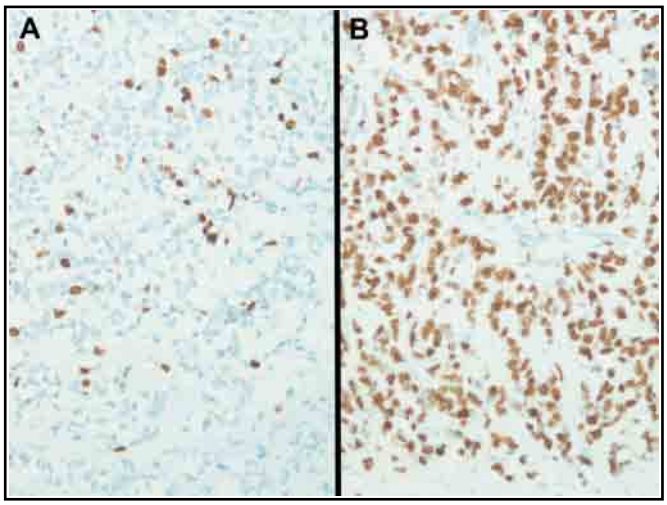


Fig 4. Good demonstration of Ki-67 on the UK NEQAS distributed breast samples. (A) lower percentage of moderate to high intensity and (B) high percentage of high intensity of Ki-67 staining. Stained using the Ventana 30-9 pre-dilute antibody, on the Ventana ULTRA with CC1 standard retrieval.

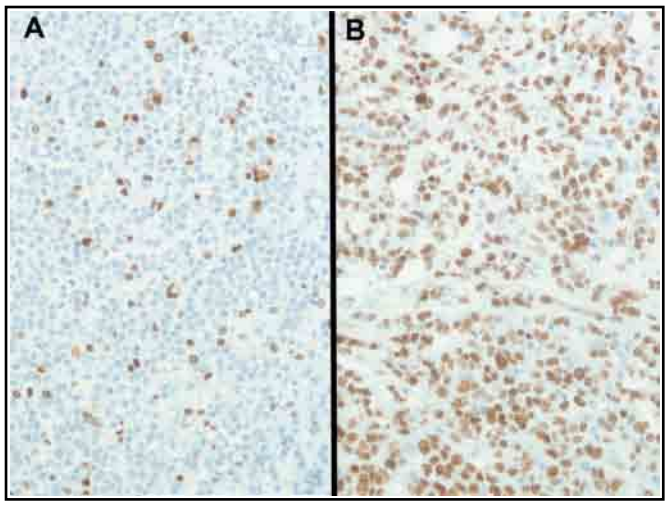


Fig 5. Good demonstration of Ki-67 in the UK NEQAS distributed breast sections 'A' & 'B'. The example shows moderate to strong staining in both samples with the expected percentage of tumour cells expressing: (A) low percentage and (B) high percentage of positive tumour cells. Same method as Fig 2.

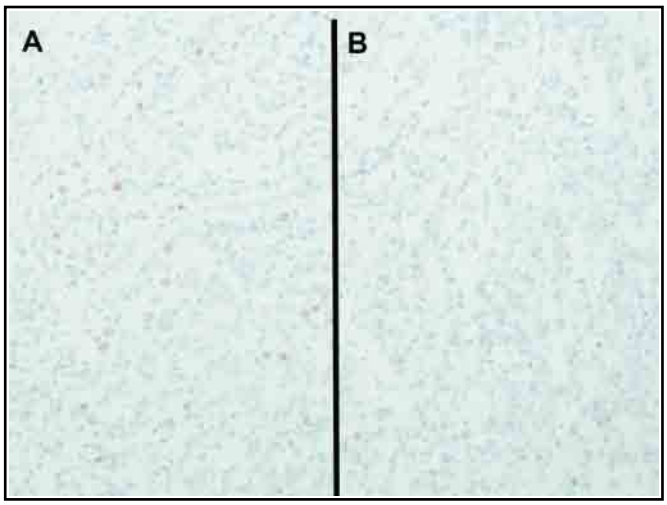


Fig 6. Unacceptable demonstration of Ki-67 in the UK NEQAS distributed breast samples A & B (compare to Figs 4 & 5). Both sections show very weak staining with far fewer nuclei staining positive than expected. Stained with the Dako MIB1 antibody (1:200), on the Ventana Benchmark ULTRA, CC1 for 32 minutes.

Selected Images showing Optimal and Sub-optimal Immunostaining

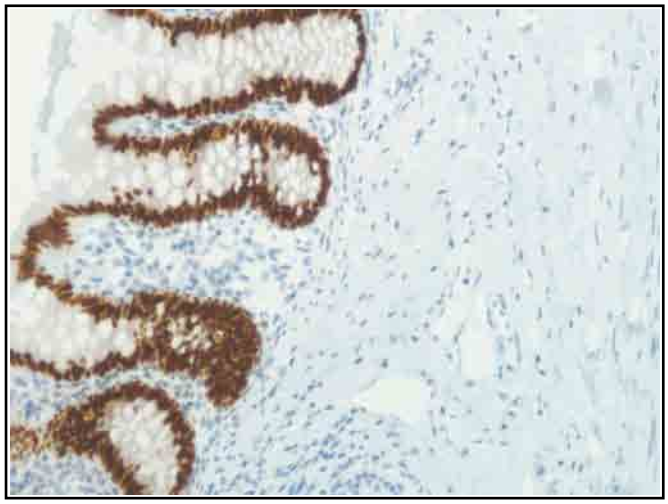


Fig 7. Good demonstration of CDX2 on the UK NEQAS distributed appendix section, showing strong nuclear staining in virtually all the epithelial cells, while the background remains clean. Stained with the Cell Marque EPR2764Y antibody, 1:500, on the Ventana Benchmark XT with CC1 standard retrieval.

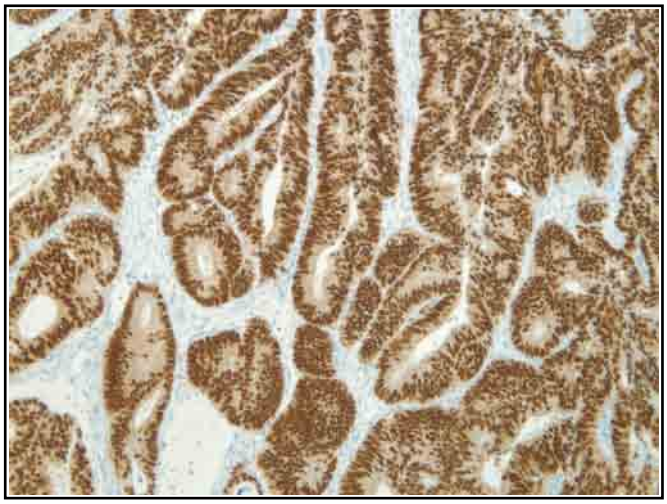


Fig 8. Optimal staining for CDX2 on the UK NEQAS colon adenocarcinoma. The section shows strong staining in virtually all the tumour cells. Same protocol as Fig 7.

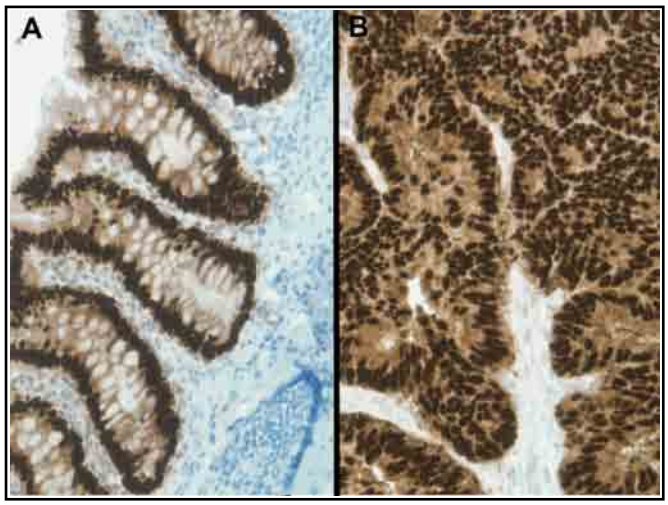


Fig 9. CDX2 staining on the UK NEQAS appendix (A) and colon adenocarcinoma (B) samples. Although the epithelial and tumour cells are staining as expected, the staining is very intense and excessive. This may be due to the antibody being too concentrated. Stained using Cell Marque antibody (1:150) on the Dako Autostainer with PT link retrieval.

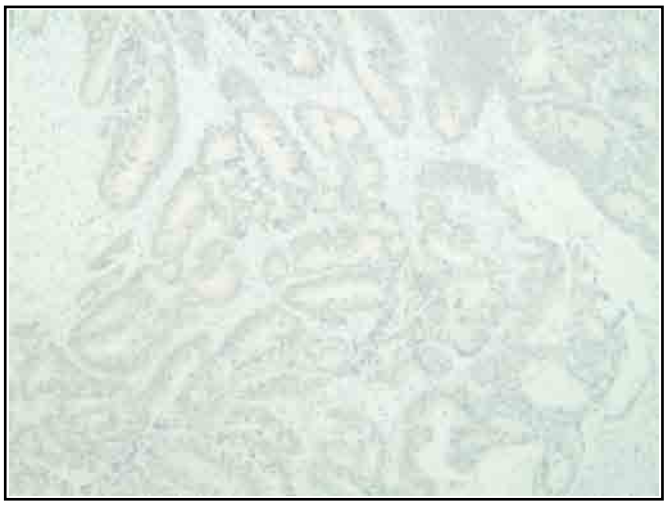


Fig 10. Sub-optimal CDX2 staining in the UK NEQAS colon adenocarcinoma section (compare to Fig 8). The tumour is virtually void of staining with only some weak diffuse staining. Stained with the Novocastra AMT28 antibody, 1:50, on the Ventana ULTRA with CC1 retrieval for 76 minutes.

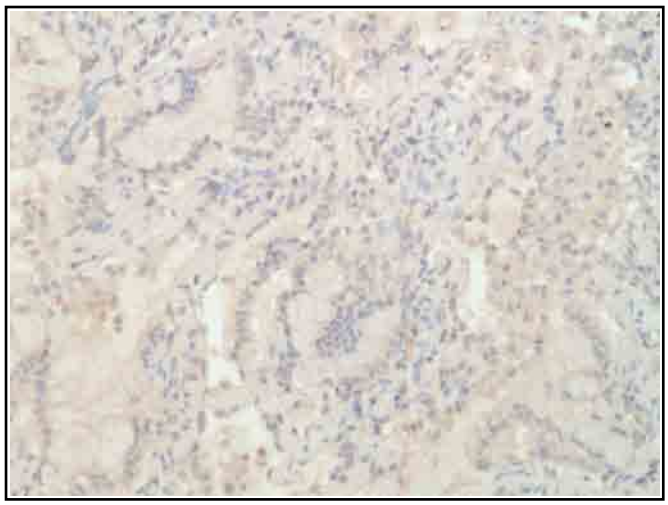


Fig 11. Unacceptable CDX2 staining in the UK NEQAS lung adenocarcinoma sample. This sample should be negative for CDX2, however, the example shows diffuse inappropriate non-specific staining in the tumour. Stained with the Novocastra AMT28 antibody, 1:50, on the Leica BondMax with ER2 for 30 minutes.

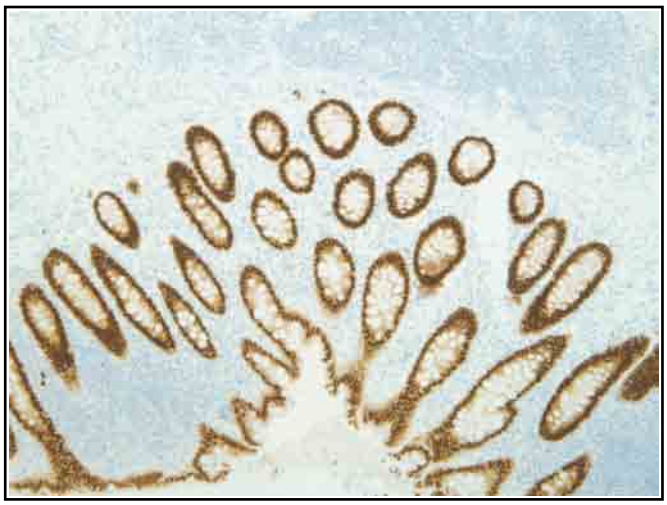
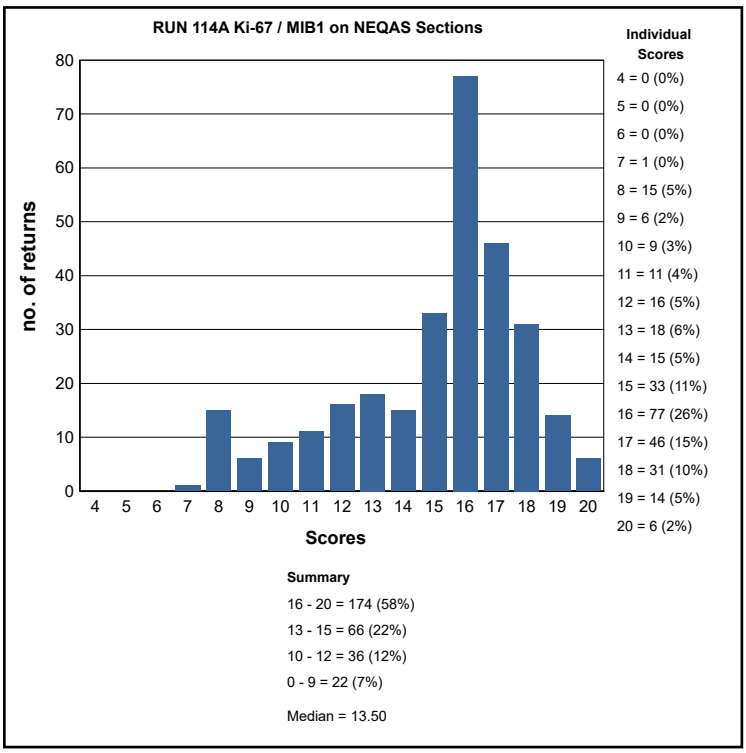
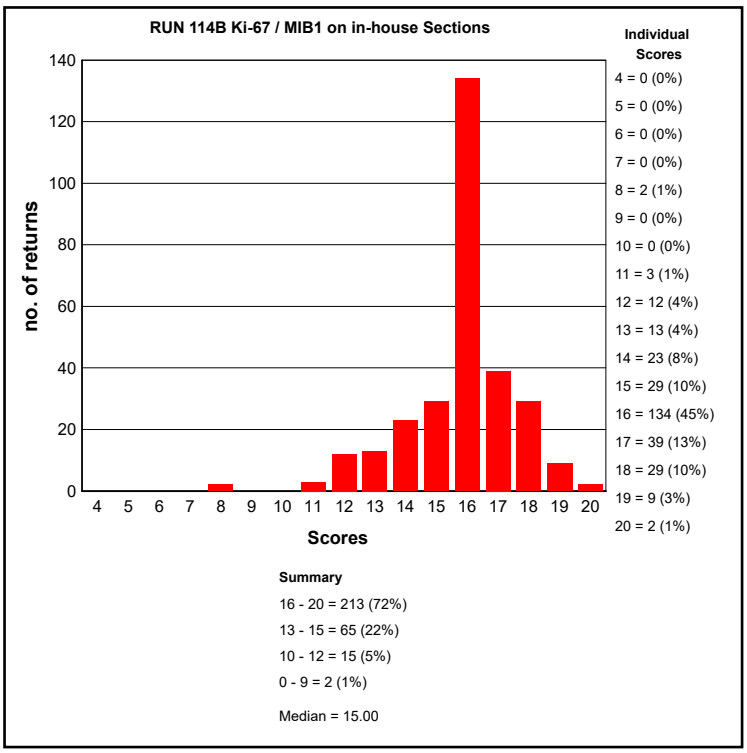
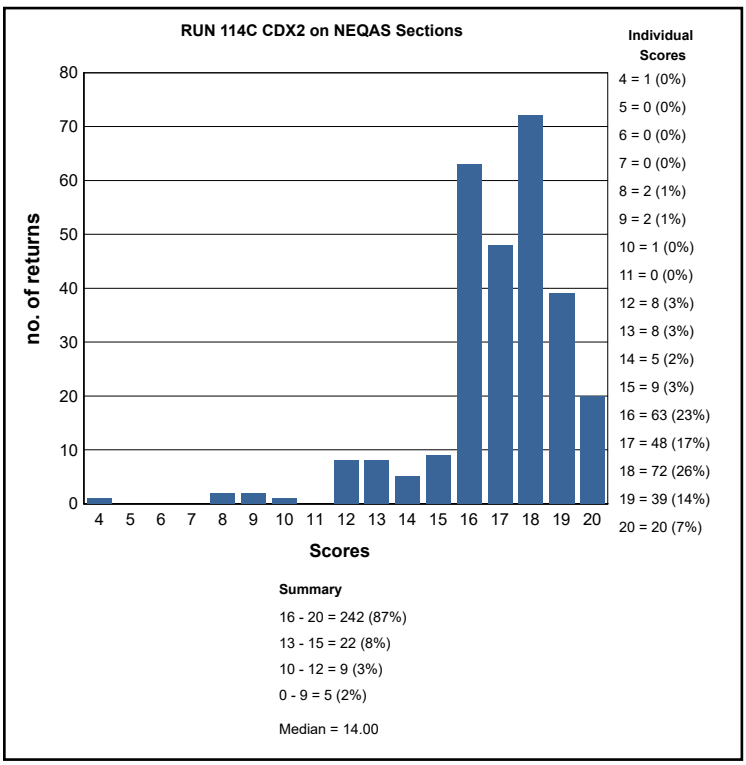


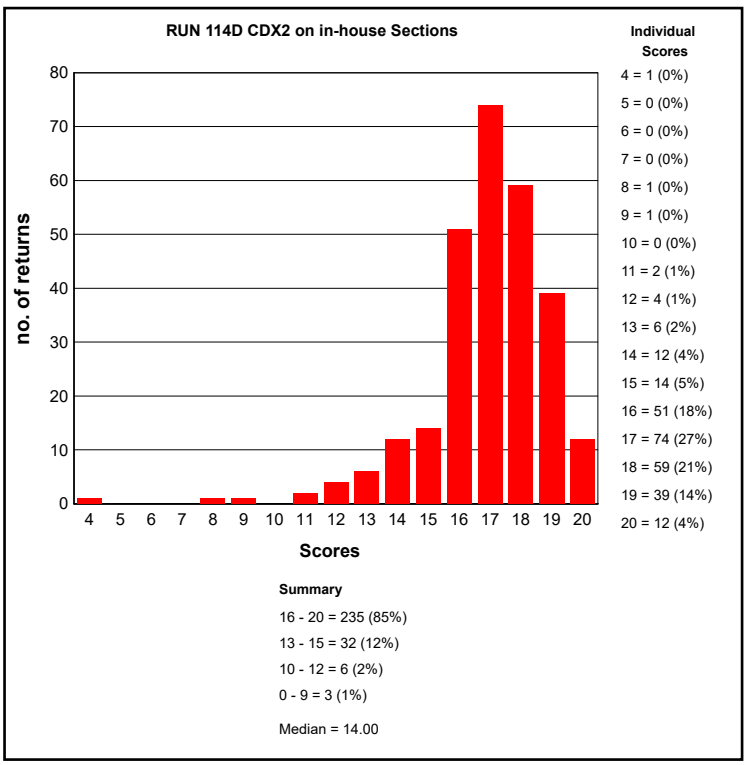
Fig 12. Good CDX2 staining of an in house appendix: The section shows strong distinct staining of all the epithelial cells while the background remains clean. Stained with the Dako DAK-CDX2 RTU antibody on the Omnis with pretreatment for 30 minutes.

GRAPHICAL REPRESENTATION OF PASS RATES









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: 114

Primary Antibody : Ki-67 / MIB1

Antibody Details	N	%
Dako M7187 (clone Ki-67)	1	100
Dako M7240 (clone MIB1)	156	70
NeoMarkers RM 9106 (clone SP6)	4	100
Novocastra NCL-Ki67 (clone MM1)	3	67
Ventana 760-2910	4	100
Other	4	100
Dako IR/IS626 (clone MIB-1)	21	86
Leica NCL-Ki67-MM1 (MM1)	1	100
Leica RTU PA0410 (MM1)	3	100
Leica PA0118 (MM1)	4	50
Leica PA0230 (K2)	23	91
Vector VP-RM04 (clone SP6)	1	100
Ventana 790-4286 (clone30-9)	60	97
Cell Marque 275R-14/15/16/17/18 (SP6)	6	83
Dako Omnis GA626 (MIB-1)	4	100

General Pathology Run: 114

Primary Antibody : CDX2

Antibody Details	N	%
Biogenex (CDX2-88)	15	67
Dako IS/IR080 (DAK-CDX2)	52	100
Epitomics (EPR2764Y)	1	0
Leica RTU PA0535 (AMT28)	15	100
Leica/Novocastra NCL-CDX2 (AMT28)	12	75
Thermo/Neomarkers (EPR2764Y)	1	100
Ventana 760-4380 (EPR2764Y)	73	100
Other	20	95
Cell Marque 235R-18 (EPR2764Y)	32	94
Dako M3636 (DAK-CDX2)	10	90
Dako Omnis GA080 (DAK-CDX2)	5	100
Leica RTU PA0375 (EP25)	24	100
Abcam AB76541 (EPR2764Y)	2	100

General Pathology Run: 114

Heat Mediated Retrieval	CDX2 N	CDX2 %	Ki-67 / MIB1 N	Ki-67 / MIB1 %
Biocare Decloaking Chamber	1	100	2	100
Dako Omnis	9	100	9	100
Dako PTLink	31	100	36	92
Lab vision PT Module	2	50	3	100
Leica ER1 20 mins	9	89	7	43
Leica ER1 30 mins	3	100	3	33
Leica ER1 40 mins	0	0	2	0
Leica ER2 10 mins	4	100	2	50
Leica ER2 20 mins	50	96	58	69
Leica ER2 30 mins	11	91	13	62
Leica ER2 40 mins	0	0	3	100
Microwave	2	100	4	75
None	2	100	0	0
Other	3	100	2	50
Pressure Cooker	4	100	5	80
Steamer	2	100	2	100
Ventana CC1 20mins	0	0	1	100
Ventana CC1 24mins	5	100	4	75
Ventana CC1 32mins	16	100	24	75
Ventana CC1 36mins	8	88	11	73
Ventana CC1 40mins	4	100	2	50
Ventana CC1 48mins	2	100	3	100
Ventana CC1 52mins	5	80	4	100
Ventana CC1 56mins	3	100	5	100
Ventana CC1 64mins	30	100	27	85
Ventana CC1 76mins	4	50	1	100
Ventana CC1 88mins	2	50	1	100
Ventana CC1 8mins	3	100	1	100
Ventana CC1 92mins	1	100	0	0
Ventana CC1 extended	1	100	1	100
Ventana CC1 mild	6	100	15	80
Ventana CC1 standard	28	93	34	100
Ventana CC2 24mins	1	100	1	100
Ventana CC2 36mins	0	0	1	100
Ventana CC2 44mins	1	0	0	0
Ventana CC2 64mins	0	0	2	50
Ventana CC2 mild	0	0	1	0
Ventana CC2 standard	2	100	0	0
Water bath 68 OC	1	100	1	100
Water bath 95-98 OC	0	0	1	100

General Pathology Run: 114

Enzyme Mediated Retrieval	CDX2 N	CDX2 %	Ki-67 / MIB1 N	Ki-67 / MIB1 %
AS PER KIT	2	100	1	100
NOT APPLICABLE	103	95	149	77
Other	1	100	1	100
Ventana Protease	0	0	1	100
Ventana Protease 1 (760-2018)	1	100	0	0

General Pathology Run: 114	CDX2		Ki-67 / MIB1	
Detection	**N**	**%**	**N**	**%**
A Menerini Polymer (MP-XCP)	1	100	1	100
AS PER KIT	25	100	18	100
BioGenex HRP (HK 519-06K)	0	0	1	100
BioGenex SS Polymer (QD 420-YIKE)	1	100	0	0
Dako EnVision FLEX (K8000/10)	9	100	7	86
Dako EnVision FLEX+ (K8002/12)	18	100	28	93
Dako Envision HRP/DAB (K5007)	3	100	4	100
Dako Envision+ HRP mouse K4004/5/6/7	1	100	2	100
Dako REAL HRP/DAB (K5001)	0	0	1	0
LabVision UltraVision ONE Polymer (TL-012/5-HDJ/T	1	0	1	100
Leica Bond Polymer Define (DS9713)	0	0	1	100
Leica Bond Polymer Refine (DS9800)	68	94	82	61
MenaPath X-Cell Plus (MP-XCP)	0	0	1	100
Novocastra Novolink PDS (RE7-140/150/280/290-K)	1	100	1	100
Other	7	100	7	100
Ventana iView system (760-091)	2	100	4	100
Ventana OptiView (760-700) + Amp. (7/860-099)	2	100	3	100
Ventana OptiView Kit (760-700)	41	98	44	82
Ventana UltraView Kit (760-500)	67	91	86	84

General Pathology Run: 114	CDX2		Ki-67 / MIB1	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	1	100	1	100
Dako Autostainer	0	0	3	67
Dako Autostainer Link 48	30	100	31	90
Dako Autostainer plus	1	100	1	100
Dako Autostainer Plus Link	2	100	4	100
Dako Omnis	9	100	9	100
LabVision Autostainer	0	0	2	100
Leica Bond Max	30	93	36	64
Leica Bond-III	50	96	52	63
Menarini - Intellipath FLX	2	100	2	100
None (Manual)	6	83	8	100
Shandon Sequenza	1	100	3	100
Ventana Benchmark GX	3	100	5	100
Ventana Benchmark ULTRA	87	94	87	85
Ventana Benchmark XT	37	92	51	82

General Pathology Run: 114	CDX2		Ki-67 / MIB1	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	40	95	32	91
BioGenex Liquid DAB (HK153-5K)	1	100	1	100
DAKO DAB+	1	100	1	100
Dako DAB+ Liquid (K3468)	2	50	4	100
Dako EnVision Plus kits	4	100	2	100
Dako FLEX DAB	26	100	34	91
Dako REAL EnVision K5007 DAB	3	100	3	100
Dako REAL K5001 DAB	0	0	1	0
Leica Bond Polymer Refine kit (DS9800)	69	94	82	62
menapath xcell kit DAB (MP-860)	1	100	2	100
Other	16	100	14	93
Sigma DAB (D5637)	1	100	1	100
Sigma DAB (D5905)	1	100	1	100
Ventana DAB	25	100	29	83
Ventana iview	1	100	3	100
Ventana Ultraview DAB	71	92	85	84

BEST METHODS - Gold Standard Antibody

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

Ki-67 / MIB1 - Method 1

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

Primary Antibody: Dako M7240 (clone MIB1)
Automation: Dako Autostainer Link 48
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako PTLink
EAR:
Chromogen: Dako FLEX DAB
Detection: Dako EnVision FLEX+ (K8002/12)

Ki-67 / MIB1 - Method 2

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

Primary Antibody:	Ventana 790-4286 (clone30-9) , 16 Mins, 20 ºC Dilution 1: 1
Automation:	Ventana Benchmark GX
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 standard
EAR:	
Chromogen:	Ventana Ultraview DAB, Time 1: 16 Mins, Time 2: 4 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 20 ºC

Ki-67 / MIB1 - Method 3

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

Primary Antibody:	Ventana 790-4286 (clone30-9) , 16 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 standard
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB, 36 ºC., Time 1: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC Prediluted

Ki-67 / MIB1 - Method 4

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

Primary Antibody:	Dako M7240 (clone MIB1) , 30 Mins, 23 ºC Dilution 1: 200
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590), PH: 7.6
HMAR:	Leica ER2 30 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), 23 ºC., Time 1: 10 Mins
Detection:	

BEST METHODS - Secondary Antibody

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

CDX2 - Method 1

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

Primary Antibody:	Dako M3636 (DAK-CDX2) , 30 Mins, 23 ºC Dilution 1: 50
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer, PH: 7.6
HMAR:	Dako PTLink, Buffer: ENVISION TR SOLUTION HIGH pH, PH: 9
EAR:	
Chromogen:	Dako FLEX DAB, 23 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 35 Mins, 23 ºC Prediluted

CDX2 - Method 2

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

Primary Antibody:	Ventana 760-4380 (EPR2764Y)
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	
EAR:	
Chromogen:	Ventana DAB
Detection:	

CDX2 - Method 3

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

Primary Antibody:	Leica RTU PA0535 (AMT28)
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)
Detection:	Leica Bond Polymer Refine (DS9800)

CDX2 - Method 4

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

Primary Antibody:	Cell Marque 235R-18 (EPR2764Y) , 32 Mins, 42 ºC Dilution 1: 500
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 standard
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

The Breast Hormonal Receptor Module Run 114

Merdol Ibrahim and Suzanne Parry

Antigen Assessed:	Progesterone Receptor (PR)
Tissue Sections circulated:	Composite slide consisting of 3 breast carcinomas with different levels of receptor expression and normal tonsil
Number of Registered Participants:	272
Number of Participants This Run	260 (96%)

Circulated Tissue
The table below shows the staining characteristics of the tissue sections circulated during Run 114. This composed of three infiltrating ductal carcinomas (IDCs) with differing levels of receptor expression along with a section of tonsil. The staining of the breast tumours were characterised using the Leica (clone 1A6), Dako (clone PgR 636) and Ventana (clone 1E2) antibodies.

Sections	% Positivity	Expected Staining Intensity	Allred / Quick Score
A. IDC	>95%	High	8
B. IDC	50-70%	Mid	4 (the expression level varied depending on the serial section received)
C. IDC	0%	Negative	0
D. Tonsil	≤ 3%	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-House Tissue 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. 11-66% tumour positivity with 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 maximum 'borderline' pass (scores10-12/20).

Introduction

Expression of the steroid hormone receptors, oestrogen receptor-alpha (ER-α) and progesterone receptor (PR, types A and B) in breast epithelial cells is important in the development and function of the normal breast (Anderson). They also play a key-role in proliferative and neoplastic diseases of the breast (Cui et al.).

When examined by immunocytochemical staining, approximately 75% of primary breast cancers express ER-α, and of those approximately 50% co-express PR. Hormone receptor status is a good predictor of response to anti-oestrogen based treatments such as Tamoxifen and aromatase-inhibitors (Fisher et al). PR expression is under the control of ER-α, and because of this it has been proposed that PR expression in a tumour indicates the presence of functional ER-α (Cui et al); moreover, it potentially defines a subpopulation of patients with superior response to tamoxifen (Osborne et al); conversely, there is evidence that ER-α positive tumours that are PR negative are best treated with aromatase-inhibitors in preference to the ER-antagonists (Cui et al.). Finally, patients whose breast tumours are ER-α negative but PR positive respond equally well to anti-oestrogen based treatments as do those whose tumours are ER-α positive (Ciocca and Elledge). All

these factors lead to the conclusion that correct PR status is becoming increasingly important. Correct staining protocols and validated staining techniques are therefore vital to avoid false ER and PR staining (Rhodes et al. and Ibrahim et al.), which can have a direct impact on patient treatment regime

Choice of Tissue for Assessments

This assessment consisted of three invasive breast tumours of varying PR expression level (samples A-C). A tonsil (sample D) was also included and used as a further negative control to help gauge the specificity of the test.

NEQAS tissue samples were tested by staining every 50th section from the relevant tissue blocks using the Leica 1A6 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 and included Leica (clone 1A6), Dako (clone PgR 636) and Ventana (clone 1E2). It should be noted that the submitted 1E2 clone showed nuclear staining in the germinal centres of the tonsil section in less than 2% of cells.

Features of Optimal Immunostaining (Figs1,2,3,4,6 &10)

- 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 not excessive
- No background staining of connective tissues or inappropriately localised staining

Features of Suboptimal Immunostaining (Figs5,7,8 & 9)

- False positive/negative staining
- Relatively weak nuclear staining of the receptor positive tumours
- Excessive cytoplasmic staining
- Excessive background staining of connective tissue elements
- Inappropriate staining of some cells e.g. lymphocytes, fibroblasts

NEQAS Section Assessment Results

260 laboratories submitted their slides for the PR assessment. The acceptable pass rate has fallen slightly since the previous PR run (Run 112). Please see table below. The most popular antibody used in this assessment run was the Ventana 1E2 clone, used by 65 participants with a pass rate of 83% and showed an acceptable pass rate of 83%. There were some laboratories using the 1E2 clone exhibiting false positive staining in the negative tumour. (Fig 8). The second most popular antibody was the Leica/Novocastra PgR 16A clone, used by 40 participants with an acceptable pass rate of 93%.

The results are summarised in the table below:

PR Pass Rates :		
	Run 112	**Run 114**
Acceptable	88%(N=242)	85% (N=221)
Borderline	8%(N=21)	10% (N=25)
Unacceptable	4%(N=10)	5% (N=14)

The borderline and failed marks for this assessment Run 114 were mostly due to weak staining, particularly in the mid-expressing tumour and false positive staining in the negative tissue seen mostly with users of the 1E2 clone. A few labs failed due to inappropriate or excessive background. Staining.

In-House Tissue Assessment Results

99% of participants also submitted their in-house controls for assessment. Overall these showed a lower acceptable pass rate, with more participants receiving a borderline pass. The results are summarised in the table below:

In-House Pass Rates Run 114:	
Acceptable	76%(N=194)
Borderline	21%(N=54)
Unacceptable	3%(N=8)

Many of the borderline passes were due to the lab not providing a composite control consisting of a high, a mid and a negative-expressing tumour, which is a requirement by UK NEQAS IHC. Other labs lost marks due to poor tissue quality, inappropriate non-specific staining, false positive staining in the negative control or excessive background.

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 PR may have been 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 control is much stronger.

Another factor to take into consideration is that the sensitivity of a participant's assay may be acceptable on their own tissue section, but may not be optimal. A protocol should be in place that will stain effectively on both the NEQAS samples and the labs own samples.

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.

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 PR; 2) re-tested and validated staining of every 50th section from the relevant tissue blocks using the Leica 1A6 clone; 3) re-tested and validated samples by the relevant commercial companies to further verify the expected level of staining.
- 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) PR-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.

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Acknowledgments

We would like to thank Dako, Roche and Leica for agreeing to stain the UK NEQAS Breast hormonal receptor 'Gold standard' slides with their respective assays.

Selected Images showing Optimal and Sub-optimal Immunostaining

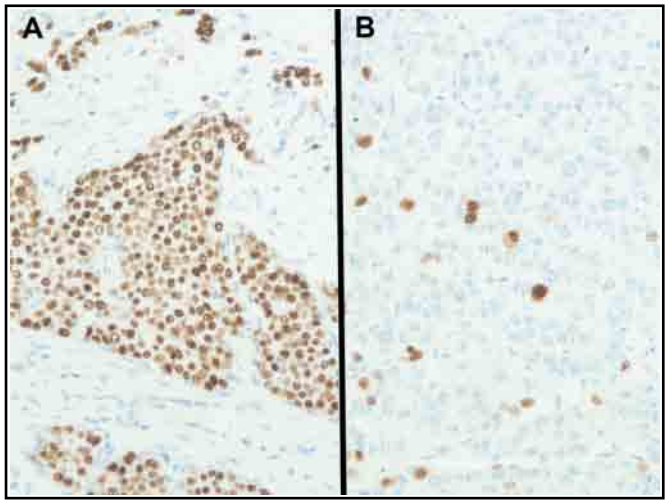


Fig 1. Optimally stained UK NEQAS distributed (A) high and (B) low-moderate PR expressing tumours. (A) Moderate-strong intense staining in over 95% of neoplastic cells and (B) varying intensity of PR staining in approximately 20% of neoplastic cells. Stained with the Novocastra 16+SAN27 (A&B) antibody, 1:50, on the Leica Bond III with ER1 for 20 minutes.

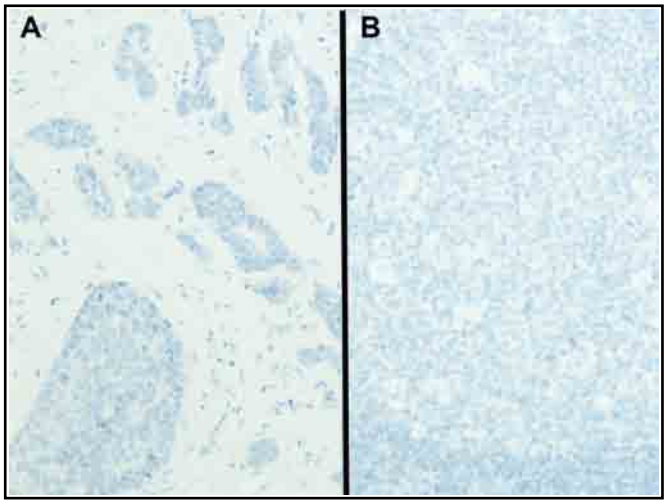


Fig 2. Expected level of expression of PR in the UK NEQAS distributed samples: Both the PR (A) negative tumour and (B) tonsil remain unstained. Same protocol as Fig 1.

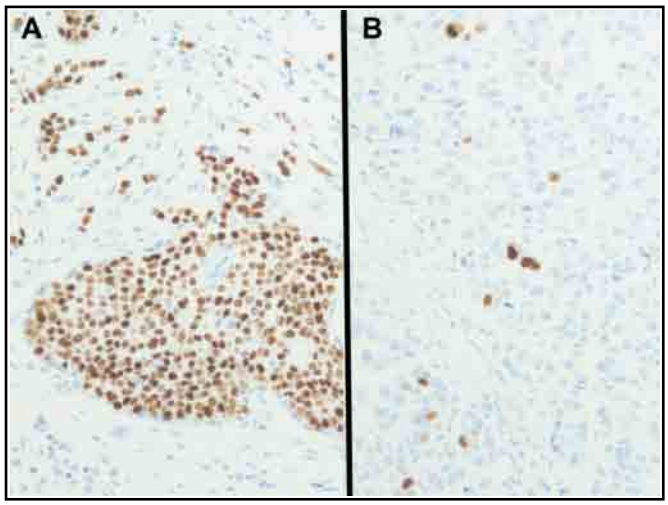


Fig 3. Optimal demonstration of PR in the UK NEQAS (A) high and (B) low-mid expressing tumours, showing the expected level of staining in both samples. (A&B) Stained with the Dako RTU PgR 636 clone, on the Dako Autostainer and pre-treatment in the PT link with high pH buffer for 15 minutes.

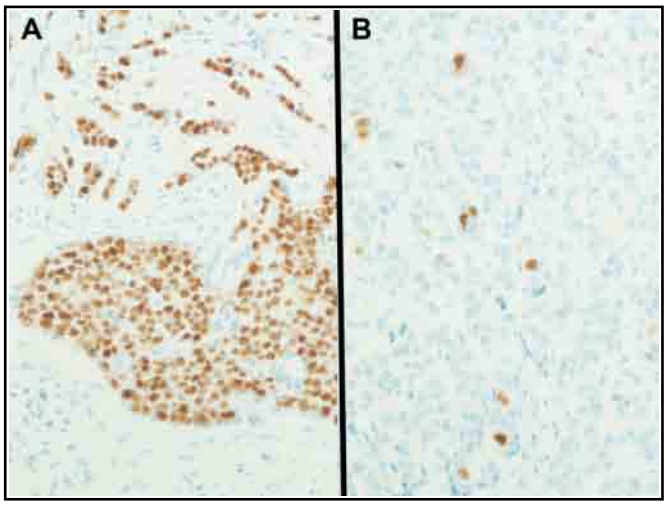


Fig 4. (A&B) Optimal level of PR expression in the UK NEQAS distributed samples stained with the Ventana 1E2 clone. (A) High expressing tumour shows moderate-strong intense staining in over 95% of neoplastic cells, while (B) low-mid-expressing tumour shows staining in less than 25% of tumour cells. Stained on the Benchmark ULTRA with CC1 retrieval for 64 minutes.

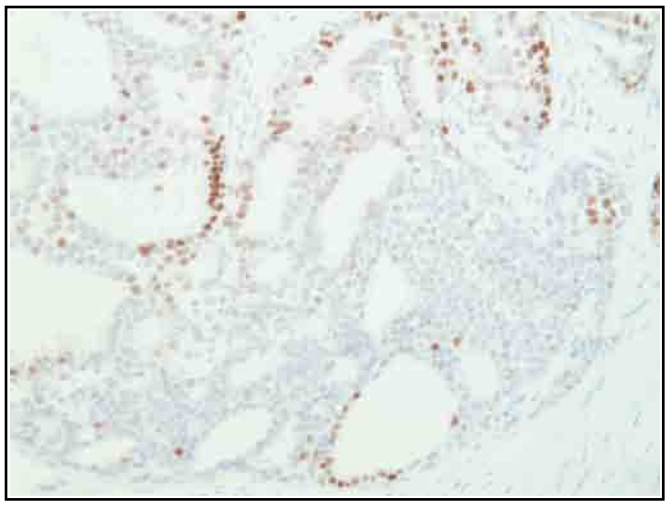


Fig 5. Unacceptable staining of the UK NEQAS high PR-expressing tumour (compare to Figs 1A, 3A & 4A). Staining is weak with a much lower percentage of PR-positive cells. The NEQAS mid-expressing tumour on the same slide showed no staining, indicating sub-optimal methodology.

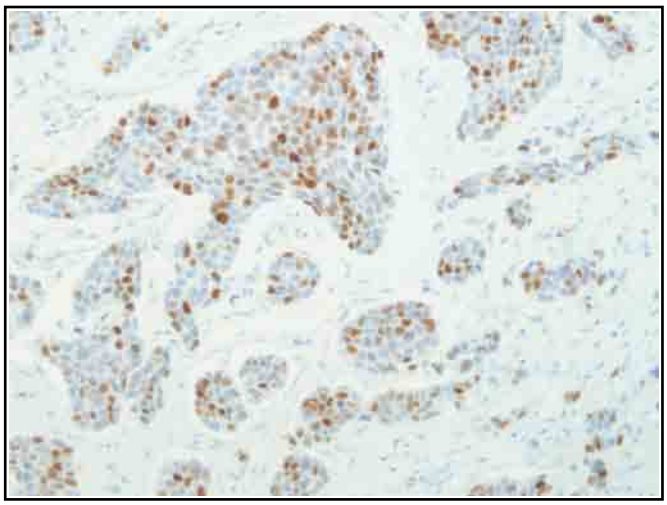


Fig 6. Optimal demonstration of PR in the UK NEQAS mid-expressing tumour (block 2): The section shows the expected level of varying intensity of positive staining in approximately 30% of neoplastic cells. Stained with the Ventana 1E2 clone on the Benchmark XT with CC1 mild retrieval and UltraView detection.

Selected Images showing Optimal and Sub-optimal Immunostaining

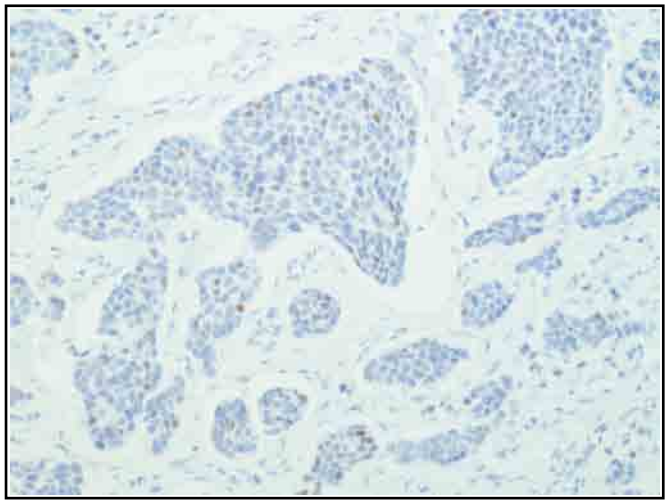


Fig 7. Sub-optimal demonstration of PR in the UK NEQAS mid-expressing tumour (block 2) (compare to Fig 6): The intensity and percentage of PR-positive tumour cells is much lower than expected. Over-diluted antibody is the most likely reason. Stained with the Novocastra PgR 312 (16) clone (1:500), on the Leica Bond III with ER1 for 30 mins.

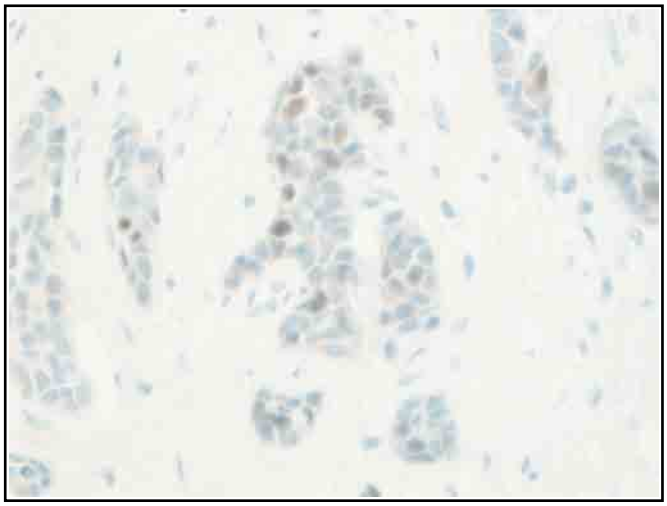


Fig 8. Unacceptable demonstration of PR in the UK NEQAS negative tumour, which appears to show false-positive staining. The UK NEQAS tonsil on the same slide also showed more lymphocyte staining than expected. Stained with the Ventana 1E2 pre-diluted antibody on the Ventana ULTRA with CC1 retrieval for 64 minutes and UltraView detection.

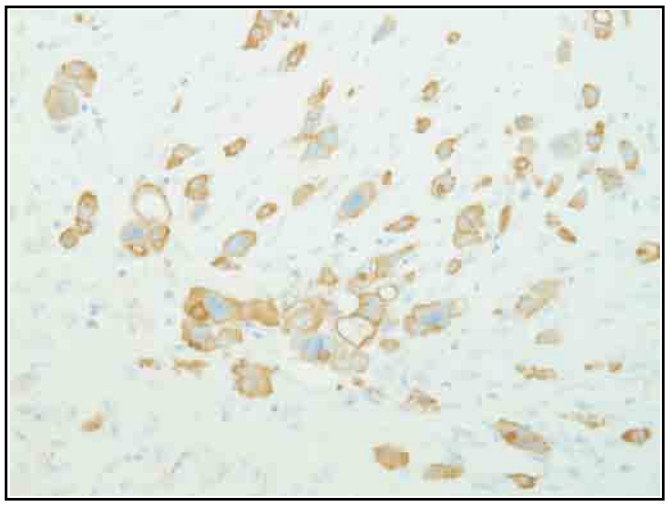


Fig 9. Inappropriate staining in the UK NEQAS negative-expressing tumour. Sample should be negative, but the staining present is cytoplasmic-membranous. This may have been caused by contamination of the antibody. Stained with the Dako RTU PgR (A&B) antibody on the Dako Autostainer with retrieval in the PT link.

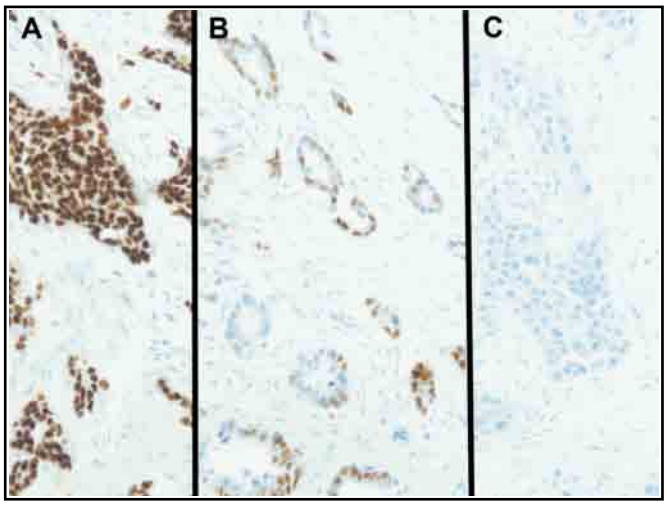
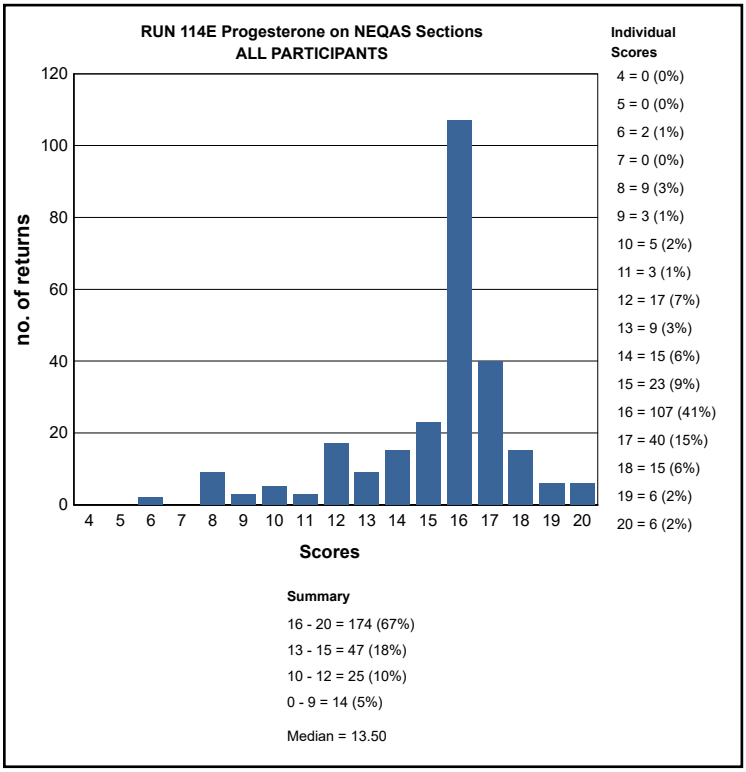
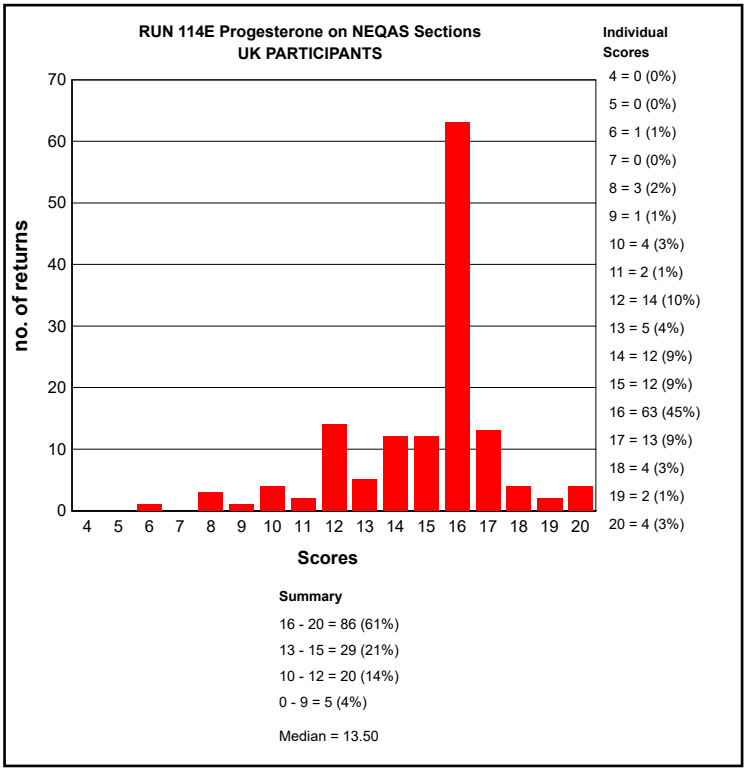
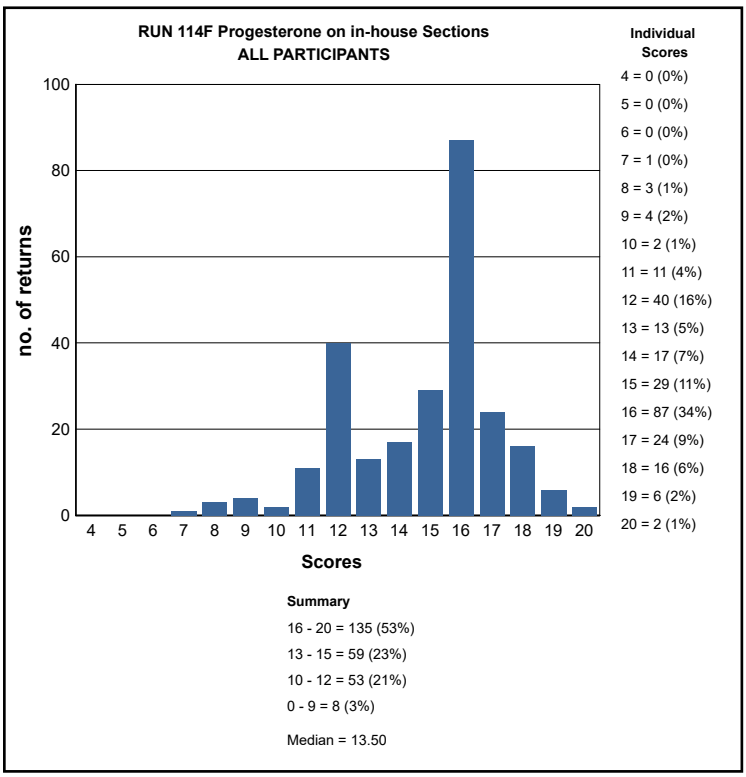


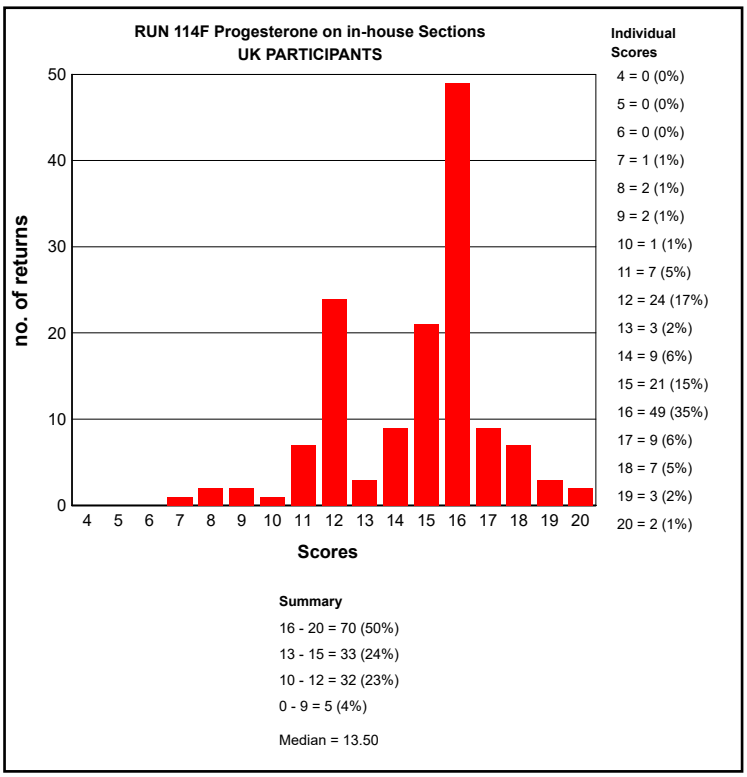
Fig 10. Good example of an in house multi-block control for PR. (A) high, (B) mid, and (C) negative expressing tumours. Stained with the Dako PgR 636 (A&B) antibody, 1:80, on the Dako Autostainer and retrieval in the PT link with high retrieval buffer for 20 minutes at high temperature.

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: 114

Primary Antibody : Progesterone

Antibody Details	N	%
Dako M3569 (PgR 636) (A&B)	34	79
Dako K1904 (PgR 1294 (b))	1	100
NeoMarkers RM-9102-S (SP2) (A&B)	2	100
Novocastra NCL-PGR (1A6) (A&B)	3	67
Novocastra NCL-PGR-312 (16) (A)	14	86
Novocastra NCL-L-PGR-312 (16) (A)	40	93
Ventana 790-4296 (1E2) (A&B)	33	79
Novocastra NCL-L-PGR-AB (16+SAN27) (A&B)	9	100
Novocastra RTU-PGR-312 (16) (A)	3	100
Novocastra PA0312 (16) (A)	12	100
Novocastra NCL-L-PGR/2 (1A6) (A&B)	2	0
Novocastra NCL-PGR-AB (16+SAN27)	5	100
Ventana 790-2223 (1E2) (A&B)	65	83
Dako N1630 RTU (PgR 636) (A&B)	3	67
Dako IR068 (PgR 636) (A&B)	12	92
Dako IS068 (PgR 636) (A&B)	1	100
Zytomed Sytems SA-RBK-02 (SP42) (A&B)	1	100
Ventana 790-4324 (SP2) (A&B)	5	100
Cell Marque 323R-16 (A)	1	0
Biocare Medical CRM302C (SP2) (A&B)	1	100
Other	4	50
Dako M3568 (PgR 1294)	2	100

Breast Steroid Hormone Receptor Run: 114

Automation — Progesterone

	N	%
Dako Autostainer Link 48	27	93
Dako Autostainer plus	2	100
Dako Autostainer Plus Link	3	33
Dako Omnis	4	100
LabVision Autostainer	3	100
Leica Bond Max	28	93
Leica Bond X	1	100
Leica Bond-III	43	88
Menarini - Intellipath FLX	1	100
None (Manual)	2	50
Other	1	100
Shandon Sequenza	2	100
Ventana Benchmark GX	6	100
Ventana Benchmark ULTRA	85	76
Ventana Benchmark XT	48	85

Breast Steroid Hormone Receptor Run: 114

Heat Mediated Retrieval — Progesterone

	N	%
Biocare Decloaking Chamber	1	100
Dako Omnis	4	100
Dako PTLink	28	86
Lab vision PT Module	3	100
Leica ER1 20 mins	14	86
Leica ER1 30 mins	24	92
Leica ER1 40 mins	2	50
Leica ER2 10 mins	2	100
Leica ER2 20 mins	26	92
Leica ER2 30 mins	3	100
Leica ER2 40 mins	1	100
Microwave	2	100
None	1	0
Other	2	100
Pressure Cooker	4	100
Pressure Cooker in Microwave Oven	1	100
Ventana CC1 16mins	3	67
Ventana CC1 24mins	1	100
Ventana CC1 32mins	9	78
Ventana CC1 36mins	20	90
Ventana CC1 40mins	1	100
Ventana CC1 48mins	3	100
Ventana CC1 52mins	6	50
Ventana CC1 56mins	1	100
Ventana CC1 64mins	36	69
Ventana CC1 72mins	1	100
Ventana CC1 76mins	1	100
Ventana CC1 8mins	1	100
Ventana CC1 extended	3	100
Ventana CC1 mild	14	93
Ventana CC1 standard	33	82
Ventana CC2 64mins	1	100
Ventana CC2 mild	1	100
Water bath 95-98 OC	2	50

Breast Steroid Hormone Receptor Run: 114

Enzyme Mediated Retrieval — Progesterone

	N	%
AS PER KIT	7	86
NOT APPLICABLE	159	84
Ventana Protease 1 (760-2018)	1	100

Breast Steroid Hormone Receptor Run: 114		
Detection	**Progesterone**	
	N	**%**
AS PER KIT	11	100
Biocare polymer (M4U534)	1	0
Dako EnVision FLEX (K8000/10)	8	100
Dako EnVision FLEX+ (K8002/12)	18	78
Dako Envision HRP/DAB (K5007)	4	100
Dako Envision+ HRP mouse K4004/5/6/7	1	100
Dako REAL HRP/DAB (K5001)	1	100
Leica Bond Polymer Refine (DS9800)	67	91
MenaPath X-Cell Plus (MP-XCP)	1	100
NOT APPLICABLE	2	50
Novocastra Novolink PDS (RE7-140/150/280/290-K)	1	100
Other	4	100
Ventana iView system (760-091)	3	100
Ventana OptiView Kit (760-700)	14	86
Ventana UltraView Kit (760-500)	118	80

Breast Steroid Hormone Receptor Run: 114		
Chromogen	**Progesterone**	
	N	**%**
AS PER KIT	19	95
DAKO DAB+	1	100
Dako EnVision Plus kits	3	33
Dako FLEX DAB	24	92
Dako REAL EnVision K5007 DAB	4	100
Dako REAL K5001 DAB	1	100
Leica Bond Polymer Refine kit (DS9800)	67	91
menapath xcell kit DAB (MP-860)	1	100
NOT APPLICABLE	1	0
Other	8	75
Ventana DAB	7	86
Ventana Enhanced Alk. Phos. Red Detection Kit	1	100
Ventana iview	2	100
Ventana Ultraview DAB	117	79

BEST METHODS

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

Progesterone - Method 1

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

Primary Antibody: Novocastra NCL-L-PGR-AB (16+SAN27) (A&B) , 15 Mins Dilution 1: 50
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER1 20 mins
EAR: NOT APPLICABLE
Chromogen: Leica Bond Polymer Refine kit (DS9800)
Detection: Leica Bond Polymer Refine (DS9800)

Progesterone - Method 2

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

Primary Antibody: Dako IR068 (PgR 636) (A&B) , 20 Mins, ambient ºC Prediluted
Automation: Dako Autostainer Link 48
Method: Dako FLEX kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako PTLink, Buffer: DAKO target retrieval high pH, PH: 9
EAR:
Chromogen: Dako FLEX DAB, ambie ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection: Dako EnVision FLEX (K8000/10) , 20 Mins, ambient ºC Prediluted

Progesterone - Method 3

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

Primary Antibody: Novocastra NCL-L-PGR-312 (16) (A) , 30 Mins, 23 ºC Dilution 1: 150
Automation: Dako Autostainer Link 48
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer, PH: 7.6
HMAR: Dako PTLink, Buffer: ENVISION TR HIGH pH SOLUTION, PH: 9
EAR:
Chromogen: Dako FLEX DAB, 23 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection: Dako EnVision FLEX+ (K8002/12) , 35 Mins, 23 ºC Prediluted

Progesterone - Method 4

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

Primary Antibody:	Ventana 790-2223 (1E2) (A&B) , 16 Mins, 37 ºC Prediluted
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, PH: 36, Time 1: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 36 ºC

The Breast HER2 IHC Module Run 114

Keith Miller and Suzanne Parry

Antigen Assessed:	HER2
Sections Circulated:	Composite slide consisting of 4 breast carcinoma cell lines (see table below)
Number of Registered Participants:	279
Number of Participants this Run	266 (95%)

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 with additional technical feedback. As well as taking into account the expected range of cell line membrane staining (30-90% see above), 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 summarised in the table below:

	Pass Rates Run 114:	
	Neqas Section	In-House
Acceptable	70% (N=187)	61% (N=160)
Borderline	15% (N=41)	29% (N=75)
Unacceptable	14% (N=38)	10% (N=25)

As with previous runs the most popular antibody was the Ventana 4B5, used by 96% of participants and showed an overall acceptable pass rate of 85%. 14 labs are using the Dako HercepTest (23% acceptable pass rate), and 18 labs are using the Leica Oracle kit 83% acceptable pass rate). 46 laboratories used lab-devised methods, using a variety of antibodies, pre-treatment methods and platforms. These labs showed an acceptable pass rate of 34%. All data is based on the methodology information submitted by participants, but unfortunately not all labs provided their methodology data.

References

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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
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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 Recommendations 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 IHC 'Gold standard' samples with their respective kits/assays.

Selected Images showing Optimal and Sub-optimal Immunostaining

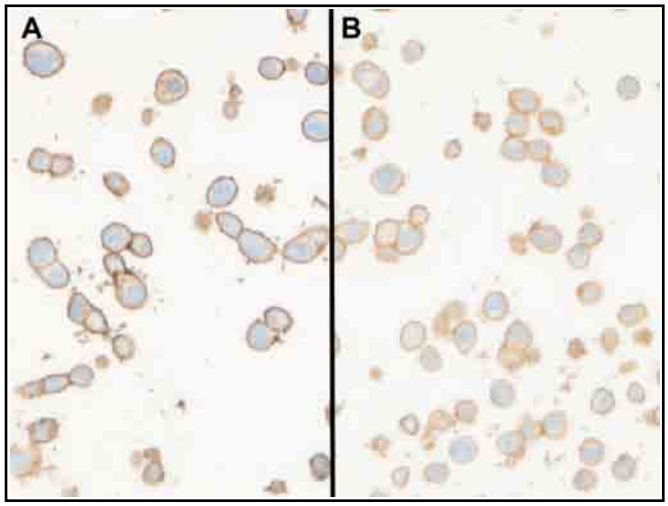


Fig 1. Appropriate staining of the UK NEQAS SK-BR3 (3+) cell line. (A & B) Strong and complete circumferential membrane. Stained using (A) the Dako HercepTest on the Autostainer with PT link pre-treatment and (B) Leica Oracle kit on a BondMax as per recommendations.

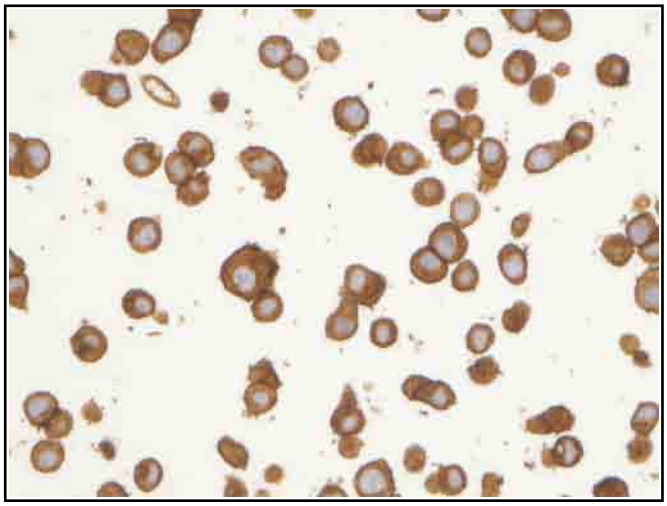


Fig 2. Unacceptable staining of the UK NEQAS 3+ cell line. Section shows excessive cytoplasmic staining, making it difficult to read the membranes. Staining carried out using a lab-devised method with the Novocastra CB11 antibody, 1:100, on the Dako Autostainer and pre-treatment in the PT link with high pH buffer.

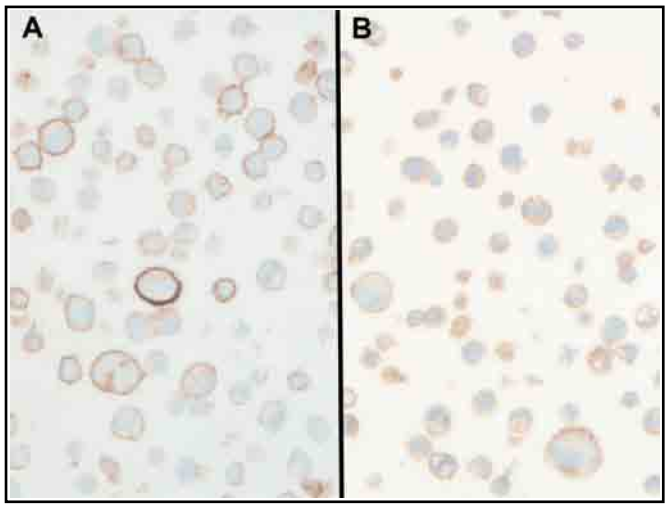


Fig 3. Two examples showing the expected level of staining of the UK NEQAS MDA-MB-453 (2+) cell line. (A & B) The majority of cells show weak to moderate complete membrane staining. Stained using (A) the Ventana Pathway 4B5 and (B) the Leica Oracle kit on a BondMax.

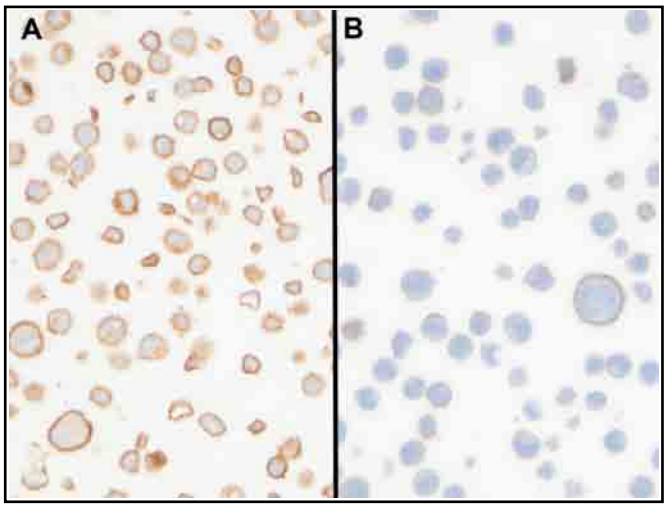


Fig 4. Unacceptable staining of the UK NEQAS 2+ distributed cell lines. (A) Excessive membrane staining, which is more representative of 3+ staining. Slide stained using a lab-devised method. (B) Shows excessive counterstain, and making it difficult to interpret the membranes (method not provided).

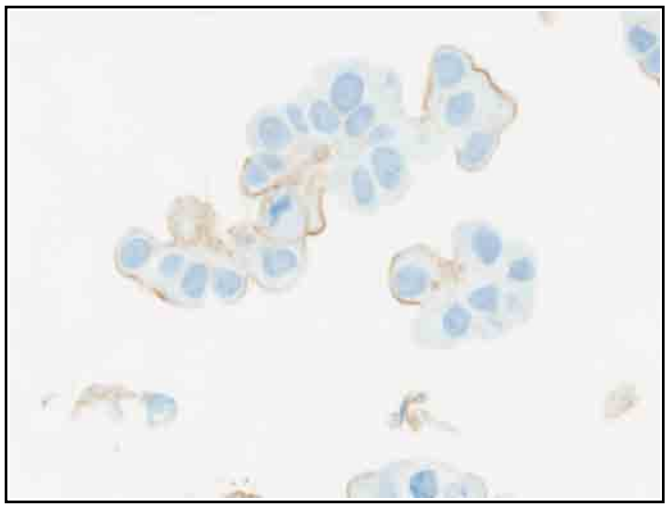


Fig 5. Expected level of staining in the UK NEQAS MDA-MB-175 (1+) cell line, which is partial membranous. Section stained with the Dako HercepTest on the Autostainer and pre-treatment in the PT link.

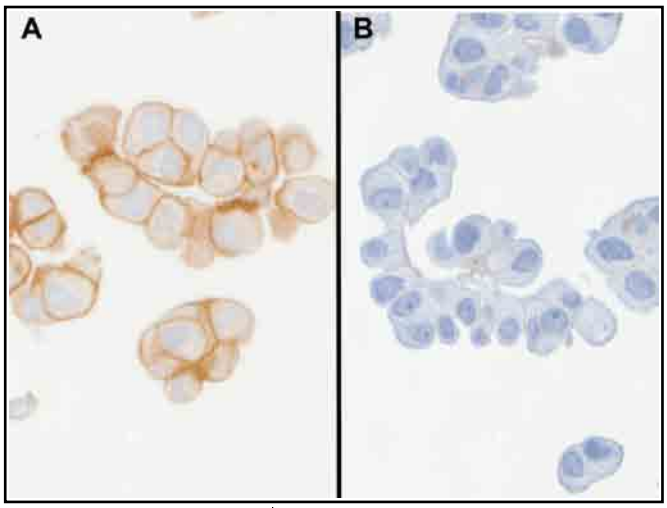


Fig 6. Two examples showing unacceptable staining of the UK NEQAS MDA-MB-175 (1+) cell line. In (A) the staining is too high and more representative of 2+staining, while in (B) any possible staining is masked by the grey counterstain.

Selected Images showing Optimal and Sub-optimal Immunostaining

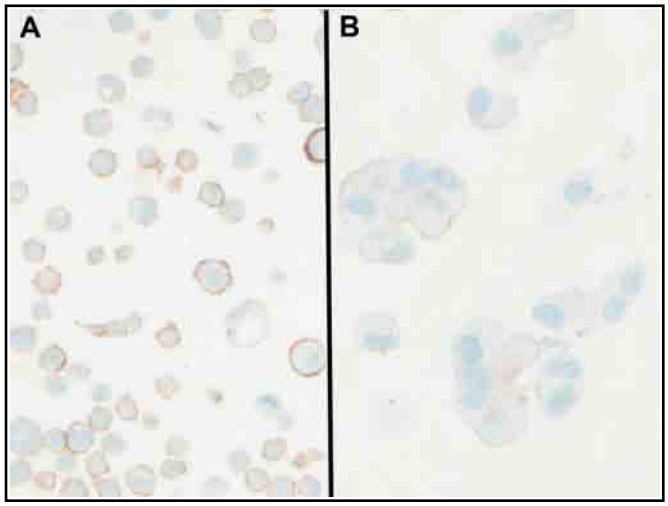


Fig 7. Acceptable staining of the UK NEQAS (A) 2+ and (B) 1+ cell lines stained by a laboratory using the Ventana 4B5 assay on the Benchmark GX.

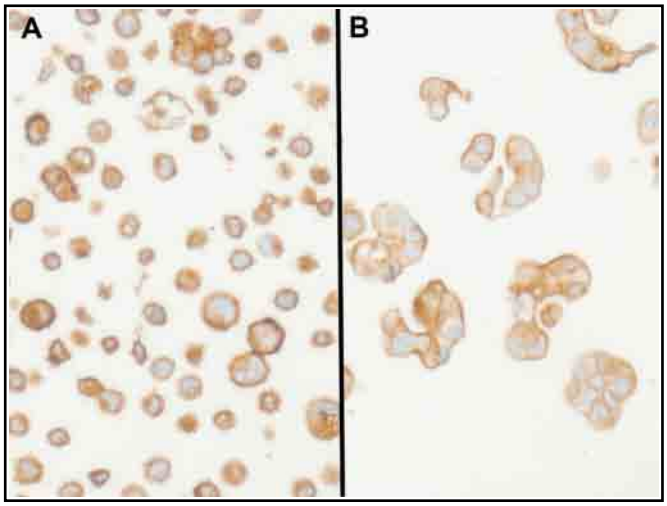


Fig 8. Images showing unacceptable staining of the UK NEQAS (A) 2+ and (B) 1+ cell lines stained by a laboratory whose slide serial sections following those in Fig 7. Section was stained using the Leica Oracle kit, but not as per recommendations by the supplier.

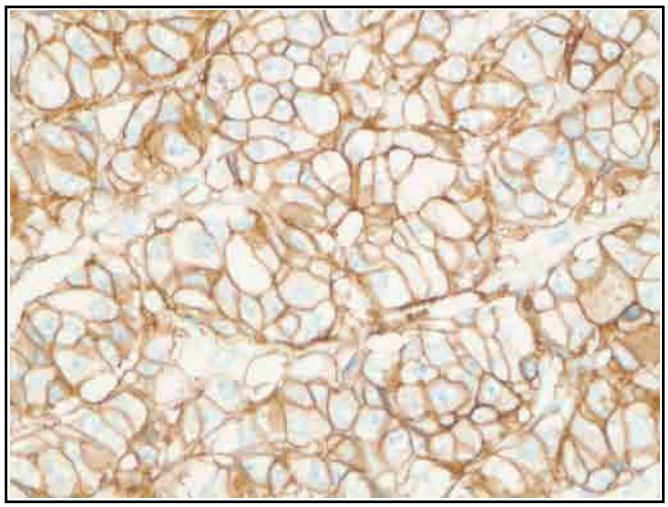


Fig 9. Good example of a participant's 3+ in-house control submitted for assessment. Section shows strong complete membranous staining with a clean background. Stained with the Ventana 4B5 assay.

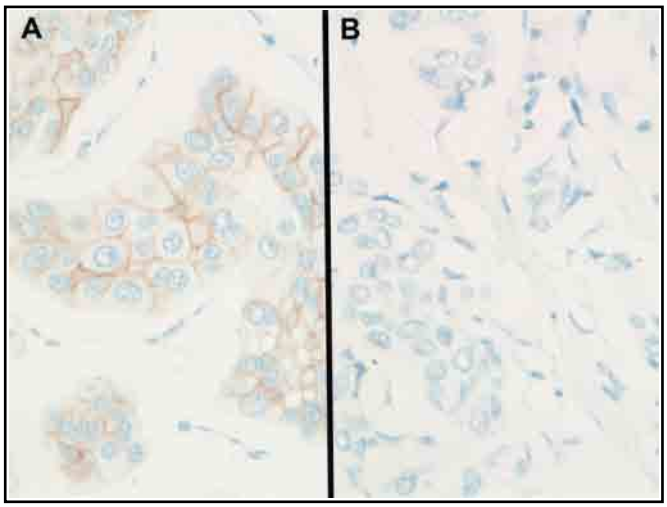
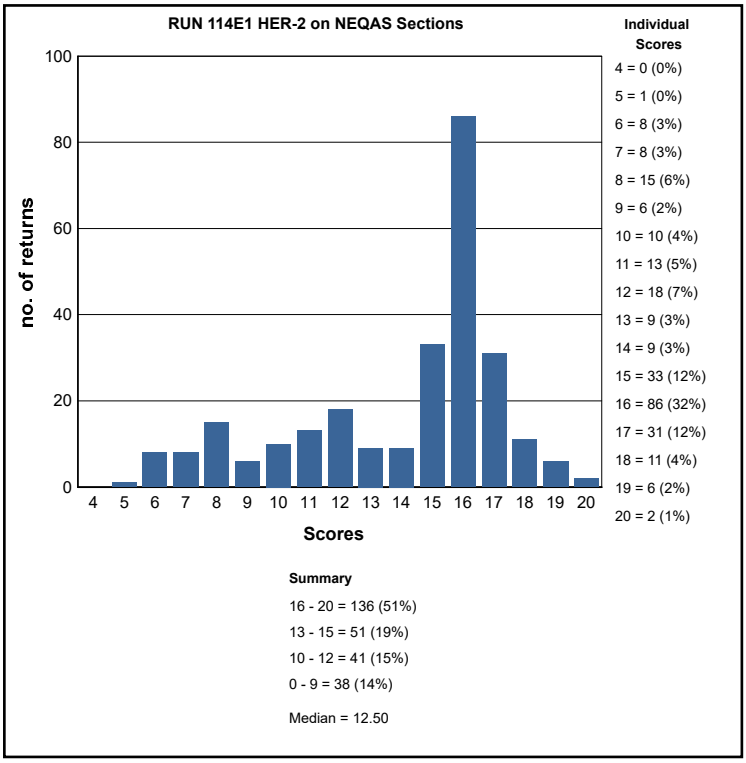
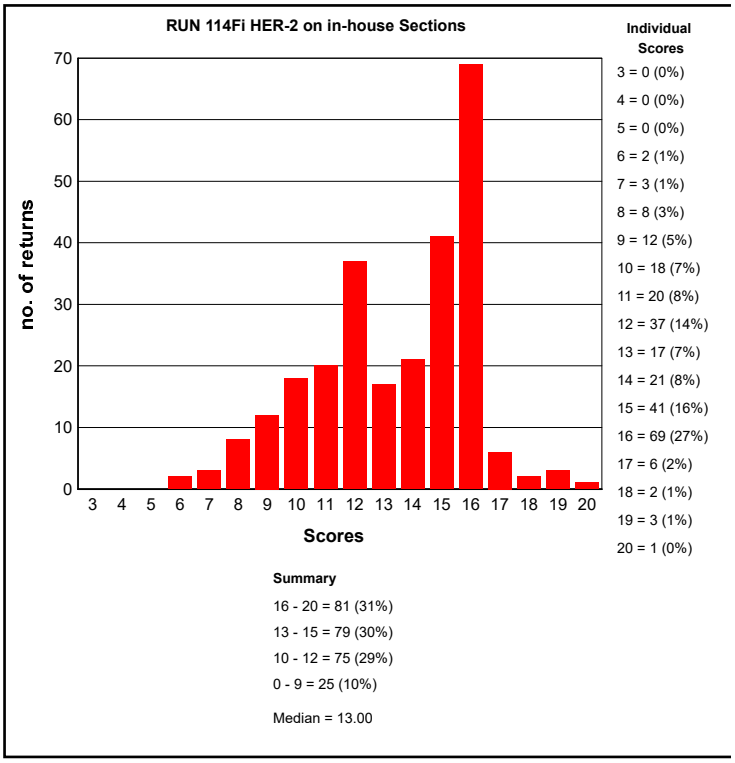


Fig 10. Good in house breast controls optimally stained with HER2. (A) 2+ and (B) negative breast tumours (same protocol as Fig 9).

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: 114

Primary Antibody	N	%
Dako HercepTest K5204 (poly)	3	0
Dako HercepTest K5205 (poly)	1	0
Dako HercepTest K5207 (poly)	3	33
Dako A0485 C-erB-2 (poly)	18	28
Cell Marque CMA 601 (CB11)	2	0
Ventana Pathway 790-100 (4B5)	13	92
Labvision / Neomarkers RM-9103 (SP3)	3	0
Biogenex AM134-5M (CB11)	1	0
Leica Oracle HER2 Bond IHC (CB11)	18	83
Dako Link HercepTest SK001 (poly)	7	57
BioGenex (EP1045Y) rb mono	1	100
Ventana Confirm 790-4493 (4B5)	37	78
Ventana Pathway 790-2991 (4B5)	130	85
Novocastra NCL-L-CB11 (CB11)	7	14
Biocare CME 342 A,B (EP1045Y)	3	33
Other	10	30
Cell Marque 237R (SP3)	1	100

Breast HER2 ICC Run: 114

Automation	N	%
BioGenex GenoMX 6000i	1	0
Dako Autostainer	3	0
Dako Autostainer Link 48	13	38
Dako Autostainer plus	1	100
Dako Autostainer Plus Link	3	67
Dako Omnis	1	100
Leica Bond Max	15	27
Leica Bond-III	18	89
Menarini - Intellipath FLX	1	0
None (Manual)	14	7
Other	5	0
Shandon Sequenza	1	0
Ventana Benchmark GX	12	75
Ventana Benchmark ULTRA	91	84
Ventana Benchmark XT	78	87

Breast HER2 ICC Run: 114

Heat Mediated Retrieval	N	%
Biocare Decloaking Chamber	6	0
Dako Omnis	1	100
Dako Pascal	1	0
Dako PTLink	16	38
Lab vision PT Module	2	0
Leica ER1 10 mins	3	67
Leica ER1 20 mins	8	25
Leica ER1 25 mins	14	93
Leica ER1 30 mins	1	0
Leica ER2 20 mins	3	33
Microwave	6	17
None	5	80
Other	5	60
Pressure Cooker	2	0
Ventana CC1 16mins	3	100
Ventana CC1 20mins	3	0
Ventana CC1 24mins	1	100
Ventana CC1 32mins	17	82
Ventana CC1 36mins	39	87
Ventana CC1 40mins	2	100
Ventana CC1 48mins	1	0
Ventana CC1 52mins	3	100
Ventana CC1 56mins	9	89
Ventana CC1 64mins	4	100
Ventana CC1 72mins	1	0
Ventana CC1 8mins	5	80
Ventana CC1 mild	69	86
Ventana CC1 standard	14	86
Ventana CC2 36mins	1	100
Water bath 95-98 OC	8	13

Breast HER2 ICC Run: 114

Detection	N	%
AS PER KIT	18	78
Biocare polymer (M4U534)	1	0
Biocare SLAB (STU HRP 700H,L10)	1	0
BioGenex SS Polymer (QD 420-YIKE)	1	0
BioGenex SS Polymer (QD 430-XAKE)	1	0
Dako HerCep Test (K5204)	2	0
Dako EnVision FLEX (K8000/10)	4	25
Dako EnVision FLEX+ (K8002/12)	8	38
Dako Envision HRP/DAB (K5007)	3	33
Dako HerCep Test Autor (K5207)	1	0
Dako HerCep Test Autor (SK001)	4	100
LabVision UltraVision ONE Polymer (TL-012/5-HDJ/T	1	0
Leica Bond Polymer Define (DS9713)	1	100
Leica Bond Polymer Refine (DS9800)	19	42
Other	10	20
Ventana iView system (760-091)	5	100
Ventana OptiView Kit (760-700)	10	90
Ventana UltraView Kit (760-500)	157	82

Breast HER2 ICC Run: 114

Enzyme Retrieval	N	%
AS PER KIT	17	65
NOT APPLICABLE	128	74
Other	1	0
Ventana Protease	1	100
Ventana Protease 1 (760-2018)	2	100

Breast HER2 ICC Run: 114

Chromogen	N	%
AS PER KIT	39	72
BioGenex Liquid DAB (HK153-5K)	2	0
Dako DAB+ Liquid (K3468)	1	0
Dako DAB+ REAL Detection (K5001)	1	0
Dako EnVision Plus kits	1	0
Dako FLEX DAB	12	50
Dako REAL EnVision K5007 DAB	4	25
Leica Bond Polymer Refine kit (DS9800)	21	43
NOT APPLICABLE	1	0
Other	17	35
Ventana DAB	6	100
Ventana iview	3	100
Ventana Ultraview DAB	150	83

BEST METHODS

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

HER-2 - Method 1

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

Primary Antibody:	Ventana Pathway 790-2991 (4B5)
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)

HER-2 - Method 2

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

Primary Antibody:	Ventana Confirm 790-4493 (4B5) , 16 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark GX
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.6
HMAR:	Ventana CC1 mild, Buffer: Cell Conditioning 1, PH: 8.4
EAR:	
Chromogen:	Ventana Ultraview DAB, 37 ºC., Time 1: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC Prediluted

HER-2 - Method 3

Participant scored 20/20 (UK NEQAS Slide) and 15/20 (In House slide) using this method.

Primary Antibody:	Ventana Pathway 790-2991 (4B5)
Automation:	Ventana Benchmark XT
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)

The Gastric HER2 IHC Module Run 114

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**. 1+ or 0 or 2+ depending on the serial section received **C**. 1+ or 0 **D**. 3+
Number of Registered Participants	65
Number of Participants this Run	61 (94%)

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 by the assessment team 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	**1+ or 0 (or 2+)**	Non-Amplified (Amplified)
C	**2+**	Amplified
D	**3+**	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 1,3, 5 &7)

- 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 Suboptimal or Unacceptable Staining: (Figs 2,4, 6 & 8)

- 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. These are summarised in the table below:

NEQAS Pass Rates Run 114:	
Acceptable	83% (N=51)
Borderline	10% (N=6)
Unacceptable	7% (N=4)

There is continued improvement in the acceptable pass rate for Run 114; up to 83% from 73% seen at Run113. There was also a vast improvement from Run 112, which showed an acceptable pass rate of 65%. 4 labs (7%) still failed the assessment, and this was due to either weak membrane staining or over-staining. In the clinical setting false negative results will lead to suitable patients not being put forward for Herceptin therapy, and equally concerning is that many patients may be over-treated: For instance, a HER2 IHC result of 3+ will automatically mean that the patient is put forward for Herceptin therapy. Also, as IHC 2+ cases are also reflex tested, incorrect over-staining could mean that more samples than necessary are being put forward for in-situ hybridisation reflux testing.

Most labs are using the recommended standardised protocols for their particular automated systems, such as CC1 Mild antigen retrieval on the Ventana XT with the 4B5 pre-diluted antibody clone. However, a few labs are not using the recommended protocols, and this may be the cause of either weaker or stronger membrane staining seen at assessment.

All participants apart from 2 labs also submitted their in-house controls. The in-house results are summarised in the following table:

In-House Pass Rates Run 114:	
Acceptable	70% (N=41)
Borderline	22% (N=13)
Unacceptable	7% (N=4)

The overall pass rates on the in-house controls was lower than on the Neqas samples. More labs received a borderline pass on the in-house samples. Several of these borderline passes were due to labs not submitting the requested composite control material, consisting of 3+, 2+ and 1+/0 expressing gastric/breast control tumour samples. These labs were therefore given a maximum score of 12/20. Other labs were marked down for poor tissue quality or fixation. 1 of the sections that failed the assessment showed excessive cytoplasmic and background staining.

Methodologies

The majority of labs are using the Ventana 4B5 standardised kit for their gastric HER2 testing: In this particular assessment run the Ventana assay was used by 66% of participants with an acceptable pass rate of 87%, 5 % of the participants used the Dako Hercept test with 100% acceptable pass rate.

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 also 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.

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 IHC 'Gold standard' samples with their respective kits/assays and also for providing the ISH.

Selected Images showing Optimal and Sub-optimal Immunostaining

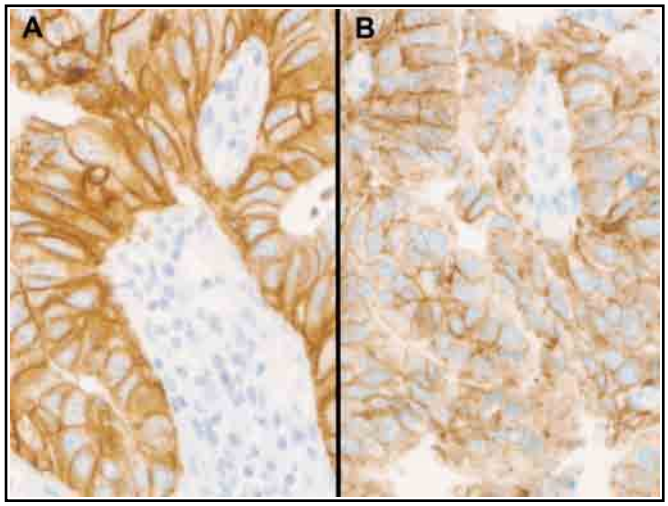


Fig 1. (A, B) Two examples showing the expected 3+ HER2 expression in the UK NEQAS gastric tumour sample 'A'. Stained using (A) Dako HercepTest with antigen retrieval in the PT link and (B) with the Ventana 4B5 Pathway on the Benchmark XT, CC1 mild.

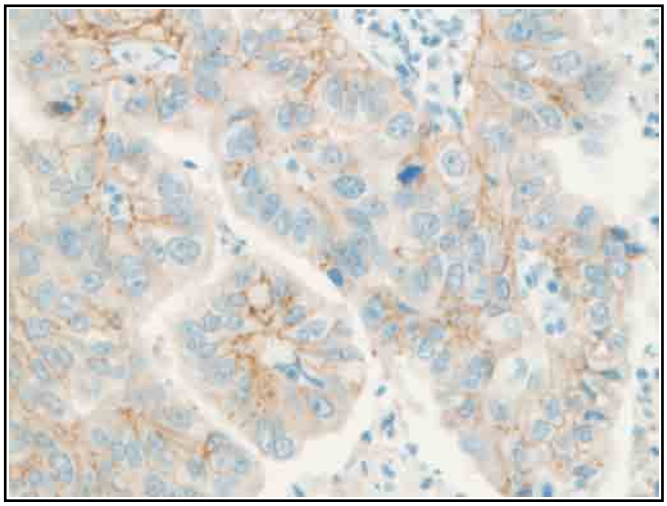


Fig 2. Unacceptable level of staining the UK NEQAS gastric 3+ tumour sample 'A'. The staining is weak and more representative of a 2+. Stained using a lab-devised method with the Ventana 4B5 antibody on the Leica bond system, with ER2 retrieval for 20 minutes.

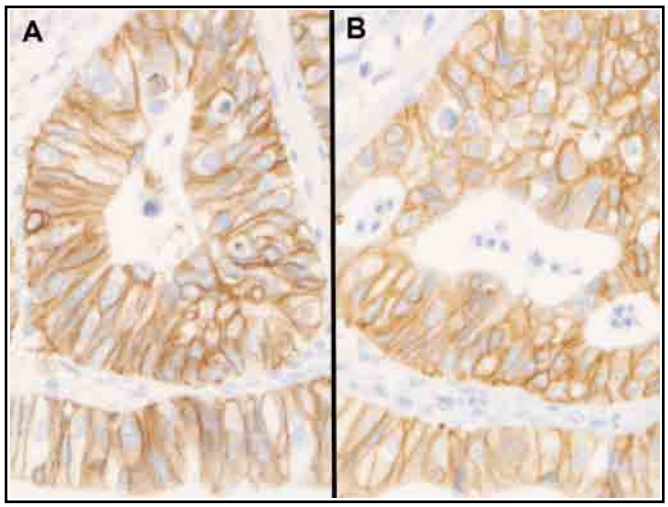


Fig 3. (A, B) Two examples showing the expected 3+ level of HER2 staining in the UK NEQAS distributed sample 'B'. Stained with (A) Ventana 4B5 pathway on the Benchmark XT, and (B) Dako HercepTest.

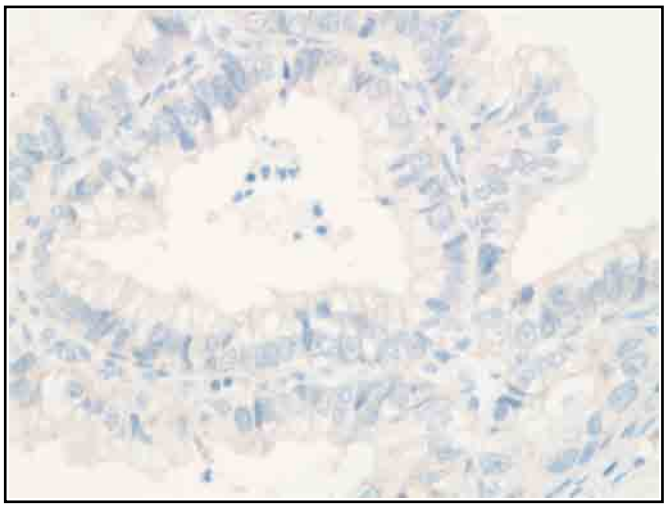


Fig 4. Unacceptable staining in the UK NEQAS 3+ sample 'B'. Staining is very weak and is therefore more representative of a weak 2+ rather than a 3+. Stained using the Leica Oracle kit as per supplier protocol recommendations.

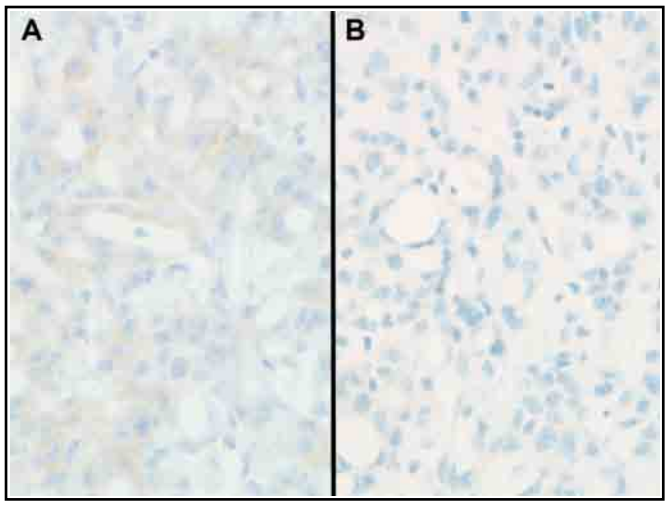


Fig 5. (A, B) Acceptable level of HER2 staining on the UK NEQAS distributed gastric sample 'C'. Expression level varied from 0 to 1+ depending on the block and serial section distributed. (A) 1+ expression, stained with the Dako HercepTest and (B) negative example, stained with the Ventana 4B5 Pathway.

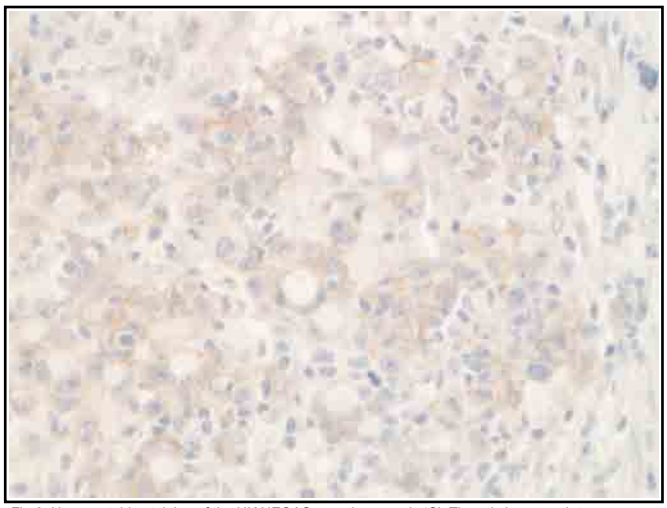


Fig 6. Unacceptable staining of the UK NEQAS negative sample 'C'. There is inappropriate excessive membrane staining, more representative of a 2+. Section also shows background staining. Stained with the Labvision / Neomarkers SP3 clone (1:50), on a Dako Autostainer, and Labvision PT link with high pH buffer pre-treatment.

Selected Images showing Optimal and Sub-optimal Immunostaining

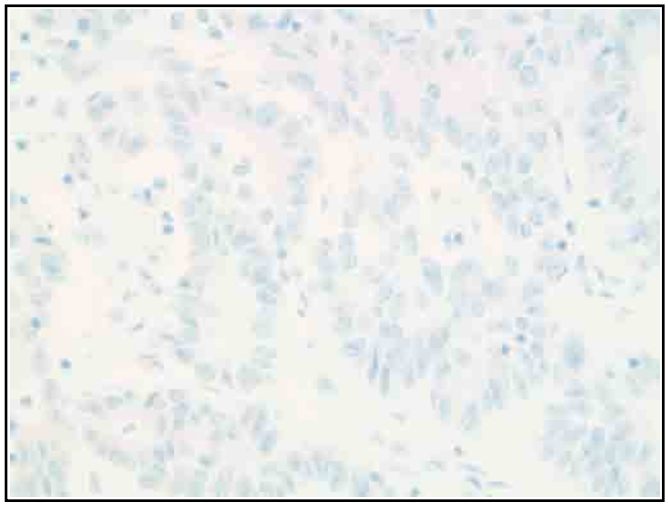


Fig 7. Acceptable level of expression of the UK NEQAS negative sample 'D'. As expected the section shows no membrane staining and has a clean background. Stained with the Ventana 4B5 assay.

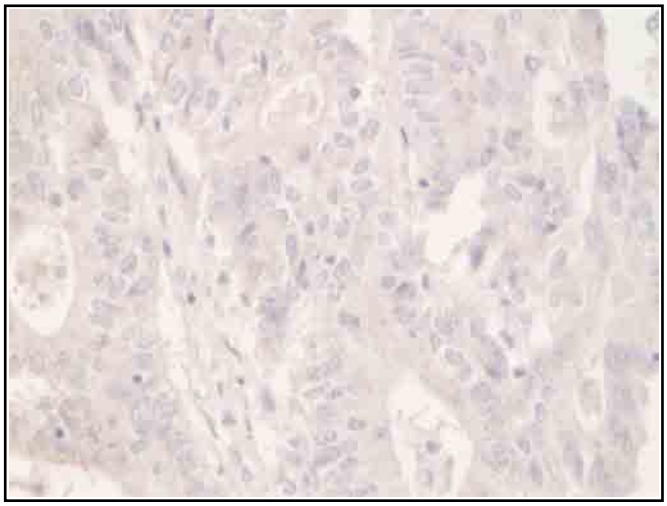


Fig 8. Unacceptable staining of the UK NEQAS negative sample 'D'. Section shows excessive cytoplasmic and background staining. Same protocol as Fig 6.

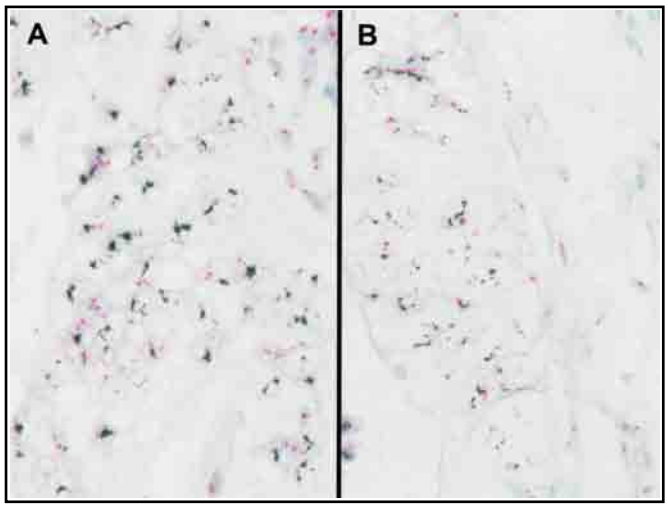


Fig 9. DDISH carried out on the UK NEQAS samples 'A' and 'B', showing both to be amplified using the Ventana DDISH assay.

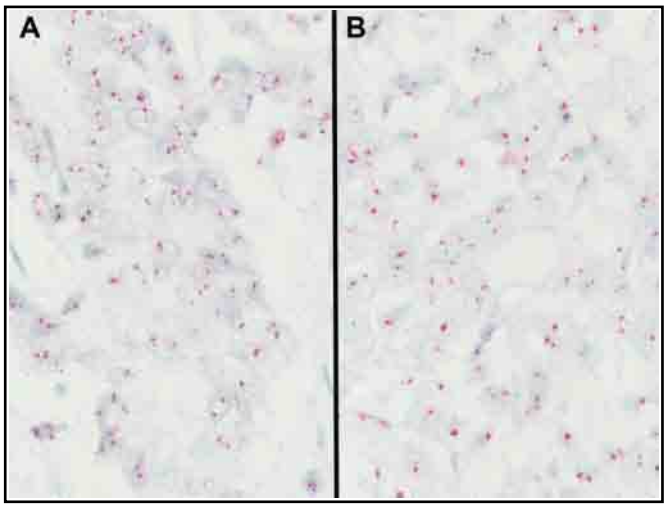
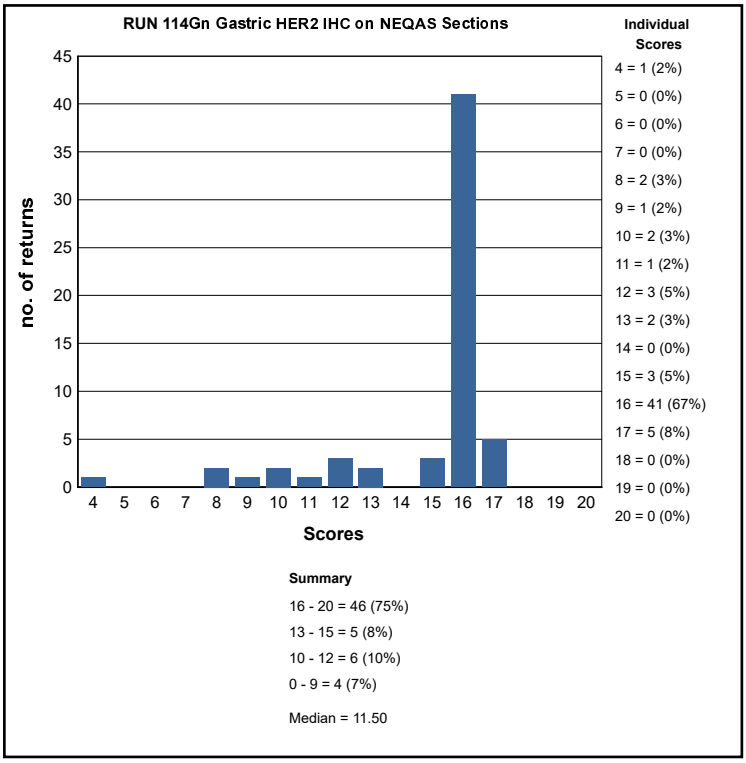
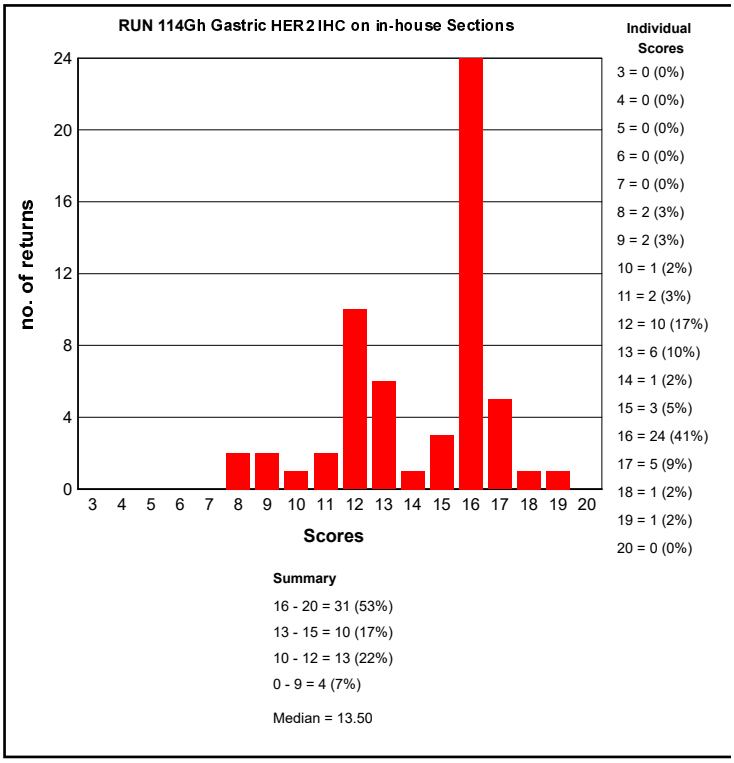


Fig 10. DDISH carried out on the UK NEQAS samples 'C' and 'D', showing both to be non-amplified using the Ventana DDISH assay.

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: 114

Primary Antibody	N	%
Labvision / Neomarkers RM-9103 (SP3)	1	0
Leica Oracle HER2 Bond IHC (CB11)	1	0
Dako Link HercepTest SK001 (poly)	3	100
Ventana Pathway 790-100 (4B5)	4	75
Ventana Pathway 790-2991 (4B5)	38	87
Other	1	0
Ventana Confirm 790-4493 (4B5)	10	90

Gastric HER2 ICC Run: 114

Automation	N	%
Dako Autostainer Link 48	1	100
Dako Autostainer plus	1	0
Dako Autostainer Plus Link	2	100
Leica Bond-III	1	0
Other	1	0
Ventana Benchmark GX	2	100
Ventana Benchmark ULTRA	27	89
Ventana Benchmark XT	22	86

Gastric HER2 ICC Run: 114

Heat Mediated Retrieval	N	%
Dako PTLink	3	100
Lab vision PT Module	1	0
Leica ER1 25 mins	1	0
Leica ER2 20 mins	1	0
Ventana CC1 16mins	1	100
Ventana CC1 20mins	1	0
Ventana CC1 24mins	3	100
Ventana CC1 32mins	6	83
Ventana CC1 36mins	12	83
Ventana CC1 56mins	2	100
Ventana CC1 64mins	3	67
Ventana CC1 mild	19	95
Ventana CC1 standard	4	100

Gastric HER2 ICC Run: 114

Detection	N	%
AS PER KIT	4	75
Dako HerCep Test Autor (SK001)	2	100
Leica Bond Polymer Refine (DS9800)	1	0
Other	1	0
Ventana iView system (760-091)	2	100
Ventana OptiView Kit (760-700)	5	100
Ventana UltraView Kit (760-500)	41	85

Gastric HER2 ICC Run: 114

Enzyme Retrieval	N	%
AS PER KIT	4	75
NOT APPLICABLE	26	73
Ventana Protease 1 (760-2018)	1	100

Gastric HER2 ICC Run: 114

Chromogen	N	%
AS PER KIT	9	89
Dako FLEX DAB	2	100
Leica Bond Polymer Refine kit (DS9800)	1	0
Other	2	0
Ventana DAB	3	100
Ventana Ultraview DAB	41	85

BEST METHODS

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

Gastric HER2 IHC - Method 1

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

Primary Antibody:	Ventana Pathway 790-2991 (4B5)
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)

Gastric HER2 IHC - Method 2

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

Primary Antibody:	Dako Link HercepTest SK001 (poly) Prediluted
Automation:	Dako Autostainer Link 48
Method:	Other
Main Buffer:	AS PER KIT
HMAR:	Dako PTLink, PH: 6
EAR:	
Chromogen:	AS PER KIT
Detection:	AS PER KIT Prediluted

Gastric HER2 IHC - Method 3

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

Primary Antibody:	Ventana Confirm 790-4493 (4B5) , 24 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 mild
EAR:	
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

The Lymphoma Module Run 114

David Blythe and Dawn Wilkinson

	Gold Standard	Second Antibody
Antigens Assessed:	BCL-2	Ki-67
Tissue Sections circulated:	Follicular lymphoma and reactive tonsil	Reactive hyperplasia - tonsil
Number of Registered Participants:	210	
Number of Participants this Run	203 (97%)	

Introduction

Gold Standard: BCL-2

The BCL-2 molecule is an oncogene product (oncoprotein) blocking apoptotic cell death. In lymphoid tissues BCL-2 is expressed by mantle zone B-cells and all effector T-cells, while germinal centre B-cells lack this protein at an immunodetectable level. BCL-2 immunoreaction results in an eccentric cytoplasmic signal since it is localised in the nuclear envelope, endoplasmic reticulum and mitochondrial membrane in positive cells. The up regulation of BCL-2 expression in lymphoid nodular structures is a specific feature of most follicular lymphomas resulting from the t(14;18) chromosomal translocation when the BCL-2 gene is inserted into the juxtaposition to the highly active IgH gene

Features of Optimal Immunostaining (Figs1,2,5 & 6):

- Strong cytoplasmic staining of most lymphocytes except germinal centre B-cells
- Eccentric ring-like appearance of immunostaining within individual cells with a wide range of expression levels adjacent cells resulting in a dynamic staining pattern
- Minimal or lacking extracellular membrane staining

Features of Sub-optimal Immunostaining (Fig 3&4):

- Weak, uneven, diffuse, partially missing staining
- High background or non-specific staining of cell types not expected to stain

References:

1. Zutter M, Hockenbery D, Silverman GA, Korsmeyer SJ. Immunolocalization of the bcl-2 protein within hematopoietic neoplasms. Blood 1991, 78:1062-1068.
2. Ngan B-Y, Chen-Levy Z, Weiss LM et al. Expression in non-Hodgkin's lymphoma of the bcl-2 protein is associated with the t(14;18) chromosomal translocation. New Engl J Med 1988, 318: 1638-1644

Second Antigen: Ki-67

(For summary please refer to General module)

Features of Optimal Immunostaining (Figs 7,8,11 &12)

- Intense and well-localised nuclear staining of 80-90% of the germinal centre B-cells
- Intense staining of the basal epithelial cells
- Clean background with no non-specific staining

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

- Weak, uneven, or partially missing staining of relevant cells
- Diffuse staining
- High background or non-specific staining of cell types not
- expected to stain.

References:

1. P A Hall, et al. The prognostic value of Ki67 immunostaining in non-Hodgkin's lymphoma. J Pathol 1988; 154:223-35.
2. C-Y HSU et al. Tonsil surface epithelium is ideal for monitoring Ki-67 immunohistochemical staining. Histopathology 2013; 63, 810-816.

Assessment Summary:

BCL-2

203 laboratories submitted their slides for the BCL-2 assessment. Pass rates are shown in the following table:

NEQAS Pass Rates Run 114:BCL-2	
Acceptable	74% (N=152)
Borderline	22% (N=45)
Unacceptable	3% (N=6)

Similarly to previous results, weak staining was the main reason for marks being deducted and therefore receiving a borderline pass. In some cases, where there was very little staining at all, the labs received a failed score. This was not particularly attributable to a particular antibody clone, but mostly due to an inappropriate dilution or antigen retrieval protocol. All laboratories except one submitted an in-house control with a higher acceptable pass rate of 88% compared to the NEQAS scores for this run, with no laboratories failing on their in-house material.

A variety of antibodies and automated platforms were used in the assessment. However, overall the most popular antibody clone was the Dako 124 (M0887), which was used by 86 participants and showed an acceptable pass rate of 67%. The Ventana (124) was the second most popular antibody choice, which showed an acceptable pass rate of 77%.

Ki-67

201 laboratories submitted their slides for the Ki-67 assessment. The pass rates were very good with an acceptable pass rate of 93%. All participating laboratories except for 1 laboratory also submitted in-house material. These showed similar acceptable pass rates to that of the NEQAS stained sections. Results are summarised in table below:

NEQAS Pass Rates Run 114:Ki-67	
Acceptable	93% (N=186)
Borderline	7% (N=14)
Unacceptable	0% (N=1)

It was noted that the laboratories that received a borderline score or a fail (1 laboratory) was due to sub-optimal staining of Ki67 resulting in the germinal centre B cells only weakly being demonstrated . The laboratory that failed had extremely weak staining in the reactive tonsil combined with some cells expected to stain not being demonstrated. This was probably due to a problem with the dilution for the primary antibody or an inappropriate antigen retrieval method. The most popular primary antibody for this run was the Dako M7240 (MIB-1) which was used by 99 participants and showed an acceptable pass rate of 87%. Another popular choice of antibody was the Ventana RTU 30-9 clone (790-4286):42 participants used this clone which showed a100% acceptable pass rate.

Selected Images showing Optimal and Sub-optimal Immunostaining

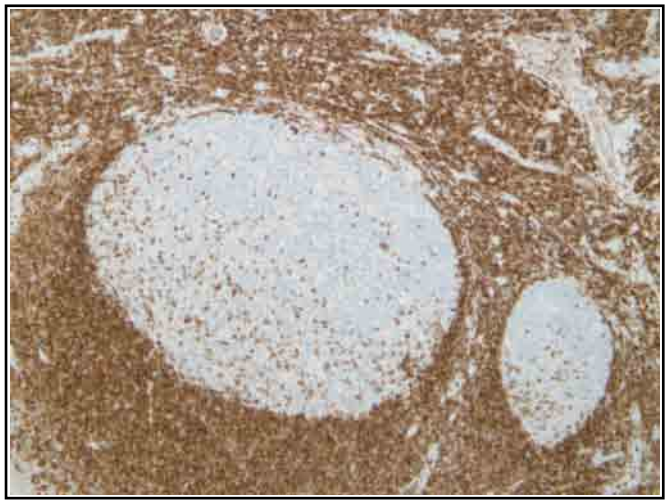


Fig 1. Optimal demonstration of BCL2 in the reactive tonsil section, showing strong cytoplasmic staining of the peripheral B-cells and intra-follicular T-cells. Section stained with the Novocastra BCl2/100/D5 antibody, 1:20, on the Leica Bond III, ER2 20 minutes.

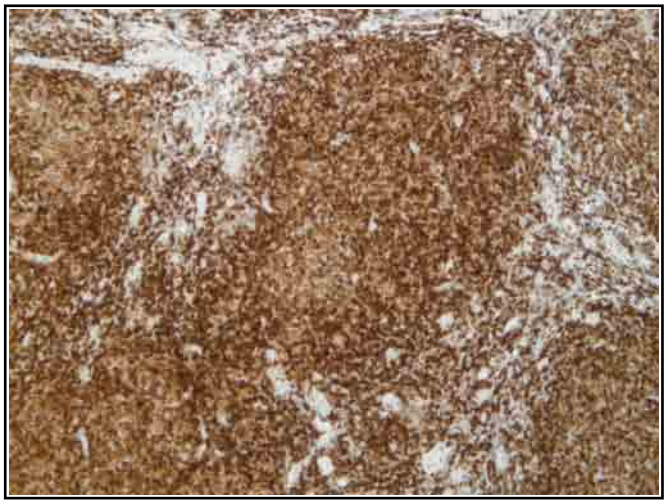


Fig 2. Good demonstration of BCl2 in the UK NEQAS follicular lymphoma. Virtually all the tumour cells show strong crisp and well localised cytoplasmic staining. Same protocol as Fig 1.

Fig 3. Poor demonstration of BCl2 in the UK NEQAS tonsil section (compare to Fig 1). The staining is weak and many of the cells expected to stain are not demonstrated. Section stained with the Dako FLEX 124 pre-diluted antibody on the Dako Omnis with 30 minutes pre-treatment.

Fig 4. Sub-optimal demonstration of BCL2 in the UK NEQAS follicular lymphoma (compare to Fig 2). The example shows weak, patchy and diffuse staining. Same protocol as Fig 3.

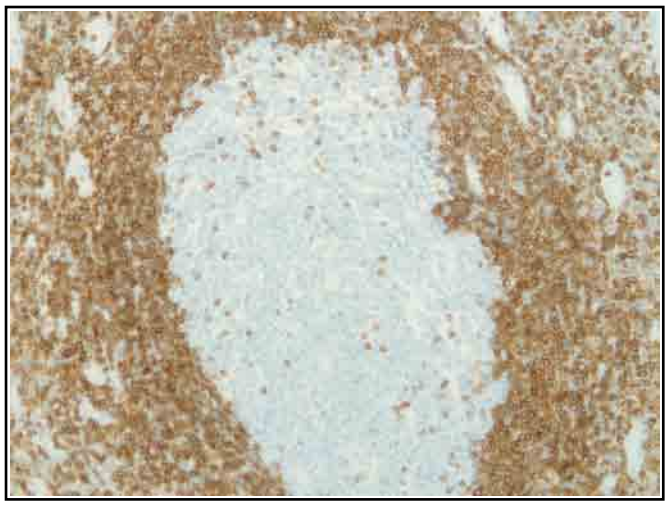


Fig 5. Optimal staining of BCL2 in the reactive tonsil section. The staining is strong and well localized in the mantle zone B-cells and peri- and intra-follicular T-cells. Stained with the Dako 124 antibody, 1:20, on the Autostainer with pre-treatment in the PT link with high pH buffer.

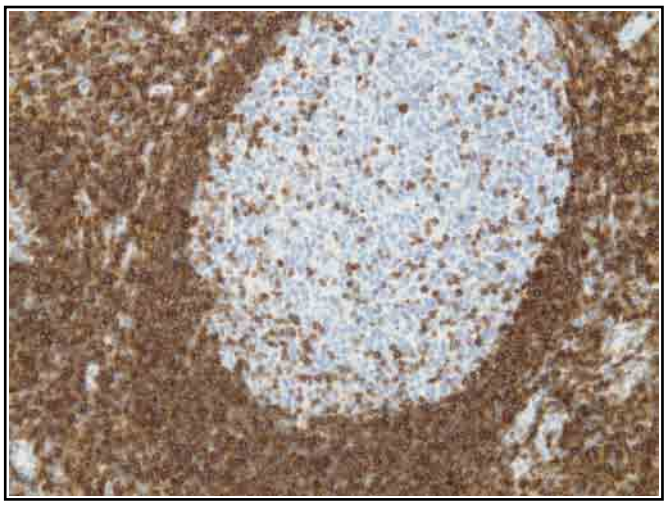


Fig 6. Good example of an in house reactive tonsil control stained with BCl2, showing strong crisp and well-localised staining of the peripheral B-cells and intra-follicular T-cells (same protocol as Fig 5).

Selected Images showing Optimal and Sub-optimal Immunostaining

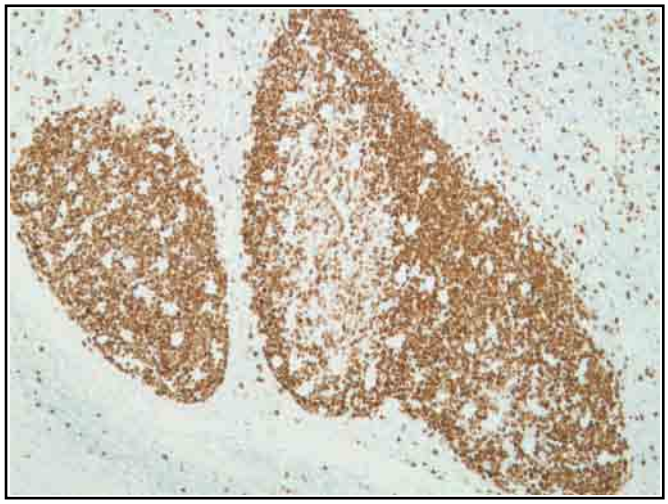


Fig 7. Optimal demonstration of Ki-67 in the UK NEQAS distributed reactive tonsil section, showing strong well-localised, nuclear staining with a clean background. Section stained on a Ventana Benchmark XT using the Ventana 30-9 RTU antibody, and CC1 mild antigen retrieval, UltraView detection kit.

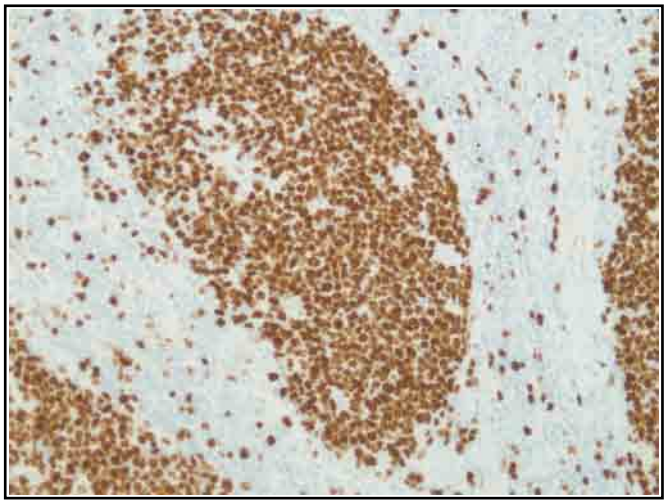


Fig 8. Good example of Ki-67 in the UK NEQAS distributed reactive tonsil section. The nuclear staining is strong and well-localised, while the background remains clean. Stained with the Dako MIB-1 antibody, 1:100 on the Autostainer with pre-treatment in the PT link, high pH buffer.

Fig 9. Sub-optimal staining of Ki-67 in the UK NEQAS reactive tonsil section (compare to Figs 7 & 8). The majority of the germinal centre B-cells are demonstrated but the staining is very weak. Section stained with the Dako MIB-1 clone (1:300), on a Leica Bond III with ER2 retrieval for 20 minutes.

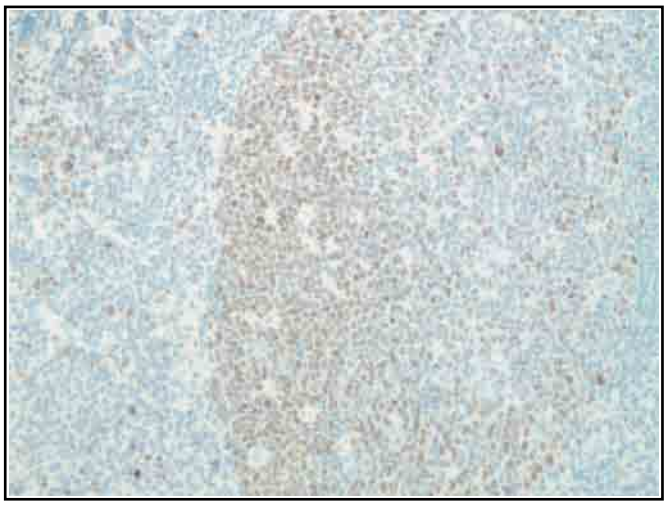


Fig 10. Another example of poor Ki-67 staining in the UK NEQAS reactive tonsil section (compare to Figs 7 & 8). Not only is the staining weak, but some of the cells expected to stain are not demonstrated. Same protocol as Fig 9.

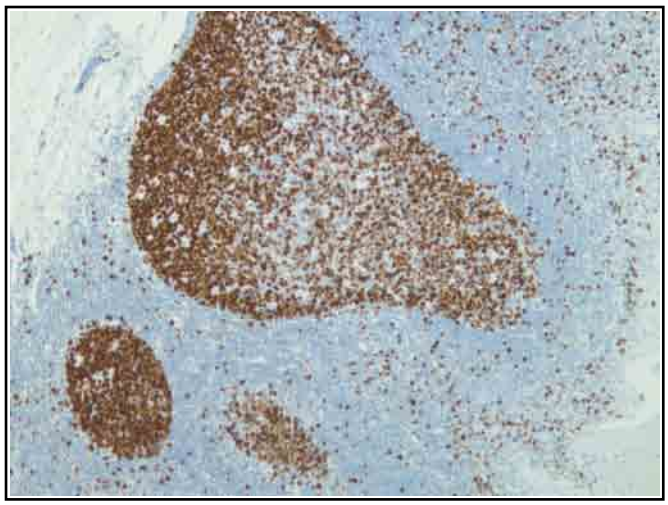


Fig 11. Good demonstration of Ki-67 on an in house tonsil section. The staining is strong and well-localised, while the background remains clean. Section stained with the Dako Omnis pre-diluted MIB1 antibody, on the Omnis with low pH retrieval for 30 minutes.

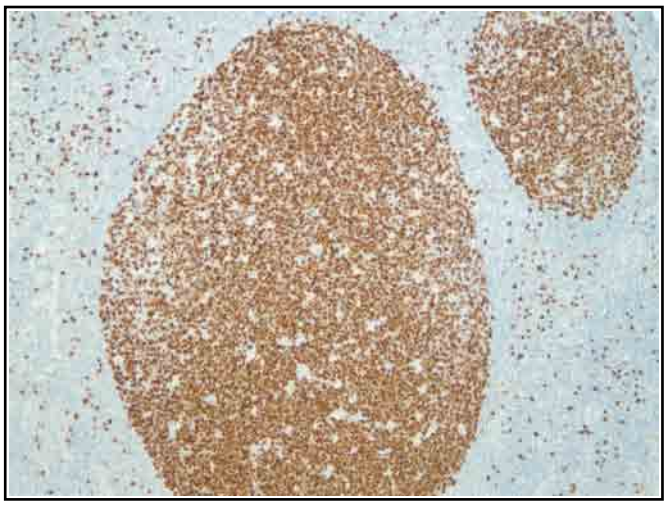
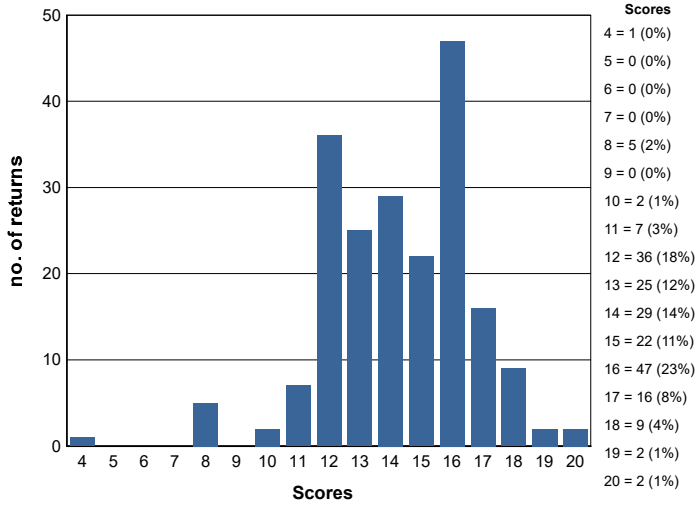


Fig 12. Good example of an in-house tonsil control stained with Ki-67, showing strong and well-localised nuclear staining and a clean background. Same protocol as Fig 8.

GRAPHICAL REPRESENTATION OF PASS RATES

RUN 114L BCL2 on NEQAS Sections

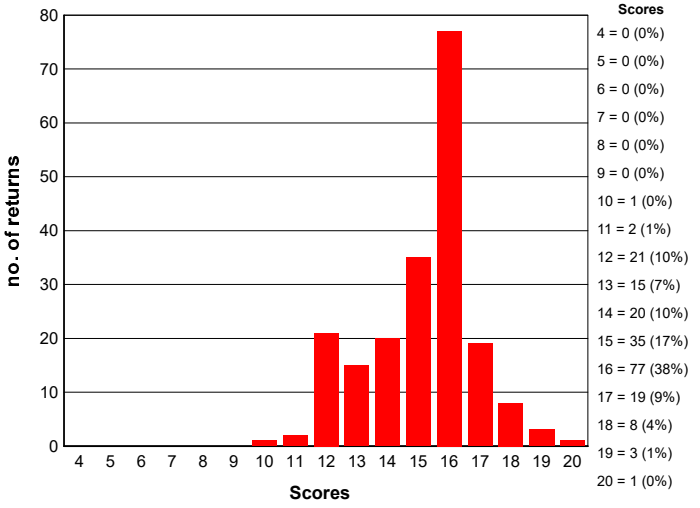


Individual Scores
4 = 1 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 0 (0%)
8 = 5 (2%)
9 = 0 (0%)
10 = 2 (1%)
11 = 7 (3%)
12 = 36 (18%)
13 = 25 (12%)
14 = 29 (14%)
15 = 22 (11%)
16 = 47 (23%)
17 = 16 (8%)
18 = 9 (4%)
19 = 2 (1%)
20 = 2 (1%)

Summary
16 - 20 = 76 (37%)
13 - 15 = 76 (37%)
10 - 12 = 45 (22%)
0 - 9 = 6 (3%)

Median = 14.00

RUN 114M BCL2 on in-house Sections

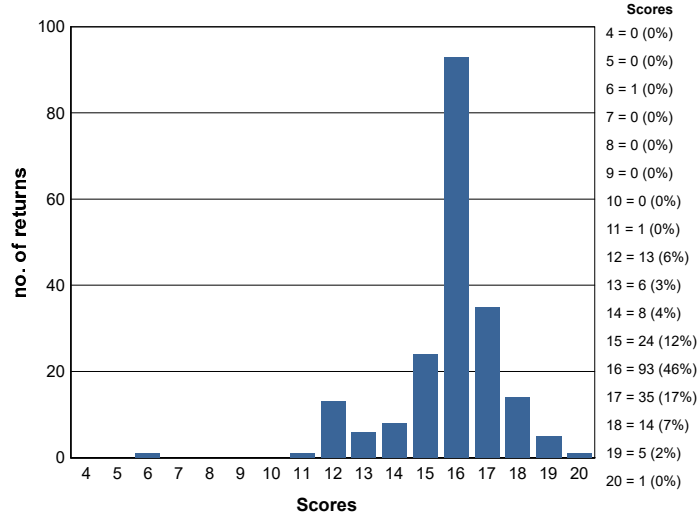


Individual Scores
4 = 0 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 0 (0%)
8 = 0 (0%)
9 = 0 (0%)
10 = 1 (0%)
11 = 2 (1%)
12 = 21 (10%)
13 = 15 (7%)
14 = 20 (10%)
15 = 35 (17%)
16 = 77 (38%)
17 = 19 (9%)
18 = 8 (4%)
19 = 3 (1%)
20 = 1 (0%)

Summary
16 - 20 = 108 (53%)
13 - 15 = 70 (35%)
10 - 12 = 24 (12%)
0 - 9 = 0 (0%)

Median = 15.00

RUN 114N Ki-67/MIB1 on NEQAS Sections

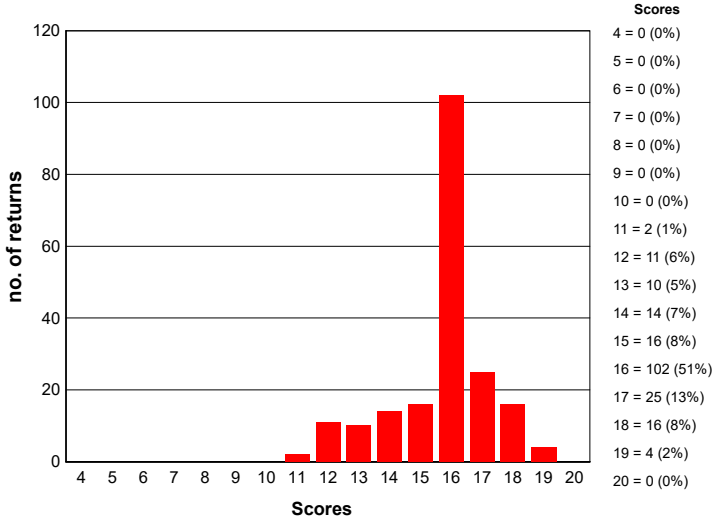


Individual Scores
4 = 0 (0%)
5 = 0 (0%)
6 = 1 (0%)
7 = 0 (0%)
8 = 0 (0%)
9 = 0 (0%)
10 = 0 (0%)
11 = 1 (0%)
12 = 13 (6%)
13 = 6 (3%)
14 = 8 (4%)
15 = 24 (12%)
16 = 93 (46%)
17 = 35 (17%)
18 = 14 (7%)
19 = 5 (2%)
20 = 1 (0%)

Summary
16 - 20 = 148 (74%)
13 - 15 = 38 (19%)
10 - 12 = 14 (7%)
0 - 9 = 1 (0%)

Median = 15.00

RUN 114P Ki-67/MIB1 on in-house Sections



Individual Scores
4 = 0 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 0 (0%)
8 = 0 (0%)
9 = 0 (0%)
10 = 0 (0%)
11 = 2 (1%)
12 = 11 (6%)
13 = 10 (5%)
14 = 14 (7%)
15 = 16 (8%)
16 = 102 (51%)
17 = 25 (13%)
18 = 16 (8%)
19 = 4 (2%)
20 = 0 (0%)

Summary
16 - 20 = 147 (74%)
13 - 15 = 40 (20%)
10 - 12 = 13 (7%)
0 - 9 = 0 (0%)

Median = 15.00

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: 114

Primary Antibody : BCL2

Antibody Details	N	%
Dako M0887 (124)	86	67
Labvision/Neomarkers MS-123-P (100/D5)	1	0
Leica/Novocastra NCL-BCl-2 (BCl2/100/D5)	15	73
Ventana (SP66) 790-4604	18	94
Ventana 760-4240 (124)	4	100
Other	1	100
Cell marque CMC329 (124)	2	50
Dako FLEX IR614 (124)	14	79
Leica/Novocastra NCL-BCl-2-486 (3.1)	8	88
Leica NCL-L-BCL-2 (bcl2/100/D5)	2	50
Leica RTU (BCl2/100/D5) PA0117	18	94
Ventana (124) 790-4464	22	77
Cell Marque 226R-26 (SP66)	5	80
Biogenex NU541-UC (E17)	1	0
Abcam ab32124 (E17)	1	100

Lymphoma Run: 114

Primary Antibody : Ki-67/MIB1

Antibody Details	N	%
Dako M7187 Ki67 (Ki-S5)	1	100
Dako Omnis GA626 (MIB-1)	4	100
Leica/Novocastra NCL-Ki67 (MM1)	3	67
Ventana 760-2910 (K2)	3	100
Other	3	100
Dako FLEX RTU IR626 (MIB1)	13	100
Leica/Novocastra RTU PA0118 (MM1)	3	100
Leica/Novocastra RTU PA0230 (K2)	16	94
Ventana RTU 790-4286 (30-9)	42	100
Vector VP-RM04 (SP6)	1	100
Menapath MP-325-CRM (SP6)	2	100
Cell Marque 275R-14/15/16 (SP6)	3	100
Cell Marque RTU 275R-17/18 (SP6)	2	100
Dako M7240 (MIB-1)	99	87
Leica/Novocastra RTU PA0410 (MM1)	1	100

Lymphoma Run: 114

Heat Mediated Retrieval	BCL2 N	BCL2 %	Ki-67/MIB1 N	Ki-67/MIB1 %
Biocare Decloaking Chamber	1	0	1	100
Dako Omnis	5	40	8	100
Dako PTLink	23	87	21	95
Lab vision PT Module	1	0	1	100
Leica ER1 10 mins	1	0	0	0
Leica ER1 20 mins	5	40	4	75
Leica ER1 30 mins	6	67	1	100
Leica ER1 40 mins	0	0	1	100
Leica ER2 10 mins	1	0	1	100
Leica ER2 20 mins	42	98	40	85
Leica ER2 30 mins	8	100	8	100
Leica ER2 40 mins	1	100	2	100
Microwave	1	0	0	0
Other	1	100	0	0
Pressure Cooker	2	50	3	67
Pressure Cooker in Microwave Oven	1	100	0	0
Ventana CC1 24mins	1	100	5	100
Ventana CC1 32mins	7	57	21	100
Ventana CC1 36mins	2	50	7	71
Ventana CC1 40mins	3	100	4	75
Ventana CC1 48mins	8	88	1	100
Ventana CC1 52mins	1	100	2	100
Ventana CC1 56mins	4	75	3	100
Ventana CC1 64mins	31	74	22	100
Ventana CC1 72mins	3	67	0	0
Ventana CC1 76mins	6	33	0	0
Ventana CC1 80mins	1	0	0	0
Ventana CC1 88mins	1	100	1	100
Ventana CC1 92mins	2	100	0	0
Ventana CC1 extended	7	86	1	100
Ventana CC1 mild	2	50	7	86
Ventana CC1 standard	15	60	17	100
Ventana CC2 44mins	0	0	1	100
Ventana CC2 64mins	0	0	1	100
Water bath 95-98 OC	2	0	2	50

Lymphoma Run: 114

Enzyme Mediated Retrieval	BCL2 N	BCL2 %	Ki-67/MIB1 N	Ki-67/MIB1 %
AS PER KIT	2	100	4	75
NOT APPLICABLE	110	73	94	91

Lymphoma Run: 114	BCL2		Ki-67/MIB1	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	11	64	13	77
BioGenex SS Polymer (QD 420-YIKE)	1	100	1	100
Dako EnVision FLEX (K8000/10)	5	80	4	100
Dako EnVision FLEX+ (K8002/12)	16	88	18	100
Dako Envision HRP/DAB (K5007)	3	33	1	100
Dako Envision+ HRP mouse K4004/5/6/7	1	100	0	0
Dako REAL HRP/DAB (K5001)	1	0	1	0
Leica Bond Polymer Define (DS9713)	2	100	0	0
Leica Bond Polymer Refine (DS9800)	58	86	55	85
MenaPath X-Cell Plus (MP-XCP)	1	0	1	100
Other	4	50	6	83
Ventana iView system (760-091)	1	0	3	100
Ventana OptiView (760-700) + Amp. (7/860-099)	5	40	2	100
Ventana OptiView Kit (760-700)	41	76	36	100
Ventana UltraView Kit (760-500)	47	72	55	95

Lymphoma Run: 114	BCL2		Ki-67/MIB1	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	1	100	1	100
Dako Autostainer Link 48	21	81	19	95
Dako Autostainer Plus Link	4	50	4	100
Dako Omnis	5	60	7	100
LabVision Autostainer	2	50	1	100
Leica Bond Max	31	87	26	88
Leica Bond-III	36	89	36	83
Menarini - Intellipath FLX	2	0	2	50
None (Manual)	1	0	2	50
Ventana Benchmark GX	2	100	2	100
Ventana Benchmark ULTRA	64	66	66	97
Ventana Benchmark XT	29	79	31	94

Lymphoma Run: 114	BCL2		Ki-67/MIB1	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	26	85	26	85
BioGenex Liquid DAB (HK153-5K)	1	100	1	100
Dako EnVision Plus kits	2	100	2	100
Dako FLEX DAB	22	73	21	100
Dako REAL EnVision K5007 DAB	2	50	1	100
Dako REAL K5001 DAB	1	0	1	0
Leica Bond Polymer Refine kit (DS9800)	58	86	53	87
menapath xcell kit DAB (MP-860)	1	0	1	100
Other	14	50	10	100
Sigma DAB (D5905)	0	0	1	0
Ventana DAB	20	75	21	100
Ventana iview	2	50	2	100
Ventana Ultraview DAB	48	71	57	95
Vision BioSystems Bond X DAB	1	100	0	0

BEST METHODS - Gold Standard Antibody

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

BCL2 - Method 1

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

Primary Antibody: Dako M0887 (124) , 20 Mins, 23 ºC Dilution 1: 200
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 20 mins
EAR:
Chromogen: AS PER KIT, Time 1: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 15 Mins, 23 ºC Prediluted

BCL2 - Method 2

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

Primary Antibody: Leica RTU (BCl2/100/D5) PA0117 , 15 Mins, RT ºC Prediluted
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 2: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins, RT ºC Prediluted

BCL2 - Method 3

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

Primary Antibody:	Dako M0887 (124) , 30 Mins, 21 ºC Dilution 1: 50
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: flex high ph
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 21 ºC., Time 1: 10 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 30 Mins, 21 ºC Prediluted

BCL2 - Method 4

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

Primary Antibody:	Ventana (124) 790-4464
Automation:	Ventana Benchmark XT
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700)

BEST METHODS - Secondary Antibody

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

Ki-67/MIB1 - Method 1

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

Primary Antibody:	Dako M7240 (MIB-1) , 32 Mins, 37 ºC Dilution 1: 1:100
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC2 64mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB, 37 ºC., Time 1: 4 Mins, Time 2: 4 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC

Ki-67/MIB1 - Method 2

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

Primary Antibody:	Dako FLEX RTU IR626 (MIB1)
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	AS PER KIT
HMAR:	Dako PTLink
EAR:	
Chromogen:	AS PER KIT
Detection:	Dako EnVision FLEX+ (K8002/12)

Ki-67/MIB1 - Method 3

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

Primary Antibody:	Leica/Novocastra RTU PA0230 (K2) , 15 Mins, 24 ºC Prediluted
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800), 24 ºC., Time 1: 10 Mins, Time 2: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, 24 ºC Prediluted

Ki-67/MIB1 - Method 4

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

Primary Antibody:	Ventana RTU 790-4286 (30-9) Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 32mins
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	Ventana OptiView Kit (760-700) Prediluted

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	Gold Standard	Second Antibody
Antigens Assessed:	GFAP	Prolactin
Tissue Sections circulated:	Glioblastoma.	Normal pituitary.
Number of Registered Participants:	61	
Number of Participants this Run	58 (95%)	

Introduction

Gold Standard: 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.

Second Antigen: Prolactin

Prolactin is an adenohypophyseal polypeptide hormone produced in cells of the anterior pituitary gland of most mammals. Prolactin producing cells constitute about 17% of all normal human anterior pituitary cells. Elevated counts of these cells have been observed in pregnant women, newborns and in multiparous women. Prolactin is a pituitary hormone involved in the stimulation of milk production, salt and water regulation, growth, development and reproduction.
Anti-Prolactin is a useful marker in classification of pituitary tumours and the study of pituitary disease. It reacts with prolactin-producing cells. Such prolactin-producing cells can also be found in prostate epithelium.
Malignant pituitary adenomas or pituitary carcinomas arise from and consist of adenohypophysial cells. They can produce various hormones such as ACTH, Growth hormone, TSH, FSH, LH and Prolactin. Tumours that do not consist of adenohypophysial cells neither produce nor contain pituitary hormone, and thus immuno-peroxidase techniques are helpful in distinguishing from those pituitary tumours that store various hormones in the cell cytoplasm. The multihormonal nature of pituitary adenomas has been well established. In these tumours, growth hormone-producing and prolactin producing cells were the most frequently observed combination.

Features of Optimal Immunostaining:
- Specific cytoplasmic staining in the prolactin producing cells
- Clean background with no non-specific staining
- Good contrast with counterstain

Features of Suboptimal Immunostaining:
- Weak or uneven staining of the hormone
- Diffuse, poorly-localised or nuclear staining
- Excessive background or non-specific specific staining
- Weak or overtly heavy counterstain

References:
1. Asa SL, et al. Prolactin cells in human pituitary. A quantitative immunocytological analysis. Arch Pathol Lab Med 1982; 106:360
2. Heitz PU. Multihormonal pituitary adenomas. Horm Res 1979; 10:1
3. Duello TM, et al. Immunocytochemistry of prolactin-producing human pituitary adenomas. Amer J Anat 1980; 158:463
4. Kalyanaraman UP, et al. Prolactin-secreting pituitary oncocytoma with galactorrhea-amenorrhea syndrome. A histologic, ultrastructuraland immunocytochemical study. Cancer 1980; 45:1584
5. Kovacs K, et al. Immunocytology of the human pituitary. In: DeLellis (ed.). Diagnostic Immunochemistry. New York: Masson Publ. 1980;3
6. Mukai K and Rosai J. Applications of immunoperoxidase techniques in surgical pathology. Fenoglio and Wolff (eds.). New York Masson Publ 1980; 1:3

Assessment Summary:

For the 2016/2017 EQA year there are currently 61 participants registered for the neuropathology module. This represents a small decrease from last year (65). For this run 3 laboratories did not submit any slides at all; the remaining 58 submitted their Gold (GFAP) slides. For the 2nd antigen 11 labs did not return a Prolactin stained slide, but only 4 requested an alternative - CD34. This left a total of 217 slides for assessment: **G = 58, H = 58, J = 51, K = 50.**

A single participant had submitted their GFAP slides (**G&H**) but then subsequently decided to withdraw from the scheme. For completeness these 2 slides were included in all analyses.

The overall pass rate was 88% (191 slides), 10% were borderline (21), and 2% (5) were failed by the assessors. With an average score of 16/20 for all 217 slides.

GFAP (G&H)

No participant failed on the NEQAS (**G**) slide, although 10% (6) of labs were scored as borderline. Four employed the Dako polyclonal, and two the Dako monoclonal (6F2 clone). Although these were by far the most popular antibodies used, with a combined usage of 65% (43% and 22% respectively). Pre-treatments and dilutions varied for both reagents, but four of the six used the Dako Autostainer Link 48. All slides were marked down due to weak and/or patchy staining.
A single participant failed their in-house (**H**) GFAP, Dako monoclonal, no dilution given, with Ventana CC1 36 mins, on a Ventana Benchmark ULTRA platform, no other details given although interestingly the laboratory marked themselves 16/20.
On closer inspection it was noticed that the in-house tissue at the bottom of the slide had been marked with a cross, but there was no accompanying comments. It was therefore assessed as normal.

Neil Bilbe

Prolactin (J&K)

As mentioned above, only 51 participants submitted slides for the 2nd antigen (**J&K**) of which 4 returned slides stained with CD34. All the CD34 slides scored between 14 - 20, with an average score of 17.

There were four slides assessed as failing on the Prolactin, two each for the NEQAS (**J**) and in-house (**K**), these were by the same two participants. One had used the Dako polyclonal (68% of labs used this) and another the Thermo Scientific pre-diluted monoclonal. Neither used any retrieval. The first showed excessive non-specific staining, and the second was only weakly demonstrating the hormone. Only one slide scored 20 on the NEQAS pituitary section, using Prolactin. This participant used the Dako polyclonal, but employed a PT Link pretreatment stage, on the Dako Autostainer Link 48. Although Dako recommends no pretreatment with their polyclonal (A0569) it appears that many labs still use a PT step.

Selected Images showing Optimal and Sub-optimal Immunostaining

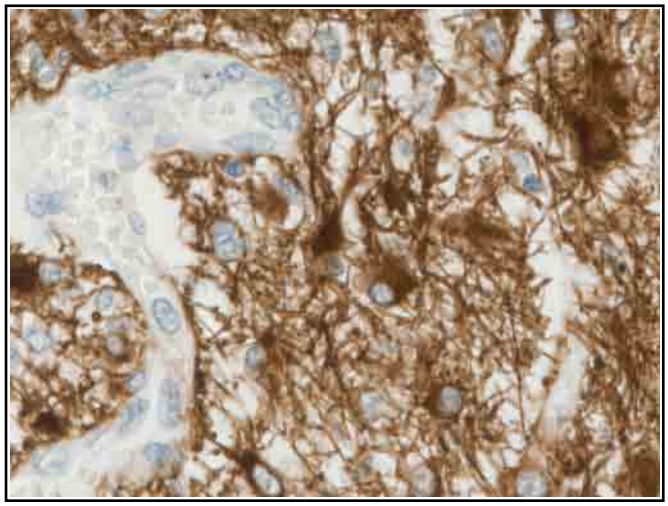


Fig 1. Excellent GFAP demonstration in the NEQAS section of glioblastoma. The tumour and processes are clearly defined, and there is minimal background staining. Dako polyclonal, 1:2000, with Leica ER1 for 20 mins, on a Leica Bond-III, and a Leica Bond Polymer Refine kit second layer.

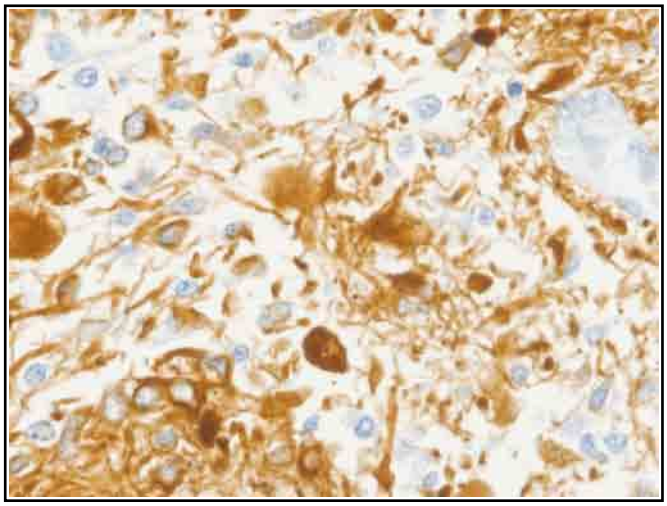


Fig 2. Sub-optimal GFAP demonstration in the NEQAS section. Staining is somewhat weak (compare Fig 1) and patchy. This was assessed as a borderline score, but adequate for diagnosis. Dako polyclonal, 1:2000, with no retrieval. Dako Autostainer Link 48, using the Dako EnVision FLEX+ kit.

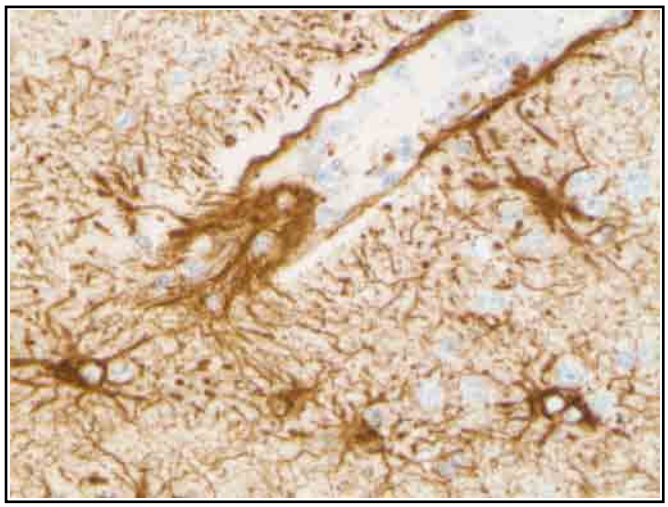


Fig 3. Optimal GFAP staining on the NEQAS tumour (2nd piece). The cell bodies and processes are nicely demonstrated, and the background is clean. RTU Dako Omnis polyclonal, with the Dako Omnis HMAR for 30 mins, the Omnis platform and the Dako EnVision FLEX high pH (Omnis) kit.

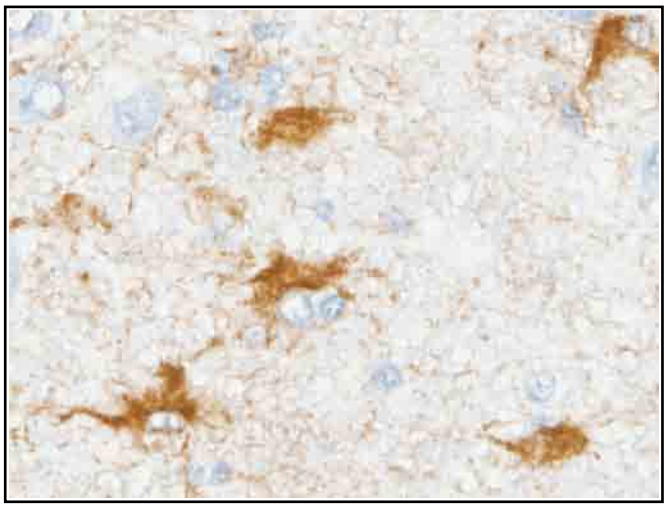


Fig 4. Sub-optimal GFAP on the NEQAS tumour. Staining is weak and patchy, some processes are also absent. The slide was awarded a low borderline mark. Dako polyclonal, no dilution given, no retrieval, Dako Autostainer Link 48, and the Dako EnVision FLEX+ it.

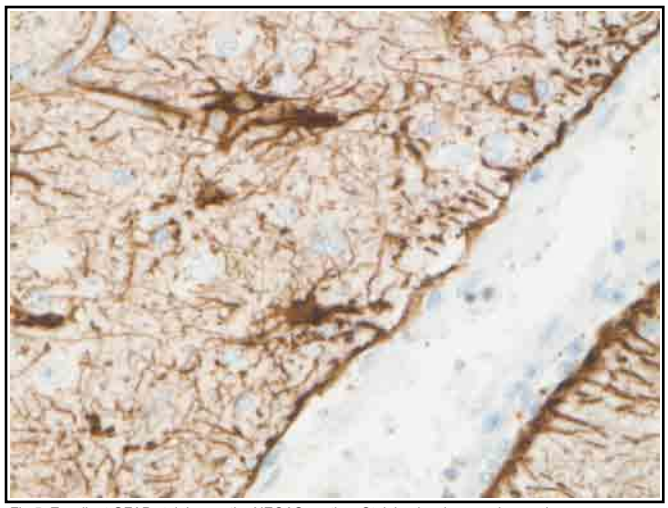


Fig 5. Excellent GFAP staining on the NEQAS section. Staining is crisp, precise, and very selective. The counterstain intensity is ideal. Dako polyclonal, 1:5000, for 15 mins, with the Leica ER2 HMAR 20 mins, on a Leica Bond-III, and a RTU Leica Bond Polymer Refine kit.

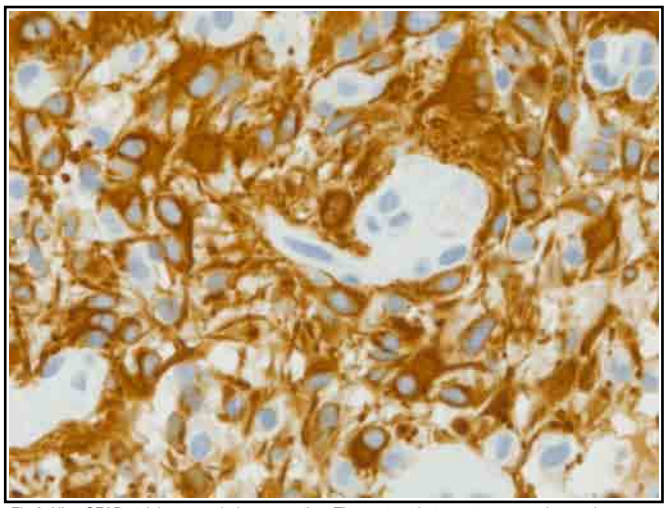


Fig 6. Nice GFAP staining on an in-house section. The contrast between tumour and normal tissues is good, although there is a slight hint of morphological damage. Dako polyclonal, 1:3200, 20 mins, using a Dako water bath @ 95°C, on a Dako Autostainer Link 48, and a Dako FLEX+ kit.

Selected Images showing Optimal and Sub-optimal Immunostaining

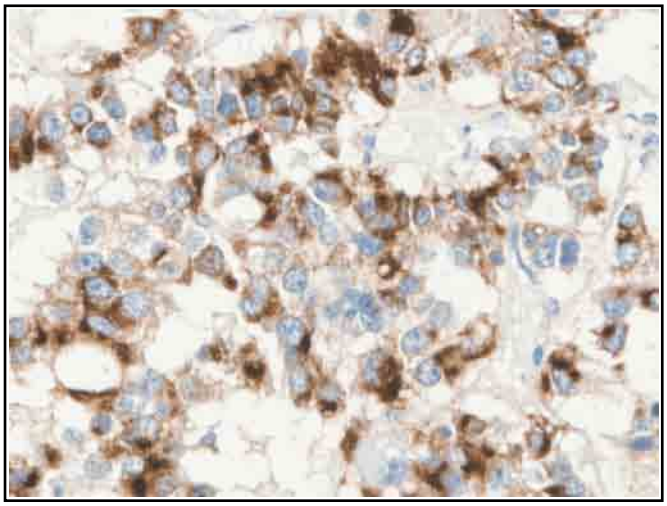


Fig 7. Excellent staining of prolactin in the NEQAS pituitary section. Staining is crisp and cytoplasmic, in the correct portion of the gland and cell numbers. The background is relatively unstained. Dako polyclonal, 1:800, 15 mins, no retrieval, on a Leica Bond-III, with a Leica Bond Polymer Refine kit.

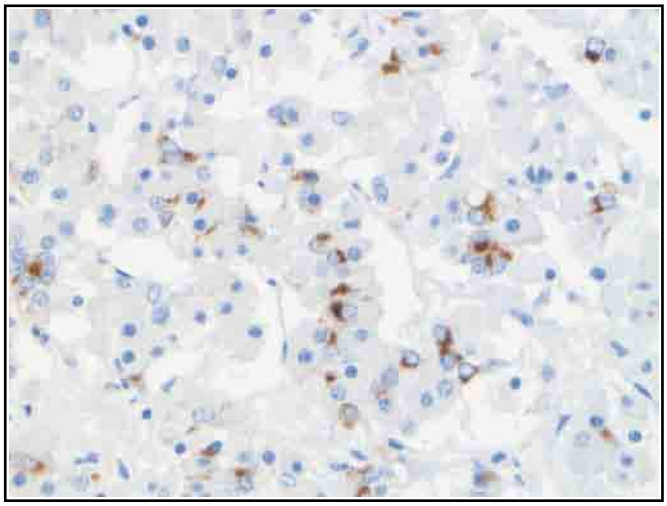


Fig 8. Sub-optimal demonstration of prolactin in the NEQAS sample. Staining is weak, and not all appropriate cells were positive. Overall this was assessed as borderline. Dako poly, 1:1500, 8 mins, no retrieval, Leica Bond-III, RTU Leica Bond Polymer Refine kit, 8 mins, plus a Bond DAB enhancer.

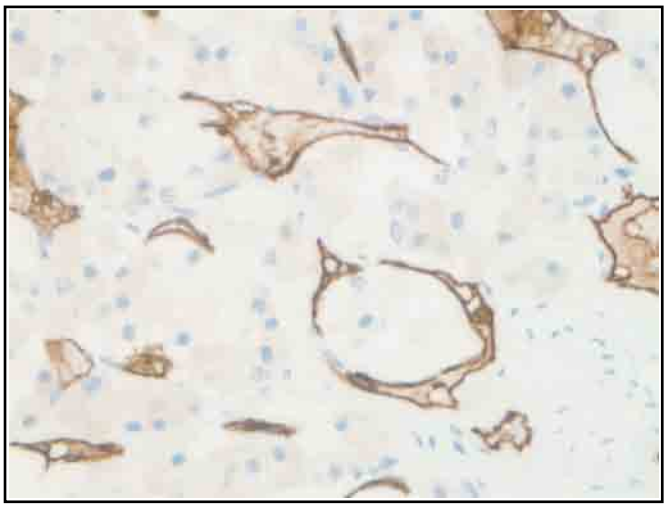


Fig 9. Excellent CD34 staining on the NEQAS pituitary section. This was given as the alternative antibody to those labs not stocking prolactin (12%), and illustrates that not stocking the requested antibody does not preclude laboratories from submitting slides for EQA assessment.

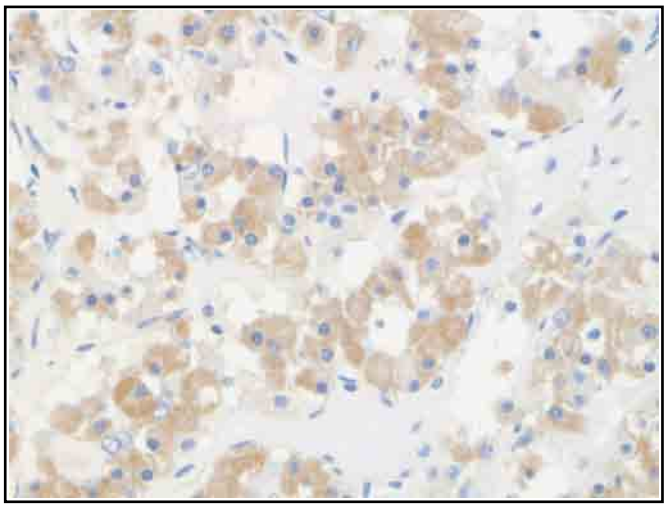


Fig 10. Sub-optimal prolactin staining on the NEQAS pituitary sample. There is a high degree of non-specific demonstration of prolactin, in cells which should not contain the hormone. The slide therefore failed. Dako polyclonal, 1:1000, 15 mins, no HMAR, Leica Bond-III, and RTU Leica Bond Polymer Refine kit.

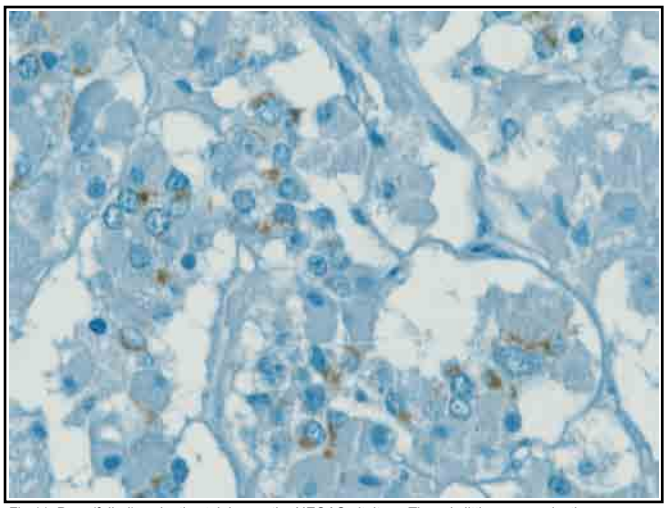


Fig 11. Poor (failed) prolactin staining on the NEQAS pituitary. There is little or no prolactin positivity, counterstain is too heavy and tissue shows evidence of damage. ThermoScientific monoclonal SPM108, prediluted, 16 mins, no retrieval, Ventana Benchmark XT, and a Ventana UltraView Kit.

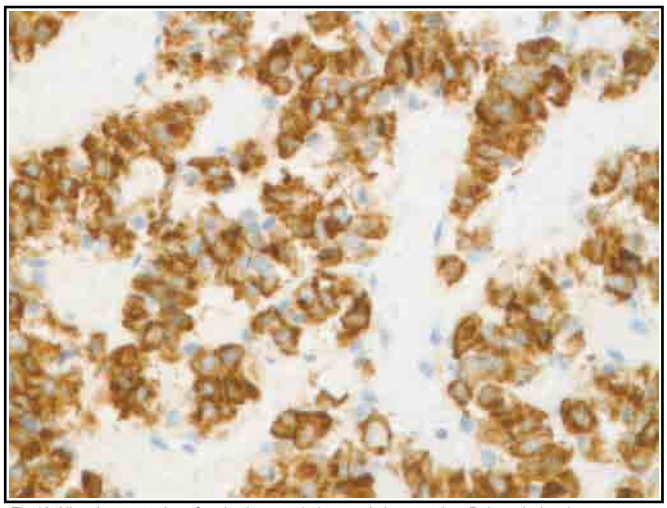
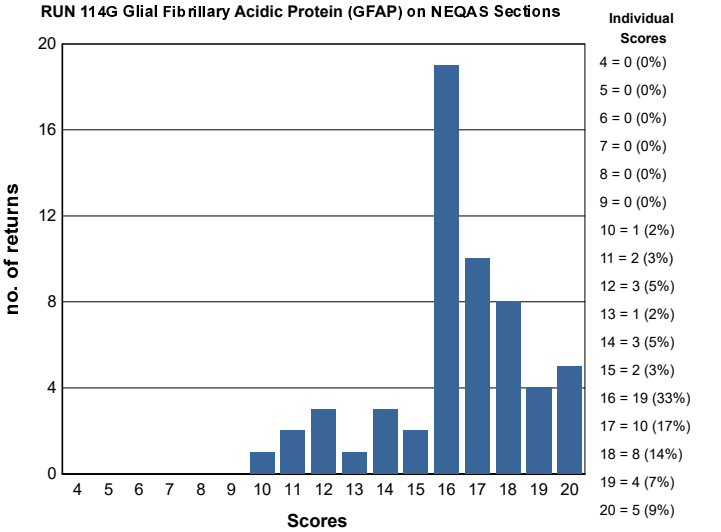
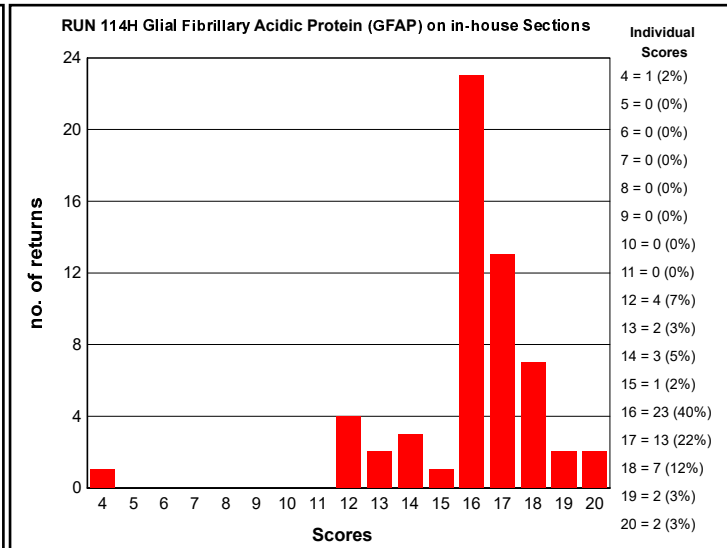
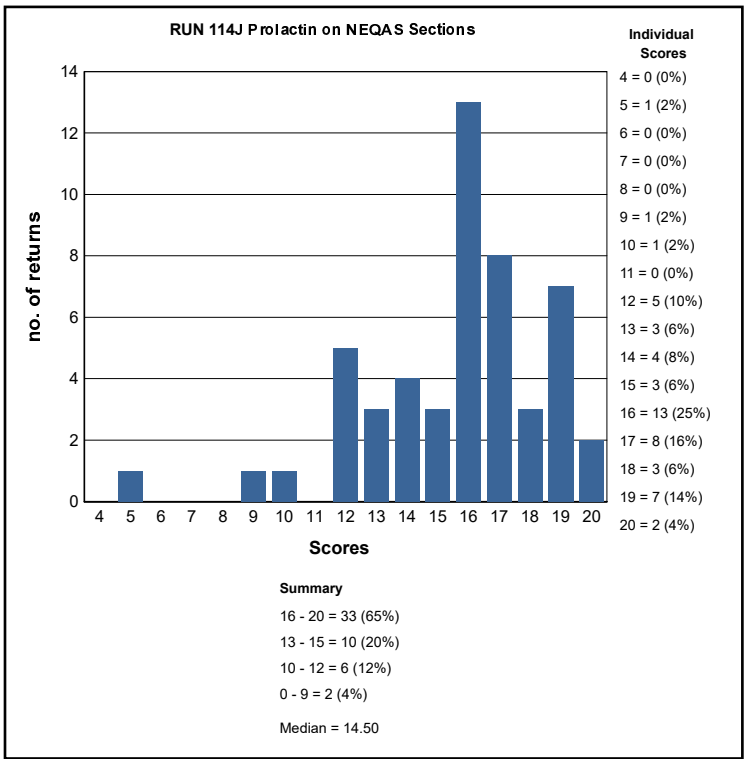


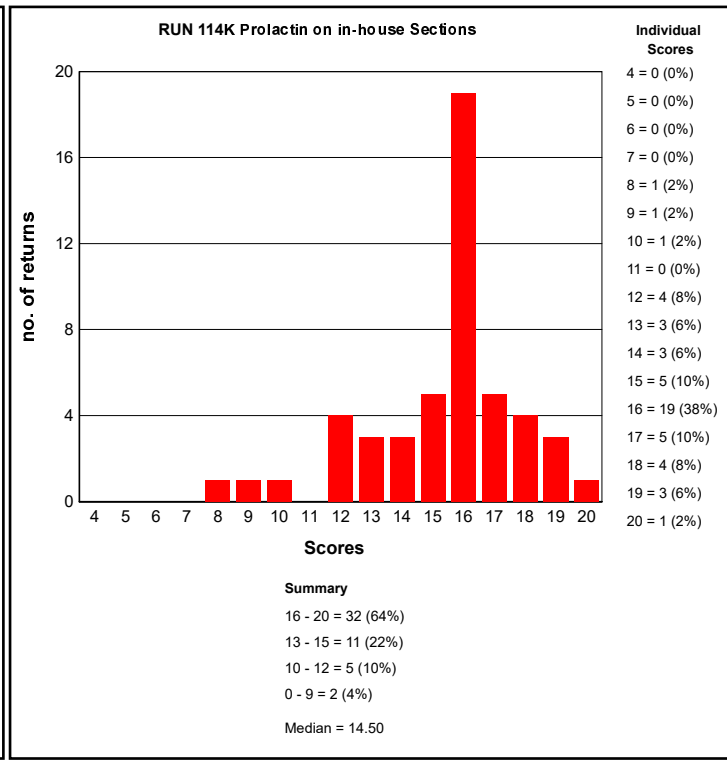
Fig 12. Nice demonstration of prolactin on an in-house pituitary section. Dako polyclonal, 1:4000, 20 mins, with the Dako Omnis high pH retrieval, 30 mins, on the Dako Omnis, with a RTU Dako EnVision FLEX kit, 20 mins.

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: 114

Primary Antibody : Glial Fibrillary Acidic Protein (GFAP)

Antibody Details	N	%
Dako M0761 (6F2)	13	85
Dako Z0334 (R Poly)	25	84
Immunon 490740RB	1	100
Novocastra NCL-GFAP-GA5 (GA5)	2	100
Sigma G3895 (GA5)	2	100
Zymed/Invitrogen 08-1021 (ZCG29)	1	100
Other	1	100
Dako IR524 (R Poly)	2	100
Novocastra PA0026 RTU (GA5)	3	100
Ventana 760-4345 (EP672Y)	5	100
Cell Marque (EP672Y) 258R	2	100
Dako Omnis GA524 (R Poly)	1	100

Neuropathology Run: 114

Primary Antibody : Prolactin

Antibody Details	N	%
Biogenex MU 031 UC (clone BGX031A)	3	67
DakoCytomation A0569, (polyclonal)	32	84
Novocastra NCL-PRO (Clone INN-hPRL-3)	2	100
Other	3	67
Cell Marque 210A-16 (Polyclonal)	2	100
Ventana 760-2803 (Polyclonal)	5	80

Neuropathology Run: 114

Heat Mediated Retrieval	Glial Fibrillary Acidic Protein (GFAP) N	%	Prolactin N	%
Dako Omnis	3	100	2	100
Dako PTLink	5	60	6	67
Leica ER1 10 mins	1	100	1	100
Leica ER1 20 mins	3	100	0	0
Leica ER1 30 mins	2	100	0	0
Leica ER2 20 mins	5	100	1	100
Microwave	2	100	1	100
None	11	82	16	75
Other	2	50	0	0
Ventana CC1 16mins	1	100	1	100
Ventana CC1 20mins	1	100	0	0
Ventana CC1 24mins	0	0	2	100
Ventana CC1 32mins	1	100	2	100
Ventana CC1 36mins	2	100	2	50
Ventana CC1 40mins	1	100	0	0
Ventana CC1 48mins	1	100	0	0
Ventana CC1 56mins	0	0	1	100
Ventana CC1 64mins	4	100	1	100
Ventana CC1 76mins	1	100	0	0
Ventana CC1 8mins	2	100	2	100
Ventana CC1 mild	4	100	1	100
Ventana CC1 standard	3	100	2	100
Water bath 95-98 OC	1	100	1	100

Neuropathology Run: 114

Enzyme Mediated Retrieval	Glial Fibrillary Acidic Protein (GFAP) N	%	Prolactin N	%
AS PER KIT	0	0	1	100
Dako Proteinase K (S3020)	1	0	0	0
Enzyme digestion + HIER	0	0	1	100
NOT APPLICABLE	27	93	23	87
Other	1	100	1	100
VBS Bond Enzyme 1	4	75	0	0
Ventana Protease 1 (760-2018)	4	100	0	0

Neuropathology Run: 114 Detection	Glial Fibrillary Acidic Protein (GFAP) N	%	Prolactin N	%
AS PER KIT	6	83	6	83
Dako EnVision FLEX (K8000/10)	3	67	3	67
Dako EnVision FLEX+ (K8002/12)	4	50	3	100
Dako Envision HRP/DAB (K5007)	1	100	2	100
Leica Bond Polymer Refine (DS9800)	17	94	14	79
None	0	0	1	100
Other	1	100	1	100
Vector Elite ABC Kit (PK-7200)	1	100	0	0
Ventana OptiView Kit (760-700)	8	88	8	100
Ventana UltraView Kit (760-500)	17	100	8	63

Neuropathology Run: 114 Automation	Glial Fibrillary Acidic Protein (GFAP) N	%	Prolactin N	%
Dako Autostainer Link 48	7	43	11	82
Dako Autostainer Plus Link	1	100	0	0
Dako Omnis	3	100	2	100
Leica Bond Max	6	100	4	100
Leica Bond-III	12	92	12	75
None (Manual)	2	100	1	100
Ventana Benchmark ULTRA	19	95	10	80
Ventana Benchmark XT	8	100	9	89

Neuropathology Run: 114 Chromogen	Glial Fibrillary Acidic Protein (GFAP) N	%	Prolactin N	%
AS PER KIT	6	83	10	90
Dako DAB+ REAL Detection (K5001)	1	100	0	0
Dako EnVision Plus kits	2	50	1	100
Dako FLEX DAB	7	71	5	80
Dako REAL EnVision K5007 DAB	0	0	2	100
Leica Bond Polymer Refine kit (DS9800)	17	94	15	80
Other	4	75	4	100
Ventana DAB	3	100	2	100
Ventana Ultraview DAB	18	100	10	70

BEST METHODS - Gold Standard Antibody

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

Glial Fibrillary Acidic Protein (GFAP) - Method 1

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

Primary Antibody: Novocastra NCL-GFAP-GA5 (GA5) , 15 Mins, RT ºC Dilution 1: 200
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER1 30 mins, 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

Glial Fibrillary Acidic Protein (GFAP) - Method 2

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

Primary Antibody: Dako Z0334 (R Poly) Dilution 1: 1:2000
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)

Glial Fibrillary Acidic Protein (GFAP) - Method 3

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

Primary Antibody:	Dako Z0334 (R Poly) , 32 Mins, 36 ºC Dilution 1: 1000
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	None
EAR:	Ventana Protease 1 (760-2018), 36 ºC. Digestion Time NEQAS: 4 Mins. In-House: 4 Mins
Chromogen:	Other, 36 ºC., Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 36 ºC Prediluted

Glial Fibrillary Acidic Protein (GFAP) - Method 4

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

Primary Antibody:	Novocastra PA0026 RTU (GA5) , 15 Mins, Ambient ºC
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins, PH: 9
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), Time 2: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, AMBIENT ºC Prediluted

BEST METHODS - Secondary Antibody

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

Prolactin - Method 1

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

Primary Antibody:	DakoCytomation A0569, (polyclonal) Dilution 1: 500
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	None
EAR:	Enzyme digestion + HIER
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

Prolactin - Method 2

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

Primary Antibody:	DakoCytomation A0569, (polyclonal) , 32 Mins Dilution 1: 400
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 mild
EAR:	
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

Prolactin - Method 3

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

Primary Antibody:	DakoCytomation A0569, (polyclonal) , 20 Mins, 32 ºC Dilution 1: 4000
Automation:	Dako Omnis
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis, Buffer: Dako TRS HIGH PH
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 32 ºC., Time 1: 5 Mins
Detection:	Dako EnVision FLEX (K8000/10) , 20 Mins, 32 ºC Prediluted

Prolactin - Method 4

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

Primary Antibody:	DakoCytomation A0569, (polyclonal)
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

Neil Bilbe and Irena Srebotnik Kirbis

	Gold Standard	Second Antibody
Antigens Assessed:	CK	ER
Sample circulated; cytospins and cell block sections:	Cell lines of melanoma, breast and cervical carcinoma, effusion with mesothelial cells, macrophages, RBCs.	Cell lines of melanoma, breast and cervical carcinoma, effusion with mesothelial cells, macrophages, RBCs.
Number of Registered Participants:	81 - Cell block 57.5 (71%), Cytospin 23.5 (29%). N.B. One lab received both samples, but submitted a cytospin.	
Number of Participants this Run	79 (98%)	

Introduction

Gold Standard: Cytokeratin

Cytokeratins (CKs) are intermediate filaments found in the intracytoplasmic cytoskeleton of epithelial tissue. Only a small number of CKs have been found in non-epithelial cell types. There are two types of CKs: acidic type I and basic type II, and together these make up a family of 20 distinct keratins. The expression of each is often organ or tissue specific, therefore, they are valuable markers of epithelial differentiation and detecting the cell of origin for various tumours. Their molecular weight covers a range from 40 to 68 kDa, and generally CKs are divided into high molecular weight versus low molecular weight. However, as several CKs can be found in each individual cell, a broad spectrum (PAN) CK will stain virtually all epithelia or CK-expressing cells. Clones AE1/AE3 or MNF116 (the two main clones used by participants) are PAN markers which demonstrate a wide range of CKs. AE1/AE3 is considered a broader PAN CK marker as it made up of more high and low molecular weight CKs than MNF116 (Goddard et al). Along with other clones, these PAN CKs antibodies are routinely used in the identification of carcinomas in cytology preparations (Fowler et al, Fetsch & Abati).

References

1. MJ Goddard et al. Comparison of commercially available cytokeratin antibodies in normal and neoplastic adult epithelial and non-epithelial tissues. J Clin Pathol 1991; 44:660-6632.
2. LJ Fowler & WA Lachar Application of immunocytochemistry to cytology. Archives of Pathology & Laboratory Medicine. 2008; 132(3): 373-38.
3. PA Fetsch & A Abati Immunocytochemistry in effusion cytology. Cancer Cytopathology. 2001; 93(5): 293-308.

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.

Second Antigen: Oestrogen Receptor

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. 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 which can have a direct impact on patient treatment regime.

Normal tissues: In cervix, the basal squamous epithelial cells and stromal cells show a moderate to strong nuclear staining reaction and the intermediate and superficial squamous epithelial cells show a weak to moderate nuclear staining reaction. Positive nuclear labelling is observed in the mammary gland, tonsil (weak focal staining of squamous epithelial cells and germinal centre cells), uterus (endometrium) and lung mesenchymal and alveolar lining cells. Granulocytes, macrophages and prostate fibromuscular stromal cells are occasionally labelled in the cytoplasm. Non-specific staining of necrotic tissue and secretions in the lung is occasionally seen.

Abnormal tissues: Numerous studies on breast cancer tissue sections have shown Anti-Erα to be reliable and effective for the demonstration of ERα status. Occasionally lymphoid tumours and non-lymphoid neoplasms such as melanoma were labelled, and pancreatic insulinomas.

Features of Optimal Immunostaining:

- 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:

- Inappropriate non-specific nuclear staining in the negative tumour
- Weak or lower expression of nuclear staining of the 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.

References

1. Mauri FA, Veronese S, Frigo B, Girlando S, Losi L, Gambacorta M, et al. ER1D5 and H222 (ER-ICA) antibodies to human estrogen receptor protein in breast carcinomas. Appl Immunohistochem 1994;2:157-63.
2. Goulding H, Pinder S, Cannon P, Pearson D, Nicholson R, Snead D, et al. A new immunohistochemical antibody for the assessment of estrogen receptor status on routine formalin-fixed tissue samples. Hum Pathol 1995;26:291-4.
3. Shaw JA, Udokang K, Mosquera J-M, Chauhan H, Jones JL, Walker RA. Oestrogen receptors alpha and beta differ in normal human breast and breast carcinomas. J Pathol 2002;198:450-7.
4. Kumar V, Green S, Stack G, Berry M, Jin J-R, Chambon P. Functional domains of the human estrogen receptor. Cell 1987;51:941-51.
5. Elledge RM, Fuqua SAW. Ch. 31: Estrogen and Progesterone Receptors. In: Diseases of the Breast. Harris JR et al. eds. Philadelphia: Lippincott Williams & Wilkins 2000:471-85.
6. Use of monoclonal antibody for assessment of estrogen receptor content in fine-needle aspiration biopsy specimen from patients with breast cancer. Masood S. Arch Pathol Lab Med. 1989 Jan; 113(1):26-30.
7. P. Konofaos et al. The role of ThinPrep cytology in the evaluation of estrogen and progesterone receptor content of breast tumours. Surgical Oncology, Volume 15, Issue 4, December 2006, Pages 257–266.

References (cell blocks in cytology)

1. Xin Jing, et al,. Morphologic and Immunocytochemical Performances of Effusion Cell Blocks Prepared Using 3 Different Methods. (2013) American Journal of Clinical Pathology, *139,* 177-182
2. Nithyananda A. Nathan, et al, Cell Block Cytology. Improved Preparation and Its Efficacy in Diagnostic Cytology. (2000) American Journal of Clinical Pathology, *114,* 599-606.
3. Hirofumi Sakamoto, et al, Use of Liquid-Based Cytology (LBC) and Cell Blocks from Cell Remnants for Cytologic, Immunohistochemical, and Immunocytochemical Diagnosis of Malignancy. Open Journal of Pathology, Vol. 2 No. 3 (2012), Article ID: 21116, 8 pages

Assessment Summary:

Out of 82 labs registered for the 2016/2017 EQA year Cytology module there were 81 laboratories registered for participation in run 114, and 79 of them submitted at least one slide for assessment. A total of 310 slides were scored by the assessors: **R= 79, S=77, T=78, U=76.**

Majority of participants request cell blocks for NEQAS slides (71%). For the in-house controls (T & U) the levels are FFPE (51%), cell blocks (21%), and cytospins (16%). Other cytology slides remain constantly low, LBC (5%) and smears (6%). The overall pass rate was 91.6% (284 slides), borderline 7.4% (23), with 1% (3) failing the assessment. An average score achieved for all 310 slides was 16/20.

Examples of the immunocytochemical reactions on NEQAS slides prepared in a reference/supplier laboratory

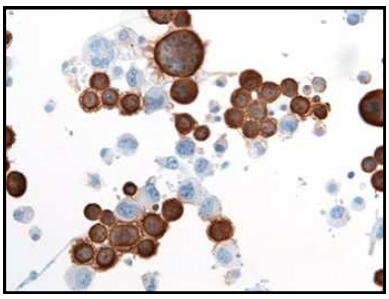


Fig 1: MNF116 on cytospin prepared from 114 R

Fig 2: MNF116 on cell block prepared from 114 R

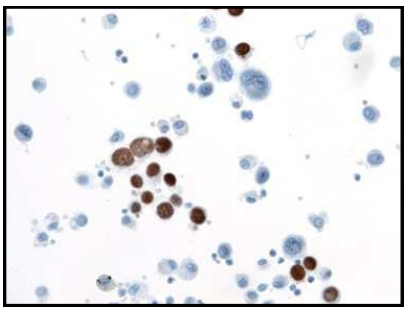


Fig 3: ER on cytospin prepared from 114 T

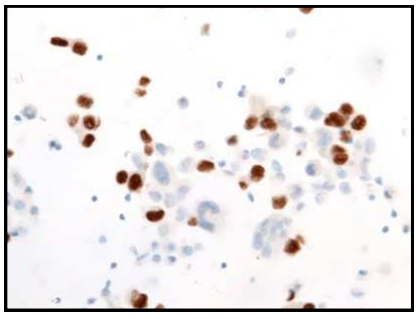


Fig 4: ER on cell block prepared from 114 T

Summary Table:

Slide	Antigen/(N)	Pass	Borderline	Fail
R (NEQAS)	CK (79)	(72) 91%	(6) 8%	(1) 1%
S (In-House)	CK (77)	(68) 88%	(8)10%	(1) 1%
T (NEQAS)	ER (78)	(73) 94%	(5) 6%	(0) 0%
U (In-House)	ER (76)	(71) 93%	(4) 5%	(1) 1%
Total (Average)		92%	7%	1%

Assessment Outcomes:

The most noticeable finding in this assessment was the high % of borderline scores on CK in-house slides (**S**) 10%, and NEQAS slides (**R**) 8% and therefore the lower pass rate for the in-house slides (**S**) of 88%, compared to the other 3 sets (R, T, and U).

There were 4 were cell blocks (7 %) and 2 cytospins (8 %) among 6 NEQAS (R) slides assessed as borderline.

For the 8 in-house control slides the sample given by the participants were: tissue x 2, cytospin x 2, cell block x 3, LBC x 1.

CK (R & S)

Only one NEQAS (**R**) slide failed the assessment a cytospin preparation using a Cell Marque CK7 concentrate (dilution not given, no retrieval), on a Leica Bond Max and the Refine kit. The slide showed non-specific nuclear staining with very weak cytoplasmic staining (see Fig 5 below). Some assessors questioned whether in fact the correct antibody had been used.

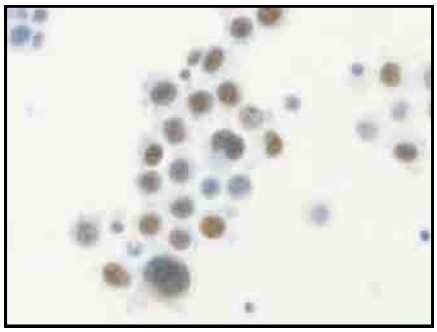


Fig 5: CK on NEQAS cytospin: 114 R - nuclear staining ++

The only failed in-house slide (**S**) was a LBC slide from lung sample. The main reason for low score/marks in this case was the absence of any noticeable positive ICC reaction. The protocol employed was the Leica CK7 (sole user), with ER1, and the Leica Bond III and Refine kit. The same participant achieved 13 on the NEQAS cytospin.

ER (T & U)

There was no NEQAS (**T**) slides failed the assessment; while a single in-house slide (**U**) prepared as a cell block from a pleural effusion was assessed as inadequate due to unacceptable background most probably caused by excessive pretreatment (Dako PT Link) using the RTU Dako 1D5 ER clone (sole user) on the Autostainer Link 48 and the EnVision Flex kit.

NEQAS slides assessed as borderline (R & T)

There were 8/77 (10%) CK in-house control slides (**S**) assessed as borderline, while NEQAS CK (**R**) and ER (**T**) slides had levels of 8% and 6% respectively. Although this CK in-house feature was the most noticeable it is interesting to compare the sample types (cell block v cytospin)

Table of cell block and cytospin borderline results:

Slide	Antigen (N)	Cell block (57)	Cytospin (24)	Total
R (NEQAS)	CK (79)	4 (7%)	2 (8%)	6 (8%)
T (NEQAS)	ER (78)	2 (4%)	3 (13%)	5 (6%)
Total (Average)		6 (5.5%)	5 (11.5%)	11 (7%)

Selected Images showing Optimal and Sub-optimal Immunostaining

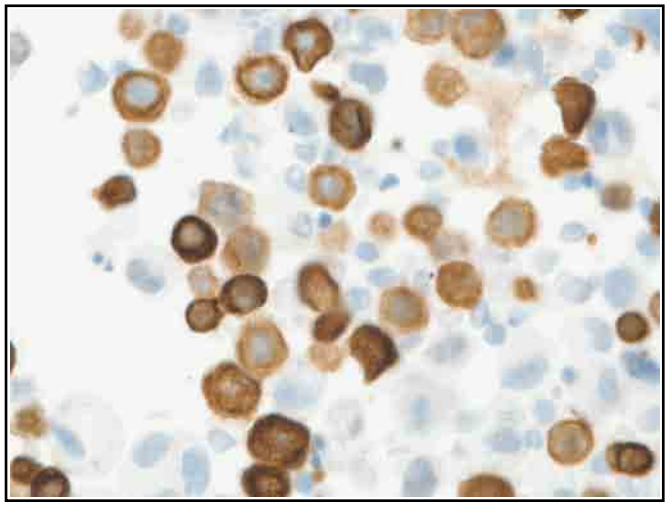


Fig 1. Optimal cytokeratin (CK) demonstration on a NEQAS cell block sample. There is crisp cytoplasmic staining and a clean background. Dako AE1/AE3, 1:200, 20 mins, with a Dako PT Link, high pH for 20 mins on the Dako Autostainer Link 48, using a RTU Dako EnVision FLEX+ kit.

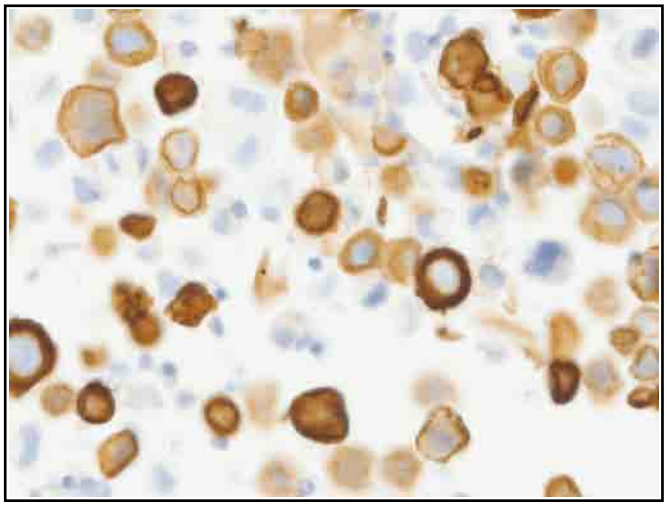


Fig 2. Sub-optimal CK on a NEQAS cell block section. The tumour cells are weakly stained and not as well defined (compare Fig 1). Dako monoclonal AE1/AE3, 1:100, 28 mins, with Ventana CC1 mild for 30 mins, on the Ventana Benchmark XT and a prediluted UltraView Kit for 28 mins.

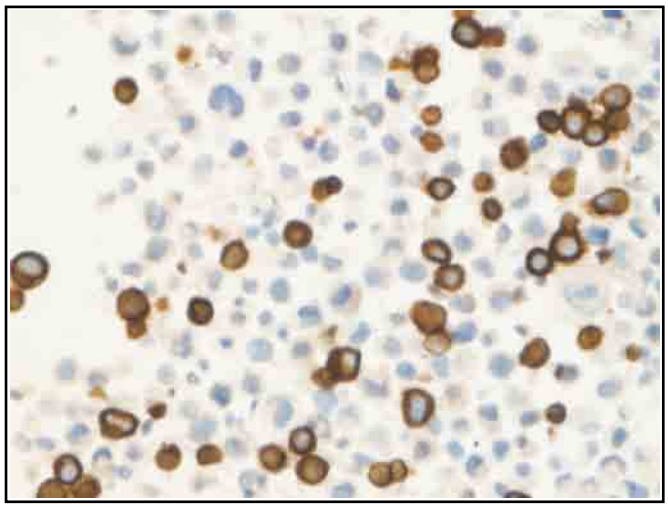


Fig 3. Sub-optimal CK on a NEQAS cell block sample. There is some non-specific staining. This was assessed as a low pass, but adequate for diagnosis. Novocastra NCL-L-AE1/AE3, 1:200, with Leica ER2 20 mins, on a Leica Bond-III, and the Leica Bond Polymer Refine kit.

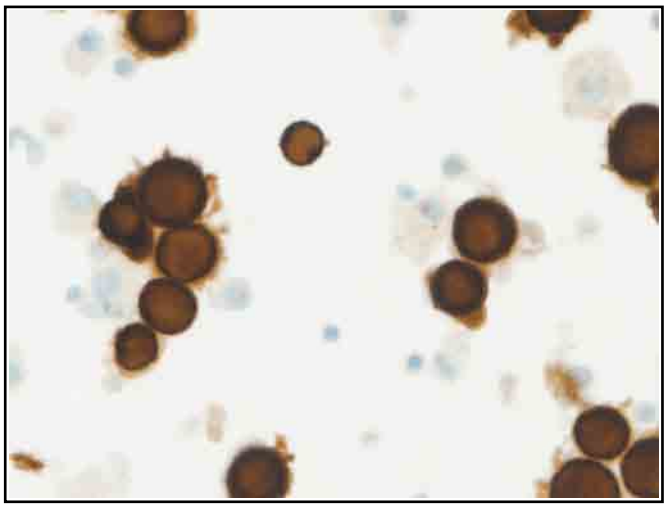


Fig 4. Sub-optimal CK staining on a NEQAS cytospin preparation. Staining is slightly heavy, and there is a hint of background, otherwise nicely demonstrated. Ventana AE1/AE3/PCK26, pan CK, with Ventana CC1 20mins, on a Ventana Benchmark ULTRA, and the Ventana UltraView Kit (760-500).

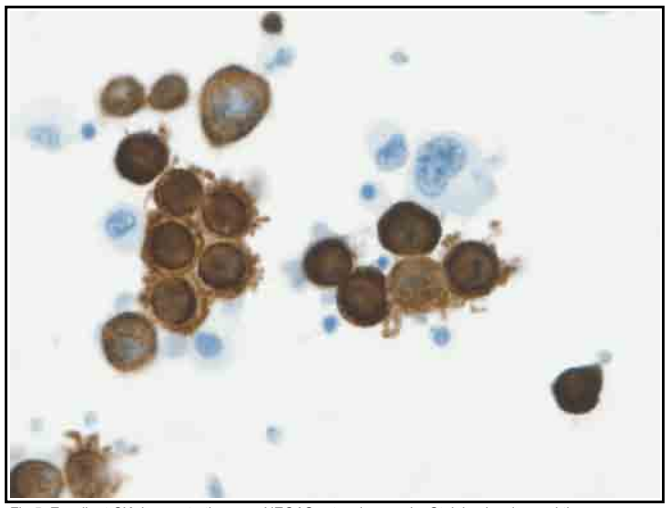


Fig 5. Excellent CK demonstration on a NEQAS cytospin sample. Staining is crisp and the background clean. Dako AE1/AE3, 1:100, 60 mins, with a MW pretreatment in hot citrate buffer pH6, for 15 mins using a manual method. It is rare to see this quality of staining, with this type of protocol.

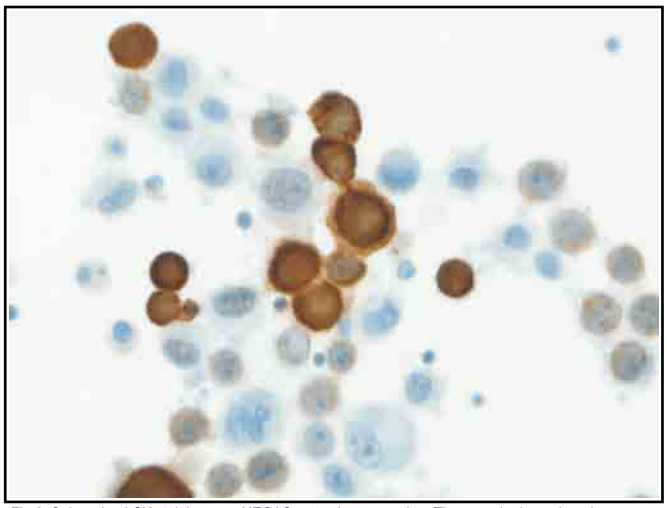


Fig 6. Sub-optimal CK staining on a NEQAS cytospin preparation. There are both weak and non-specific reactions. Slide assessed as a low pass, whereas the previous slide (Fig 5), a high pass. Dako AE1/AE3, diluted 1:3000, 16 mins, no PT, on Ventana Benchmark GX, and the Ventana OptiView kit.

Selected Images showing Optimal and Sub-optimal Immunostaining

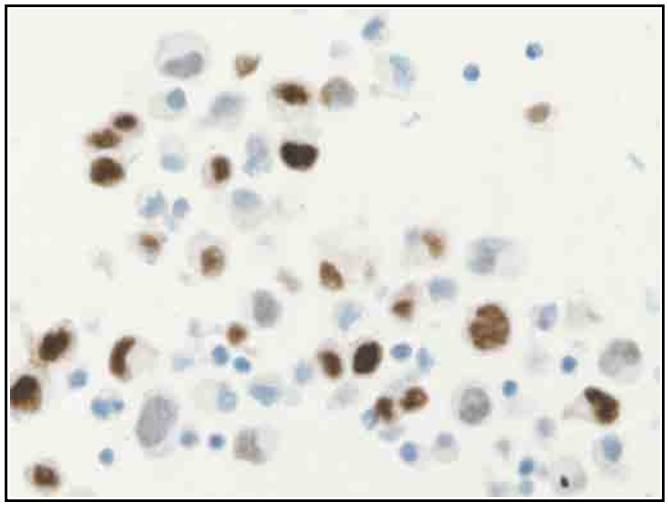


Fig 7. Sub-optimal Oestrogen demonstration on a NEQAS cell block sample. Staining is patchy and in some areas non-specific. Novocastra NCL-L-ER- 6F11, 1:20, 15 mins, with Leica ER1 40 mins, on a Leica Bond Max, and a RTU Leica Bond Polymer Refine kit for 8 mins.

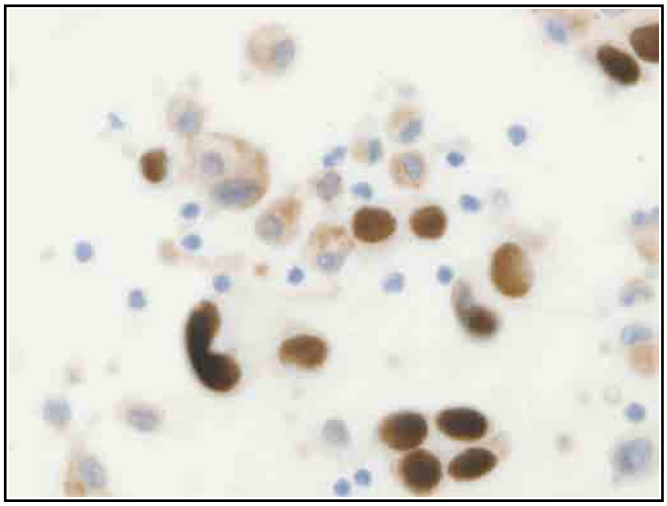


Fig 8. Sub-optimal ER demonstration on a NEQAS cytospin sample. Non-specific staining is more pronounced, and therefore assessed as borderline. Novocastra NCL-ER-6F11, 1:80, without PT, on the Leica Bond-III, and a Leica Bond Polymer Refine kit at RT.

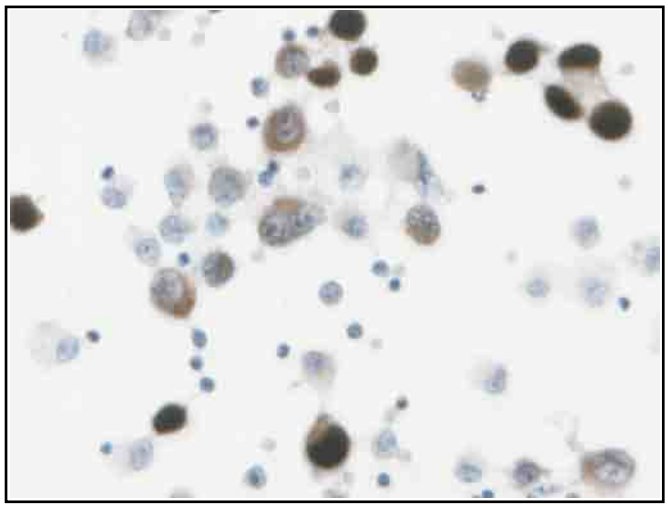


Fig 9. Sub-optimal ER demonstration on a NEQAS cytospin. The slide is dirty, and the counterstain heavy, and again assessed as borderline. Novocastra NCL-ER-6F11, 1:100, 60 mins, using a pressure cooker, EDTA buffer at pH8, manual method, with a secondary layer of Dako REAL HRP/DAB kit.

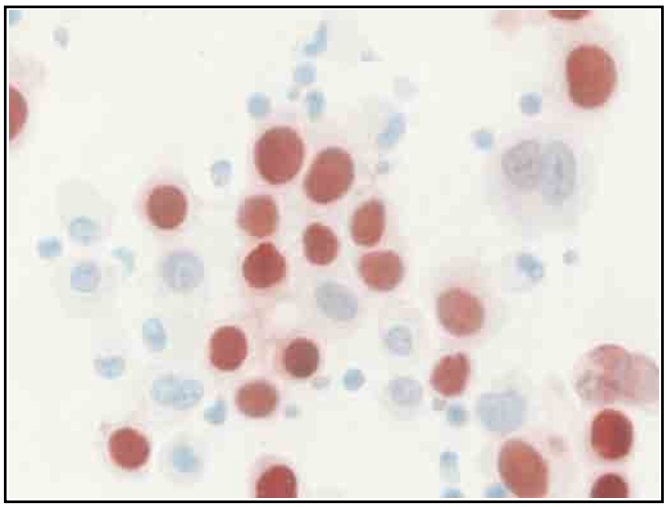


Fig 10. Sub-optimal ER demonstration on a NEQAS cytospin. There is a background blush of non-specific chromogen, though most cells are nicely stained. Leica/Novocastra NCL-L-ER-6F11, 1:80, 30 mins, Leica ER1 10 mins, on a Leica Bond Max, and a RTU Leica Bond Polymer Refine kit 8 mins.

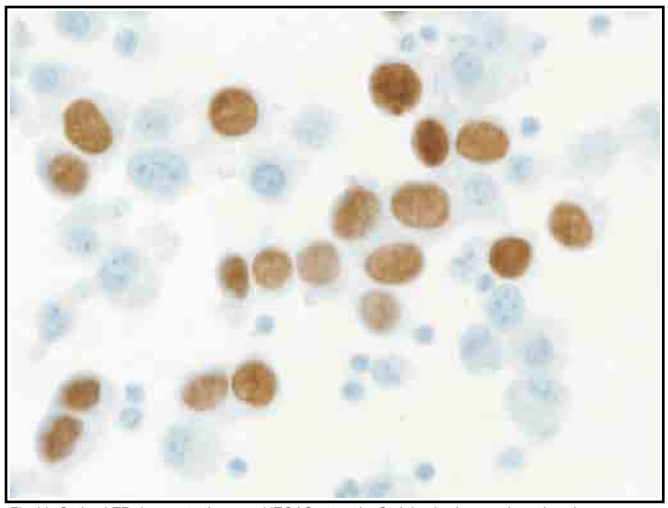


Fig 11. Optimal ER demonstration on a NEQAS cytospin. Staining is clean and precise, the background is clean and the counterstain intensity excellent. Ventana 790-4324 (SP1), 24 mins, Ventana CC1 32mins, on a Ventana Benchmark XT, with a RTU Ventana UltraView Kit.

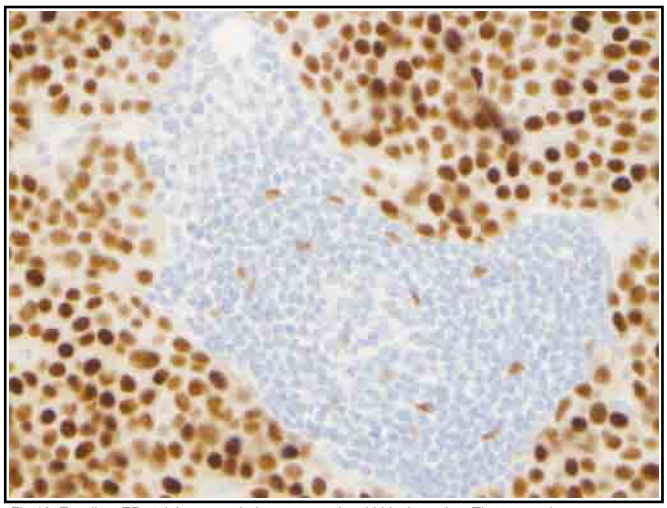
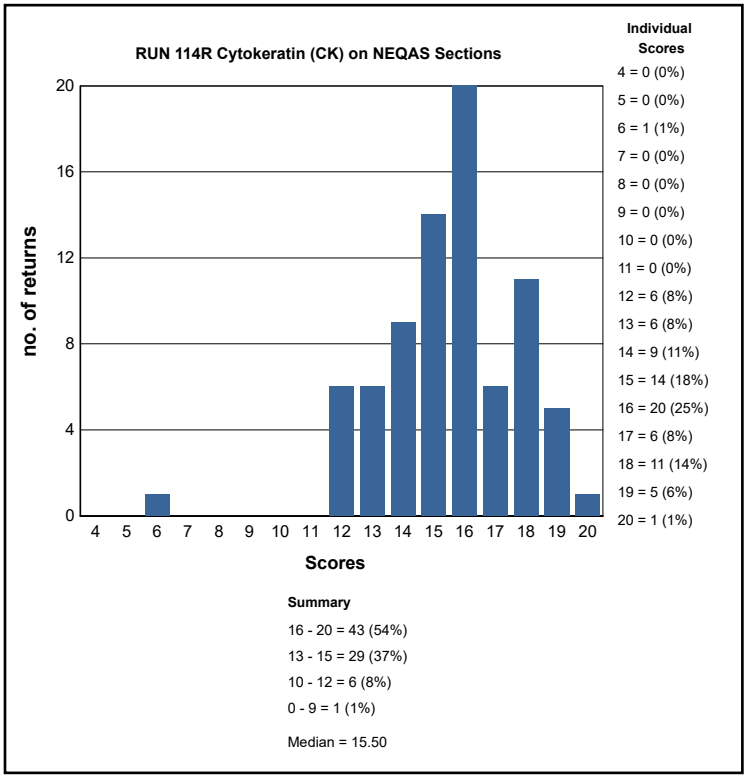
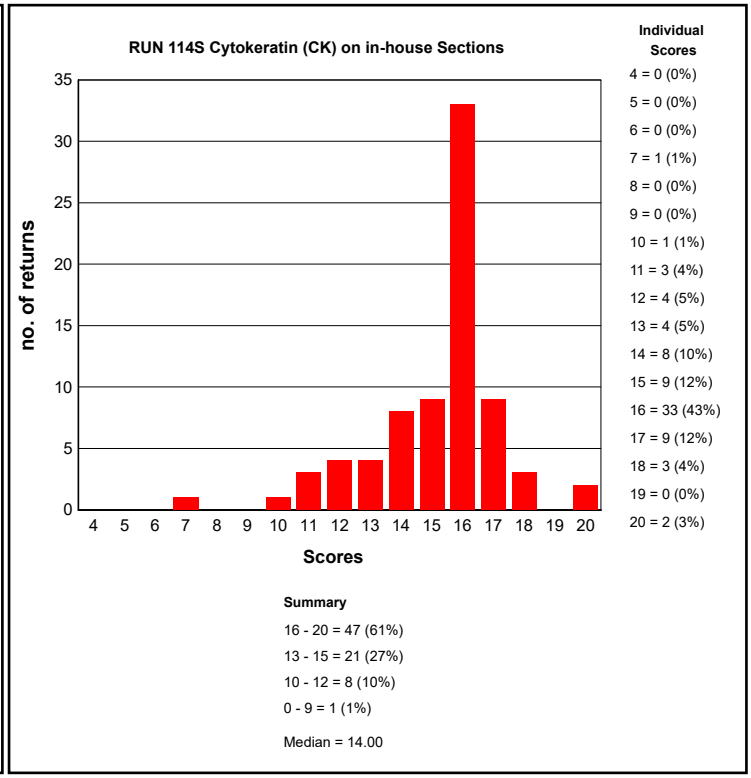
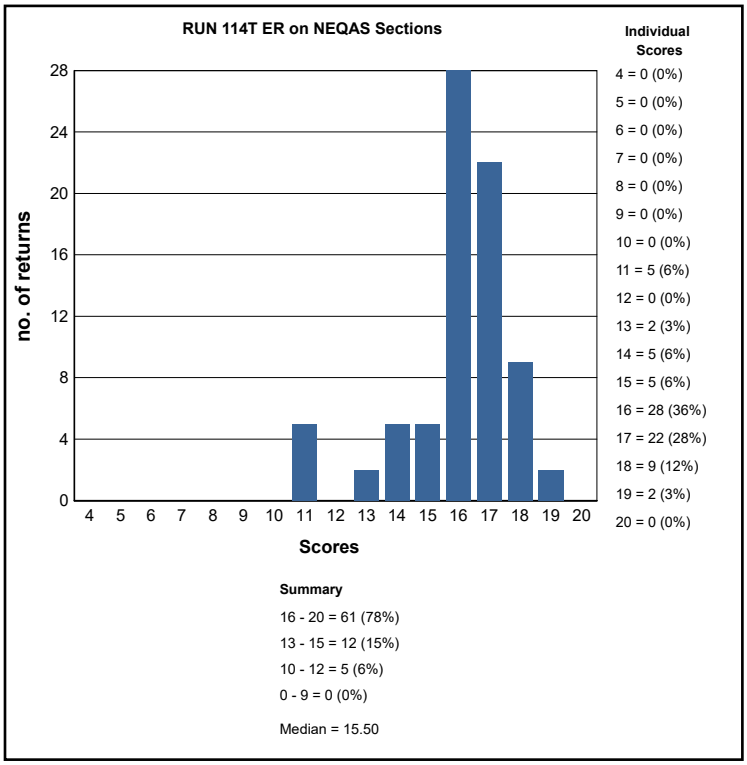


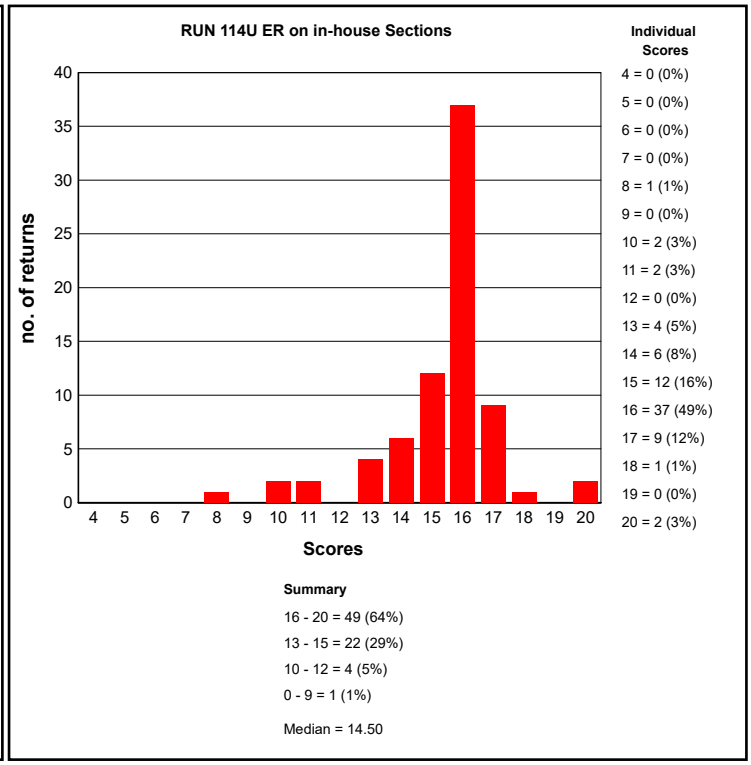
Fig 12. Excellent ER staining on an in-house control multi-block section. The tumour is beautifully demonstrated, and the rest is clean. RTU Ventana 790-4324 (SP1), mins, Ventana CC1 standard for 60 mins, on a Ventana Benchmark XT, and a prediluted Ventana UltraView Kit, for 8 mins at 37°C.

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: 114

Primary Antibody : Cytokeratin (CK)

Antibody Details	N	%
Becton Dickinson 349205 (CAM5.2)	3	100
BioGenex MU071-UC (clones AE1/AE3)	1	100
Dako M3515 (AE1/AE3)	17	82
Dako M0821(MNF116)	28	100
Vector Labs VP-C420 (C-11)	1	100
Leica/Novocastra RTU PA0909 (AE1/AE3)	1	100
Leica/Novocastra NCL-L-AE1/AE3	3	100
Ventana 760 2135 (AE1/AE3/PCK26)	1	100
Ventana 760 2595 AE1/AE3/PCK26	5	60
Other	2	100
Cell marque 307M-95 (CK7)	1	0
Cell Marque 313M- (AE1/AE3)	1	100
Leica/Novocastra NCL-AE1/AE3	1	100
Biomedicals BMA-T-1302	1	100
Ventana CONFIRM 790-4373 (34BE12)	1	100
Dako FLEX RTU IR053 (AE1/AE3)	4	100
ImmunoBS MM-1012 (CK cocktail)	1	100
Ventana 790-4555 (CAM 5.2)	2	100
Leica/Novacastra NCL- L-CK5/6/8/18 (Multi 5D3/LP34	2	100
Dako Omnis FLEX GA053 (AE1/AE3)	2	50

Cytology Run: 114

Primary Antibody : ER

Antibody Details	N	%
Cell Marque 249-R (SP1)	1	0
Dako M7047 ER (1D5)	2	100
Dako M3634 (SP1)	4	100
Dako (EP1) RTU FLEX IR084	6	100
Dako FLEX (1D5) IR/IS657	1	100
Dako (EP1) M3643	4	100
Leica/Novocastra NCL-ER-6F11 (6F11)	4	75
Leica/Novocastra NCL-L-ER- 6F11	13	92
Leica/Novocastra NCL-ER-6F11/2	4	75
Thermo Fisher/ Neomarkers RM 9101-S (SP1)	4	100
Vector VP-E613/4 (6F11)	1	100
Ventana 250- 2596 ER (6F11)	1	100
Ventana 790-4324 (SP1)	19	95
Ventana 790-4325 (SP1)	12	100

Cytology Run: 114

Primary Antibody : Cytokeratin (CK)

Antigen Retrieval	N	%
YES	28	35
NO	51	65
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	28	
Not Specified	0	

Cytology Run: 114

Primary Antibody : ER

Antigen Retrieval	N	%
YES	39	49
NO	40	51
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	39	
Not Specified	0	

Cytology Run: 114

Heat Mediated Retrieval

Cytology Run: 114

Heat Mediated Retrieval

Cytology Run: 114

Enzyme Mediated Retrieval

Cytology Run: 114

Enzyme Mediated Retrieval

Cytology Run: 114

Detection	Cytokeratin (CK)		ER	
	N	%	N	%
AS PER KIT	5	100	8	100
BioGenex HRP (HK 519-06K)	1	100	0	0
Dako EnVision FLEX (K8000/10)	3	67	2	100
Dako EnVision FLEX+ (K8002/12)	6	100	5	100
Dako Envision HRP/DAB (K5007)	1	100	1	100
Dako Envision+ HRP mouse K4004/5/6/7	2	50	1	0
Dako REAL HRP/DAB (K5001)	0	0	1	0
Leica Bond Polymer AP Red Detection (DS9305)	1	100	0	0
Leica Bond Polymer Define (DS9713)	0	0	1	100
Leica Bond Polymer Refine (DS9800)	18	94	13	92
NOT APPLICABLE	0	0	1	100
Other	4	75	3	67
Ventana iView system (760-091)	3	100	1	100
Ventana OptiView (760-700) + Amp. (7/860-099)	1	0	0	0
Ventana OptiView Kit (760-700)	11	91	5	100
Ventana UltraView Kit (760-500)	19	95	30	93

Cytology Run: 114

Automation	Cytokeratin (CK)		ER	
	N	%	N	%
BioGenex GenoMX 6000i	1	100	0	0
Dako Autostainer Link 48	10	90	11	91
Dako Autostainer Plus Link	1	100	1	100
Dako Omnis	3	67	1	100
LabVision Autostainer	1	100	0	0
Leica Bond Max	9	89	7	86
Leica Bond-III	13	100	10	90
None (Manual)	1	100	1	0
Ventana Benchmark GX	3	100	4	100
Ventana Benchmark ULTRA	23	91	25	96
Ventana Benchmark XT	13	85	17	94

Cytology Run: 114

Chromogen	Cytokeratin (CK)		ER	
	N	%	N	%
AS PER KIT	7	100	10	90
BioGenex Liquid DAB (HK153-5K)	1	100	0	0
DAKO DAB+	1	0	2	50
Dako DAB+ Liquid (K3468)	1	100	1	0
Dako FLEX DAB	9	89	7	100
Dako REAL EnVision K5007 DAB	0	0	1	100
Leica Bond Polymer Refine kit (DS9800)	21	95	14	93
Other	5	60	3	100
Ventana DAB	9	89	4	100
Ventana Enhanced Alk. Phos. Red Detection Kit	1	100	1	100
Ventana iview	2	100	3	100
Ventana Ultraview DAB	21	95	31	94

BEST METHODS - Gold Standard Antibody

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

Cytokeratin (CK) - Method 1

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

Primary Antibody:	Dako M3515 (AE1/AE3) , 20 Mins, 23 ºC Dilution 1: 200
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: FLEX TRS HIGH PH
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 23 ºC., Time 1: 10 Mins, Time 2: 23 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 20 Mins, 23 ºC Prediluted

Cytokeratin (CK) - Method 2

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

Primary Antibody:	Dako M0821(MNF116) , 24 Mins, 36 ºC Dilution 1: 100
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	None
EAR:	Ventana Protease 1 (760-2018), 36 ºC. Digestion Time NEQAS: 12 Mins. In-House: 12 Mins
Chromogen:	Ventana Ultraview DAB, RT ºC., Time 1: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, RT ºC Prediluted

Cytokeratin (CK) - Method 3

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

Primary Antibody:	Dako M0821(MNF116) , 24 Mins, 37 ºC Dilution 1: 250
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	None
EAR:	Ventana Protease 1 (760-2018), 37 ºC. Digestion Time NEQAS: 8 Mins. In-House: 8 Mins
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700)

Cytokeratin (CK) - Method 4

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

Primary Antibody:	Dako M0821(MNF116) , 15 Mins, 22 ºC Dilution 1: 400
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590), PH: 7.6
HMAR:	None
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), 22 ºC., Time 1: 10 Mins, Time 2: 10 Mins
Detection:	Dako Envision+ HRP mouse K4004/5/6/7 , 8 Mins, 22 ºC Prediluted

BEST METHODS - Secondary Antibody

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

ER - Method 1

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

Primary Antibody:	Ventana 790-4325 (SP1) , 48 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark XT
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)

ER - Method 2

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

Primary Antibody:	Dako (EP1) RTU FLEX IR084
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	AS PER KIT
HMAR:	Dako PTLink
EAR:	
Chromogen:	AS PER KIT
Detection:	AS PER KIT

ER - Method 3

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

Primary Antibody:	Dako (EP1) RTU FLEX IR084 , 20 Mins, RT ºC Prediluted
Automation:	Dako Omnis
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis, Buffer: 0
EAR:	
Chromogen:	DAKO DAB+, RT ºC., Time 1: 5 Mins
Detection:	AS PER KIT , 20 Mins, RT ºC Prediluted

ER - Method 4

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

Primary Antibody:	Leica/Novocastra NCL-L-ER- 6F11 , 16 Mins, 37 ºC Dilution 1: 100
Automation:	Ventana Benchmark GX
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	
EAR:	
Chromogen:	AS PER KIT
Detection:	AS PER KIT

The Alimentary Tract Module: GIST Run 114

Suzanne Parry

	First Antibody	Second Antibody
Antigens Assessed:	CD117	DOG-1
Tissue Sections circulated:	Normal appendix, GIST and Desmoid.	
Number of Registered Participants:	112	
Number of Participants this Run	110 (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, 3 & 6)**

- Good localisation of CD117 to mast cells in the appendix and desmoid sections
- Good localisation of CD117 to interstitial cells of Cajal
- Good localisation of CD117 to cells of the GIST
- No staining of the desmoid tumour

Features of Sub-optimal Immunostaining: **(See Figs 2, 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
- Staining of the desmoid tumour

Second Antibody: DOG-1

Discovered on GIST 1" (DOG-1) antibody was initially described in 2004[4] and has now started to be recognized as a more specific marker of GISTs than CD117 [4,5,6]. A recent study has shown DOG-1 sensitivity of 99% and found to be strong and easier to interpret than CD117[6]. The groups also showed that seven CD117 negative GIST cases were found to be DOG-1 positive, and six of the tumours had PDGFRA mutations. Furthermore, DOG-1 is not expressed in Kaposi sarcomas of the GI tract which have been shown to stain for CD117[7]. In more cases, combined CD117 and DOG-1 immunohistochemistry has been suggested as a sufficient panel to confirm diagnosis, with CD117 and DOG-1 negative cases tested further with a panel of antibodies, including SMA, Desmin, S100. Molecular analysis should be considered[6].

Features of Optimal Immunostaining (See Figs 7, 8 & 12)

- Good localisation of DOG-1 to cells of the GIST (Fig 7)
- Good localisation of DOG-1- to the interstitial cells of Cajal
- No staining of desmoid tumour

Features of Suboptimal Immunostaining (See Figs 9, 10 & 11)

- Weak and/or patchy staining of the tumour cells of the GIST (Fig 9)
- Excessive background or non specific staining
- Staining of the desmoid tumour
- Staining of the mast cells (Note: Mast cells are not expected to stain with DOG-1)

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 DOG1 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.

Assessment Summary:

Results from the **CD117** assessment showed an overall acceptable pass rate of 93% on the Neqas distributed material. The main reason for sub-optimal marks was 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 84% of participants and showed an acceptable pass rate of 92% in this assessment Run.

NEQAS Pass Rates Run 114:CD117	
Acceptable	93% (N=102)
Borderline	5% (N=5)
Unacceptable	3% (N=3)

The **DOG-1** antibody was chosen as the secondary antibody for this current run 114 assessment. The acceptable pass rate was slightly lower than that of the CD117 assessment. Results are summarised in the table below:

NEQAS Pass Rates Run 114: DOG-1	
Acceptable	85% (N=88)
Borderline	13% (N=13)
Unacceptable	2% (N=2)

Slightly more labs received borderline passes for their DOG-1 staining, and similarly to the CD117 assessment, this was due to weak or non-specific and background staining. Only 2 labs failed the assessment due to very weak staining which was considered non-diagnostic (one example shown in Fig 9). The Leica K9 clone was again the most popular antibody, used by 70% of participating labs (N=70), and this showed a pass rate of 87%. The Ventana SP31 clone was also popular, used by 15% of labs (N=15), and showed an acceptable pass rate of 87%. The overall acceptable pass rate on the participants' in-house controls was slightly lower than on the Neqas material for both the CD117 and DOG-1 assessments: More labs received a borderline rather than an acceptable pass. This was mainly because these labs had not submitted the recommended control material including a GIST and normal epithelium or appendix, and therefore marks were deduced. The fail rate on the in-house material was the same as for the Neqas material, and again the failed slides were due to very weak staining. It was encouraging to see that most labs are using a composite control with a positive GIST tumour along with normal tissue.

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. Novelli M, et al. DOG-1 and CD117 are the antibodies of choice in the diagnosis of gastrointestinal stromal tumours. Histopathology 2010, 57(2): 259-270.
6. Miettinen et al, Immunohistochemical spectrum of GISTs at different sites and their differential diagnosis with a reference to CD117 (KIT). Mod Pathol. 2000

Selected Images showing Optimal and Sub-optimal Immunostaining

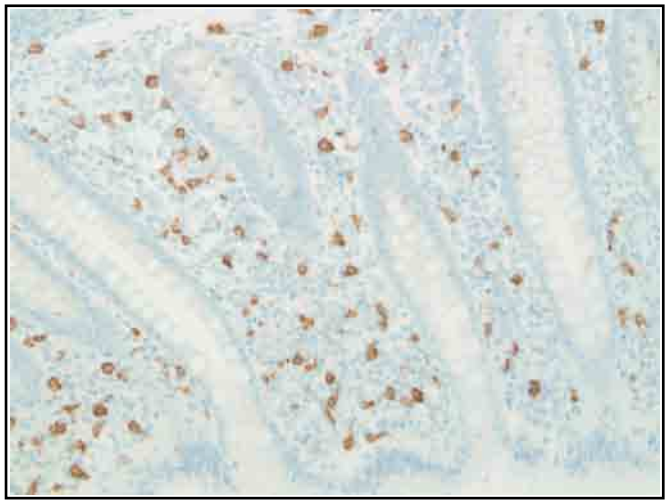


Fig 1. Good demonstration of CD117 in the UK NEQAS distributed appendix. Mast cells show distinct membranous staining, while the background remains clean. Stained with the Ventana pre-diluted 9.7 clone on the Ventana ULTRA, CC1 64 mins pre-treatment and OptiView detection.

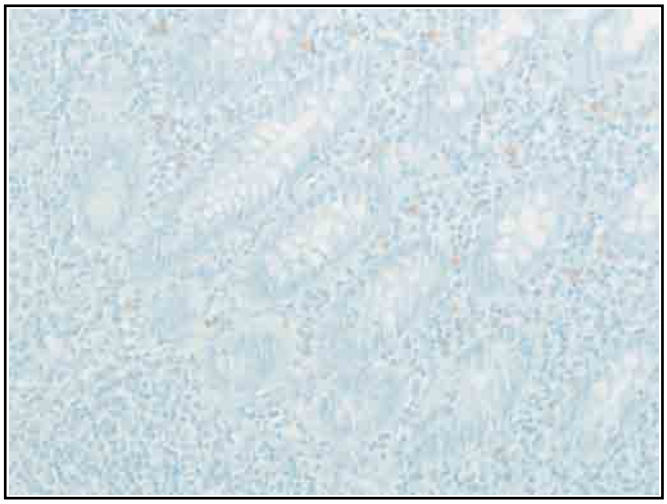


Fig 2. Sub-optimal demonstration of CD117 in the UK NEQAS distributed appendix (compare to Fig 1). Staining of mast cells is weak with fewer than expected cells demonstrated, possibly due to insufficient pre-treatment. Stained with the Dako polyclonal antibody on the Ventana ULTRA, without antigen retrieval.

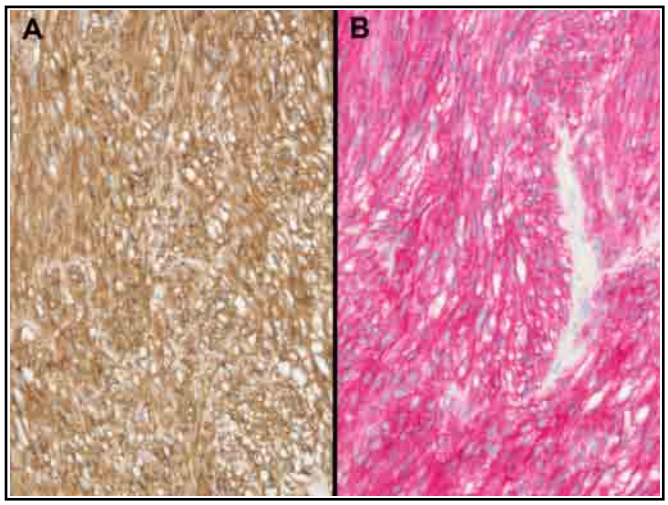


Fig 3. Two examples of good CD117 staining in the UK NEQAS distributed GIST. Both examples show strong cytoplasmic and membranous staining in the tumour cells. Both stained using the Dako polyclonal antibody on the Leica Bond with ER2 antigen retrieval for 20 minutes with (A) DAB chromogen and (B) Refine Red detection.

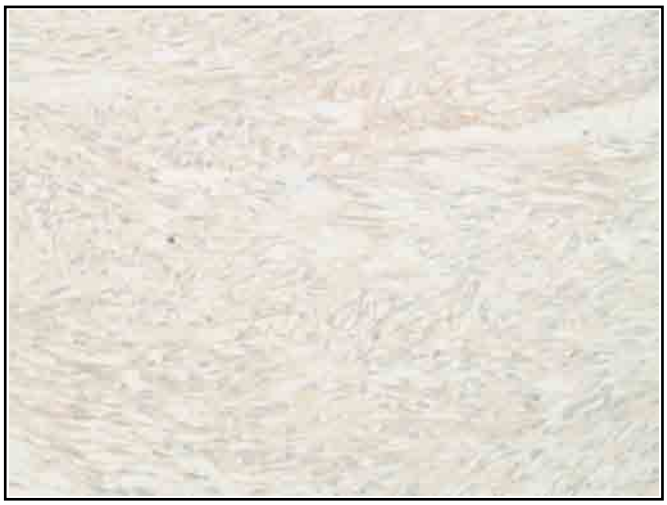


Fig 4. Poor demonstration of CD117 in the UK NEQAS distributed GIST section (compare to Figs 3A&B). Overall the staining is very weak. The appendix section on the same slide was also negative in the mast cells which are expected to be positive. Stained with the Dako polyclonal antibody, 1:70, and CC1 antigen retrieval for 36 minutes on the Ventana ULTRA.

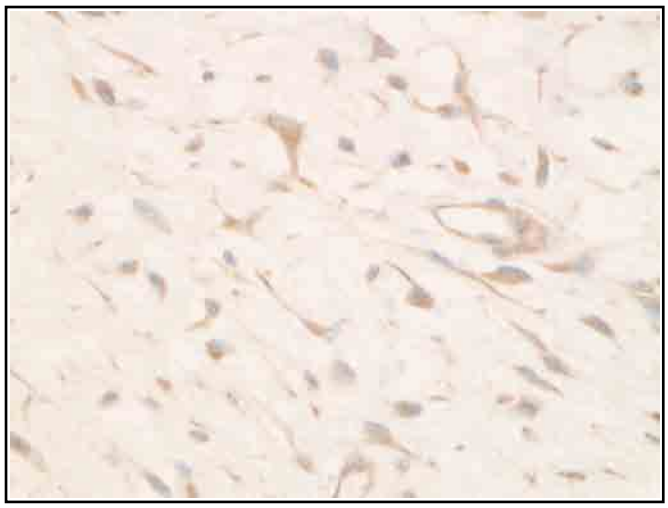


Fig 5. Unacceptable CD117 staining in the UK NEQAS distributed desmoid. This examples shows non-specific and background staining. Stained using the Dako polyclonal antibody, 1:100, on the Leica Bond III with ER2 antigen retrieval for 20 minutes.

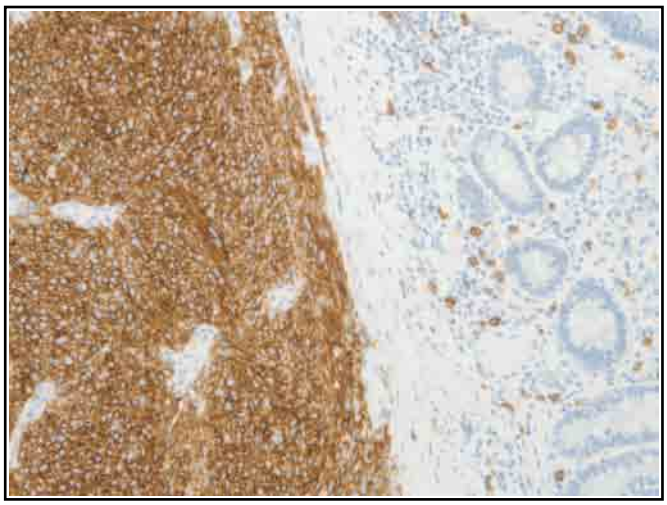


Fig 6. Good example of an in-house CD117 control. Section shows good strong staining of the GIST, and also includes normal epithelium to demonstrate the strong distinct staining of the mast cells.

Selected Images showing Optimal and Sub-optimal Immunostaining

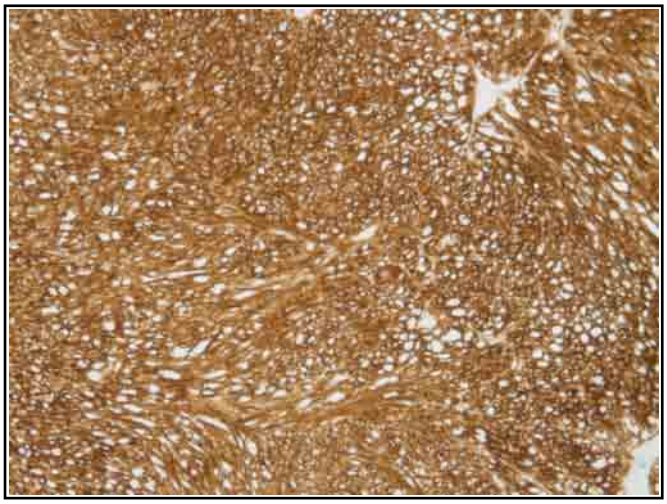


Fig 7. Good demonstration of DOG-1 in the UK NEQAS distributed GIST. There is strong crisp staining in the tumour cells. Stained with the Leica K9 antibody, 1:25, on the Ventana Benchmark XT with CC1 mild antigen retrieval and UltraView detection kit.

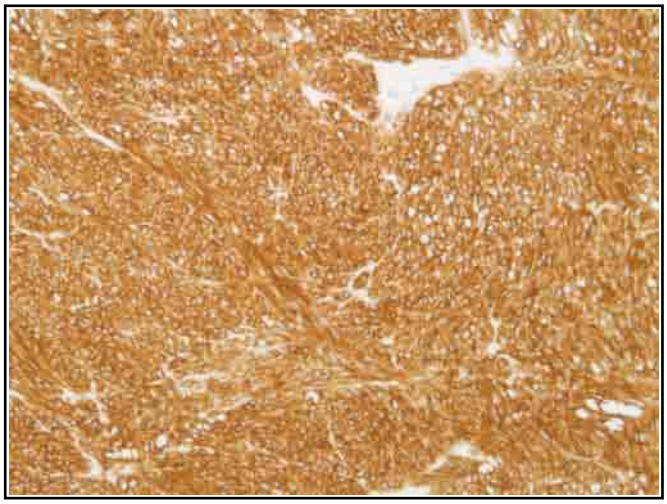


Fig 8. Optimal demonstration of DOG-1 in the UK NEQAS distributed GIST, with strong cytoplasmic expression in all the tumour cells. Stained with the Leica K9 antibody, 1:100, on the Dako Autostainer with pre-treatment in the PT link with high pH buffer for 20 minutes.

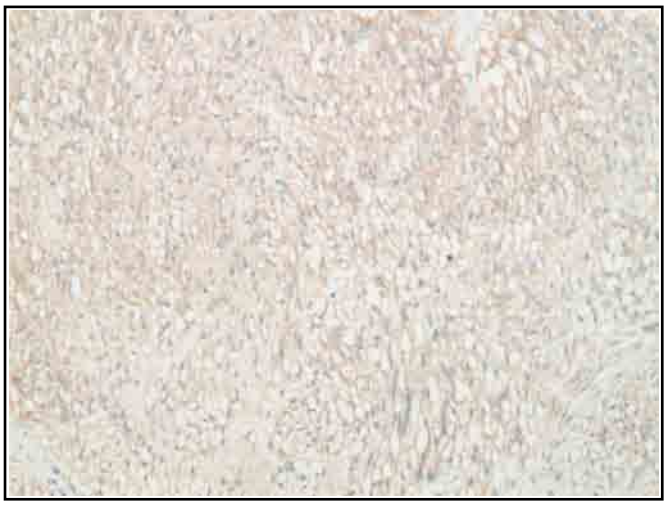


Fig 9. Poor demonstration of DOG-1 in the UK NEQAS GIST (compare to Figs 7&8). The staining is much weaker than expected, most likely due to the antibody being too dilute. Section stained with the Leica K9 antibody, 1:200, on the Leica BondMax with ER2 for 20 minutes.

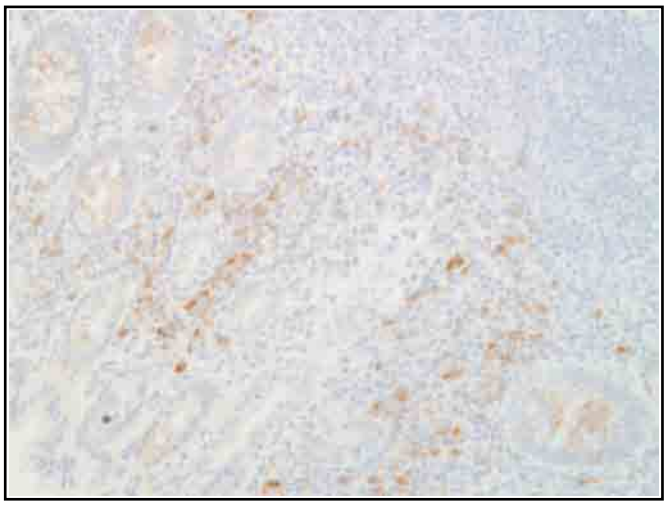


Fig 10. Poor demonstration of DOG-1 in the UK NEQAS distributed appendix. Although the example is negative for DOG-1, the section shows inappropriate non-specific staining of plasma cells. Stained with the Abcam TMEM16A antibody on the Dako autostainer with antigen retrieval in the PT link.

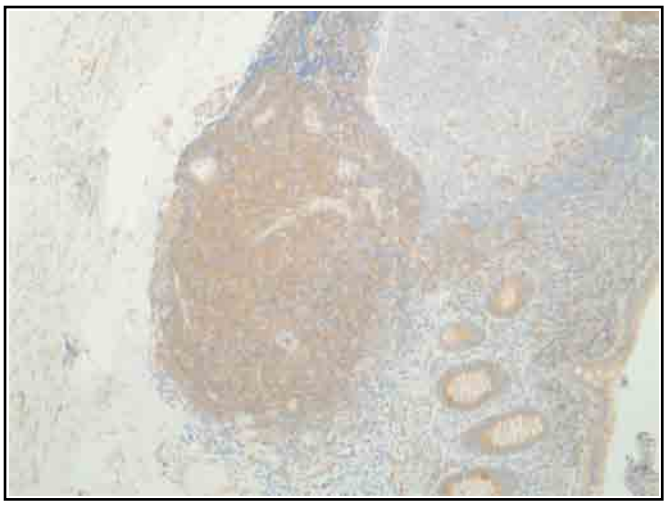


Fig 11. Sub-optimal demonstration of DOG-1 in the UK NEQAS distributed appendix, showing inappropriate and non-specific staining in the lymphocytes and background. Stained with the CellMarque SP31 pre-diluted antibody, on the Leica Bond III with ER2 for 30 minutes.

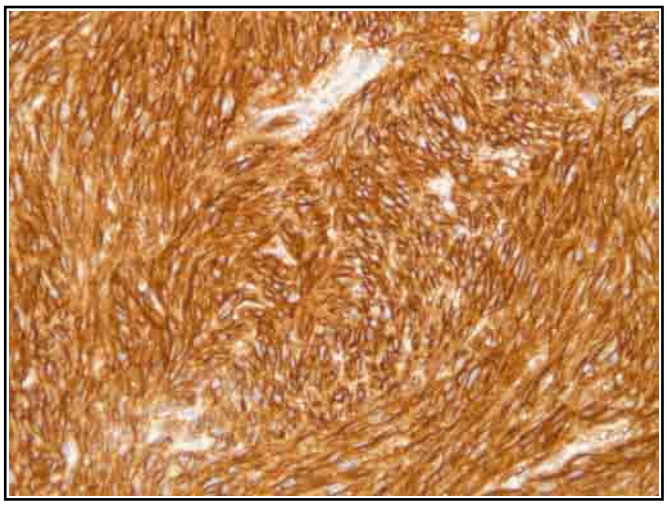
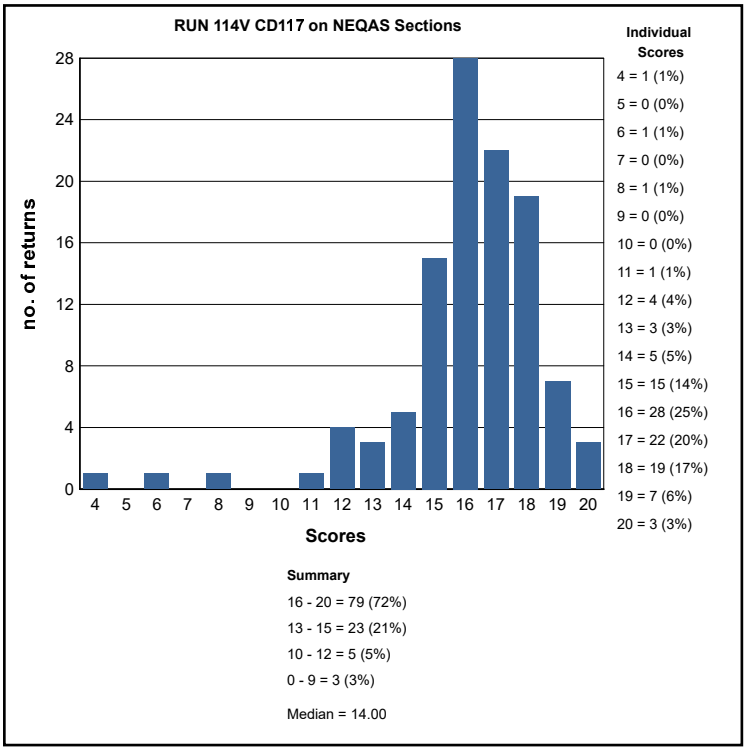
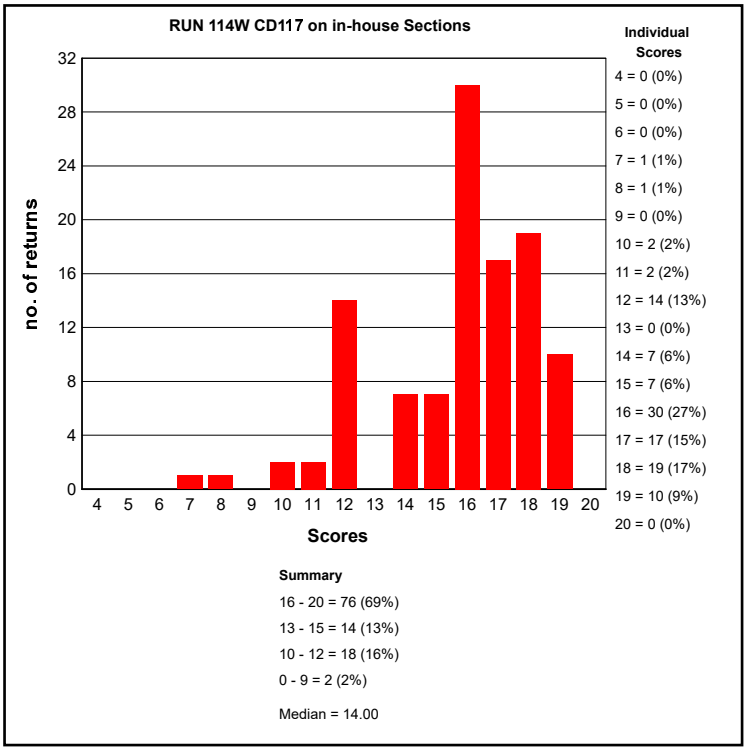
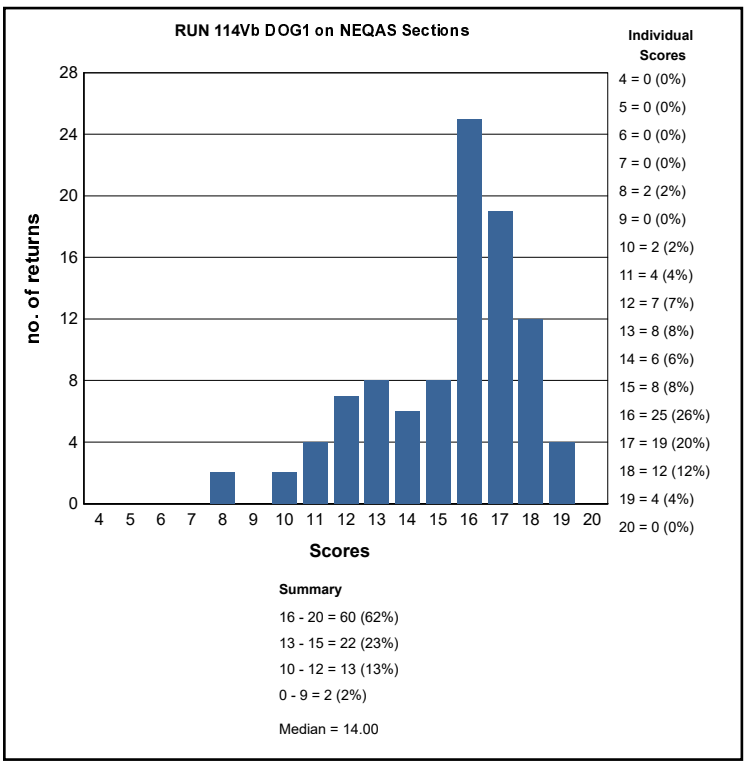


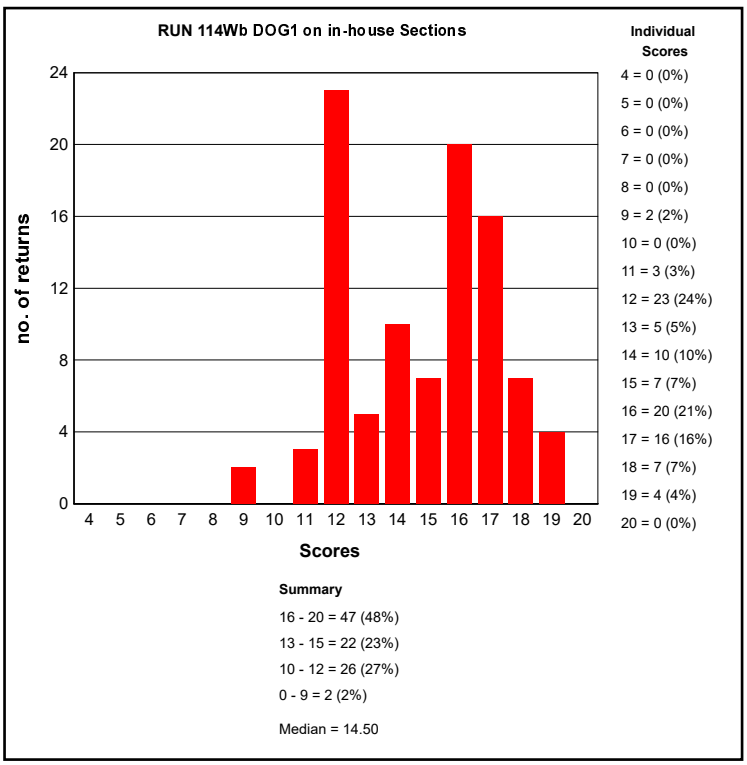
Fig 12. Good example of the in-house GIST control stained with DOG-1. The example shows strong and crisp cytoplasmic staining. Stained with the Leica K9 antibody, 1:75, on the Dako Omnis with high pH retrieval buffer.

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: 114

Primary Antibody : CD117

Antibody Details	N	%
Dako A4502 (rb poly)	92	92
Leica/Novocastra NCL-CD117 (T595)	1	100
Ventana 790-2939 (rb poly)	1	0
Cell Marque 117R/S-xx (YR145)	3	100
Ventana 790-2951 (9.7)	10	100
Epitomics AC-0029 (EP10)	1	100
Leica RTU (EP10) PA0007	2	100

Alimentary Tract Pathology Run: 114

Primary Antibody : DOG1

Antibody Details	N	%
Biocare CM 385 (1.1)	1	0
Cell Marque 244R-14/15/16 (SP31)	1	100
Cell Marque 244R-17/18 (SP31)	2	50
Leica NCL-L-DOG-1 (K9)	49	88
Leica PA0219 (K9)	21	86
Thermo RM-9132-R7 (SP31)	1	100
Other	3	100
Spring Biosciences M3311 (SP31)	1	100
Abcam TMEM16A (ab53212)	1	100
Ventana (SP31) 760-4590	15	87
Menarini MP-385-CM01/1	1	0

Alimentary Tract Pathology Run: 114

Heat Mediated Retrieval	CD117 N	CD117 %	DOG1 N	DOG1 %
Dako Omnis	1	100	2	100
Dako PTLink	14	93	14	93
Leica ER1 10 mins	1	100	0	0
Leica ER1 20 mins	4	100	5	60
Leica ER1 30 mins	4	100	0	0
Leica ER2 10 mins	1	100	0	0
Leica ER2 20 mins	20	95	26	88
Leica ER2 30 mins	7	100	4	75
None	2	0	1	100
Pressure Cooker	1	100	1	100
Ventana CC1 16mins	1	100	0	0
Ventana CC1 24mins	0	0	2	100
Ventana CC1 32mins	8	88	7	57
Ventana CC1 36mins	5	60	2	100
Ventana CC1 40mins	2	100	0	0
Ventana CC1 48mins	3	100	2	100
Ventana CC1 52mins	1	100	0	0
Ventana CC1 56mins	4	100	1	100
Ventana CC1 64mins	13	100	15	73
Ventana CC1 76mins	1	100	0	0
Ventana CC1 88mins	1	100	1	100
Ventana CC1 8mins	0	0	1	100
Ventana CC1 mild	7	86	4	100
Ventana CC1 standard	9	100	8	88
Ventana CC2 32mins	0	0	1	100
Ventana CC2 mild	0	0	1	0

Alimentary Tract Pathology Run: 114

Enzyme Mediated Retrieval	CD117 N	CD117 %	DOG1 N	DOG1 %
AS PER KIT	1	100	0	0
NOT APPLICABLE	75	93	61	85

Alimentary Tract Pathology Run: 114	CD117		DOG1	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	8	100	7	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)	2	100	2	100
Dako EnVision FLEX+ (K8002/12)	7	100	7	86
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 Intense R Detection (DS9263)	1	100	1	100
Leica Bond Polymer Refine (DS9800)	34	97	33	85
Other	2	50	3	67
Ventana iView system (760-091)	1	100	1	100
Ventana OptiView (760-700) + Amp. (7/860-099)	1	100	0	0
Ventana OptiView Kit (760-700)	23	96	18	83
Ventana UltraView Kit (760-500)	28	82	22	82

Alimentary Tract Pathology Run: 114	CD117		DOG1	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	1	100	1	100
Dako Autostainer Link 48	12	100	11	91
Dako Autostainer Plus Link	2	50	2	100
Dako Omnis	1	100	2	100
LabVision Autostainer	0	0	1	100
Leica Bond Max	12	100	13	69
Leica Bond-III	25	96	23	91
Ventana Benchmark GX	1	100	1	100
Ventana Benchmark ULTRA	35	89	28	89
Ventana Benchmark XT	21	90	16	63

Alimentary Tract Pathology Run: 114	CD117		DOG1	
Chromogen	**N**	**%**	**N**	**%**
A. Menarini Liquid Stable DAB kit	1	100	0	0
AS PER KIT	15	100	13	85
BioGenex liquid DBA (HK-124-7K)	0	0	1	100
Dako EnVision Plus kits	1	100	2	100
Dako FLEX DAB	10	90	10	90
Leica Bond Polymer Refine kit (DS9800)	34	97	34	85
Other	6	100	4	100
Ventana DAB	12	92	9	78
Ventana iview	1	100	1	100
Ventana Ultraview DAB	30	83	24	75

BEST METHODS - Gold Standard Antibody

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

CD117 - Method 1

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 pH 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

CD117 - Method 2

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

Primary Antibody: Dako A4502 (rb poly) , 15 Mins, 37 ºC Dilution 1: 200
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), RT ºC., Time 1: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins, RT ºC Prediluted

CD117 - Method 3

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

Primary Antibody:	Dako A4502 (rb poly) , 15 Mins, 21 ºC Dilution 1: 150
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 30 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, 21 ºC

CD117 - Method 4

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

Primary Antibody:	Ventana 790-2951 (9.7) , 32 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700)

BEST METHODS - Secondary Antibody

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

DOG1 - Method 1

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

Primary Antibody:	Leica NCL-L-DOG-1 (K9) , 20 Mins Dilution 1: 75
Automation:	Dako Omnis
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis, Buffer: Dako High pH TRS
EAR:	
Chromogen:	Dako FLEX DAB, Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Other , 10 Mins Prediluted

DOG1 - Method 2

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

Primary Antibody:	Leica PA0219 (K9)
Automation:	Leica Bond-III
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)

DOG1 - Method 3

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

Primary Antibody:	Leica NCL-L-DOG-1 (K9) , 32 Mins 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
Detection:	Ventana UltraView Kit (760-500) Prediluted

DOG1 - Method 4

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

Primary Antibody:	Leica NCL-L-DOG-1 (K9) , 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: High pHTRS, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 20 ºC., Time 1: 10 Mins, Time 2: 10 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 15 Mins, 20 ºC Prediluted

Lynch Syndrome/HNPCC Run 114

Keith Miller and Suzanne Parry

	Gold Standard	Second Antibody
Antigens Assessed:	MLH1	PMS2
Tissue Sections circulated:	Positive and negative colonic tumours plus normal appendix	
Number of Registered Participants:	94	
Number of Participants This Run:	80 (85%)	

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 **MLH1** (1st Antibody) and **PMS2** (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, 3, 5, 6, 7, 9, & 11))

Appendix: (Figs 1 & 7)

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 4 & 9)

Strong nuclear staining in the tumour cells.

- Strong nuclear staining in the lymphocytes and stromal cells.

Tumour with loss of MMR protein: (Figs 6 & 11)

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

Features of Suboptimal Immunostaining: (Figs 2, 4,8,10 &12)

Appendix: (Figs 2 & 8)

- 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:

- 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 **MLH1** assessment were lower than the previous time this antibody was assessed, with 66% of labs achieving an acceptable pass, and a further 21% of participants receiving a borderline score (10-12/20), and therefore an overall pass of 87%. There was a fail rate of 14%, which was higher than the previous assessment for MLH1 which was 6% (Run 112). However, similarly to previous runs, the main reason for a failed assessment was due to either weak staining or inappropriate non-specific staining. The Ventana M1 clone was the most popular choice of antibody, and showed a pass rate of 67%. The Leica/Novocastra ES05 clone was also popular , used by 14 labs and showed a pass rate of 71%.

The **PMS2** assessment showed a similar pass rate to that of the MLH1 antibody in this assessment run: 75% of participants received an acceptable pass, a similar result to the previous run. The fail rate for MLH1 was higher than previous run up to 14% compared to 6%. Conversely the PMS 2 failure rate was the same from the previous run. Weak staining was the main reason for failure or borderline scores (depending on the severity). The Ventana EPR3947 clone was the most popular choice of PMS2 antibody used in this assessment by 33 labs, showed a pass rate of 79%. The BDPharmingen A16-4 and the DakoEP51 clones were also commonly used, and they showed pass rates of 73% and 60% respectively.

In-House Control Tissue Recommendations

The majority of laboratories submitted appendix tissue as their in house control sample. This is an ideal control to demonstrate the intensity of nuclear staining, and to highlight the gradation of intensity of epithelial cell staining down the crypts. However, better still would be to use an appendix alongside a colon tumour of known mismatch marker expression: This is a feedback comment often given to participants from our team of assessors. Furthermore, to include both positive and negative tumours along with the appendix would be the ideal multi tissue control as this would highlight any false positivity (in the negative colonic tumour) and also weak or under staining in both the appendix and positive colonic tumours. Several laboratories did submit excellent examples of this latter type of composite control; 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.

References

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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.

Selected Images showing Optimal and Sub-optimal Immunostaining

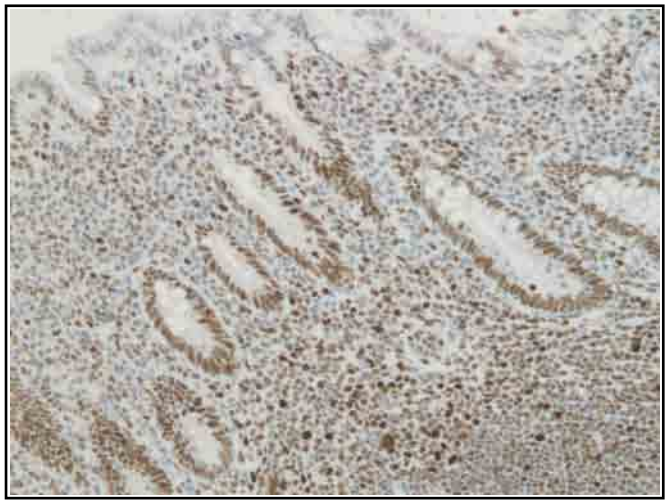


Fig 1. Optimal demonstration of MLH1 in the UK NEQAS distributed appendix. Strong MLH1 staining of the epithelial and stromal cells, with fading of staining intensity towards the luminal surface of the epithelial crypts. Stained with the Novocastra ES05 antibody, 1:50, on the Leica BondMax, ER2 30 minutes.

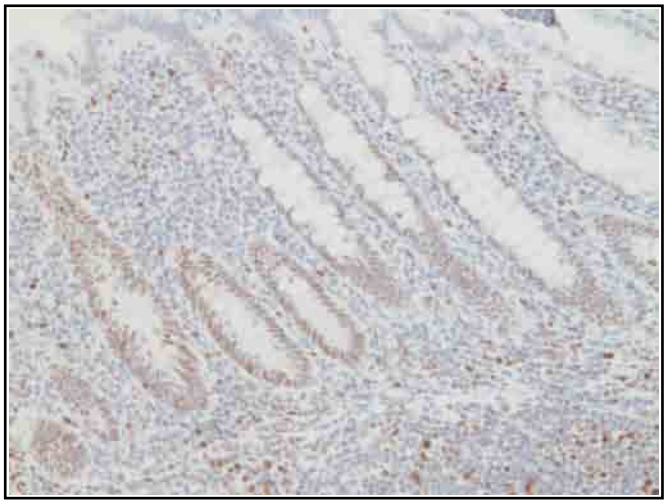


Fig 2. Slightly weak demonstration of MLH1 in the UK NEQAS distributed appendix (compare to Fig 1), although most of the lymphocytes and epithelial crypts are still demonstrated. Similar protocol as Fig 1, but using ER2 for 20 minutes and a shorter antibody incubation time of 15 minutes. Extending both of these factors may improve the staining intensity.

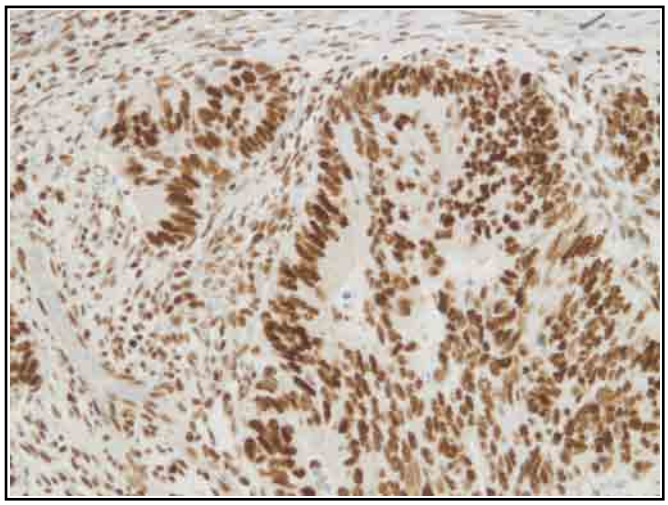


Fig 3. Good demonstration of MLH1 in the UK NEQAS distributed positive colonic tumour, showing strong staining in both the tumour cells and the stromal cells and lymphocytes. Stained using the Dako ES05 antibody, 1:50, on the Dako Autostainer with PT link retrieval for 20 minutes.

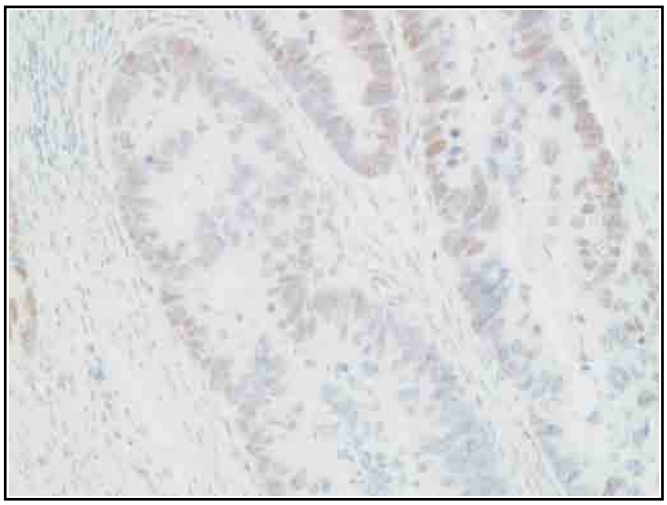


Fig 4. Unacceptable demonstration of MLH1 in the UK NEQAS distributed positive colonic tumour (compare to Fig 3). The staining is weak and many of the tumour cells are not staining at all. Stained with the Leica RTU ES05 antibody, on the Leica Bond III with ER2 for 30 minutes.

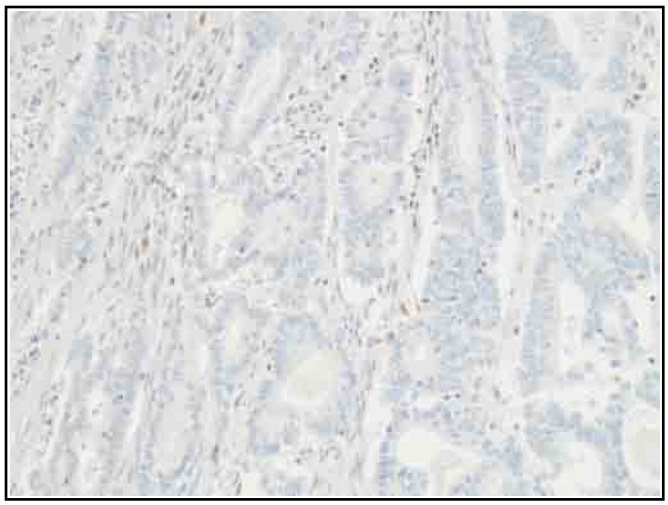


Fig 5. Acceptable demonstration of MLH1 in the UK NEQAS negative colonic tumour. As expected, the tumour cells are negative, while the intratumoural lymphocytes and stromal cells are strongly stained. Same protocol as Fig 3.

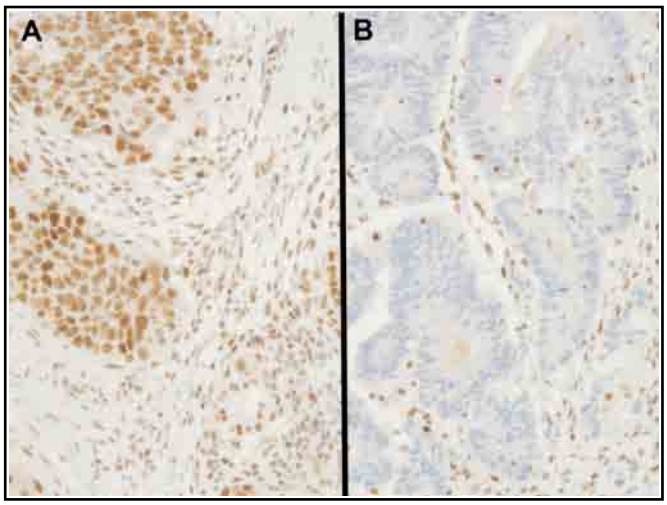


Fig 6. Good example of an in house control for MLH1 submitted for assessment. The slide consisted of both positive and negative-expressing tumours. Stained with the Dako ES05 antibody, 1:25, on the Leica Bond III with ER2 60 mins.

Selected Images showing Optimal and Sub-optimal Immunostaining

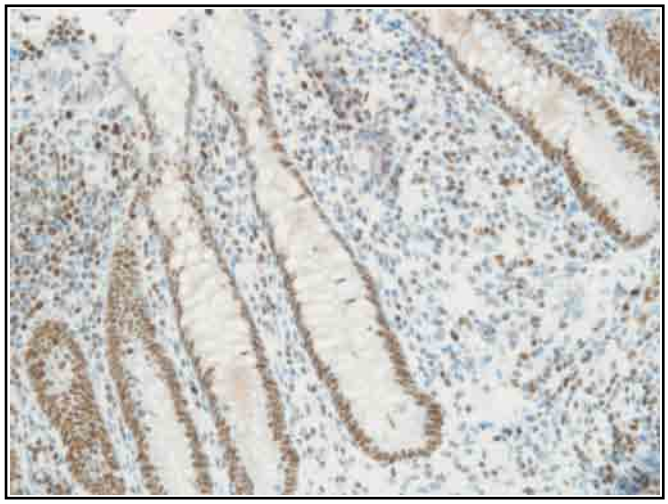


Fig 7. Optimal demonstration of PMS2 in the UK NEQAS distributed appendix. Strong PMS2 staining of the epithelial and stromal cells, with fading of staining intensity towards the luminal surface of the epithelial crypts. Stained with the Ventana EPR3947 antibody on the Benchmark XT, CC1 antigen retrieval for 80 minutes.

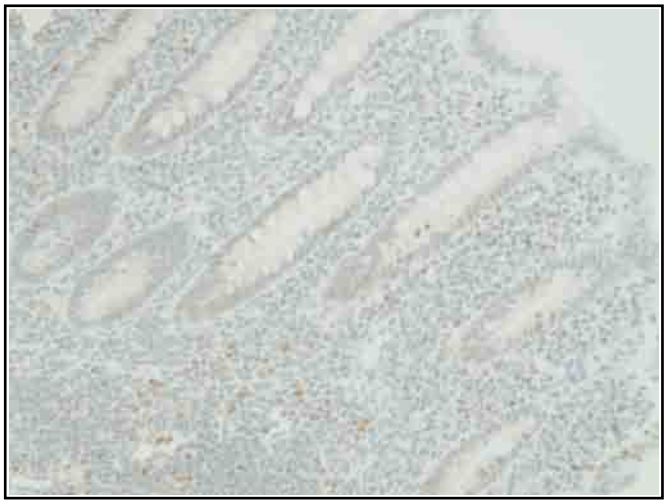


Fig 8. Poor demonstration of PMS2 on the UK NEQAS distributed appendix. The image shows weak and diffuse staining (compare to Fig 7). Stained with the Ventana EPR3947 pre-diluted antibody on the Benchmark XT with CC2 antigen retrieval for 64 minutes and UltraView detection.

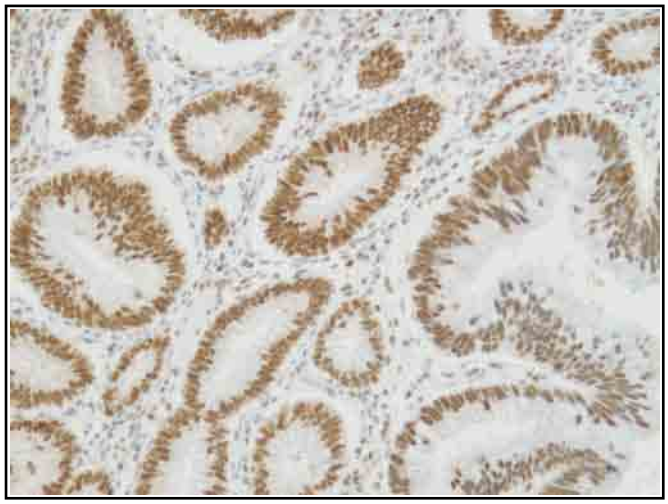


Fig 9. Optimal demonstration of PMS2 on the UK NEQAS distributed PMS2 colonic tumour, showing strong nuclear staining in both the tumour cells and the stromal cells. Stained with the Dako EP51 antibody, 1:40, on the Dako Autostainer with pretreatment in the PT link with high pH buffer for 67 minutes.

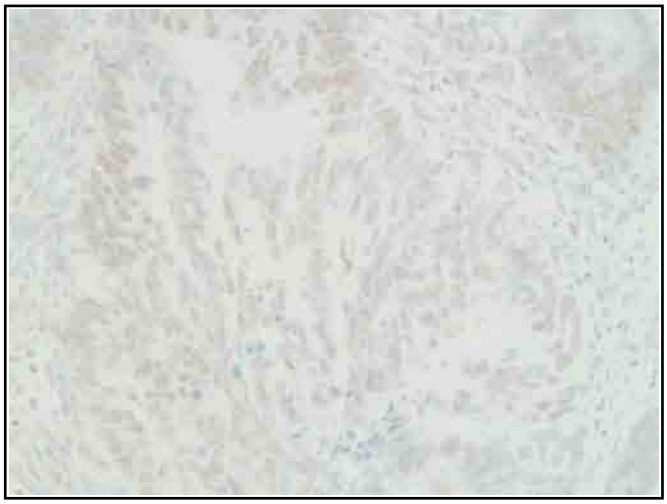


Fig 10. Unacceptable demonstration of PMS2 in the UK NEQAS distributed positive colonic tumour (compare to Fig 9). The staining is weak and diffuse, with many of the tumour cells not staining at all. Same protocol as Fig 8.

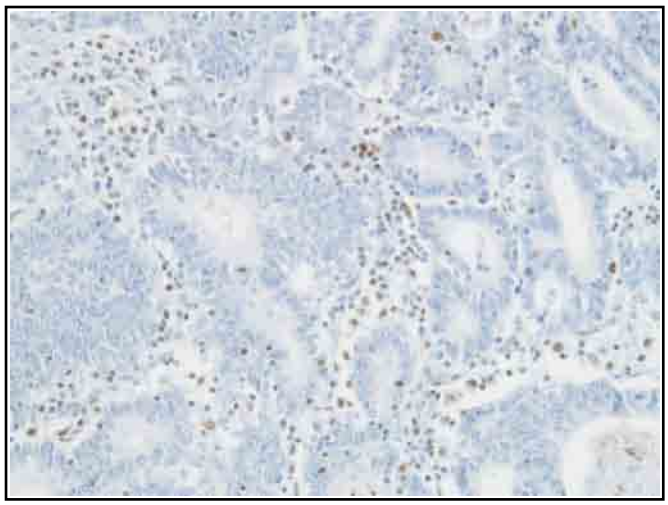


Fig 11. Optimal demonstration of PMS2 on the UK NEQAS distributed negative PMS2 colonic tumour. Tumour is unstained, whilst the intratumoural lymphocytes and stromal cells are strongly stained. Stained using the Ventana EPR3947 antibody on the Benchmark XT, CC2 64 minutes and Optiview detection.

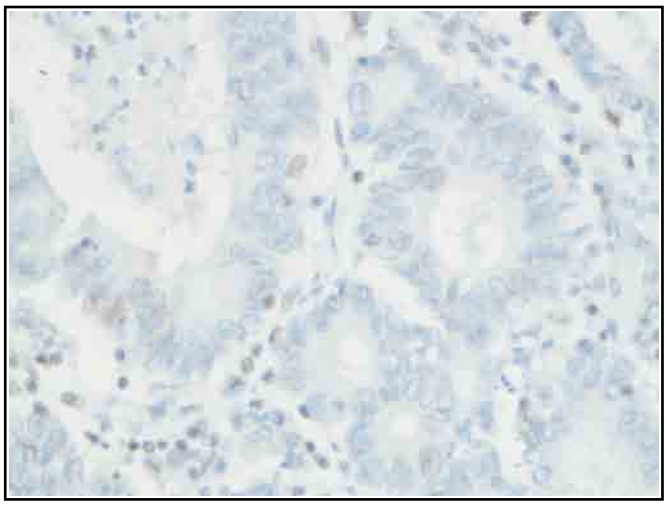
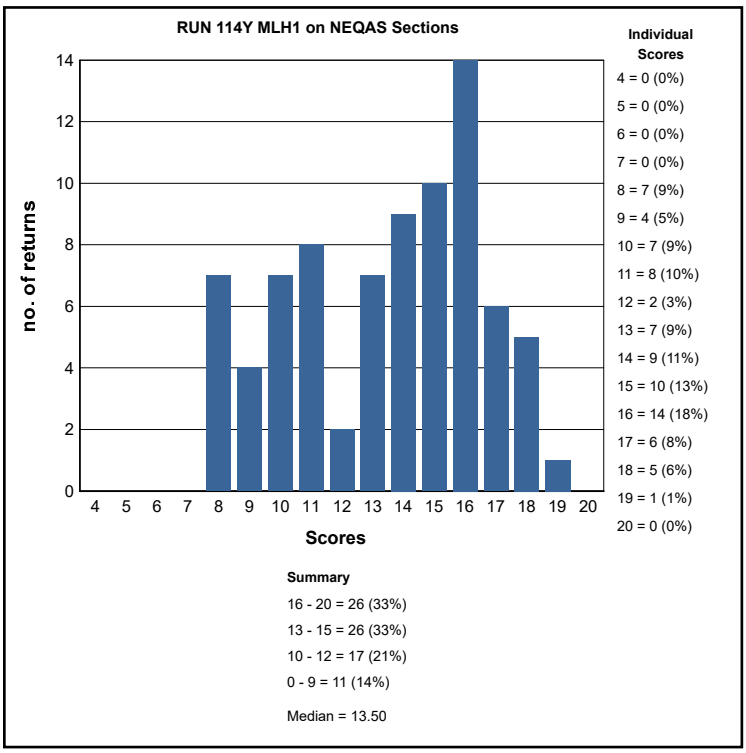
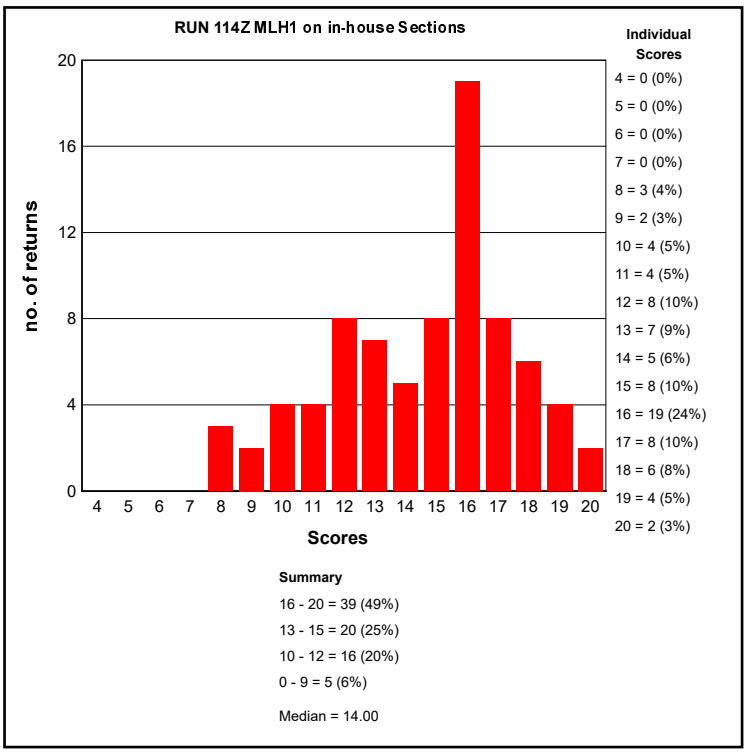
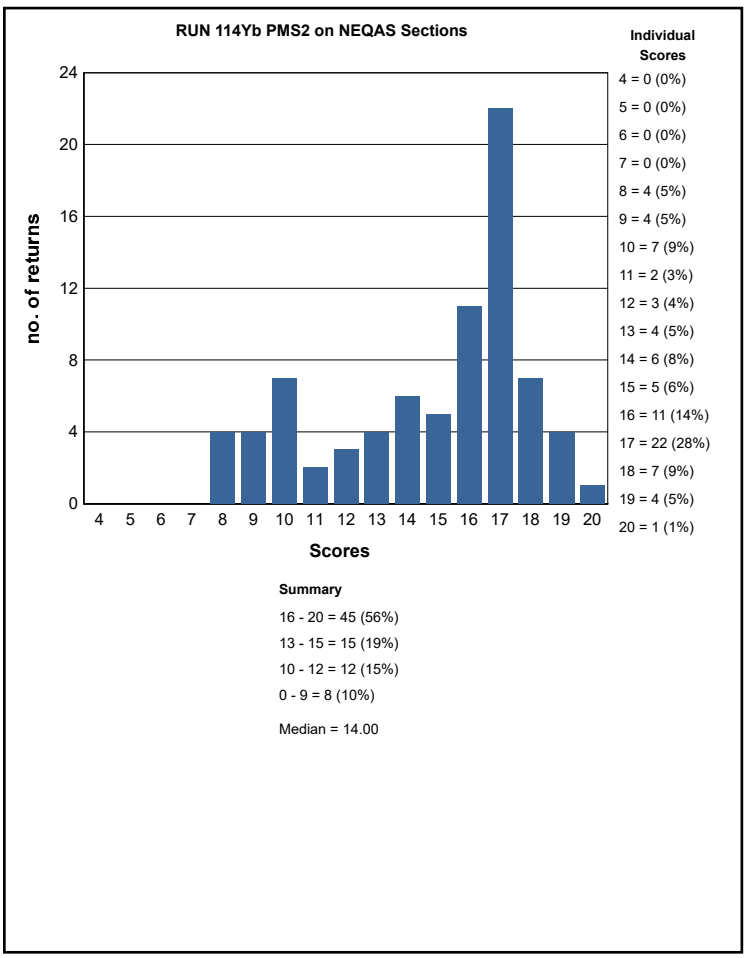


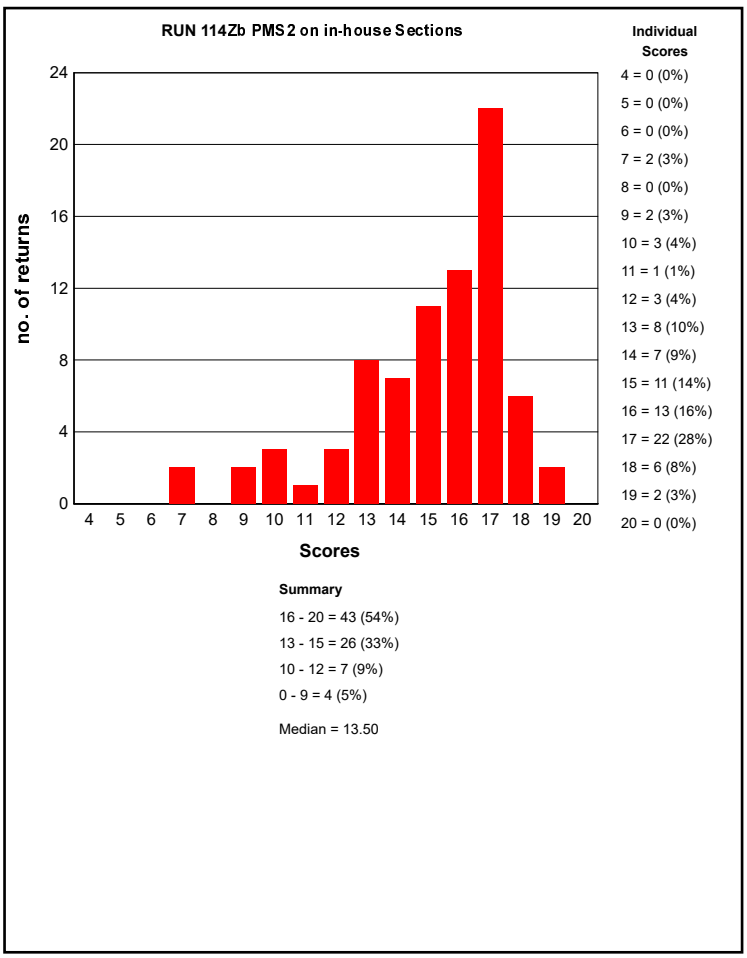
Fig 12. Unacceptable false positive staining seen in the known PMS2 negative colonic tumour. This may have been caused by inappropriate antigen retrieval. Stained with the CellMarque 16 antibody, 1:40, with pre-treatment in the microwave and manual staining using the Dako Envison kit.

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: 114

Primary Antibody : MLH1

Antibody Details	N	%
Other	2	100
BD Pharmingen (G168-15)	4	50
Biocare medical CM/PM 220 (G168-15)	1	0
Novocastra NCL-L-MLH1 (ES05)	14	71
Cell Marque CMAx/Cx (G168-728)	1	0
Dako M3640 (ES05)	9	89
Leica Bond RTU PA0610 (ES05)	4	0
Dako Flex RTU IR079/IS079 (ES05)	12	67
Ventana 790-4535 (M1)	33	67

Primary Antibody : PMS2

Antibody Details	N	%
BD Bio/Pharmingen 556415 (A16-4)	11	73
Cell Marque 288R -17/18 (EPR3947)	3	67
Leica/Novoca NCL-L-PMS2 (MOR4G)	1	0
Other	2	0
Ventana 760-4531 (EPR3947)	33	79
Cell Marque 288M -16 (MRQ28)	3	33
Dako M3647 (EP51)	15	93
Dako RTU FLEX IR087 (EP51)	10	70
Epitomics AC-0049 (EP51)	1	100

HNPCC Run: 114

Heat Mediated Retrieval	MLH1 N	MLH1 %	PMS2 N	PMS2 %
Dako Omnis	2	50	2	0
Dako PTLink	8	88	8	88
Leica ER1 20 mins	2	50	0	0
Leica ER1 30 mins	1	0	0	0
Leica ER2 20 mins	6	67	7	86
Leica ER2 30 mins	9	67	8	75
Leica ER2 40 mins	6	100	9	100
Microwave	2	0	2	0
Other	1	100	0	0
Ventana CC1 24mins	1	100	0	0
Ventana CC1 32mins	4	50	0	0
Ventana CC1 40mins	5	40	1	100
Ventana CC1 48mins	2	50	3	67
Ventana CC1 56mins	5	100	2	50
Ventana CC1 64mins	18	56	15	73
Ventana CC1 72mins	1	0	4	75
Ventana CC1 80mins	1	100	2	100
Ventana CC1 88mins	1	0	1	100
Ventana CC1 92mins	1	0	10	60
Ventana CC1 extended	0	0	1	100
Ventana CC1 standard	4	100	2	100
Ventana CC2 64mins	0	0	1	0
Ventana CC2 80mins	0	0	1	100

HNPCC Run: 114

Enzyme Mediated Retrieval	MLH1 N	MLH1 %	PMS2 N	PMS2 %
AS PER KIT	3	67	2	50
NOT APPLICABLE	42	62	42	74

HNPCC Run: 114	MLH1		PMS2	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	3	100	2	100
Dako EnVision FLEX (K8000/10)	1	100	1	0
Dako EnVision FLEX+ (K8002/12)	6	83	5	60
Dako Envision HRP/DAB (K5007)	1	0	1	0
Dako Envision+ HRP mouse K4004/5/6/7	1	100	1	100
Leica Bond Polymer Define (DS9713)	0	0	1	100
Leica Bond Polymer Refine (DS9800)	23	70	22	91
None	0	0	1	100
Other	2	50	2	50
Ventana OptiView (760-700) + Amp. (7/860-099)	7	43	10	50
Ventana OptiView Kit (760-700)	30	63	26	81
Ventana UltraView Kit (760-500)	5	60	4	50

HNPCC Run: 114	MLH1		PMS2	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	0	0	1	100
Dako Autostainer Link 48	6	83	6	83
Dako Autostainer plus	1	100	1	100
Dako Autostainer Plus Link	1	100	1	100
Dako Omnis	2	50	2	0
Leica Bond Max	10	60	8	63
Leica Bond-III	15	80	16	100
None (Manual)	2	0	2	0
Ventana Benchmark GX	2	100	2	100
Ventana Benchmark ULTRA	31	61	30	67
Ventana Benchmark XT	10	50	10	80

HNPCC Run: 114	MLH1		PMS2	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	13	77	14	86
Dako EnVision Plus kits	2	50	2	0
Dako FLEX DAB	6	83	6	83
Dako REAL EnVision K5007 DAB	2	0	2	0
Leica Bond Polymer Refine kit (DS9800)	24	71	23	91
Other	7	57	7	86
Ventana DAB	18	61	18	61
Ventana Ultraview DAB	8	50	7	57

BEST METHODS - Gold Standard Antibody

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

MLH1 - Method 1

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

Primary Antibody: Novocastra NCL-L-MLH1 (ES05) , 32 Mins, 37 ºC Dilution 1: 1/50
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: AS PER KIT

MLH1 - Method 2

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

Primary Antibody: Ventana 790-4535 (M1)
Automation: Ventana Benchmark ULTRA
Method: Ventana Optiview
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 standard
EAR:
Chromogen: Ventana Ultraview DAB
Detection: Ventana UltraView Kit (760-500)

MLH1 - Method 3

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

Primary Antibody:	Dako M3640 (ES05) Dilution 1: 80
Automation:	Leica Bond Max
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)

MLH1 - Method 4

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

Primary Antibody:	Dako Flex RTU IR079/IS079 (ES05)
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	AS PER KIT
HMAR:	Dako PTLink
EAR:	
Chromogen:	AS PER KIT
Detection:	AS PER KIT

BEST METHODS - Secondary Antibody

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

PMS2 - Method 1

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

Primary Antibody:	BD Bio/Pharmingen 556415 (A16-4) , 60 Mins Dilution 1: 300
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)

PMS2 - Method 2

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

Primary Antibody:	Dako M3647 (EP51) , 30 Mins, rt ºC Dilution 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

PMS2 - Method 3

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

Primary Antibody:	Ventana 760-4531 (EPR3947) , 60 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 extended
EAR:	NOT APPLICABLE
Chromogen:	Other
Detection:	Ventana OptiView Kit (760-700)

PMS2 - Method 4

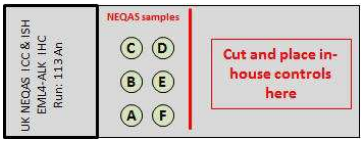
Participant scored 18/20 (UK NEQAS Slide) and 18/20 (In House slide) using this method.

Primary Antibody:	Dako M3647 (EP51) , 60 Mins, 18 ºC Dilution 1: 40
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer, PH: 7
HMAR:	Dako PTLink, Buffer: Target retrieval solution (High pH), PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 18 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Other , 15 Mins, 18 ºC Prediluted

Sample and Slide Distribution

Antibody Assessed	ALK
Samples Circulated	Composite slide (see table below)
Number Registered Participants/Submitted	50 (78%)

Fig 1 & Table 1 below illustrate the positioning of the distributed samples along with their pre-tested IHC status.



Sample code	Sample	IHC status (Roche D5F3)
A	NSCLC adenocarcinoma	+ve
B	NSCLC adenocarcinoma	-ve
C	Appendix	+ve in ganglion cells
D	**Cell line**: 100% adenocarcinoma	-ve
E	**Cell line**: Adenocarcinoma	+ve
F	Normal Lung	-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

Assessment Criteria

Same slide NEQAS & In-house controls

The UK NEQAS ICC & ISH quality control samples were placed and orientated as shown fig 1 and Table 1. The distributed slide (Fig 1) consisted of an area for participants to also cut and place their in-house control material alongside the NEQAS sample. This set-up ensured:

- Both the NEQAS & in-house controls had the same methodology applied
- Allowed easier comparison of both NEQAS & in-house controls during assessments
- Provides a more cost-effective EQA, as less reagent is used by the participant

Interpretation criteria incorporating staining intensity

The assessments consisted of assessors providing their feedback on whether each of the distributed samples were either ALK IHC positive or negative (+ve'/'-ve'). Alongside this the assessors also provided feedback on the observed intensity of staining including 3+ (high), 2+ (medium) and 1+ (low) intensity. Assessors were therefore asked to score each of the ALK IHC positive samples as: +ve (3+) OR +ve (2+) OR +ve (1+). This allowed for more informative feedback on the intensity of staining on the ALK positive samples and also reflected the different scoring systems employed by participants (see: Scoring Systems Employed by Participants and Table 3). There is also evidence that ALK IHC along with intensity (and H-score) could be used as an affective screening tool[13,14], but this is still a debatable subject requiring further clinical evidence and is quite possible related to the 'detection system' employed.

Assessment scoring guidelines

- A team of four assessors independently but simultaneously scored all of the returned slides, and provided interpretation and feedback on technical issues.
- Each of the 4 assessors also provided a possible score out of 5, with marks summed together to give a final score out of 20:
- During this assessments all participants slides were scored twice to make sure the panel were consistent in their scoring.

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.	

Assessment Results & Discussion

Features Of Acceptable Staining: (Figs 1,3, 4A, 6, 8 9 & 10)

- Moderate to strong cytoplasmic staining of the ganglion

cells in the appendix

- Moderate to strong granular staining of the positive tumour sample (C)
- Moderate to strong granular staining of the positive tumour cell line samples (E and F). The ALK negative cells within the mixed cell line (sample F) should remain unstained.
- No staining in the negative tumour sample (B)
- No staining in the normal lung sample (D)
- No background or inappropriately localised staining

Features Of Suboptimal or Unacceptable Staining: (Figs 2,4B,5 & & 7)

- Weak or no staining of the ganglion cells in the appendix
- False negative or absence of staining of known ALK positive tumour cells
- Non-specific staining, including background and excessive tyramide staining

Pass Rates

There was an increase in the number of participating labs compared to the previous assessment; from 47 to 50 labs. The pass rates were lower 58% compared with over 80% in previous run, for an excellent pass on the Neqas distributed tissue, and 6 labs failed on the Neqas material compared to no laboratories failing in the previous run. This was mainly due to either false negative staining in the positive tumour or excessive tyramide deposit.

The most popular antibody of choice was the Ventana/Roche (D5F3) with 80% of participants using this antibody and showed an 85% acceptable pass rate. The second most popular antibody was the Novocastra NCL-ALK (5A4) with 10% of laboratories using this clone with an acceptable pass rate of 60%.

In-house Control Results

All participants taking part in this assessment, also submitted in-house controls with the majority placing their samples alongside the NEQAS sample. Only 58% (n=29) of participants were assessed as having an acceptable/excellent level of staining and good choice of in-house control, with 40% (n=20) attaining a borderline score and only 2% (n=1) having an unacceptable result. It was encouraging to see most laboratories are including a composite control. There was still 2 laboratories using a lymphoma control in the lung setting. **Lymphoma** is not recommended as a control in the lung setting.

Participating labs are provided with instructions on the recommendations set out by UK NEQAS ICC for the choice of in-house controls. (See recommendations below). Any labs that do not provide this composite control tissue is scored a maximum borderline mark of 12/20.

ALK IHC Control Recommendation

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

Appendix may also be used alongside the lung tumour controls (fig 6 H-I) to gauge both the sensitivity & specificity, but should not be used 'alone' as a single control.

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).

- 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.

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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.

Selected Images showing Optimal and Sub-optimal Immunostaining

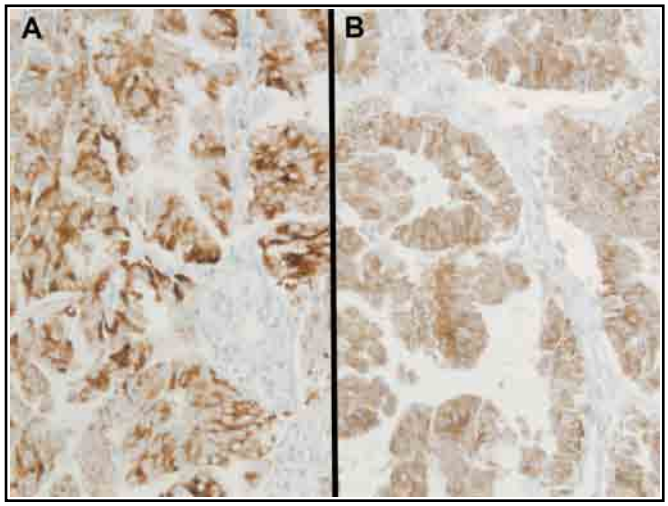


Fig 1. Two good examples from the UK NEQAS distributed ALK-positive sample 'A'. (A & B) Both show strong membranous and cytoplasmic staining of the neoplastic cells. Stained using (A) Ventana D5F3 assay with the recommended protocol, and (B) Novocastra 5A4 antibody, 1:100, on the Dako Autostainer with retrieval in the PT link (high pH buffer).

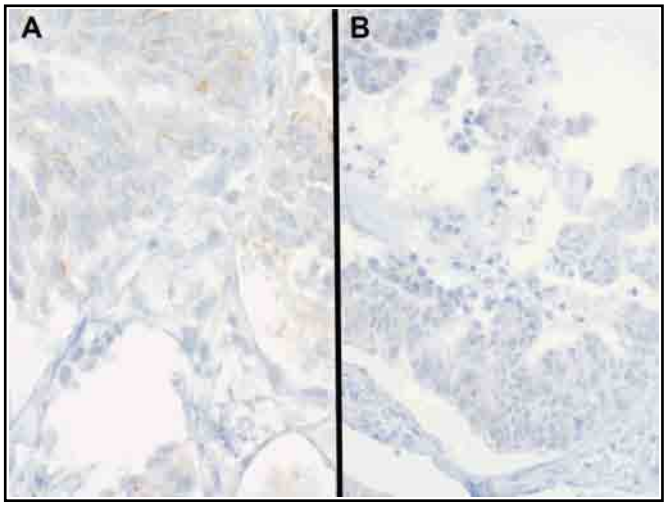


Fig 2. Sub-optimal staining on the UK NEQAS distributed ALK-positive tumour sample 'A' (compare with Fig 1). The staining is much weaker than expected. Both sections stained on the Ventana platform using (A) Ventana D5F3 assay, and (B) Novocastra 5A4 antibody, 1:50, and OptiView detection with no additional amplification kit.

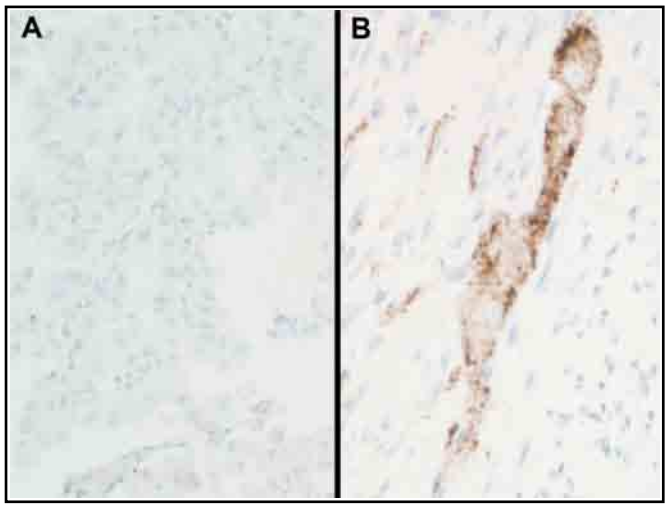


Fig 3. Acceptable ALK IHC result in the UK NEQAS distributed (A) negative tumour sample 'B' and (B) appendix sample 'C'. The appendix shows the expected positive staining of ganglion cells and axons. Both sections stained using the Ventana D5F3 assay with the recommended protocol.

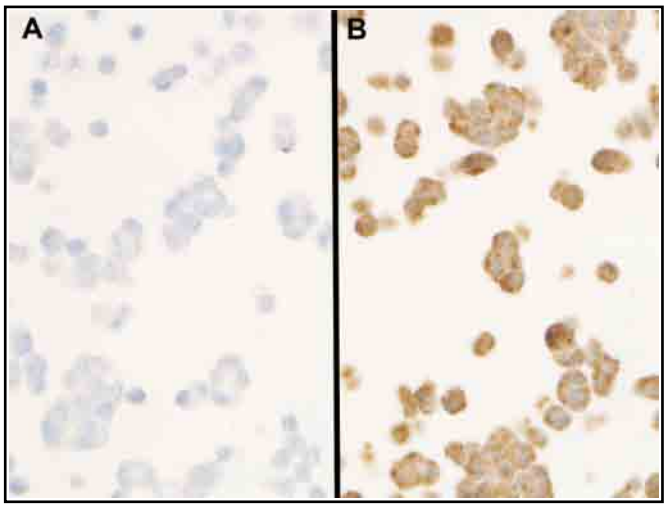


Fig 4. Two examples of ALK staining in the UK NEQAS distributed negative cell line (sample 'D'). (A) Expected ALK-negative sample, whereas (B) shows excessive non-specific staining, and was deemed unacceptable. (A) Stained using the Ventana D5F3 assay and (B) No methodology provided.

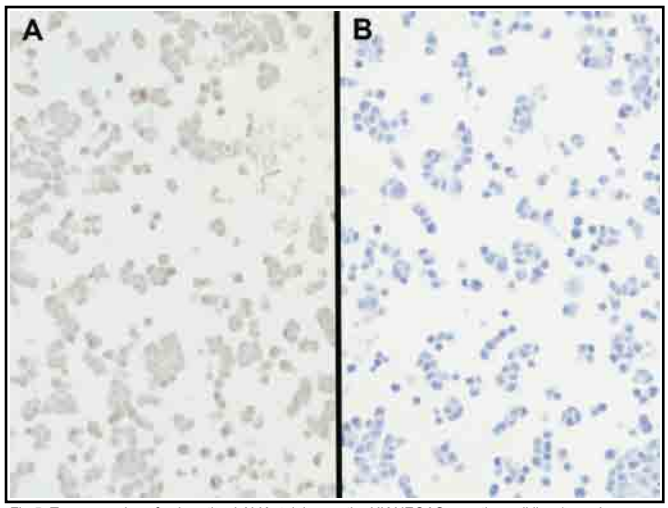


Fig 5. Two examples of sub-optimal ALK staining on the UK NEQAS negative cell line (sample 'D'). (A) False-positive non-specific staining, while (B) shows excessive counterstain.

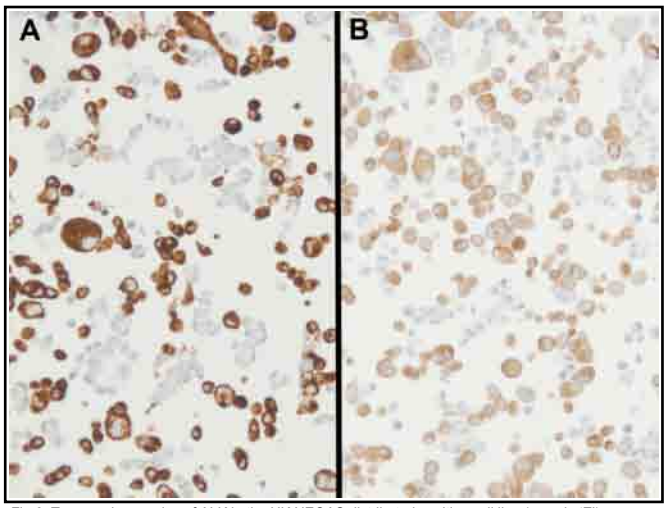


Fig 6. Two good examples of ALK in the UK NEQAS distributed positive cell line (sample 'E'). Both sections show strong membranous and cytoplasmic staining in the expected proportion of neoplastic cells. Stained (A) with the Ventana D5F3 assay and (B) with the Novocastra 5A4 antibody, 1:100, Dako Autostainer with high pH buffer in the PT link.

Selected Images showing Optimal and Sub-optimal Immunostaining

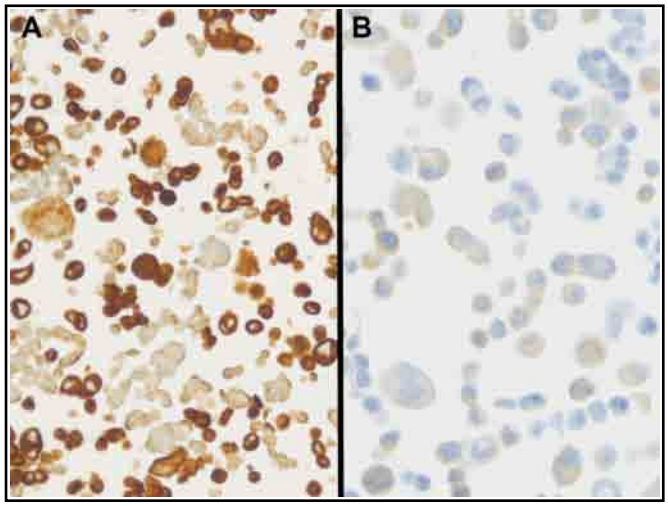


Fig 7. Two examples of sub-optimal ALK staining on the UK NEQAS positive cell line (sample E): (A) Excessive non-specific staining, while in (B) the staining is weaker with fewer cells staining positive than expected.

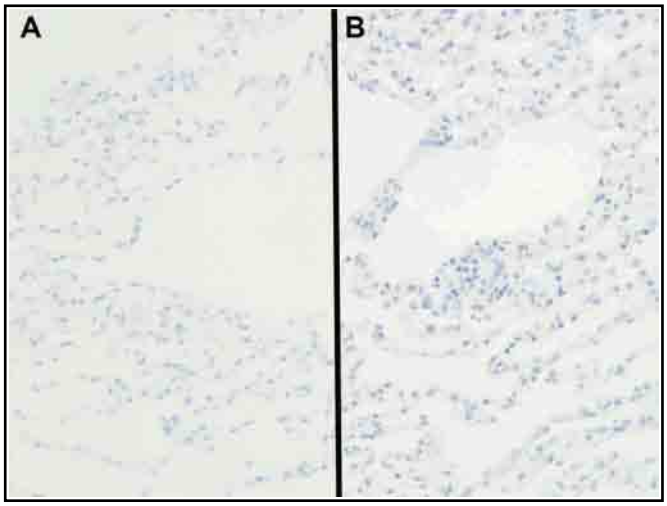


Fig 8. Two examples of the expected ALK negative result in the UK NEQAS distributed normal lung tissue. (A) stained with the Ventana D5F3 assay, and (B) stained with the Novocastra 5A4 antibody on the Dako Autostainer.

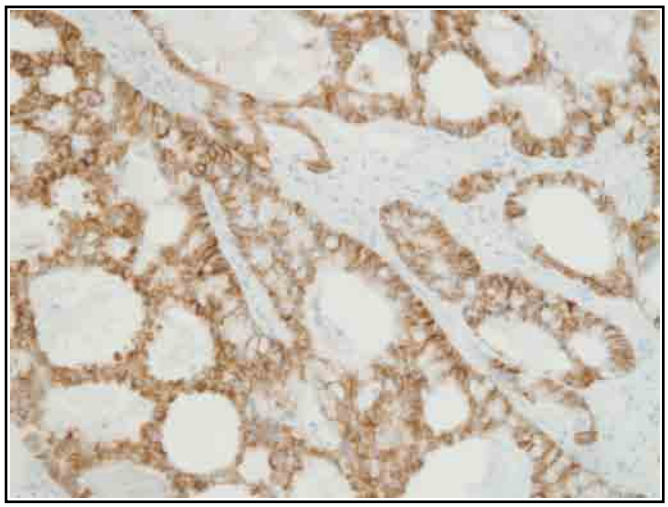


Fig 9. Good in-house NSCLC ALK positive control (see also Fig 10 for the accompanying negative tumour and appendix). Stained using the Ventana D5F3 assay on a Benchmark XT with the recommended protocols.

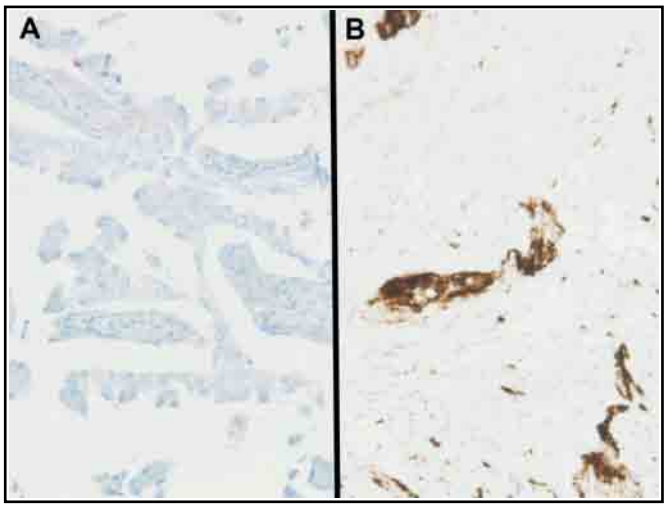
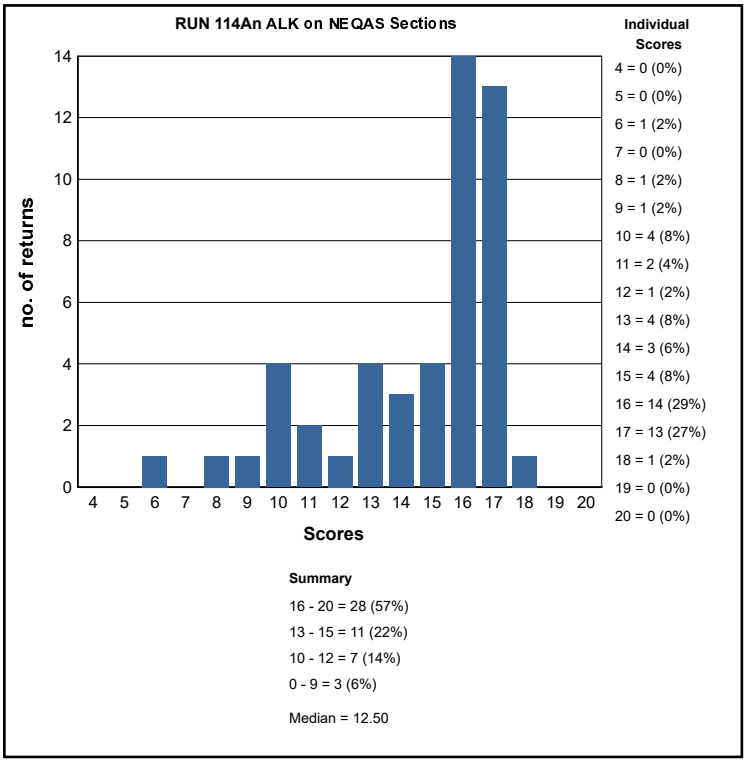
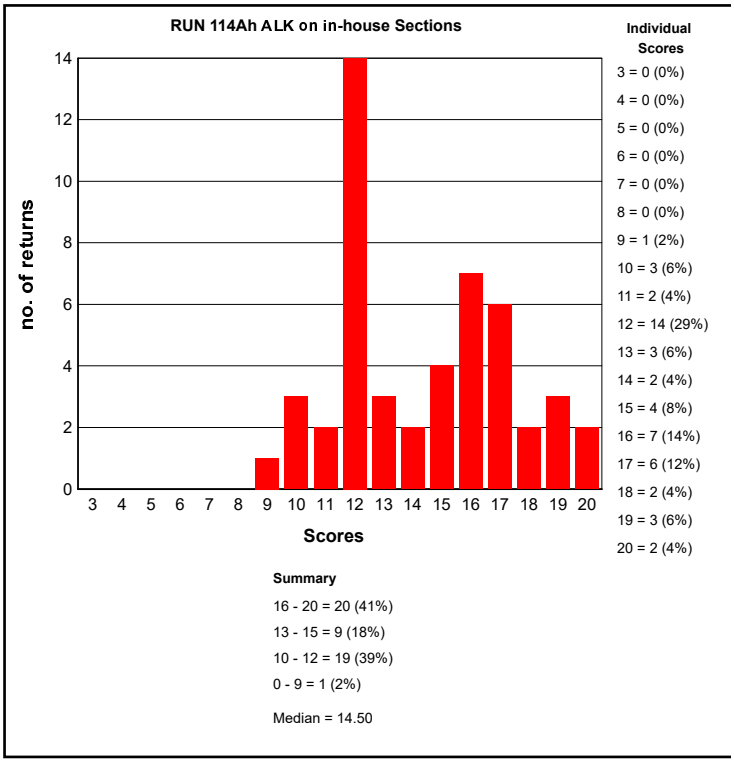


Fig 10. Good in-house (A) NSCLC ALK negative tumour and (B) appendix control (see also Fig 9 for the accompanying ALK positive tumour. Same protocol as Fig 9.

GRAPHICAL REPRESENTATION OF PASS RATES





ANTIBODIES AND OTHER TECHNICAL PARAMETERS EMPLOYED BY PARTICIPANTS IN THE ALK NSCLC 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.

ALK NSCLC Run: 114

Primary Antibody	N	%
Abcam (5A4)	1	100
Cell Sign. Tech. (D5F3)	3	67
Dako M7195 (ALK1)	1	100
Novocastra NCL-ALK (5A4)	5	60
Ventana/Roche (D5F3)	38	84

ALK NSCLC Run: 114

Automation	N	%
Dako Autostainer Link 48	3	100
Leica Bond Max	1	100
Ventana Benchmark GX	3	67
Ventana Benchmark ULTRA	15	80
Ventana Benchmark XT	26	81

ALK NSCLC Run: 114

Heat Mediated Retrieval	N	%
Dako PTLink	2	100
Leica ER2 20 mins	1	100
None	1	100
Ventana CC1 32mins	1	100
Ventana CC1 48mins	1	0
Ventana CC1 64mins	1	0
Ventana CC1 88mins	1	100
Ventana CC1 8mins	1	100
Ventana CC1 92mins	32	84
Ventana CC1 extended	3	33
Ventana CC1 standard	3	100

ALK NSCLC Run: 114

Detection	N	%
AS PER KIT	1	100
Dako EnVision FLEX (K8000/10)	1	100
Leica Bond Polymer Refine (DS9800)	1	100
Other	1	100
Ventana OptiView (760-700) + Amp. (7/860-099)	26	81
Ventana OptiView Kit (760-700)	15	73
Ventana UltraView Kit (760-500)	1	100

ALK NSCLC Run: 114

Enzyme Retrieval	N	%
NOT APPLICABLE	19	84
Ventana Protease	2	100

ALK NSCLC Run: 114

Chromogen	N	%
AS PER KIT	17	88
Dako EnVision Plus kits	1	100
Dako FLEX DAB	1	100
Leica Bond Polymer Refine kit (DS9800)	1	100
NOT APPLICABLE	1	0
Other	1	100
Ventana DAB	22	77
Ventana Ultraview DAB	4	75

BEST METHODS

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

ALK - Method 1

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

Primary Antibody:	Ventana/Roche (D5F3) Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	
Main Buffer:	
HMAR:	Ventana CC1 extended
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099)

ALK - Method 2

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

Primary Antibody:	Ventana/Roche (D5F3) , 12 Mins Prediluted
Automation:	Ventana Benchmark XT
Method:	
Main Buffer:	
HMAR:	Ventana CC1 92mins
EAR:	
Chromogen:	AS PER KIT
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099) , 12 Mins

ALK - Method 3

Participant scored 17/20 (UK NEQAS Slide) and 15/20 (In House slide) using this method.

Primary Antibody:	Novocastra NCL-ALK (5A4) , 30 Mins Dilution 1: 100
Automation:	Dako Autostainer Link 48
Method:	
Main Buffer:	
HMAR:	Dako PTLink
EAR:	NOT APPLICABLE
Chromogen:	Dako EnVision Plus kits
Detection:	Dako EnVision FLEX (K8000/10) , 10 Mins

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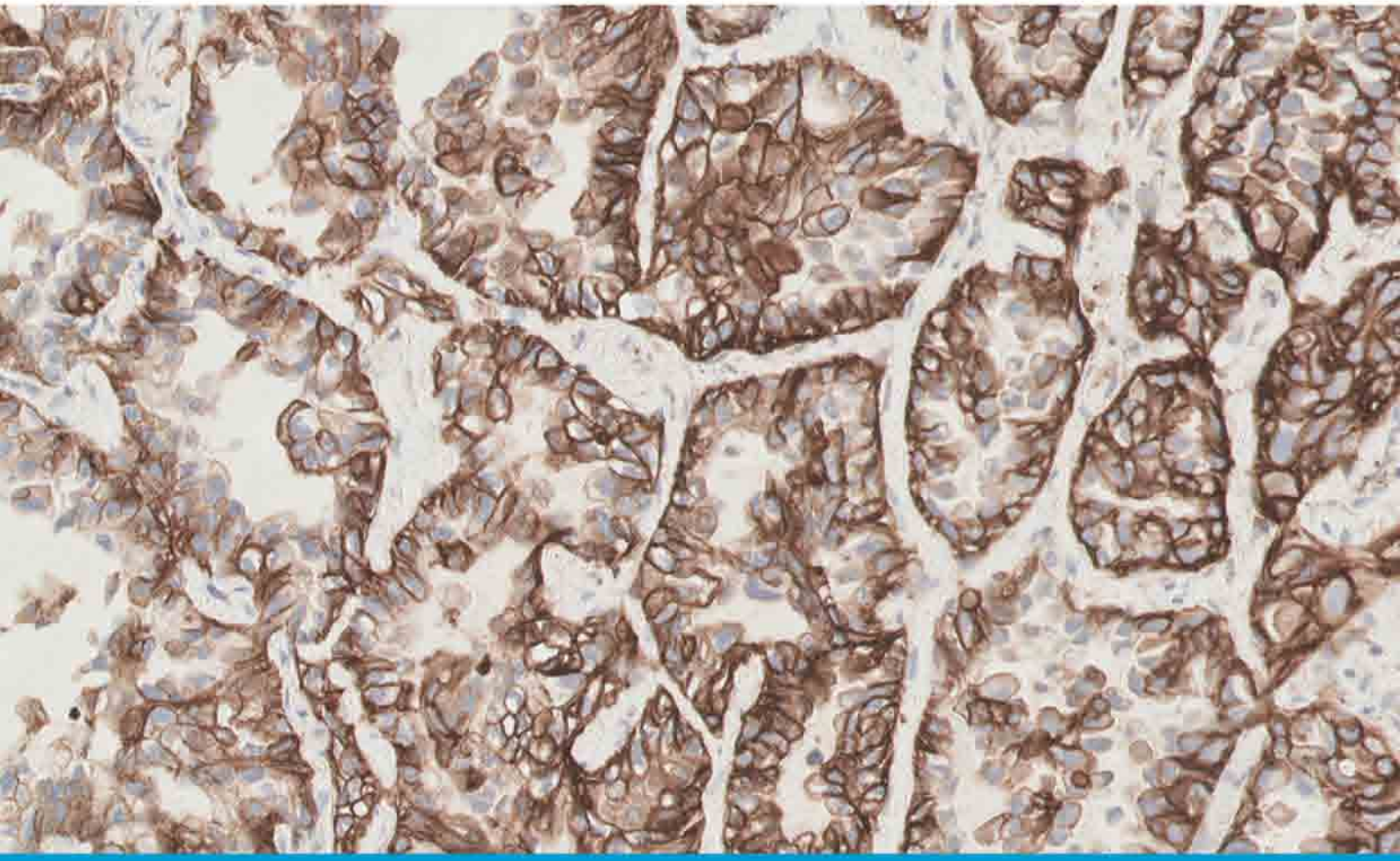


Agilent Technologies

The HER2 ISH Module - Interpretive Run 43

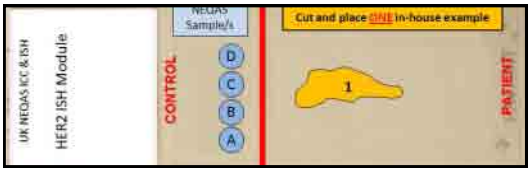
Merdol Ibrahim and Suzanne Parry

Gene Assessed:	HER2
Sections Circulated:	Composite slide consisting of 4 breast carcinoma sections (see table below)
Number of Registered Participants:	157
Number of Participants This Run	144 (92%)

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	**3+**	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 below



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.
Although single HER2 probe methods are available, such as Ventana Silver ISH (SISH)[3], the dual probe (ratio) based techniques are the method of choice, with chromogenic assays now used as abundantly as **FISH**
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.

Assessment Procedure

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

Assessment Results

Only 1 participant used a copy number alone method in the current assessment (run 43). This lab is using the Ventana SISH assay and received an excellent standard score. The rest of the participating labs are using the dual probe ratio method. A full breakdown of the ratio method pass rates is shown in Table 2. 54% of labs are using the FISH technique and 46% are using CISH method (Table 2), which was similar to the previous Run (43). The Pathvysion Vysis kit still remains the most popular FISH method, and this is used by 55% of labs using FISH methods. The Ventana DDISH was the most used chromogenic ISH technique, which was used by 75% of laboratories using CISH methods.

Overall the acceptable pass rate, irrespective of method, was 50%(≥30/36). A further 31%received a borderline pass(24-29/36). The acceptable pass rate for FISH users

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.

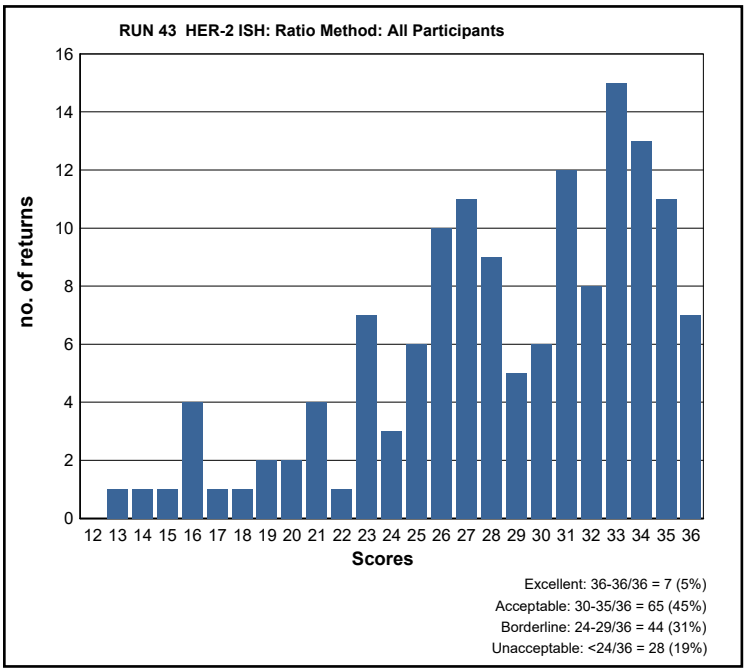
Note: Sample 'A' Is an unusual tumour with complete CEN 17 deletion, this core should be reported on copy number alone..

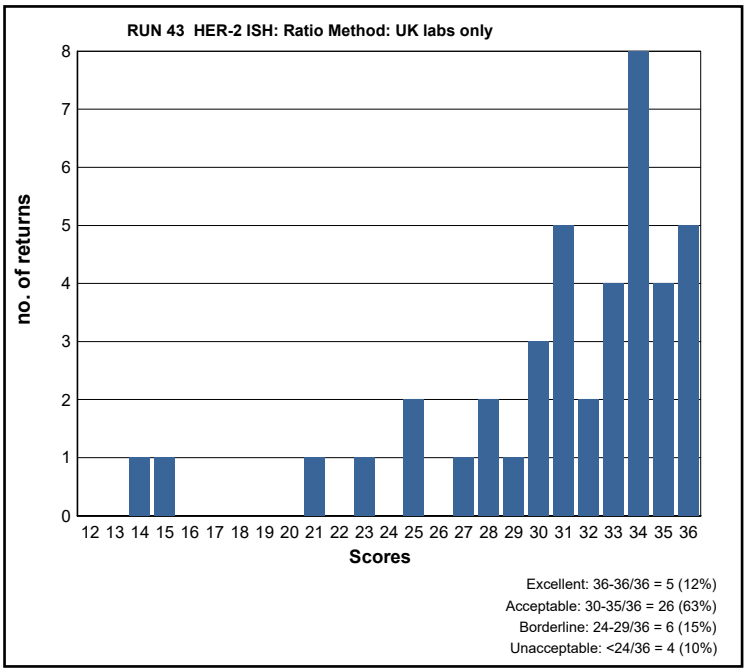
HER2 ISH Method and Probe Enumeration

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.

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

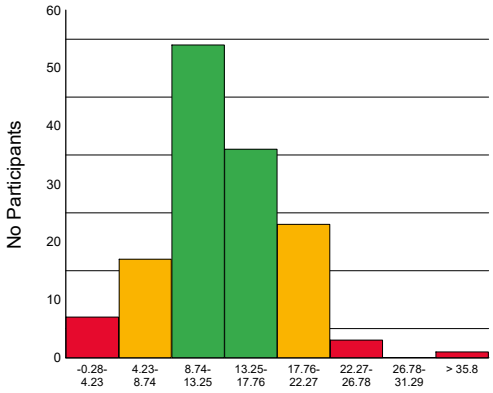
Ratio Method	N	% Pass (score ≥ 30/36)
Ratio: Dako DuoCISH	2	0%
Ratio: Dako IQFISH pharmDX	10	40%
Ratio: Dako Pharm Dx	1	100%
Ratio: In house FISH	1	0%
Ratio: Kreatech Probes	3	67%
Ratio: Leica HER2 FISH TA9217	6	67%
Ratio: Other - CISH	1	100%
Ratio: Other - FISH	5	20%
Ratio: Pathvysion Vysis Kit	45	73%
Ratio: Ventana BDISH 800-098/505	7	43%
Ratio: Ventana DDISH (780/800-4422)	50	38%
Ratio: Ventana Inform Silver ISH	4	0%
Ratio: Zytovision ZytoDot 2C	1	0%
Ratio: Zytovision ZytoLight	7	57%

Copy Method (Shown Only When Applicable)	N	% Pass (score ≥ 10/12)
Copy No.: Other	1	0%

FREQUENCY HISTOGRAMS

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

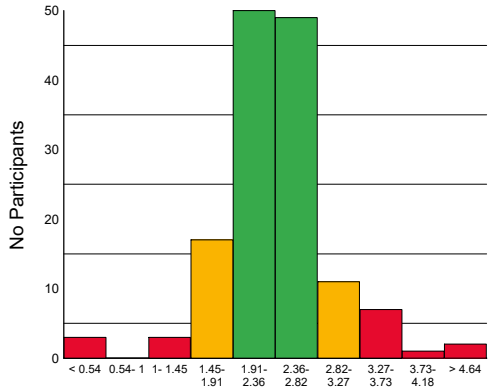
Green = mean +/- 1 Stdev Amber = mean +/- 2 Stdev Red = mean ≥ +/- 3 stdev



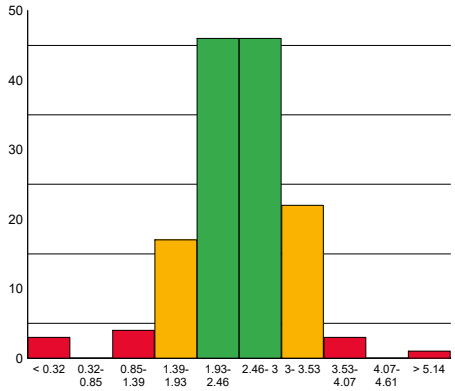
Sample A: Av. HER2 copy

Sample A: Av. Chr17

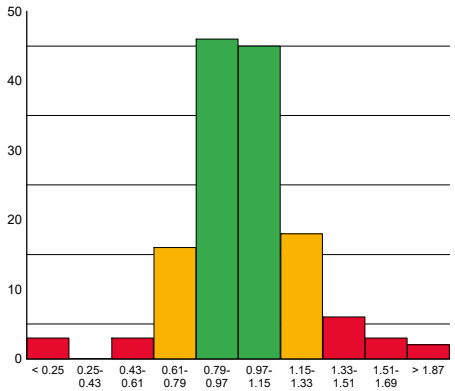
Sample A: Ratio



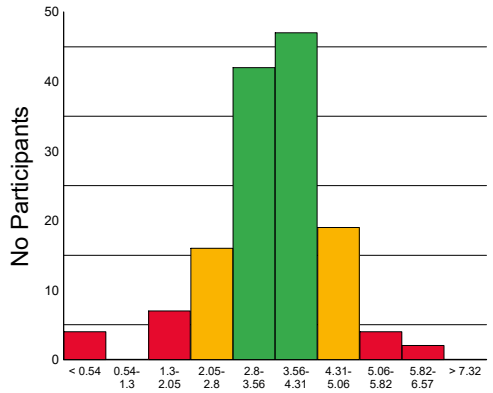
Sample B: Av. HER2 copy



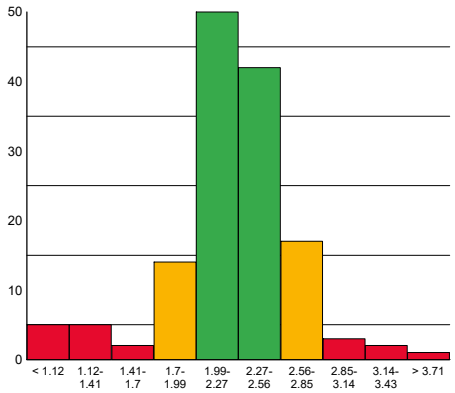
Sample B: Av. Chr17



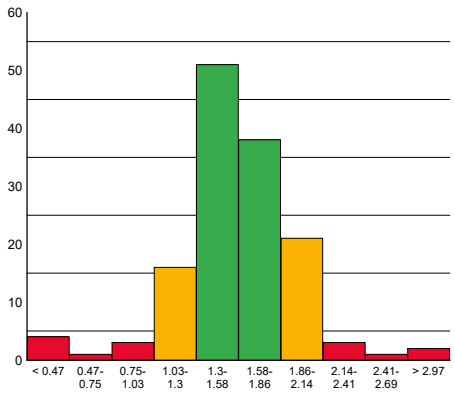
Sample B: Ratio



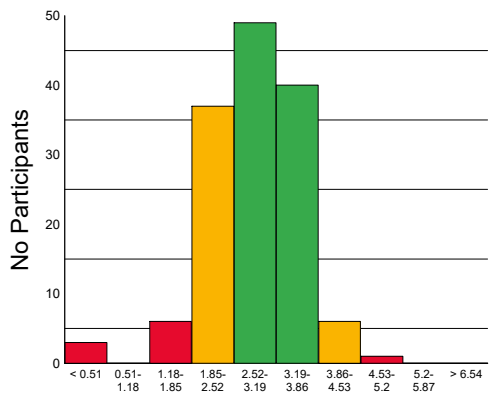
Sample C: Av. HER2 copy



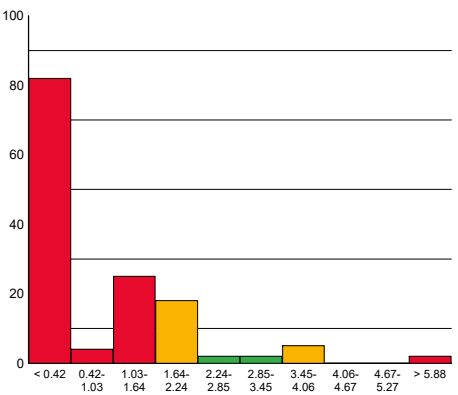
Sample C: Av. Chr17



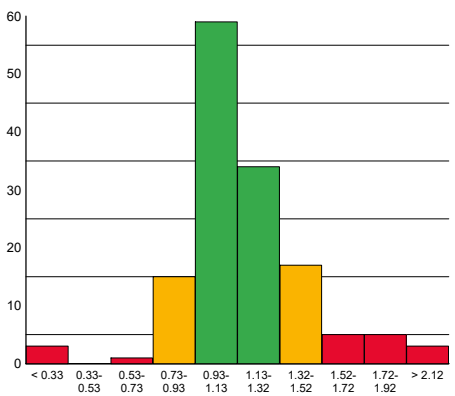
Sample C: Ratio



Sample D: Av. HER2 copy



Sample D: Av. Chr17



Sample D: Ratio

The HER2 ISH Module - Technical (Pilot) Run 43

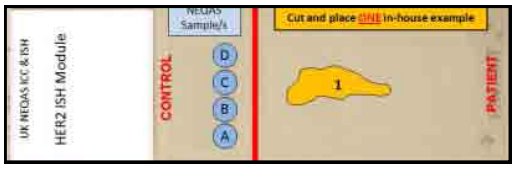
Merdol Ibrahim and Suzanne Parry

Gene Assessed:	HER2
Sections circulated:	Composite slide consisting of 4 breast carcinoma sections (see table below)
Number of Registered Participants:	179
Number of Participants Taking Part this Run	140 (78%) (75 Fluorescent and 65 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	**3+**	Amplified
B	**2+**	Non-amplified
C	**2+**	Non-amplified
D	**2+**	Non-Amplified

* Sample C showed a focal area of amplification depending on the serial section received by the laboratory
** Some sections also contained a xenograft sample which was used for validation purposes only



Tissue Section Positioning: Tissue sections were positioned on microscope slides as illustrated in the image on the left (same sample as the interpretive ISH)

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) are individually assessed for the quality of ISH staining. Assessors **do not count** the HER2/ Cen17 signals. 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 now requested for this module and participants will be required to place them on the distributed NEQAS slides. Please note that only one in-house sample is required.

Results Summary

CISH Results

Selected images Figs 7-12,show examples of the acceptable and unacceptable levels of staining of a CISH method.

The overall results from the CISH technical assessment saw an slight improvement from the previous Run (42): 48% of labs received an acceptable pass, compared to 46% in Run 42, a further 29% achieved a borderline pass in the current run. 15 labs (23%) failed the assessment, but this was an improvement on the previous run where 33% of labs failed on the UK Neqas distributed material. The failures were mostly due to weak or no Cen17 signals, which is similar to what we have been seeing in previous assessment runs. The borderline passes were mainly marked down due to weak, but still readable signals; again, mostly due to weak Cen17 signals. These observational results refer mainly to the Ventana BDISH & DDISH methods, which were used by most labs (86%) who submitted brightfield ISH slides for this technical assessment.

The Dako DuoCISH method was used by 3 labs, and these labs obtained unacceptable results , due to weak Cen 17 in some of the cores and also excessive counterstain. because the signals were weak in some of the cores. The Zytovision ZytoDot "C was used by 1 laboratory which failed this assessment due to unreadable HEr2 and CEN17 signals as well as normal cells not staining.
Important: Whichever CISH methodology being used, any laboratory experiencing staining problems should contact the relevant company for further support.

FISH Results

Images of acceptable and unacceptable levels of staining are illustrated in figures 1-6.

Overall the FISH results showed slightly lower rates than the previous assessment run (42): 63% of labs received an

The HER2 ISH Module - Technical (Pilot) Run 43

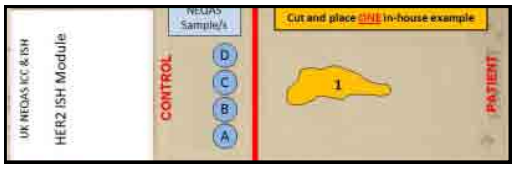
Merdol Ibrahim and Suzanne Parry

Gene Assessed:	HER2
Sections circulated:	Composite slide consisting of 4 breast carcinoma sections (see table below)
Number of Registered Participants:	179
Number of Participants Taking Part this Run	140 (78%) (75 Fluorescent and 65 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	**3+**	Amplified
B	**2+**	Non-amplified
C	**2+**	Non-amplified
D	**2+**	Non-Amplified

* Sample C showed a focal area of amplification depending on the serial section received by the laboratory
** Some sections also contained a xenograft sample which was used for validation purposes only



Tissue Section Positioning: Tissue sections were positioned on microscope slides as illustrated in the image on the left (same sample as the interpretive ISH)

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) are individually assessed for the quality of ISH staining. Assessors **do not count** the HER2/ Cen17 signals. 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 now requested for this module and participants will be required to place them on the distributed NEQAS slides. Please note that only one in-house sample is required.

Results Summary

CISH Results

Selected images Figs 7-12,show examples of the acceptable and unacceptable levels of staining of a CISH method.

The overall results from the CISH technical assessment saw an slight improvement from the previous Run (42): 48% of labs received an acceptable pass, compared to 46% in Run 42, a further 29% achieved a borderline pass in the current run. 15 labs (23%) failed the assessment, but this was an improvement on the previous run where 33% of labs failed on the UK Neqas distributed material. The failures were mostly due to weak or no Cen17 signals, which is similar to what we have been seeing in previous assessment runs. The borderline passes were mainly marked down due to weak, but still readable signals; again, mostly due to weak Cen17 signals. These observational results refer mainly to the Ventana BDISH & DDISH methods, which were used by most labs (86%) who submitted brightfield ISH slides for this technical assessment.

The Dako DuoCISH method was used by 3 labs, and these labs obtained unacceptable results , due to weak Cen 17 in some of the cores and also excessive counterstain. because the signals were weak in some of the cores. The Zytovision ZytoDot "C was used by 1 laboratory which failed this assessment due to unreadable HEr2 and CEN17 signals as well as normal cells not staining.
Important: Whichever CISH methodology being used, any laboratory experiencing staining problems should contact the relevant company for further support.

FISH Results

Images of acceptable and unacceptable levels of staining are illustrated in figures 1-6.

Overall the FISH results showed slightly lower rates than the previous assessment run (42): 63% of labs received an

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

acceptable pass which is much lower compared to 79% in run 42, and a further 13% achieved a borderline pass which is similar to previous run. The current run showed a fail rate of 24%, much higher to that of the previous run of 8%.
The main reason for unacceptable results was due to weak or no signals. We cannot be sure if this fading or total loss of signal may be due to slides not being sealed or stored correctly. However, UK Neqas does provide recommendations for this 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, used by 55% of labs for this assessment. Labs using this method showed an acceptable pass rate of 44%. A further 17 % of labs using the Vysis kit received a borderline, and 39% of Vysis users failed the assessment. The Dako IQFISH and the Zytovision Zytolight FISH kits were the next popular choices of kits, with 12% of FISH submissions stained with the Dako IQFISH and 9% with the Zytovision kit. These showed very good acceptable pass rates of 100% and 86% respectively. A variety of other kits were used, including the Dako Pharm Dx, Kreatech Probes and the leica Her2 FISH all of which performed well, although the numbers of users of these kits is 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.

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

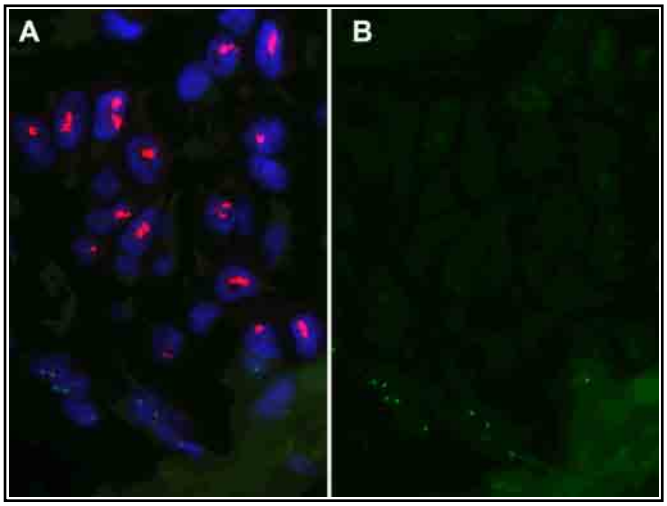


Fig 1. Acceptable FISH example from UK NEQAS distributed 'sample A' amplified case which showed (A) amplified HER2 status (copies >6) but (A,B) absence of Chr17 signal in tumour cells but, which were present in surrounding stroma. Stained using Pathvysion Vysis Kit.

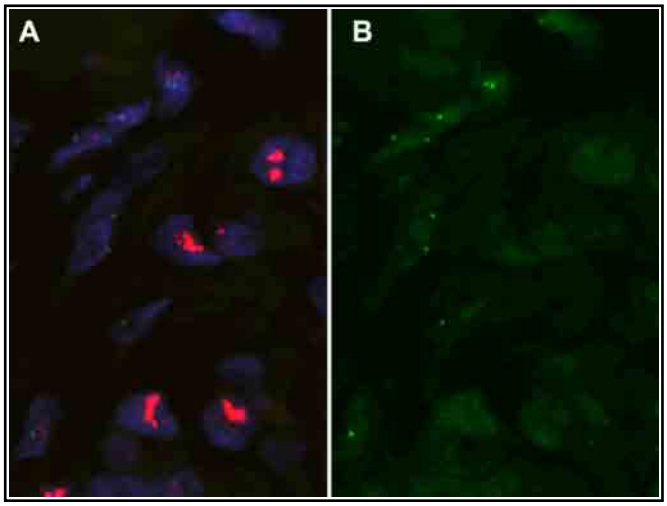


Fig 2. 'Sample A', showing the same pattern of staining as in Fig 1, but stained with the Dako IQFISH.

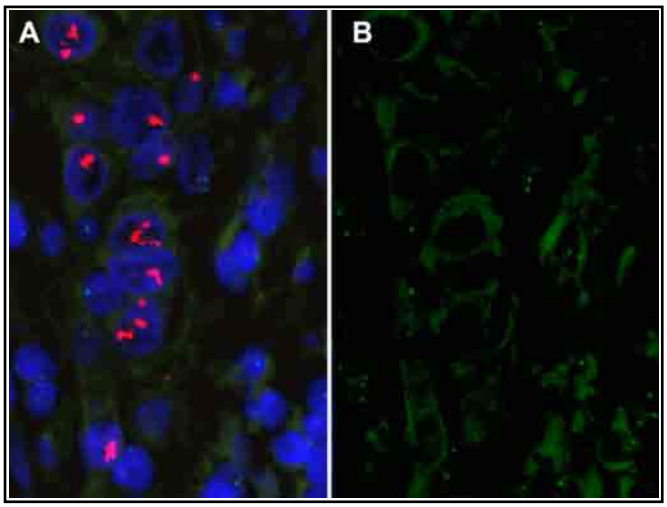


Fig 3. 'Sample A', showing the same pattern of staining as in Fig 1, but stained with the Leica HER2 FISH TA9217.

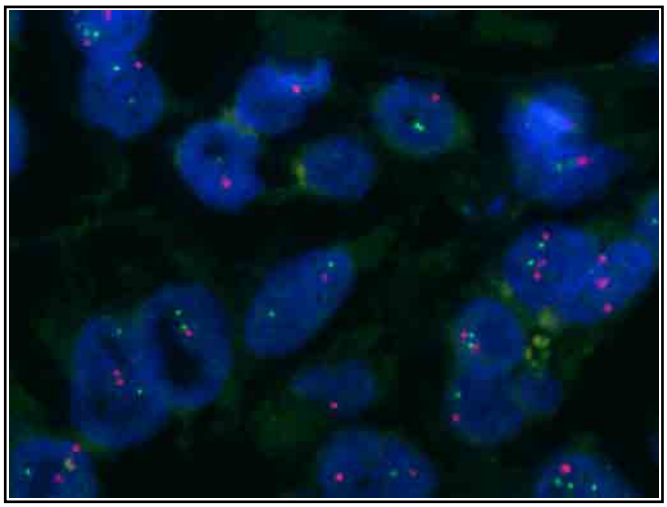


Fig 4. Acceptable FISH example from UK NEQAS distributed 'non-amplified' case (sample B), stained using Zytovision ZytoLight. Note that HER2 signals are green and Chr17 signals are red.

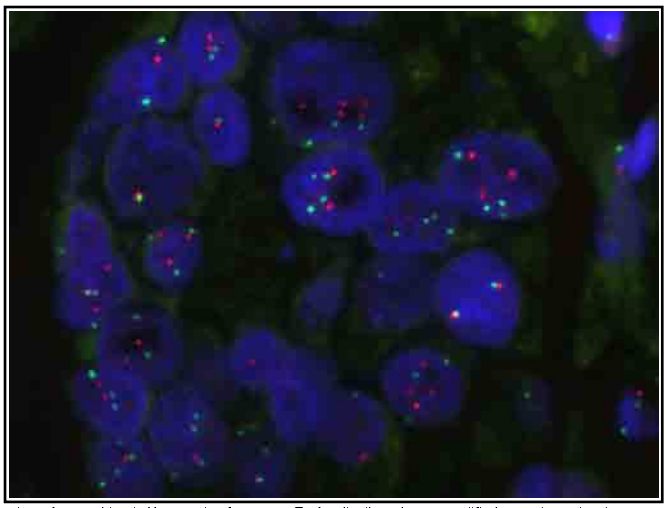


Fig 5. Acceptable FISH examples from UK NEQAS distributed 'non-amplified' case (sample D) stained using Pathvysion Vysis Kit demonstrating distinct HER2 (red) and Chr17 (green) signals.

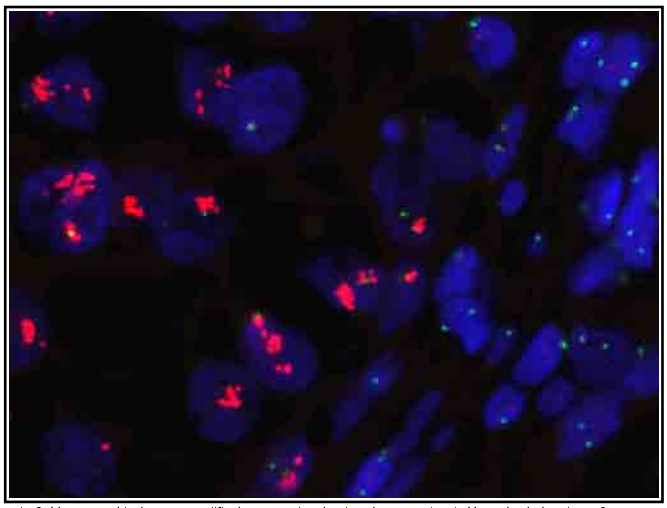


Fig 6. Very Good in-house amplified case stained using the Dako IQFISH method showing >6 HER2 copies (red signal) in the tumour cells, with stromal cells on the left side of the image acting as a good internal controls.

Selected Images showing Optimal and Sub-optimal Immunostaining

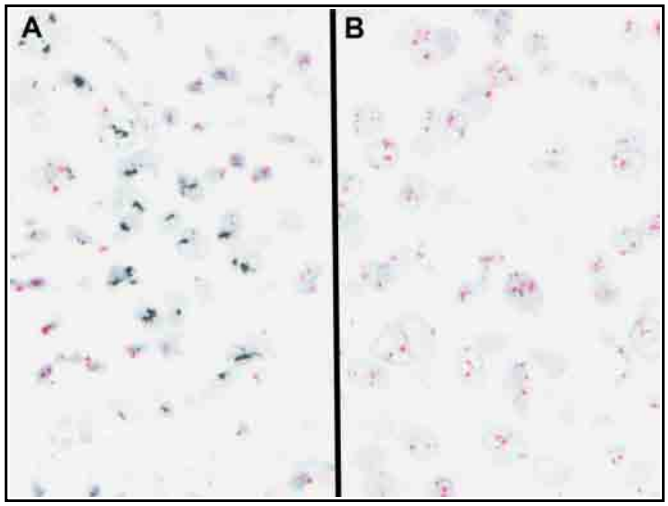


Fig 7. Acceptable Ventana DDISH in the UK NEQAS distributed samples 'A' and 'B'. (A) Sample 'A', amplified based on HER2 copies; note that sample 'A' has 'loss' of Chr17 in tumour cells but normal cells showed Chr17 expression. (B) Sample 'B'; non-amplified with distinct HER2 signals (black) and Chr17 signals (red).

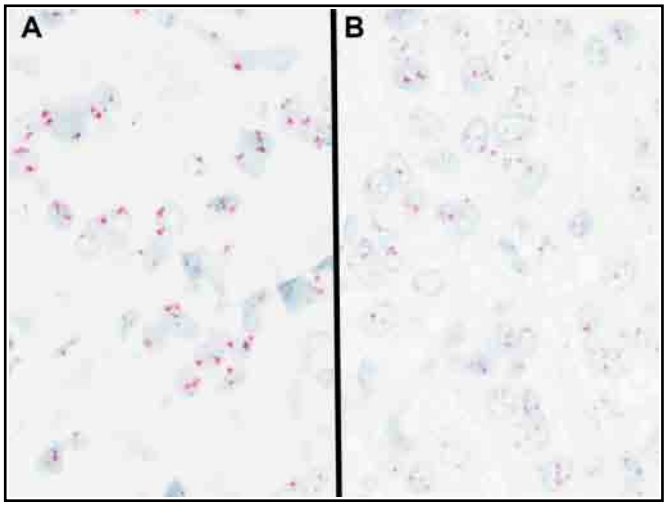


Fig 8. (A and B) Acceptable Ventana DDISH in the UK NEQAS non-amplified distributed samples C and D. Both examples show strong HER2 signals (black) and Chr17 signals (red) and the expected copy numbers per cell.

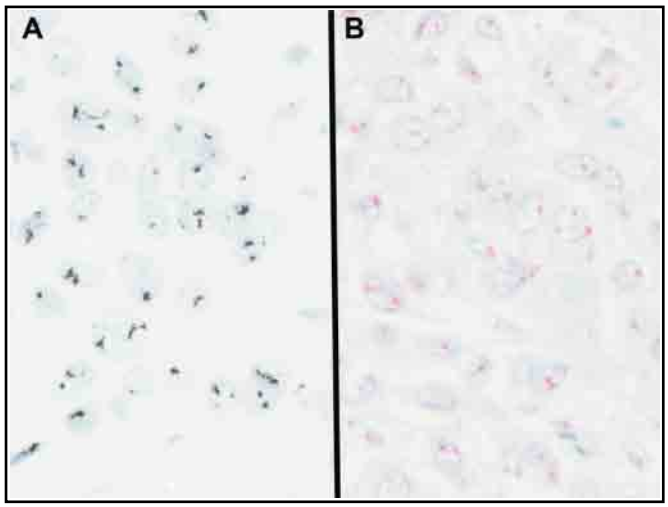


Fig 9. Two examples showing unacceptable Ventana DDISH staining in the UK NEQAS (A) Sample ' A' which was an unusual case with loss of Chr17, in this example also shows lack of signal in the normal cells. (B) Absence of HER2 signal.

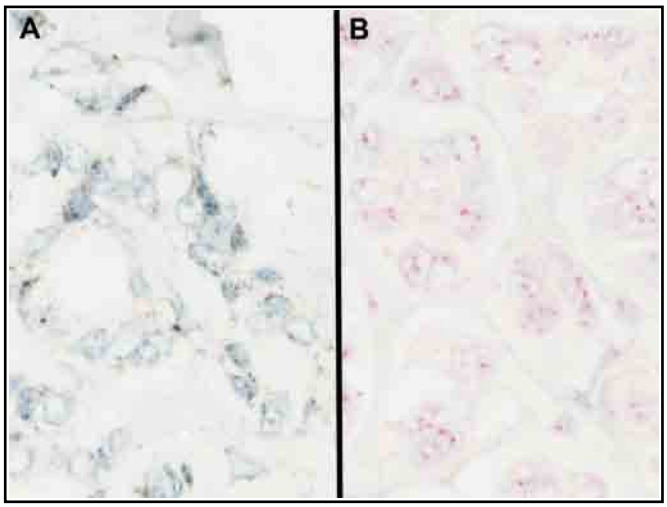


Fig 10. Unacceptable Ventana DDISH staining in the UK NEQAS samples. (A) core' C' with drying-back artefact, while (B), core' D' shows poor morphology with low and weak HER2 signals.

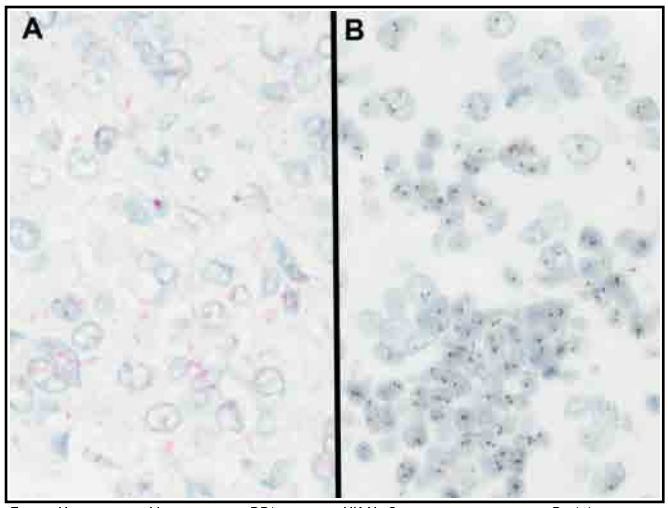


Fig 11. Unacceptable Ventana HER2 DDISH on the UK NEQAS distributed samples 'D'. (A) Excessive leaching of Chr17 and no HER2 signals, and (B) excessive silver chromogen and lack of Chr17 signals.

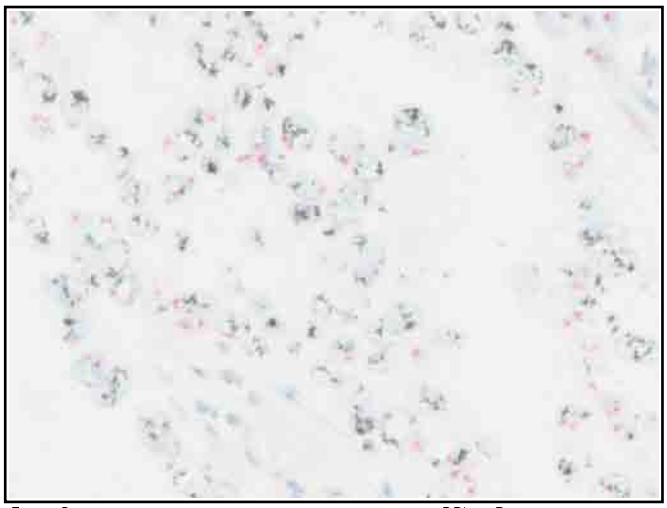
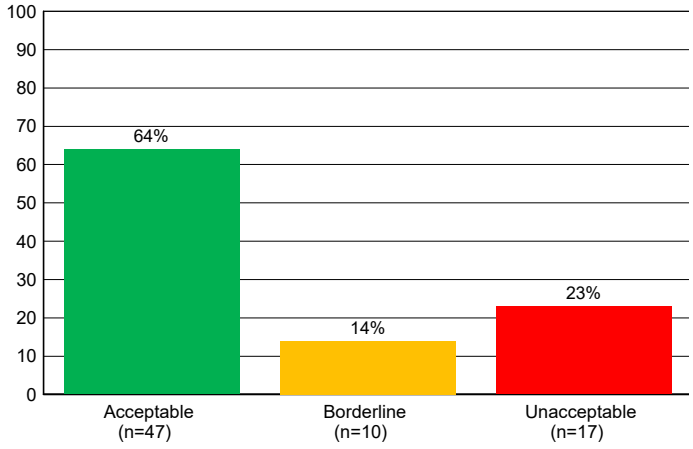


Fig 12. Good example of an amplified in house section stained with DDISH. Both the HER2 and Chr17 signals are strong and distinct.

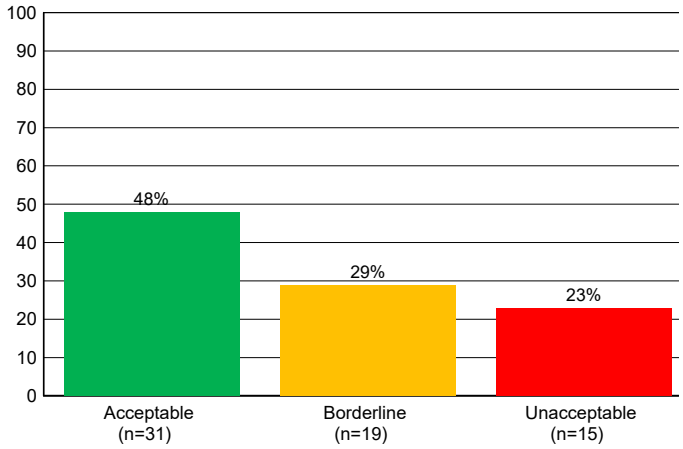
Technical ISH: Pass Rates and Methods

Overall Pass Rates

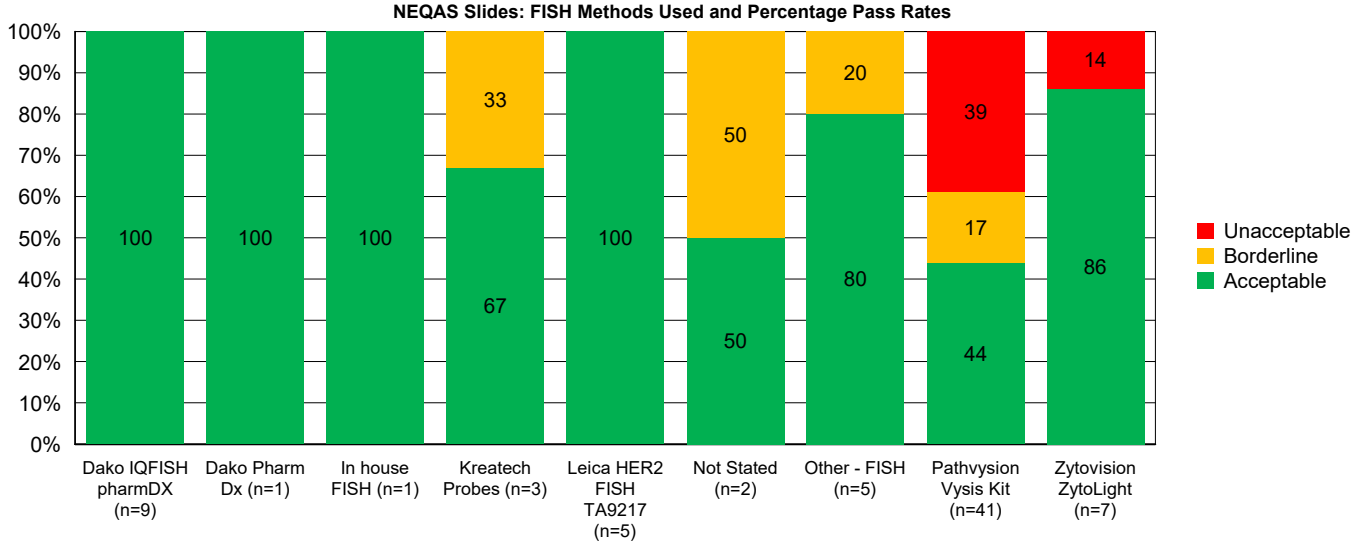
FISH NEQAS slide (n=74)



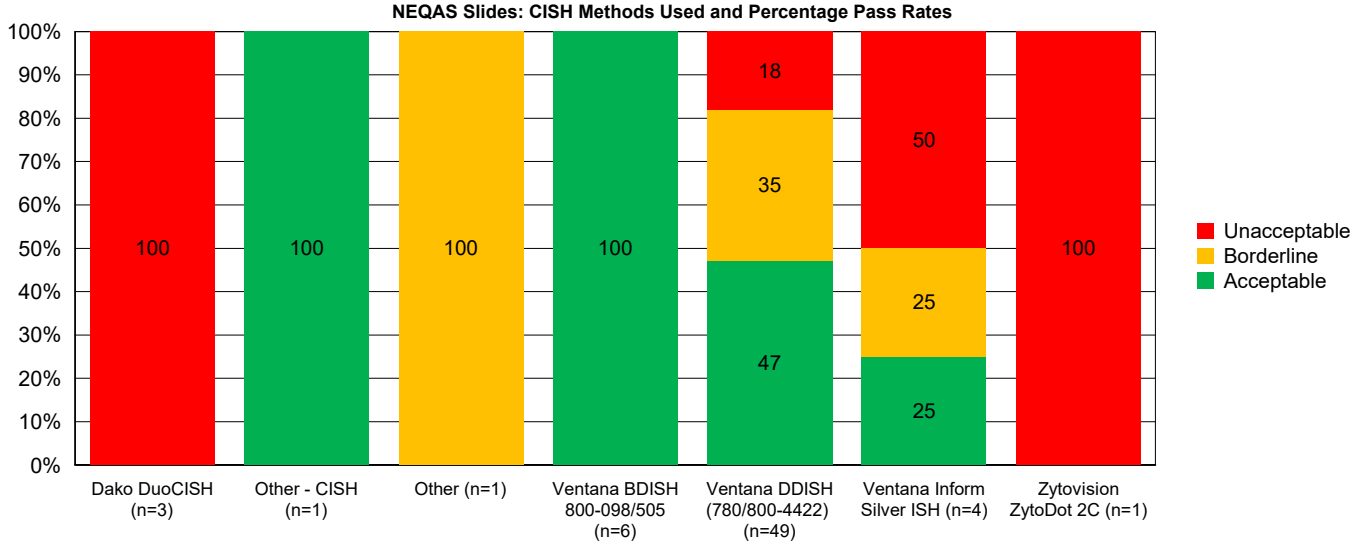
CISH NEQAS Slide (n=65)



NEQAS Slides: FISH Methods Used and Percentage Pass Rates



NEQAS Slides: CISH Methods Used and Percentage Pass Rates



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