Run 110/39

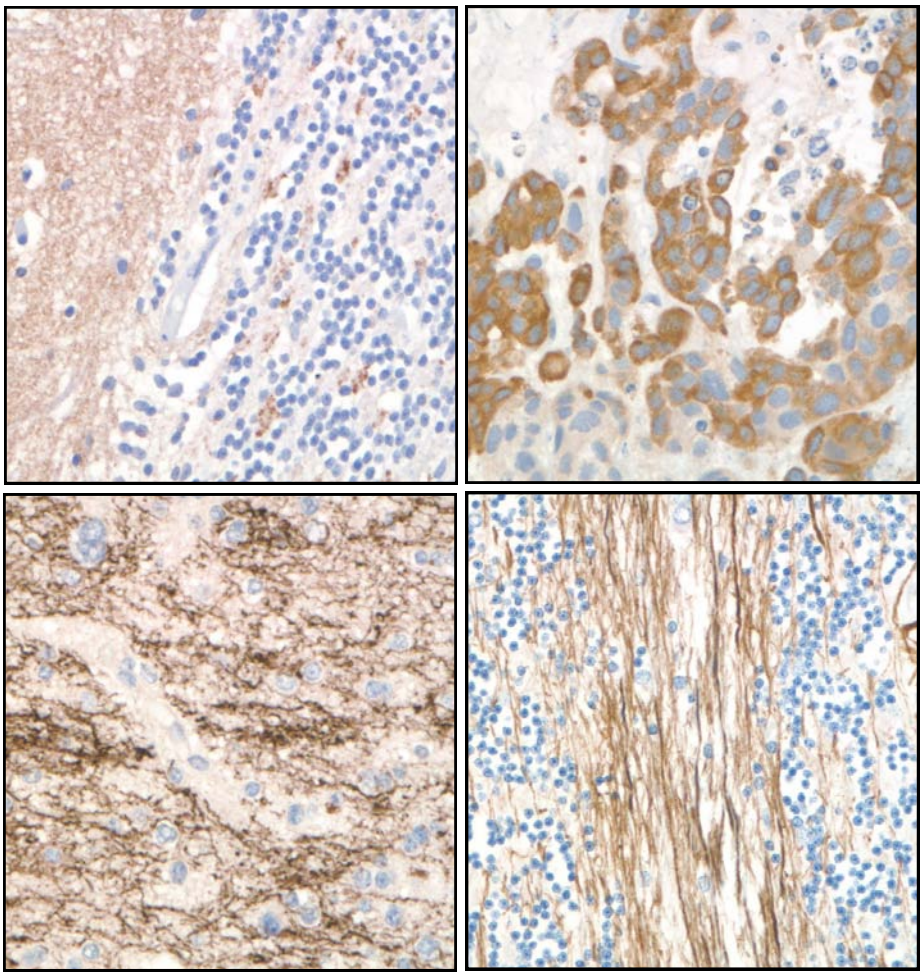
UK NEQAS
International Quality Expertise

# Immunocytochemistry

## Improving Immunocytochemistry for Over 25 Years

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

**Assessment Dates: 22nd June — 14th July 2015**



**Cover Photo: Taken from the Neuropathology Module:**

**Top Left:: Sub-optimal (weak) synaptophysin staining on the NEQAS cerebellum sample**
**Top Right: Excellent synaptophysin demonstration on an in-house tumour sample**
**Bottom Left: Optimal neurofilament protein demonstration on the NEQAS tumour section**
**Bottom Right: Excellent neurofilament staining on an in-house section of cerebellum**

### Articles / Reports



### Immunocytochemistry Modules



### In situ Hybridisation Modules



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**Reserve the Date: 6th November 2015**
**Event:** UK NEQAS ICC & ISH Participants meeting
**Where:** Hamilton House, London
**Updates:** www.ukneqasiccish.org/blog/

# General Information

UK NEQAS International Quality Expertise

CPA Accredited EQA Scheme Reference No: 044

Data shown in this article is collated from UK NEQAS ICC & ISH assessments and is presented and described 'as collected', and does not ether endorse nor denounce any particular product or method and is provided as a guide to highlight optimal and sub-optimal staining methodologies.

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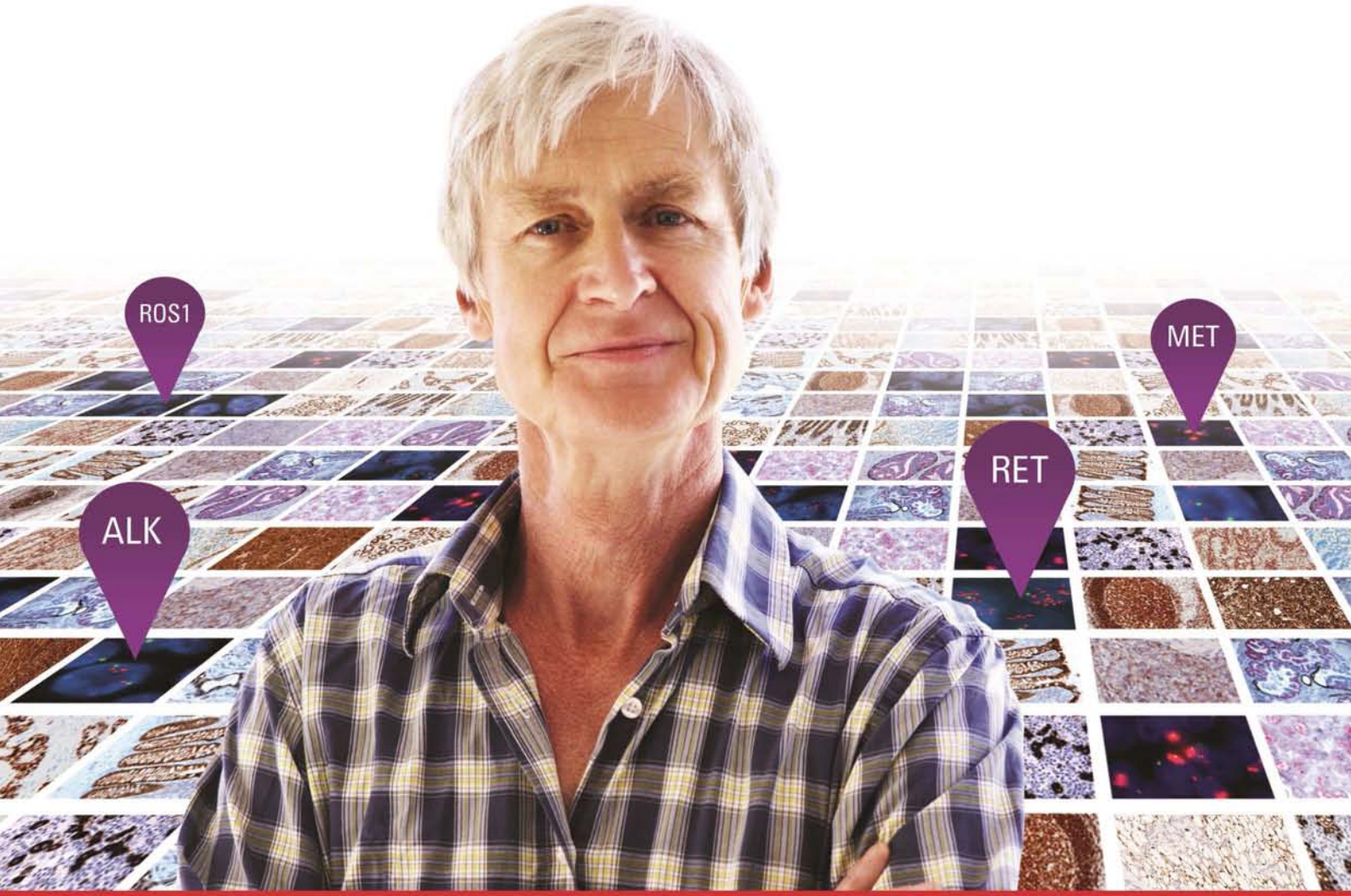
# IQFISH

## Panel for Lung Cancer

### Fastest Time to Result



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# UK NEQAS ICC & ISH User Satisfaction Survey: 2015

Merdol Ibrahim, Neil Bilbe and Keith Miller

## Method

At May 2015, approximately **596** active participants were registered with UK NEQAS ICC & ISH; an email, with a link to the online survey, was sent to each of them. The survey was open for 6 weeks – the closing date was June 12th 2015.

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

- Participant Number
- Region (UK or non-UK)
- Modules participated in during 2014 - 2015

There were 29 questions. 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** response.

Q. 25 asked participants to rate the quality of the EQA material by sample type (sections, cell lines, cell block sections and cytospins). Q. 26 change in methods based on EQA results, Q. 27 asked whether any reassessments requests had been made.

The final two questions (Q. 28 & 29) asked for the *overall* rating of our service; firstly by level of satisfaction, and secondly, with a score out of 10.

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

117 responses were received; any duplicate, incomplete and unusable, or multiple entries were removed, leaving a total of 113 replies for analysis, although 9 of these were incomplete, some data was received, and therefore included where possible.

The overall response rate to the survey was therefore approximately 19%, a decrease from the 30% last year.

**47** UK & Eire labs responded out of a total of 224 labs registered in the UK & Eire (42%), and 21% of registered labs.

**66** Non-UK & Eire (OS) replied out of a total of 372 registered with the scheme (58%), and 18% of all registered OS labs.

### Overall analysis of satisfaction

Results from the response to Q. 29 relating to overall satisfaction levels.

- Very Satisfied (39) 37.5%
- Satisfied (60) 57.6%
- Neutral (5) 4.8%
- Very/Dissatisfied 0%

Effectively, over 95% of users were either Very Satisfied or Satisfied with the service; 4.8% responded to being Neutral. There were no Dissatisfied responses.

### Response by region:

| | UK (45) | OS (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%) |

N.B. There were 9 replies with no response to Q. 28.

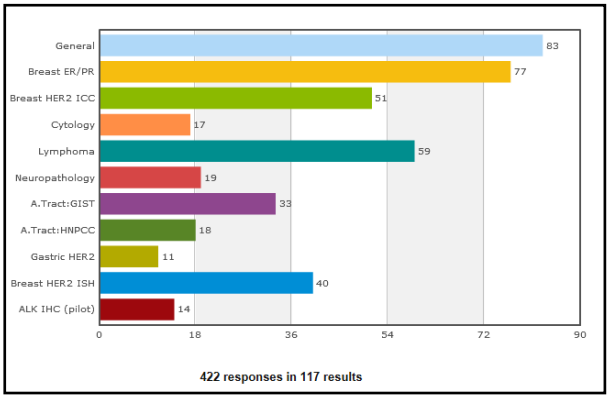
### Comparisons with data from 2014 Survey

| Responses | 2014 (%) | 2015 (%) |
|---|---|---|
| **Very Satisfied** | 34.1 | 37.5 |
| **Satisfied** | 57.3 | 57.6 |
| **All other responses** | 8.5 | 4.8 |

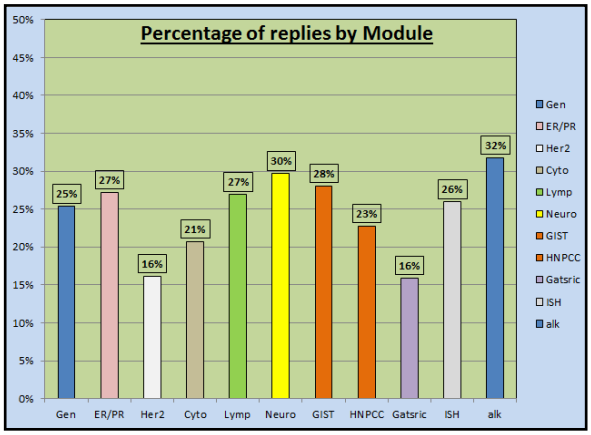
The overall levels of satisfaction remain relatively unchanged, but with an increase in the number of Very Satisfied (↑ 3.4%) responses, off-set by a decrease in Neutral responses (↓ 3.7%).

### Some module related data

The average number of modules that labs participate in is 3.7 (same as last year); the combined number of modules for all labs responding was 422, the number of labs for each module was:

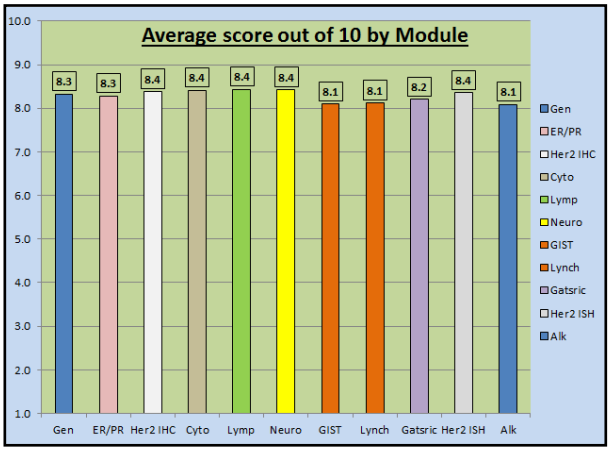



When this is compared to the actual number of labs registered for each module:



On average 25% of labs registered for a particular module responded to the survey, with a median of 26%. Lowest response 16% (Breast and Gastric Her2), to the highest 32% (ALK IHC).

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




The average score was 8.3, (8.2 last year), with a low of 8.1 (GIST & Lynch syndrome) and a high of 8.4 (Her2 IHC, cytology, neuropathology, lymphoma, and Her2 ISH ). Similar to last year, there was a narrow range of only 0.3, showing a consistent level of satisfaction across all modules.

The biggest increases in scores were for the cytology (0.4), neuropathology (0.3) and lymphoma (0.2); only the two alimentary tract modules showed a decrease (0.1), again all these are minor changes.

**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 3.5% a slight increase (3.1%), but in line with previous years: 5 year average (2010 – 2014) of 3%.

**Levels of dissatisfaction 2015**

The four standout areas from this year's survey were:

- Turnaround times: slight increase up to 8.5% from 6%
- Assessor comments: increased to 14.5% up from 10%
- Web based format of results: 6% up slightly
- This year there was an decreased level of dissatisfaction for the 'Location of Meetings' of 6% down from 7% but still the 4th highest topic for dissatisfaction

N.B. Interestingly, the feedback on the 'Number of Meetings' has improved, hopefully due to the two UK NEQAS ICC & ISH Participant meetings held in the past few years.

**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 (17 comments)
- Participant feedback and communication section (23)
- UK NEQAS ICC & ISH meetings section (10)
- Complaints about the service (6)
- Treatment of in-house & UK NEQAS samples (20)
- Assessment of in-house controls (12)
- Quality of the UK NEQAS ICC & ISH EQA material (17)
- Use of EQA results to improve in-house staining (43)
- Reassessment requests (8)
- General comments and feedback about the service (13)

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 **169** comments were received, a scheme response and comments on some of these are given further down.

The main or most common themes are summarised below:

- More feedback on possible best methods
- Too much information sent out on cover letters
- Turnaround times could be better
- More detailed comments for sub-optimal results
- Fixation differences between NEQAS – In-house
- Staining and assessment of in-house controls
- Variable section quality
- Reassessment of slides
- Same slide EQA procedure
- More antibodies for certain modules

*Plus lots of complimentary comments about the scheme*

## Summary

This year's response level of less than 20% was disappointing, particularly when compared to levels of around 25% - 30% for the preceding few years. Although it is arbitrary, perhaps not sending participants who had not replied a reminder, may have made the difference; either way, a return of **113** labs out of around **600** is both below par and slightly discouraging.

Levels of participant satisfaction remain high at c. 95% an increase from 2014. The actual level of Very Satisfied respondents also continues to increase from 27.5% (2013), 34.1% (2014) to 37.5% for 2015. This appears to be due to a reduction in the number of Neutral replies (↓ 3.7%).

# UK NEQAS ICC & ISH User Satisfaction Survey: 2015

When comparing the score out of 10 results from last year, the overall response is 8.3 (2014: 8.2); most modules were unchanged. Three went up: cytology, neuropathology and lymphoma. Two went down: GIST and Lynch syndrome. The newest module the ALK IHC module scored 8.1 the joint lowest, but it is early days for this particular module, with only one completed run at the time of survey.

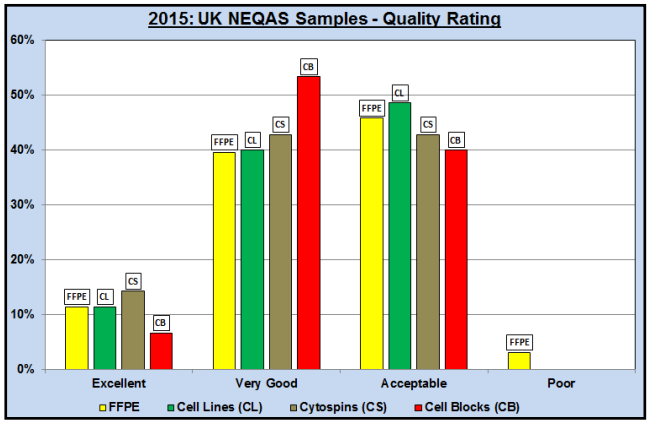
The E-Journal continues to show an increased satisfaction level, and is now 90%, up slightly from last year's 88%.

Given the overall levels of reply, the number of participants returning a rating on the quality of the UK NEQAS ICC & ISH sample quality, was relatively good:

- 96 Formalin fixed paraffin sections (FFPE)
- 70 Cell lines (CL)
- 14 Cytospins (CS)
- 15 Cell block sections (CB)

The sample quality rating again shows a steady improvement, and only the paraffin sections (FFPE) had a rating of poor (3%), whereas in 2012 all three categories (pre cell block sections) had a poor rating, averaging 5.1%. The average rating for all samples as 'excellent' has also risen from: 7.8% (2012) to 11% (2015).

**UK NEQAS EQA samples: Ratings (Q. 25)**




Given the relative numbers of labs receiving FFPE sections, compared to cytospins and cell blocks it is statistically more likely that there was a rating of poor for these samples.

**UK v OS levels of dissatisfaction**

For the three main areas of dissatisfaction: turnaround times (same), but for assessor comments (12.8% v 16.9%) , and web based format of results (4.2% v 8.4%) the UK labs had a *greater* level of dissatisfaction than the non-UK (OS) labs.

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

1. The cover letters contain too much information

*This is a valid point, and one which has already been addressed in the Run 110 cover letter, where a small table was used to highlight any module specific details. We plan to reduce the content further by removing any repetitions. Further options may be to have module specific links to a website, upload the cover letter, but not everyone has access to the internet prior to staining. This would also rely on labs knowing where to access scheme documents and/or consulting the latest Participants Manual.*

2. Would like more detailed assessor comments on sub optimal staining results

*Some of the assessment scoring sheets have been revised to allow for more detailed feedback and a greater number of comments on the reports; e.g. gastric and breast modules. We hope to roll this out to other modules. The reports now contain a 'running assessment' for the last 10 runs so that labs can see their performance over several years. Alongside the changes to the slides (see 3 below) this has meant that we are able to give better feedback.*

*We are hoping to aid labs by making available our extensive database of methods and protocols by way of a Best Methods functionality on our new website. Participants will be able to enter their reagent and platform details and obtain details of methods used by the best performing labs using the same.*

3. Same slide EQA and assessment of in-house controls

*This has been introduced during the last few runs in order to deal with the constant comment concerning the different results/quality/scores between UK NEQAS ICC & ISH material and the in-house controls. Additionally, UK NEQAS ICC & ISH has no direct influence over the in-house material, from its preparation through to the actual staining.*

*The single slide approach ensures that both samples are stained at the same time (as would happen in the clinical setting); during the assessments it enables the assessors to compare and reference the NEQAS and in-house sections easily, without having to keep searching for a separate slide. In the future we will be scoring the two sections side-by-side for most modules.*

*We appreciate that there have been a few teething problems, due to section lifting, and some of the NEQAS sections being a little high up on the slide, but long term it should allow for better overall feedback.*

4. More antibodies requested for all modules

*A frequent and consistent comment from participants. We can only reiterate what has been said previously. We survey labs every year (primarily from the UK & Eire) asking for participants' repertoire, not only of established and common antigens, but less common ones, plus there is a free text area for markers gaining popularity in the diagnostic setting.*

*As we state in the scheme documentation, there is a minimum level of usage for a marker to be used either as a Gold (up at around 90%) or a 2nd antigen (80%) otherwise the majority of labs will either not submit a slide at all, or submit an alternative antibody and therefore minimise inter-laboratory comparisons of the originally requested marker.*

*What we are looking to do, is to expand the use of alternating Golds; e.g. SMA, Desmin, SMA, Desmin so that the same antigen is not repeated for the whole year.*

*The latest survey can be accessed below:*
**www.ukneqasiccish.org/antibody-repertoire-2015/**

UK NEQAS ICC & ISH staff and management would like to thank 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

Julie Williams and Suzanne Parry

| | Gold Standard | Second Antibody |
|---|---|---|
| **Antigens Assessed:** | Desmin | p63 |
| **Tissue Sections circulated:** | Appendix and Leiomyosarcoma | Tonsil and Lung Squamous Cell Carcinoma |
| **Number of Registered Participants:** | 339 | |
| **Number of Participants this Run** | 317 (94%) | |

# Introduction

## Gold Standard: Desmin

Desmin is a 53KDa cytoplasmic intermediate filament protein that is characteristically found in all three types of muscle cells (smooth muscle, cardiac and skeletal muscle). The protein is composed of an N-terminal head-piece and a C-terminal tail-piece. It forms cytoskeletal networks across the muscle fibre at the plasma nuclear membrane border, with particular localisation at the sub-plasmalemmal region and the Z-band. Desmin's main diagnostic use is in the demonstration of rhabdomyosarcomas, leiomyomas, leiomyosarcomas (Chang et al., Pollock et al.) and other tumours with myoid differentiation, although it is important to use Desmin within a panel of antibodies.

**Features of Optimal Immunostaining:**
**Appendix: (Fig 1)**
- Strong staining of the smooth muscle layers of the muscularis propria and around the vessels in the submucosa
- Minimal background staining

**Leiomyosarcoma: (Fig 4)**
- Moderate to strong cytoplasmic staining of the tumour cells
- Minimal background staining

**Features of Suboptimal Immunostaining: (Figs 2, 3, 5 & 6)**
- Weak, uneven or no staining
- Non-specific staining of cell types not expected to stain, e.g. lymphocytes and epithelial cells (often due to excessive antigen retrieval)
- Excessive background staining (particularly in the connective tissue)

**References:**
1. T Chang et al. Immunocytochemical study of small round cell tumours in routinely processed specimens. Arch Pathol lab Med 1989; 113:1343-8
2. L Pollock et al. Desmin expression in rhabdomyosarcoma: influence of the desmin clone and immunocytochemical method. J. Clin pathol 1195;48:535-8

## Second Antigen: p63

p63 is a 63KDa nuclear homologue protein of the tumour suppressor p53 family of transcription factors, which is found in the basal and progenitor cells of many epithelial cells, including those of the breast, prostate, bladder and oesophagus (Signoretti et al.) p63 has been identified as a particularly useful aide in the differentiation between benign and malignant lesions of the prostate (Humphrey et al.), and has also shown to be a sensitive marker for squamous cell carcinomas of the lung. In breast tissue, p63 is expressed in the myoepithelial cells of normal ducts and is therefore useful within a panel of IHC markers for the assessment of breast lesions due to the differential expression of luminal versus basal and myoepithelial markers (Reis-Filho et al.)

**Features of Optimal Immunostaining:**
**Tonsil (Fig 7)**
- Strong nuclear staining in almost all of the epithelial cells
- Minimal background staining

**Lung Squamous Cell Carcinoma: (Fig 9)**
- Moderate to strong nuclear staining in virtually all of the tumour cells
- Minimal background staining

**Suboptimal Immunostaining: (Figs 8, 10 & 11)**
- Weak, uneven or no staining
- Diffuse staining
- Non-specific staining of cell types not expected to stain
- Excessive background staining

**References:**
1. Signoretti S, *et al*. p63 is a prostate basal cell marker and is required for prostate development. Am J Pathol. 2000 Dec; 157(6):1769-75.
2. Paner GP, Luthringer DJ, Amin MB. Best practice in diagnostic immunohistochemistry: prostate carcinoma and its mimics in needle core biopsies. Arch Pathol Lab Med. 2008 Sep; 132(9):1388-96.
3. Humphrey PA. Diagnosis of adenocarcinoma in prostate needle biopsy tissue. J Clin Pathol. 2007 Jan; 60(1):35-42.
4.Lerwill MF. Current practical applications of diagnostic immunohistochemistry in breast pathology. Am J Surg Pathol. 2004 Aug; 28(8):1076-91.
5. Hicks DG. Immunohistochemistry in the diagnostic evaluation of breast lesions. Appl Immunohistochem Mol Morph. 2011 Dec; 19(6):501-5.
6. Yeh IT, Mies C. Application of immunohistochemistry to breast lesions. Arch Pathol Lab Med. 2008 Mar; 132(3):349-58
7. Reis-Filho JS et al. Metaplastic breast carcinomas are basal-like tumours. Histopathology 2006 Jul:49(1):10-21

## Assessment Summary:

**Desmin** was the gold standard antigen chosen for this assessment run (110), and overall the staining was very good: 92% of labs received an acceptable pass, and a further 7% achieved a borderline pass (scores of 10-12/20). Only 3 labs failed the assessment, and this was due to very weak or no staining of the desmin filaments. For one of these labs, the reason for the very weak staining was due to the antibody being too dilute. The borderline passes were also mostly due to weaker staining than expected. A variety of antigen retrieval protocols were used by labs, with many opting for enzyme pre -treatment as recommended by both the Leica and Ventana antibody datasheets. The Dako D33 antibody datasheet recommends pre-treatment with a high pH retrieval buffer. However, several labs did not carry out any antigen retrieval, which was the reason for the weak staining in these cases. Most labs are using appendix, tonsil or bowel as their in-house controls. These showed a slightly higher pass rate, with 97% achieving an acceptable pass, and 3% received a borderline.

The second antibody assessed for this run was **p63**. The results were generally good, with 89% receiving an acceptable pass, and a further 7% achieving a borderline pass. 9 labs (3%) failed the assessment. Again, the reason for failure or a borderline mark was due to very weak or uneven staining. It was also noted by the assessors that generally the Leica 7JUL clone showed weaker staining. However, this antibody did perform well for 82% of users (N=84). Most labs are using tonsil, prostate and or breast for their in house controls, which are ideal: The tonsil should show strong staining in virtually all the squamous epithelial cells and some weak staining in scattered lymphocytes. The prostate should show moderate to strong staining in the basal cells, and p63 is strongly expressed in the myoepithelial cells of the breast .

## Selected Images showing Optimal and Sub-optimal Immunostaining

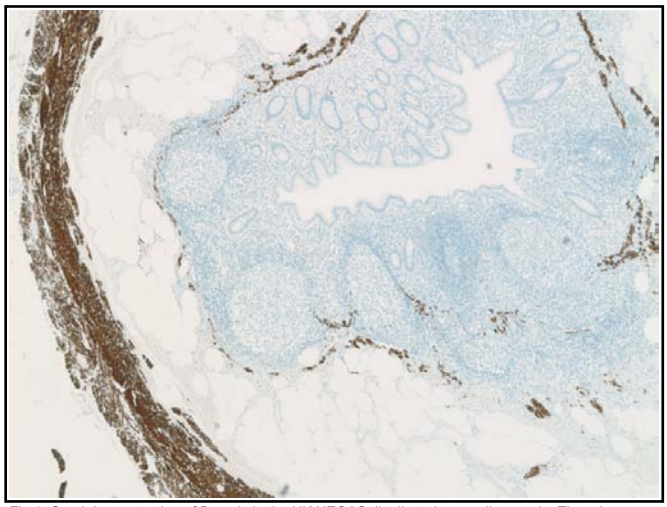


Fig 1. Good demonstration of Desmin in the UK NEQAS distributed appendix sample. There is strong staining of the smooth muscle layers of the muscularis propria and around the vessels in the submucosa. Section stained with the Ventana DER11 pre-diluted antibody on the Benchmark ULTRA with no pre-treatment.

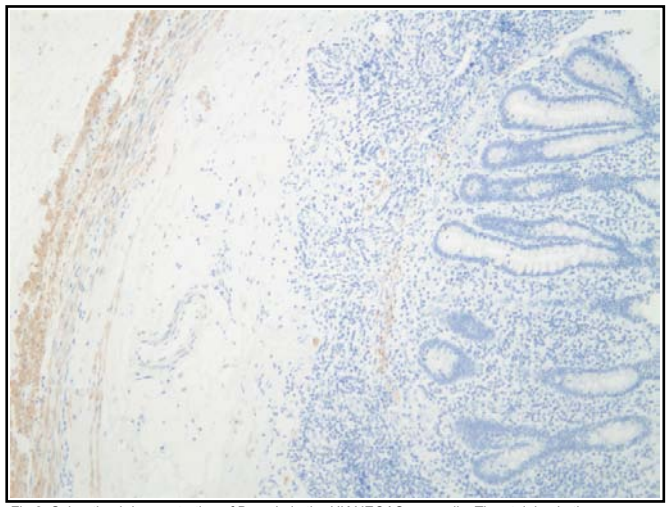


Fig 2. Suboptimal demonstration of Desmin in the UK NEQAS appendix. The staining in the smooth muscle muscularis propria is weak, with very few vessels demonstated(compare with Fig 1). Section stained with the Leica DER11 antibody, 1:200 on the Bond Max with ER1 antigen retrieval for 20 minutes.

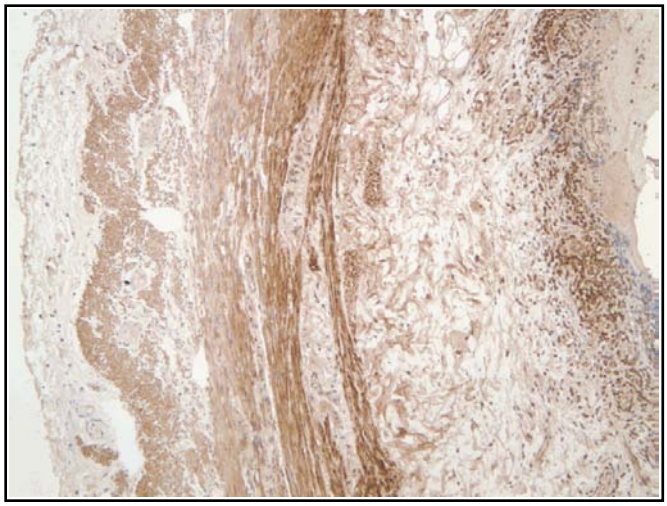


Fig 3. Suboptimal demonstration of Desmin in the UK NEQAS appendix, with excessive background staining. The slide was stained using the Dako D33 antibody, 1:50 on the Leica Bond Max with ER2 antigen retrieval for 30 minutes.

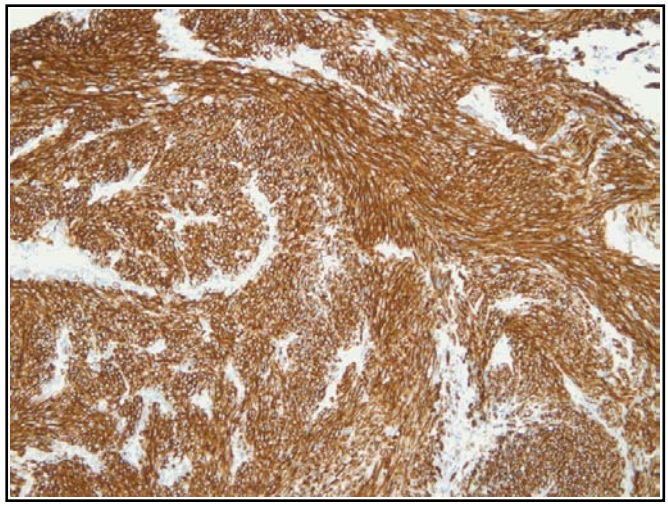


Fig 4. Good example of Desmin staining in the UK NEQAS distributed leiomyosarcoma, showing strong cytoplasmic staining in all the tumour cells. (Same methodology as Fig 1).

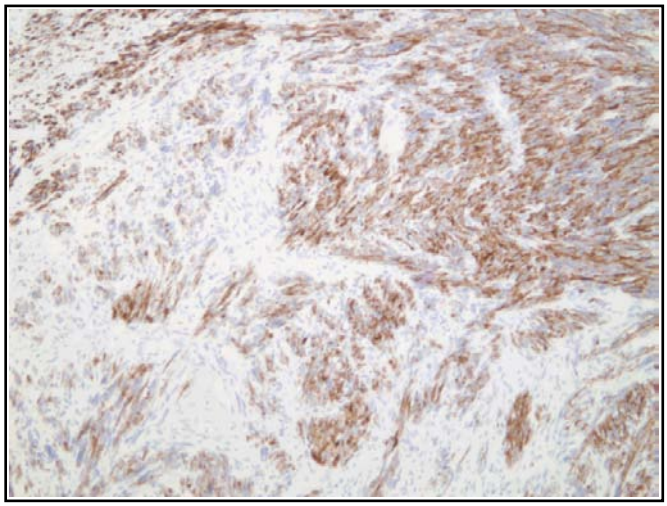


Fig 5. Poor demonstration of Desmin in the UK NEQAS distributed leiomyosarcoma tumour: The staining is patchy and many of the tumour cells expected to stain are not demonstrated (compare to Fig 4). Stained using the Leica DER11 antibody (1:100) on the Leica Bond III with VBS Bond enzyme I pre-treatment for 10 minutes.

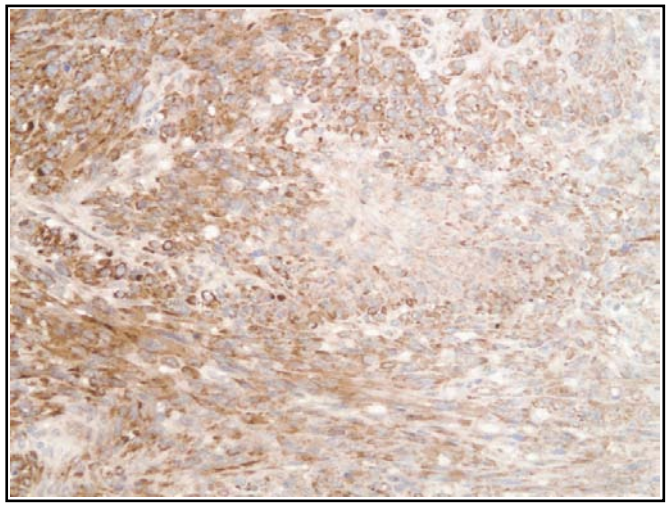


Fig 6. Suboptimal demonstration of Desmin in the UK NEQAS leiomyosarcoma section: Although most of the expected tumour cells are demonstrated, the staining is diffuse and patchy. The section was stained with the Dako D33 antibody, 1:50 on the Leica Bond Max with ER2 antigen retrieval for 30 minutes.

## Selected Images showing Optimal and Sub-optimal Immunostaining

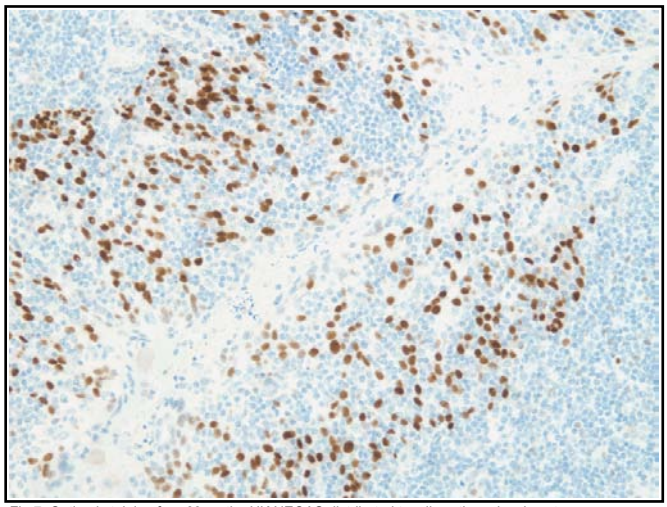


Fig 7. Optimal staining for p63 on the UK NEQAS distributed tonsil section, showing strong nuclear epithelial staining. The section was stained with the Dako 4A4 antibody, pressure cooker pre-treatment on the BioGenex platform with the BioGenex SS Link-Label detection system.

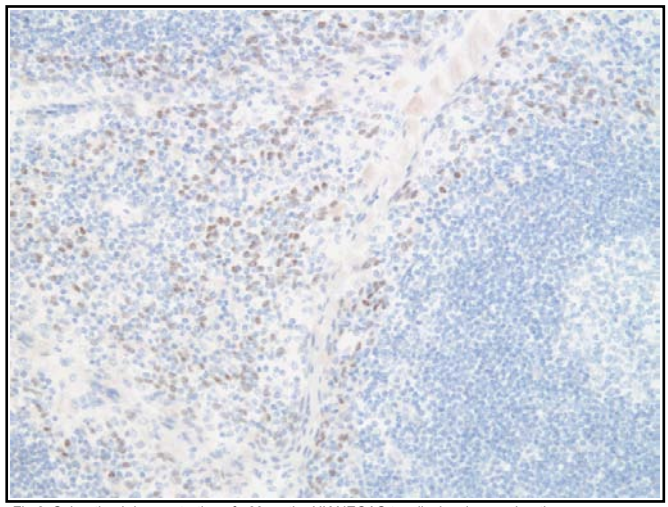


Fig 8. Suboptimal demonstration of p63 on the UK NEQAS tonsil, showing weaker than expected staining (compare to Fig 7). This is most likely due to the titre of antibody being too dilute. Section stained with the Dako 4A4 antibody at a dilution of 1:2000, and stained on the Dako Autostainer, pre-treated in the Labvision PT link with high pH buffer for 16 minutes.

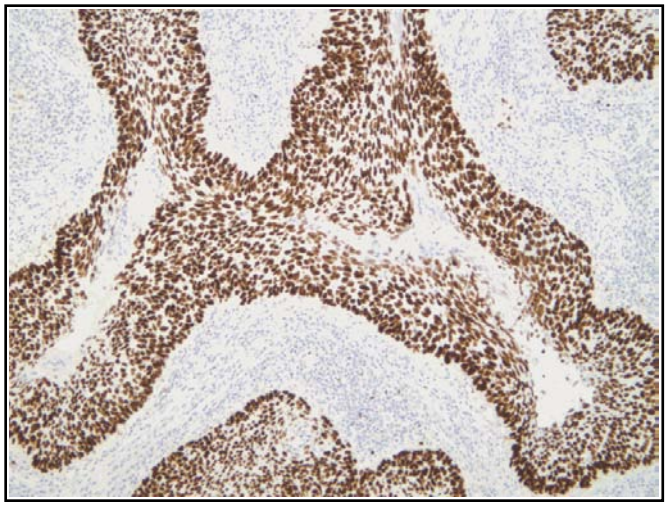


Fig 9. Optimal demonstration of p63 in the UK NEQAS distributed lung squamous cell carcinoma (SCC). There is strong nuclear staining in virtually all of the neoplastic cells. Section stained with the Biocare Medical 4A4 antibody on the Ventana Benchmark XT with CC1 retrieval for 32 minutes and Optiview detection kit.

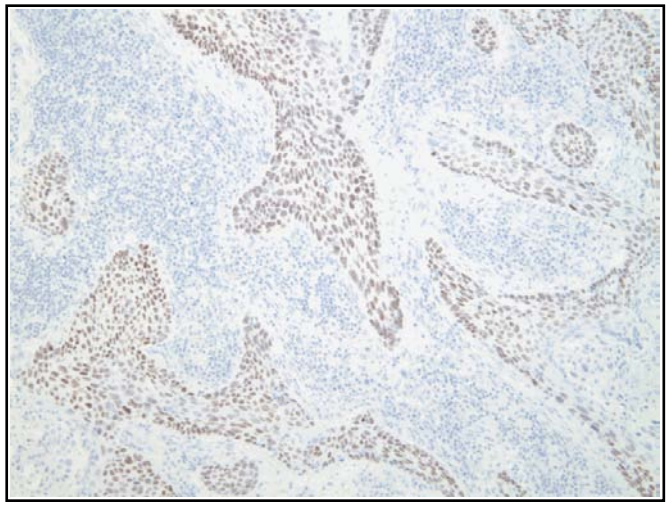


Fig 10. Sub-optimal staining of p63 in the UK NEQAS distributed lung squamous cell carcinoma section with weaker staining of tumour cells (compare to Fig 9). Section stained using the Leica 7JUL antibody with ER2 pre-treatment on the Bond III with ER2 pre-treatment for 20 minutes.

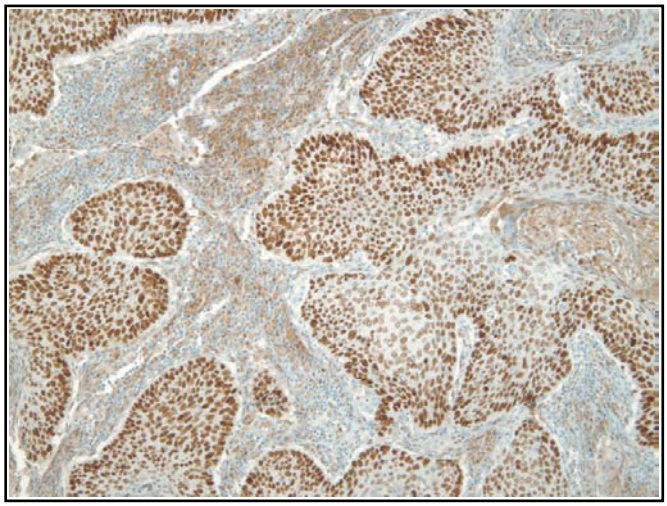


Fig 11. Sub-optimal staining of p63 in the UK NEQAS distributed lung squamous cell carcinoma (SCC). Tumour cells are stained but there is excessive background staining, which could be due to a combination of antibody incubation time (40 mins) and extended antigen retrieval. Stained using the DB Biotech V22E clone (1:50).

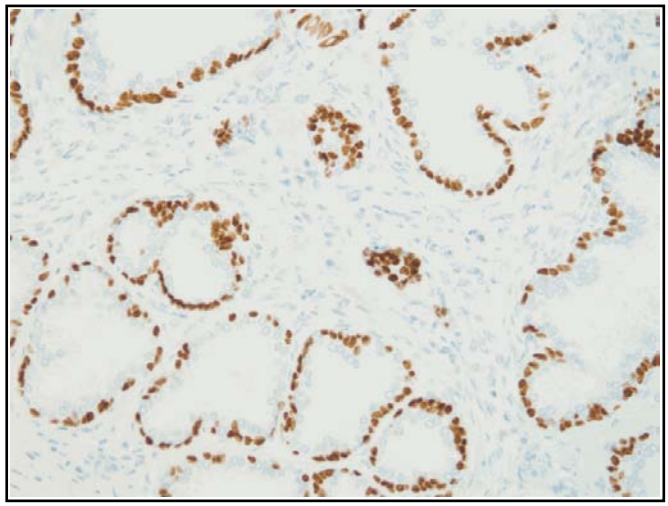
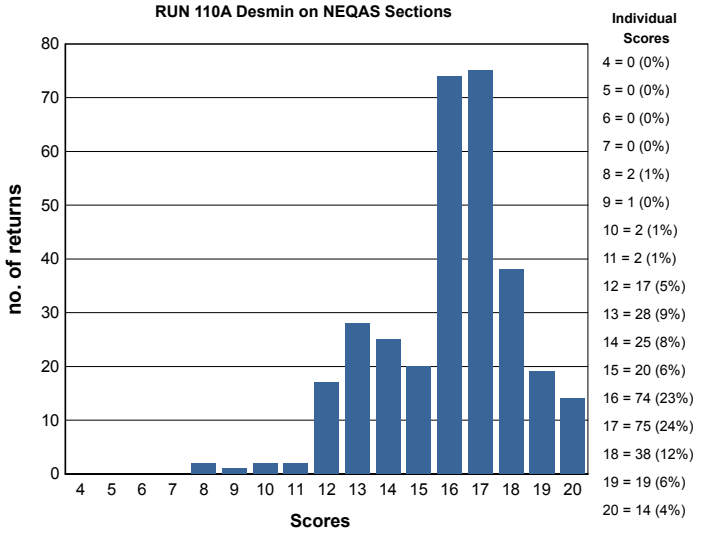
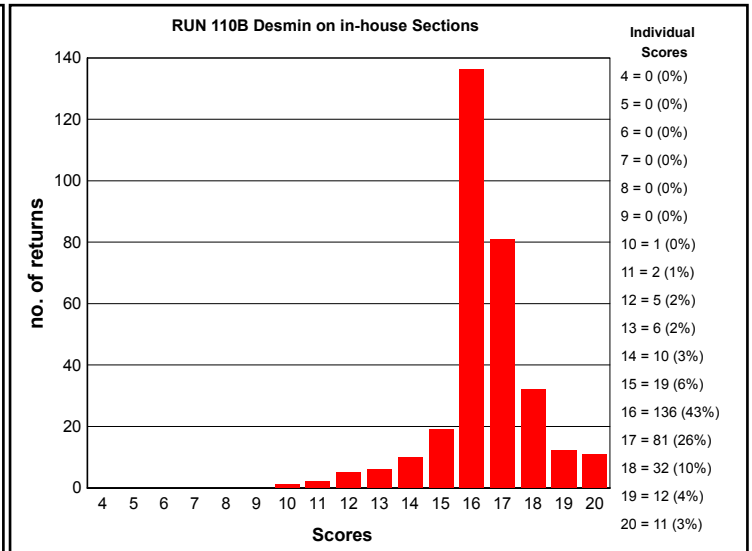
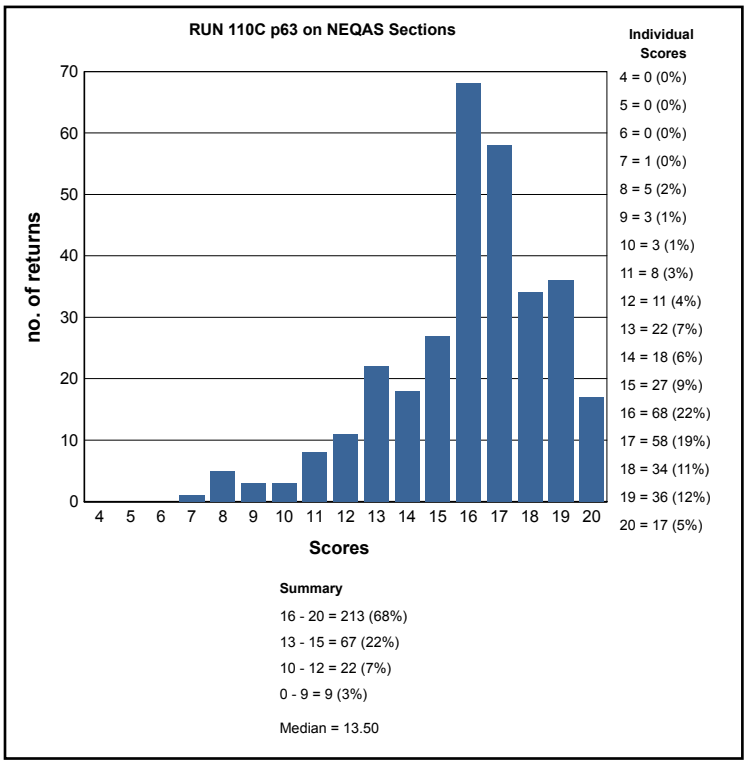


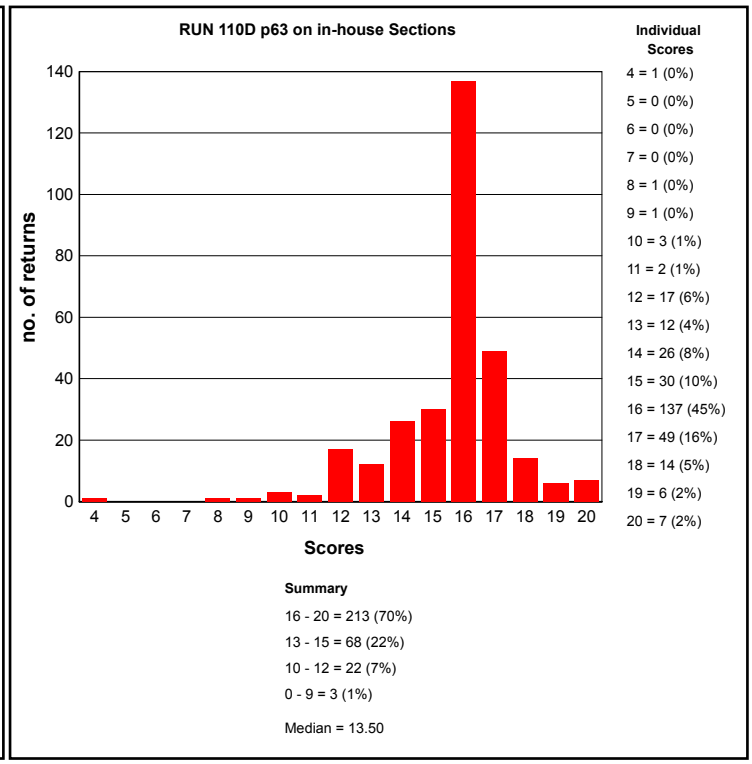
Fig 12. Good example of an in house prostate control stained with p63. The staining is strong and distinct while the background remains clean. The participant scored 20/20 for the quality of staining. Stained with the Ventana pre-diluted 454 antibody on the Benchmark GX with CC1 antigen retrieval.

## 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: 110**

**Primary Antibody :** Desmin

| Antibody Details | N | % |
|---|---|---|
| Cell Marque 243M (D33) | 3 | 67 |
| Dako IR606 RTU Flex Link (D33) | 13 | 92 |
| Dako IS606 RTU Flex Plus (D33) | 3 | 100 |
| Dako M0760 (D33) | 164 | 93 |
| Dako M724 (DER11) | 3 | 100 |
| Leica PA0032 RTU (DER11) | 17 | 94 |
| Leica/Novocastra NCL-DES (DER11) | 44 | 91 |
| Leica/Novocastra NCL-L-DES (DER11) | 12 | 75 |
| Other | 5 | 100 |
| Vector VP D502 (DER11) | 1 | 100 |
| Ventana 760 2513 (DER11) | 49 | 96 |

**General Pathology Run: 110**

**Primary Antibody :** p63

| Antibody Details | N | % |
|---|---|---|
| Biocare Medical PM163 (4A4) | 12 | 83 |
| Biogenex AM418 (4A4) | 4 | 100 |
| Dako IR662 RTU FLEX Link (DAK-p63) | 25 | 100 |
| Dako M7247 (4A4) | 7 | 86 |
| Dako M7317 (DAK-p63) | 52 | 98 |
| DBS PDM136 (4A4) | 2 | 100 |
| Leica/Novocastra NCL-L-p63 (7JUL) | 66 | 76 |
| Leica/Novocastra PA0103 RTU (7JUL) | 18 | 89 |
| Minarini MP-163-CM (4A4) | 18 | 94 |
| NeoMarkers/Thermo Ab-1 MS-1081-P (4A4) | 1 | 100 |
| Other | 29 | 79 |
| Santa Cruz sc8431 (4A4) | 5 | 100 |
| Ventana 790-4509 (454) | 61 | 98 |

**General Pathology Run: 110**

| Heat Mediated Retrieval | Desmin N | Desmin % | p63 N | p63 % |
|---|---|---|---|---|
| _Ventana Benk CC1 (Mild) | 1 | 100 | 0 | 0 |
| Biocare Decloaking Chamber | 2 | 100 | 1 | 100 |
| Dako Omnis | 4 | 100 | 5 | 100 |
| Dako Pascal | 1 | 100 | 1 | 100 |
| Dako PTLink | 43 | 93 | 39 | 97 |
| Lab vision PT Module | 6 | 83 | 6 | 67 |
| Leica ER1 10 mins | 2 | 100 | 2 | 50 |
| Leica ER1 20 mins | 10 | 80 | 4 | 50 |
| Leica ER1 30 mins | 6 | 67 | 0 | 0 |
| Leica ER2 10 mins | 5 | 80 | 2 | 100 |
| Leica ER2 20 mins | 53 | 92 | 55 | 82 |
| Leica ER2 30 mins | 13 | 85 | 25 | 76 |
| Leica ER2 40 mins | 1 | 100 | 5 | 100 |
| Microwave | 4 | 100 | 5 | 80 |
| None | 28 | 93 | 0 | 0 |
| Other | 3 | 100 | 2 | 100 |
| Pressure Cooker | 9 | 100 | 8 | 88 |
| Pressure Cooker in Microwave Oven | 0 | 0 | 1 | 100 |
| Steamer | 2 | 100 | 3 | 100 |
| Ventana CC1 16mins | 3 | 67 | 0 | 0 |
| Ventana CC1 20mins | 1 | 100 | 0 | 0 |
| Ventana CC1 24mins | 1 | 100 | 1 | 100 |
| Ventana CC1 32mins | 16 | 100 | 16 | 100 |
| Ventana CC1 36mins | 5 | 100 | 1 | 100 |
| Ventana CC1 40mins | 2 | 100 | 9 | 100 |
| Ventana CC1 44mins | 0 | 0 | 1 | 100 |
| Ventana CC1 48mins | 1 | 100 | 1 | 100 |
| Ventana CC1 52mins | 2 | 100 | 1 | 100 |
| Ventana CC1 56mins | 2 | 100 | 3 | 100 |
| Ventana CC1 64mins | 17 | 88 | 39 | 92 |
| Ventana CC1 76mins | 0 | 0 | 2 | 100 |
| Ventana CC1 88mins | 0 | 0 | 1 | 100 |
| Ventana CC1 8mins | 3 | 100 | 0 | 0 |
| Ventana CC1 92mins | 0 | 0 | 1 | 100 |
| Ventana CC1 extended | 2 | 100 | 2 | 50 |
| Ventana CC1 mild | 16 | 94 | 12 | 83 |
| Ventana CC1 standard | 29 | 97 | 39 | 97 |
| Ventana CC2 52mins | 0 | 0 | 1 | 100 |
| Ventana CC2 56mins | 1 | 100 | 0 | 0 |
| Ventana CC2 8mins | 0 | 0 | 1 | 100 |
| Ventana CC2 mild | 0 | 0 | 1 | 100 |
| Water bath 68 OC | 1 | 100 | 1 | 100 |
| Water bath 95-98 OC | 2 | 100 | 1 | 100 |

**General Pathology Run: 110**

| Enzyme Mediated Retrieval | Desmin N | Desmin % | p63 N | p63 % |
|---|---|---|---|---|
| AS PER KIT | 0 | 0 | 3 | 100 |
| BioGenex Protease | 1 | 100 | 0 | 0 |
| Dako Proteinase K (S3020) | 1 | 100 | 0 | 0 |
| NOT APPLICABLE | 107 | 93 | 97 | 89 |
| VBS Bond Enzyme 1 | 6 | 83 | 0 | 0 |
| VBS Bond Enzyme 2 | 1 | 100 | 0 | 0 |
| Ventana Protease | 4 | 100 | 0 | 0 |
| Ventana Protease 1 (760-2018) | 33 | 94 | 2 | 100 |

| General Pathology Run: 110 | Desmin | | p63 | |
|---|---|---|---|---|
| **Detection** | **N** | **%** | **N** | **%** |
| A Menerini Polymer (MP-XCP) | 0 | 0 | 1 | 100 |
| AS PER KIT | 23 | 87 | 27 | 89 |
| BioGenex SS Polymer (QD 420-YIKE) | 1 | 100 | 1 | 100 |
| Dako EnVision FLEX ( K8000/10) | 9 | 100 | 8 | 100 |
| Dako EnVision FLEX+ ( K8002/12) | 28 | 89 | 28 | 96 |
| Dako Envision HRP/DAB ( K5007) | 8 | 100 | 5 | 80 |
| Dako Envision+ HRP mouse K4004/5/6/7 | 1 | 100 | 0 | 0 |
| LabVision UltraVision ONE Polymer ( TL-012/5-HDJ/T | 1 | 0 | 2 | 50 |
| Leica Bond Polymer Define (DS9713) | 3 | 100 | 2 | 0 |
| Leica Bond Polymer Refine (DS9800) | 89 | 89 | 76 | 82 |
| MenaPath X-Cell Plus (MP-XCP) | 3 | 100 | 1 | 100 |
| None | 1 | 100 | 1 | 100 |
| NOT APPLICABLE | 1 | 100 | 0 | 0 |
| Novocastra Novolink PDS (RE7-140/150/280/290-K) | 1 | 100 | 1 | 100 |
| Other | 9 | 100 | 14 | 86 |
| Vector Elite Universal ABC (PK-6200) | 0 | 0 | 1 | 100 |
| Vector ImmPRESS Universal (MP-7500) | 1 | 100 | 0 | 0 |
| Ventana iView system (760-091) | 7 | 86 | 5 | 80 |
| Ventana OptiView Kit (760-700) | 35 | 97 | 35 | 100 |
| Ventana UltraView Kit (760-500) | 84 | 94 | 76 | 96 |

| General Pathology Run: 110 | Desmin | | p63 | |
|---|---|---|---|---|
| **Automation** | **N** | **%** | **N** | **%** |
| BioGenex GenoMX 6000i | 1 | 100 | 1 | 100 |
| Dako Autostainer | 5 | 100 | 3 | 67 |
| Dako Autostainer Link 48 | 37 | 95 | 37 | 97 |
| Dako Autostainer plus | 6 | 100 | 3 | 67 |
| Dako Autostainer Plus Link | 3 | 67 | 3 | 100 |
| Dako Omnis | 4 | 100 | 5 | 100 |
| LabVision Autostainer | 4 | 75 | 4 | 75 |
| Leica Bond Max | 49 | 88 | 41 | 83 |
| Leica Bond-III | 51 | 88 | 54 | 78 |
| Menarini - Intellipath FLX | 4 | 100 | 2 | 100 |
| None (Manual) | 6 | 100 | 6 | 100 |
| Other | 2 | 100 | 2 | 100 |
| Shandon Sequenza | 4 | 100 | 4 | 75 |
| Ventana Benchmark GX | 6 | 100 | 4 | 100 |
| Ventana Benchmark ULTRA | 68 | 94 | 72 | 96 |
| Ventana Benchmark XT | 63 | 94 | 57 | 93 |

| General Pathology Run: 110 | Desmin | | p63 | |
|---|---|---|---|---|
| **Chromogen** | **N** | **%** | **N** | **%** |
| A. Menarini Liquid Stable DAB kit | 0 | 0 | 1 | 100 |
| AS PER KIT | 29 | 97 | 41 | 95 |
| BioGenex Liquid DAB (HK153-5K) | 1 | 100 | 1 | 100 |
| Dako DAB K3468 | 1 | 100 | 1 | 0 |
| Dako DAB Liquid (K3465) | 1 | 100 | 0 | 0 |
| DAKO DAB+ | 0 | 0 | 1 | 100 |
| Dako DAB+ Liquid (K3468) | 4 | 75 | 3 | 100 |
| Dako DAB+ REAL Detection (K5001) | 3 | 100 | 0 | 0 |
| Dako EnVision Plus kits | 3 | 100 | 5 | 100 |
| Dako FLEX DAB | 36 | 92 | 30 | 97 |
| Dako REAL EnVision K5007 DAB | 7 | 100 | 6 | 83 |
| LabVision (TA-125-HD) | 0 | 0 | 1 | 100 |
| Leica Bond Polymer Refine kit (DS9800) | 93 | 88 | 82 | 78 |
| menapath xcell kit DAB (MP-860) | 3 | 100 | 1 | 100 |
| Other | 15 | 93 | 16 | 88 |
| Sigma DAB (D5637) | 1 | 100 | 1 | 100 |
| Sigma DAB (D5905) | 1 | 100 | 1 | 100 |
| Ventana DAB | 23 | 96 | 23 | 100 |
| Ventana iview | 8 | 75 | 6 | 83 |
| Ventana Ultraview DAB | 85 | 95 | 80 | 94 |

## BEST METHODS - Gold Standard Antibody

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

### Desmin - Method 1

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

**Primary Antibody:** Dako IR606 RTU Flex Link (D33) , 20 Mins Prediluted

**Automation:** Dako Autostainer Link 48

**Method:** Dako FLEX kit

**Main Buffer:** AS PER KIT

**HMAR:** Dako PTLink, PH: 9

**EAR:**

**Chromogen:** AS PER KIT

**Detection:** Dako EnVision FLEX ( K8000/10) , 20 Mins Prediluted

**Desmin - Method 2**

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

| | |
|---|---|
| **Primary Antibody:** | Dako M0760 (D33) , 30 Mins, ROOM ºC Dilution 1: 1/50 |
| **Automation:** | Dako Autostainer Link 48 |
| **Method:** | Dako FLEX+ kit |
| **Main Buffer:** | Dako FLEX wash buffer, PH: 7.6 |
| **HMAR:** | Dako PTLink, Buffer: Dako Envision High pH antigen retrieval |
| **EAR:** | NOT APPLICABLE |
| **Chromogen:** | Dako FLEX DAB, ROOM ºC., Time 1: 5 Mins, Time 2: 5 Mins |
| **Detection:** | Dako EnVision FLEX+ ( K8002/12) , 30 Mins, ROOM ºC Prediluted |

**Desmin - Method 3**

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

| | |
|---|---|
| **Primary Antibody:** | Leica/Novocastra NCL-DES (DER11) , 32 Mins, 36 ºC Dilution 1: 50 |
| **Automation:** | Ventana Benchmark ULTRA |
| **Method:** | Ventana Optiview |
| **Main Buffer:** | Ventana reaction buffer (950-300) |
| **HMAR:** | Ventana CC1 8mins |
| **EAR:** | Ventana Protease 1 (760-2018), 37 ºC. Digestion Time NEQAS: 4 Mins. In-House: 4 Mins |
| **Chromogen:** | Other, 36 ºC., Time 1: 8 Mins, Time 2: 8 Mins |
| **Detection:** | Ventana OptiView Kit (760-700) , 8 Mins, 36 ºC Prediluted |

**Desmin - Method 4**

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

| | |
|---|---|
| **Primary Antibody:** | Ventana 760 2513 (DER11) , 36 Mins, 36 ºC Prediluted |
| **Automation:** | Ventana Benchmark ULTRA |
| **Method:** | Ventana UltraView DAB |
| **Main Buffer:** | Ventana reaction buffer (950-300), PH: 7.4 |
| **HMAR:** | Ventana CC1 20mins, Buffer: Ultra CC1 (cat 950-224), PH: 8 |
| **EAR:** | Ventana Protease, 36 ºC. Digestion Time NEQAS: 8 Mins. In-House: 8 Mins |
| **Chromogen:** | Ventana Ultraview DAB, PH: 7, 36 ºC., Time 1: 8 Mins |
| **Detection:** | Ventana UltraView Kit (760-500) , 8 Mins, 36 ºC Prediluted |

## BEST METHODS - Secondary Antibody

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

**p63 - Method 1**

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

| | |
|---|---|
| **Primary Antibody:** | Dako IR662 RTU FLEX Link (DAK-p63) , 20 Mins Prediluted |
| **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 , 20 Mins Prediluted |

**p63 - Method 2**

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

| | |
|---|---|
| **Primary Antibody:** | Dako IR662 RTU FLEX Link (DAK-p63) |
| **Automation:** | Dako Autostainer Link 48 |
| **Method:** | Dako FLEX+ kit |
| **Main Buffer:** | Dako FLEX wash buffer |
| **HMAR:** | Dako PTLink |
| **EAR:** | NOT APPLICABLE |
| **Chromogen:** | Dako EnVision Plus kits |
| **Detection:** | Dako EnVision FLEX+ ( K8002/12) |

**p63 - Method 3**

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

| | |
|---|---|
| **Primary Antibody:** | Dako M7317 (DAK-p63) , 30 Mins, 25 ºC Dilution 1: 100 |
| **Automation:** | Leica Bond Max |
| **Method:** | Leica BondMAx Refine KIT |
| **Main Buffer:** | Bond Wash Buffer (AR9590), PH: 7.4 |
| **HMAR:** | Leica ER2 30 mins |
| **EAR:** | NOT APPLICABLE |
| **Chromogen:** | Leica Bond Polymer Refine kit (DS9800), 25 ºC., Time 2: 6 Mins |
| **Detection:** | Leica Bond Polymer Refine (DS9800) , 8 Mins, 25 ºC Prediluted |

**p63 - Method 4**

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

| | |
|---|---|
| **Primary Antibody:** | Ventana 790-4509 (454) |
| **Automation:** | Ventana Benchmark XT |
| **Method:** | Ventana Optiview |
| **Main Buffer:** | Ventana reaction buffer (950-300) |
| **HMAR:** | Ventana CC1 32mins |
| **EAR:** | |
| **Chromogen:** | Ventana DAB |
| **Detection:** | Ventana OptiView Kit (760-700) |

# The Breast Hormonal Receptor Module Run 110

**Suzanne Parry and Merdol Ibrahim**

| | |
|---|---|
| **Antigen Assessed:** | Oestrogen Receptor (ER) |
| **Tissue Sections circulated:** | Composite slide consisting of 3 breast carcinomas with different levels of receptor expression. Most slides also included normal breast and normal tonsil. |
| **Number of Registered Participants:** | 297 |
| **Number of Participants This Run** | 271 (91%) |

### Circulated Tissue

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

| Sections | % Positivity | Expected Staining Intensity | Allred / Quick Score |
|---|---|---|---|
| **A. IDC** | >95% | High | 8 |
| **B. IDC** | 40-60% | Medium | 5-6 (depending on the serial section received) |
| **C. IDC** | 0% | Negative | 0 |
| **D. Normal Breast** | 20-40% of normal breast glandular epithelial cells | Medium to High | 0 (Negative) |
| **E. Tonsil** | 1-5% | Weak to Medium | 0 (Negative) |

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

**General Guideline Used in The Assessment of slides:**

| Score | STAINING PATTERN |
|---|---|
| **0** | Slide not returned by participant. |
| **1 or 2** | **Unacceptable:** E.g. False positive / false negative / non-specific or inappropriate staining / lack of staining **Clinically Unacceptable.** |
| **3** | **Borderline Acceptable:** Staining weaker than expected / background staining / weak/strong counterstain, **Clinically still readable but technical improvements can be made** |
| **4 or 5** | **Acceptable:** Demonstration of the expected proportion of nuclei stained in the invasive tumours, with roughly the expected staining intensity. |
| **Marks are also deducted when correct clinical interpretation of staining may be hindered due to factors such as:** - Excessive cytoplasmic or diffuse nuclear staining - Excessively strong or weak haematoxylin counterstain - Excessive antigen retrieval resulting in morphological damage - Poor quality/inadequate choice of in-house control tissue ( poor/inadequate fixation, damaged cell morphology, over retrieval etc.) | |

**In-HouseTissue Recommendations:**

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

The required composite control should consist of the following samples:

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

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

## Introduction

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

### Choice of Tissue for Assessments

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

### ER Staining Within the Tonsil

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

### Quality Control of NEQAS Samples

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

## Assessment Results

**Features of Optimal Immunostaining: (Figs 1-6)**

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

**Features of Sub-Optimal Immunostaining: (Figs 7-11)**

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

## NEQAS Slide Results

Results for the ER assessment on the NEQAS tissue showed an acceptable pass rate of 85% (scores of ≥13-20/20), which was similar to the previous assessment (Run 109). A further 9% (n=25) received a borderline score of between 10-12/20. These borderline passes were mostly due to weak staining, particularly in the mid-expressing tumour. 17 labs (6%) failed the assessment and this was predominantly due to false positive staining in the UK NEQAS ICC negative expressing tumour.

The Ventana SP1 clone was the most popular antibody, used by 38% (n=103) participants and showed an acceptable pass rate of 97%. 59 labs (23%) used the Leica 6F11 antibody clone, and this showed an acceptable pass rate of 59%. The Dako EP1 was the next most popular antibody clone, used by 57 (21%) of participants, with an acceptable pass rate of 95%.

## In-House Tissue Results

90% of participants also submitted their in-house controls for assessment. These showed an acceptable pass rate of 71%, and a further 26% of labs received a borderline pass. 9 labs failed on their in-house submitted sections, which was due to false positive staining seen in one of the sections, which was most likely caused by excessive pre-treatment. Several labs that received a borderline pass lost marks because they did not provide a composite control consisting of high, mid and negative-expressing tumours, as required by UK NEQAS IHC.

## Comparing NEQAS Sample Scores and In-house Control Scores.

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

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

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

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

## Assessment Summary

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

As from Run 108 UK NEQAS ICC has asked labs to cut and place their own in-house control sections on the same slide as the NEQAS tissue, and therefore stain both sections together on the same slide. This has proven to be very helpful at assessment, enabling the assessors to review both sections at the same time: It was particularly interesting to see that most laboratories received a similar score for both their in-house and NEQAS samples. However, in some instances, labs received better scores on the NEQAS tissue, which was predominantly due to labs not being able to provide the required composite control for their in-house tissue; in particular, an ER mid-expressing tumour. These labs were therefore given a maximum score of 12/20 (borderline pass). Laboratories that did score highly on their NEQAS sample and only received a borderline pass due to not having an appropriate mid-expressor will have the reassurance that their NEQAS score is indicative that the level of sensitivity of their assay is acceptable.

As previously mentioned several participants scored lower on the NEQAS tissue due to weak and lower expression on the mid-expressing tumour. There are several reasons that

may have caused this:

(1) The slides are now sent out un-baked with an instruction sheet. We advise labs to cut their own in-house sections onto the slides as soon as they receive the UK NEQAS material into the laboratory. The slides will need to be drained thoroughly and then baked in the oven for 1 hour at 55-60ºC or at 37ºC overnight. We do not advocate drying slides directly on a hotplate, and we also advise to stain the slides as soon as possible on receipt into the laboratory.

(2) If the slides are stored at room temperature and not stained within at least a 2 week time frame this can affect the antigenicity of ER. 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 in-house control is much stronger/optimal.

(3) The sensitivity of your assay may be acceptable, in that it is acceptable for your own tissue sections locally and you have validated your assay, However it can indicate that the sensitivity of your assay may not be optimal.

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

**References**


1. Jennet M. Harvey, Gary M. Clark, C. Kent Osborne, and D. Craig Allred (1999) Estrogen Receptor Status by Immunohistochemistry Is Superior to the Ligand-Binding Assay for Predicting Response to Adjuvant Endocrine Therapy in Breast Cancer J Clin Oncol 17:1474-1481.
2. Robin Leake, Diana Barnes, Sarah Pinder, Ian Ellis, Liz Anderson, Tom Anderson, Ruth Adamson, Tony Rhodes, Keith Miller and Rosemary Walker (2000) J. Clin. Pathol. 2000: 634-635.
3. Anderson E. The role of oestrogen and progesterone receptors in human mammary development and tumorigenesis. Breast Cancer Res. 2002; 4:197-201.
4. Fisher B, Costantino J, Redmond C, Poisson R, Bowman D, Couture J, Dimitrov NV, Wolmark N, Wickerham DL, Fisher ER, et al. (1989) A randomized clinical trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen receptor-positive tumors. N Engl J Med 1989: 479-484.
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6. Rhodes A, Jasani B, Balaton AJ, Barnes DM, Anderson E, Bobrow LG, Miller KD (2001). Study of inter-laboratory reliability and reproducibility of estrogen and progesterone receptor assays in Europe. Documentation of poor reliability and identification of insufficient microwave antigen retrieval time as a major contributory element of unreliable assays. Am J Clin Pathol. 2001 Jan;115(1):44-58.
7. Ibrahim M, Dodson A, Barnett S, Fish D, Jasani B, Miller K. (2008) Potential for false-positive staining with a rabbit monoclonal antibody to progesterone receptor (SP2): findings of the UK National External Quality Assessment Scheme for Immunocytochemistry and FISH highlight the need for correct validation of antibodies on introduction to the laboratory. Am J Clin Pathol. 129:398-409.
8. Davies C, Godwin J, Gray R et al., (2011) Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. The Lancet 378: 771-784.


**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

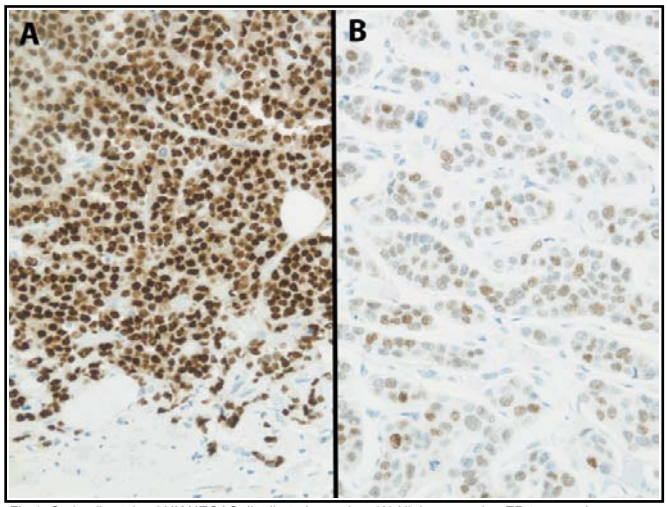



Fig 1. Optimally stained UK NEQAS distributed samples. (A) High expressing ER tumour shows intense staining in over 95% of neoplastic cells, while (B) mid-expressing tumour shows varying intensity of ER positivity in approximately 60% of neoplastic cells. Stained with the Dako EP1 antibody, 1:40 on the Dako Autostainer in the PT link, high pH buffer for 20 minutes.

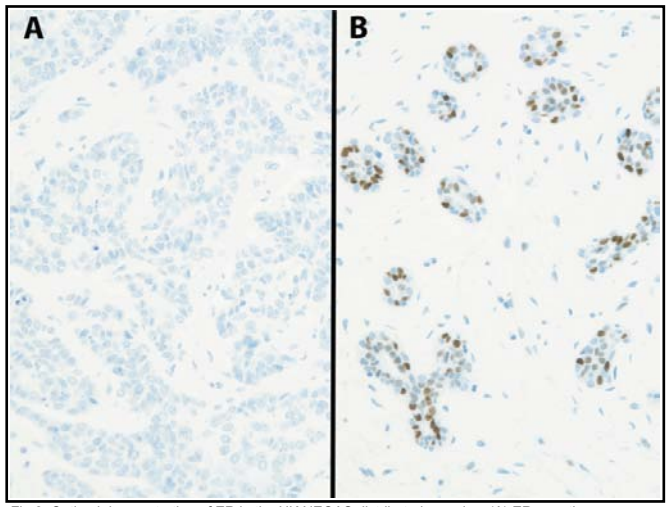



Fig 2. Optimal demonstration of ER in the UK NEQAS distributed samples. (A) ER negative tumour remains unstained. (B) As expected, only a percentage of the nuclei in the normal glands are positive. (Same protocol as Fig 1).

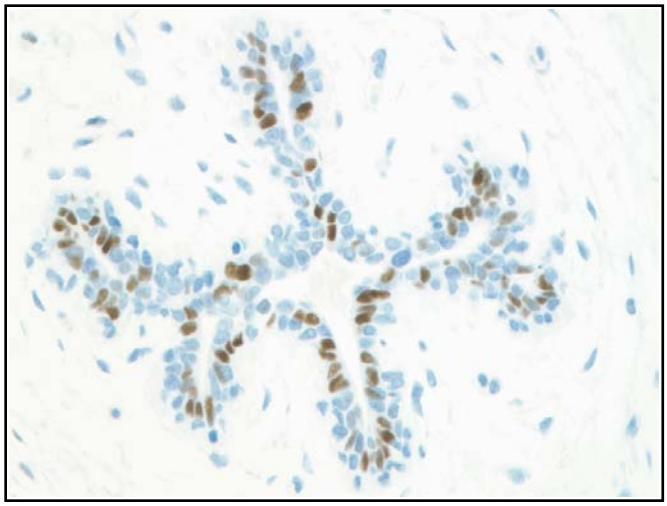


Fig 3. Higher power image demonstrating a normal breast gland stained with ER. The image shows the expected percentage of positive staining. (Same protocol as Fig 1).

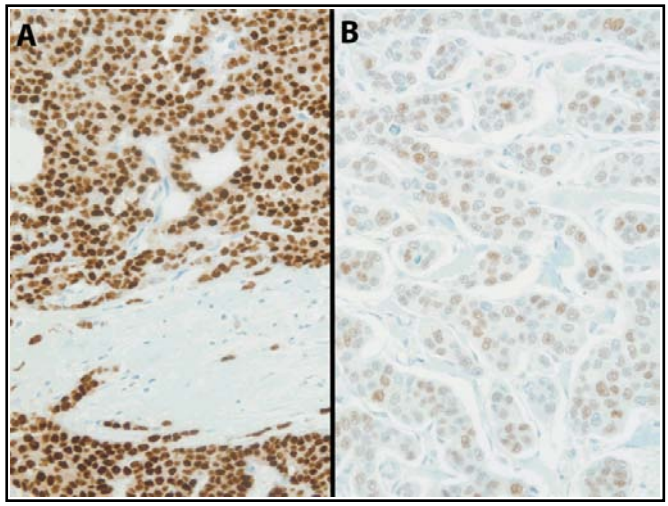



Fig 4. Optimal demonstration of ER in the UK NEQAS (A) high- and (B) mid-expressing tumours, showing the expected level of staining in both samples. Stained with the Leica 6F11 antibody, 1:30 on the Ventana platform with CC1 retrieval for 32 minutes and OptiView detection kit.

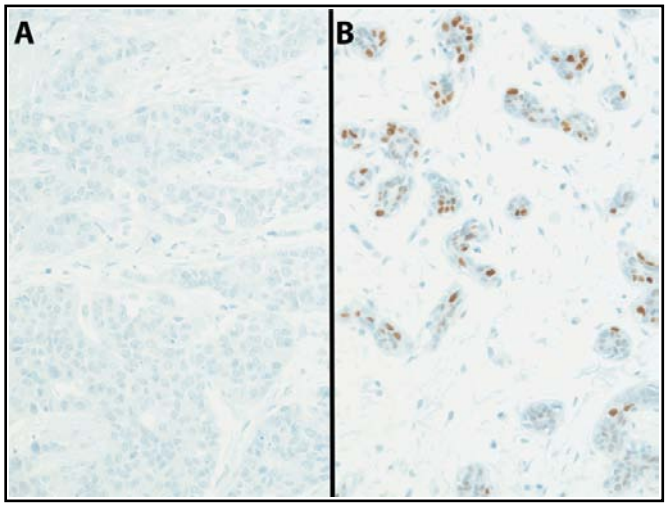



Fig 5. Optimal level of expression in the UK NEQAS distributed samples stained with the Leica 6F11 antibody clone. (A) As expected the ER negative tumour remains unstained, and (B) the normal glands show some ER positive staining (Same protocol as Fig 4).

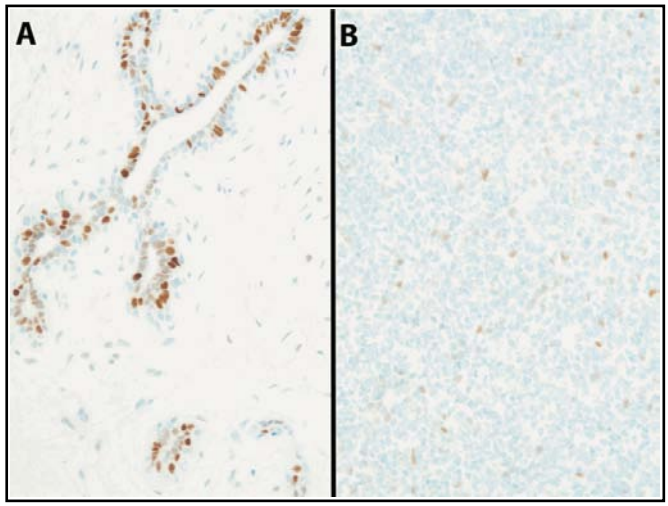



Fig 6. Good demonstration of ER on the UK NEQAS distributed (A) normal tissue and (B) tonsil with both sections showing the expected level of nuclear staining. Sections stained with the Ventana pre-diluted SP1 clone on the Ventana ULTRA with CC1 antigen retrieval for 64 minutes.

## Selected Images showing Optimal and Sub-optimal Immunostaining

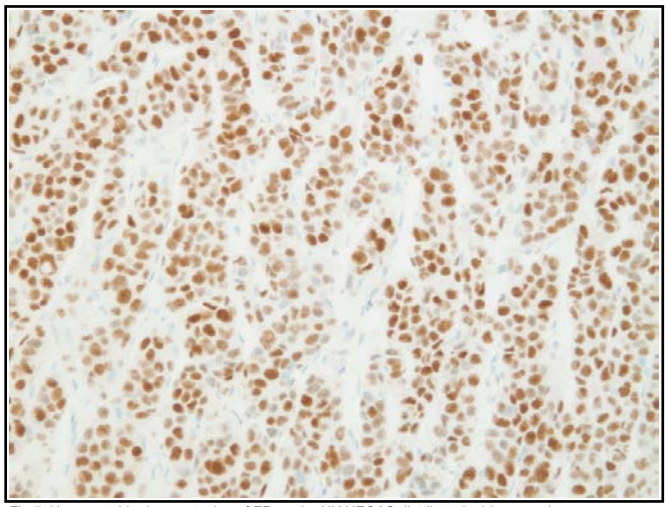


Fig 7. Unacceptable demonstration of ER on the UK NEQAS distributed mid-expressing tumour, showing much stronger and higher percentage of ER positive staining than expected (compare to Figs 1B & 4B). Stained using the Ventana SP1 clone and 76 minutes antigen retrieval. Excessive antigen retrieval is the most likely reason for the higher level of staining.

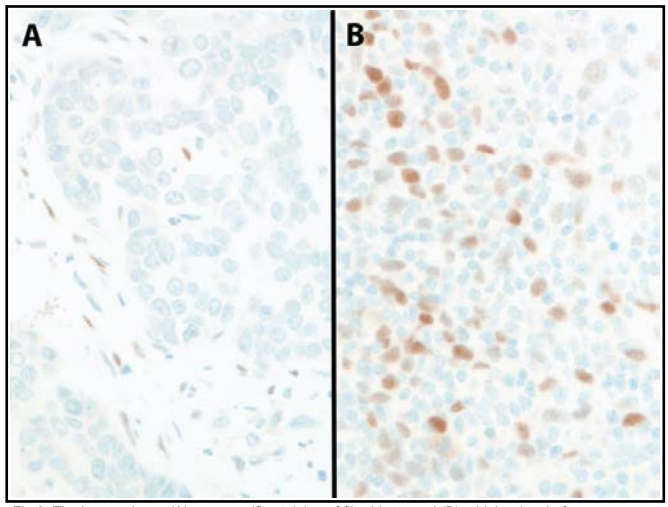



Fig 8. The image shows (A) non-specific staining of fibroblasts and (B) a higher level of lymphocytes staining in the UK NEQAS distributed tonsil. This was the same method used as Fig 7; and again, the excessive antigen retrieval is the most likely reason for the non-specific and over-staining in the tonsil.

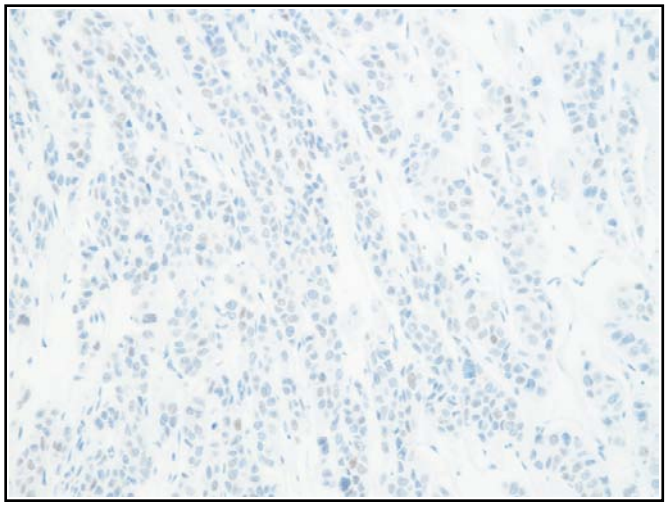


Fig 9. Unacceptable demonstration of ER on the UK NEQAS distributed mid-expressing tumour (compare to Figs 1B & 4B). The staining is very weak with far less cells expressing ER than expected. The staining was carried out using the Leica 6F11 clone at a dilution of 1:50 on the Bond III with ER1 antigen retrieval for 20 minutes.

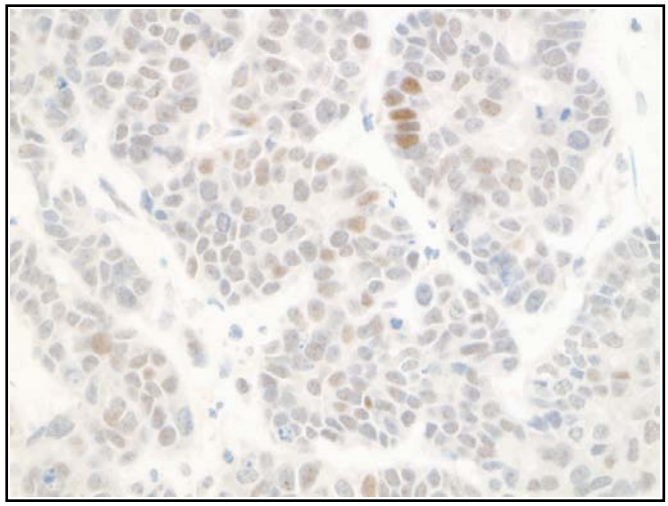


Fig 10. Unacceptable false-positive demonstration of ER in the UKNEQAS distributed ER negative tumour. A combination of high antibody concentration, antibody incubation time and use of a high pH buffer antigen retrieval may all be contributory factors. Stained with the Leica 6F11 antibody, which is recommended to be used with ER1 pre-treatment.

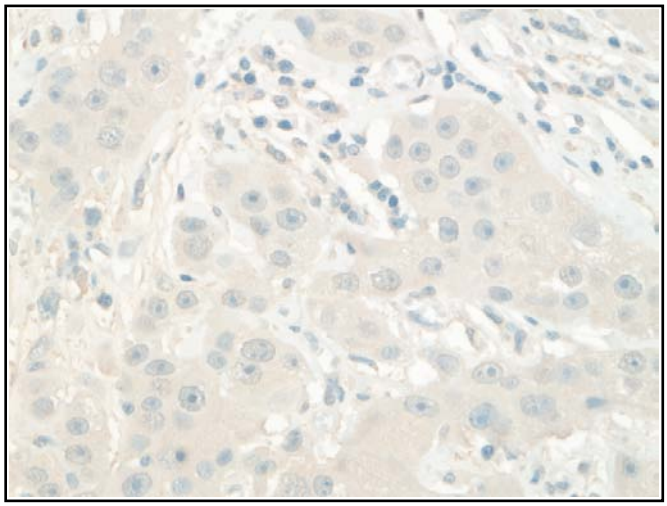


Fig 11. Poor demonstration of ER in the UK NEQAS negative section. Although the tumour cells are negative as expected, the section shows excessive cytoplasmic background staining. This is most likely due to excessive antigen retrieval. Stained with the Leica 6F11 antibody, 1:150 on the Bond Max with ER2 antigen retrieval for 30 minutes.

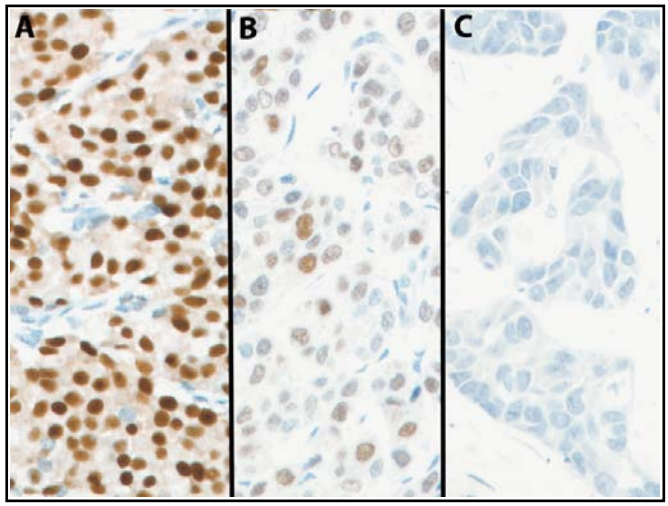
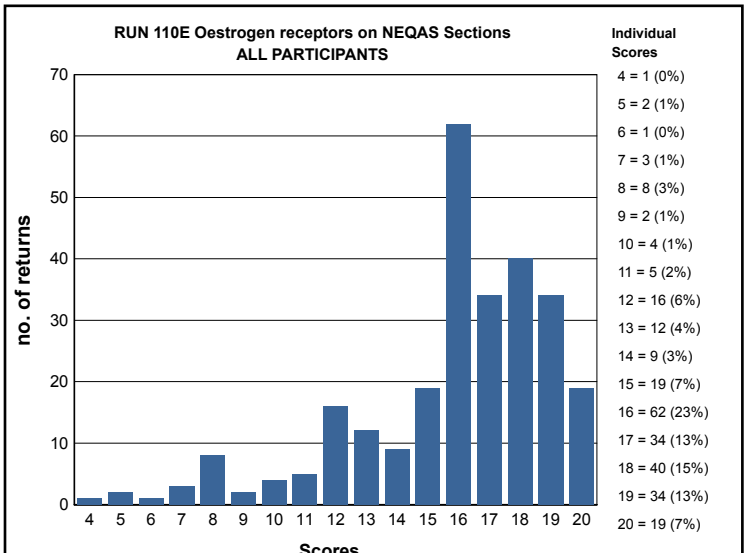
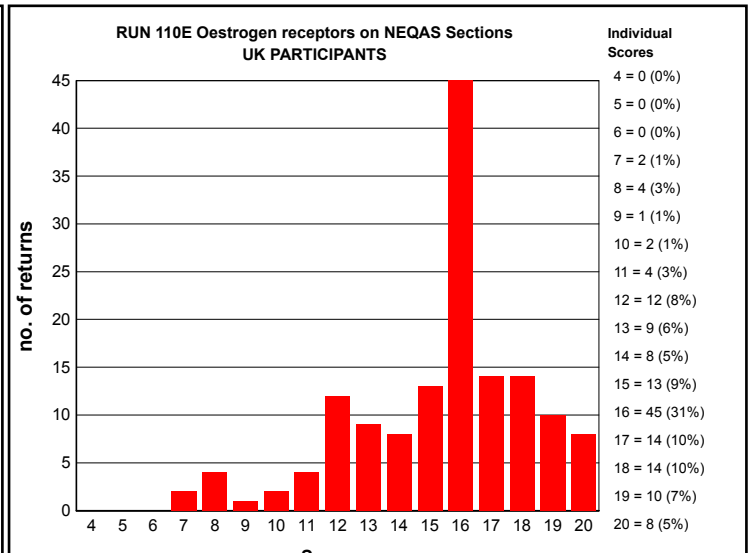
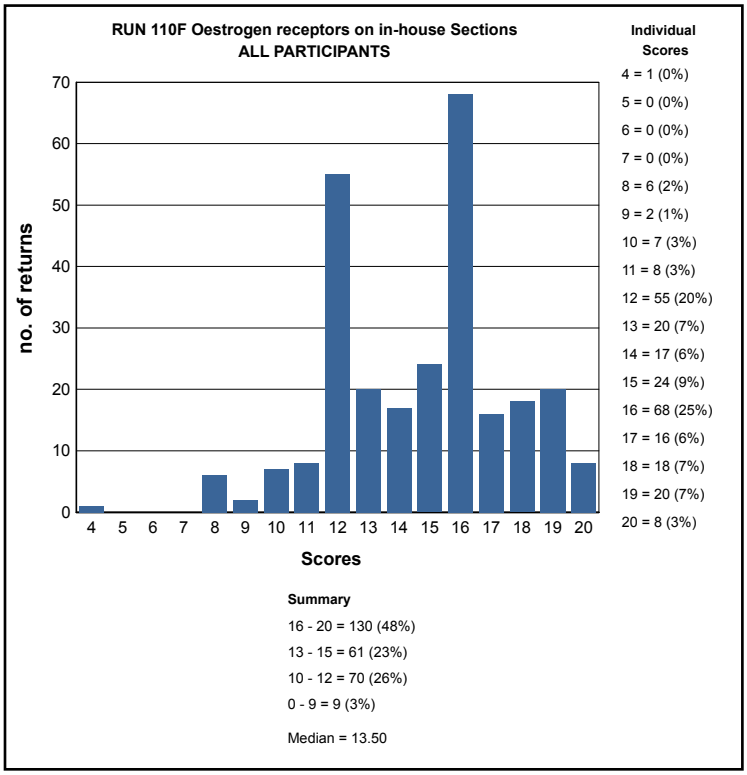


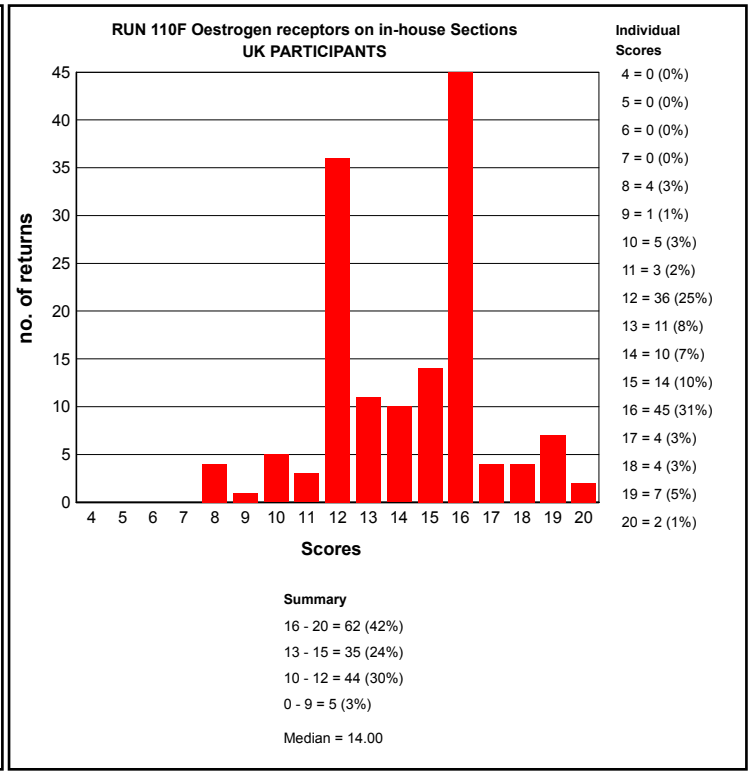

Fig 12. Good example of an 'in house' control for ER. The multi-block section contains high, mid, and negative expressing tumours ( A-C respectively). A control containing tumours of known differing expression levels is important to gauge the sensitivity of the assay.

## 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: 110**

**Primary Antibody : Oestrogen receptors**

| Antibody Details | N | % |
|---|---|---|
| Cell Marque 249-R (SP1) | 5 | 100 |
| Dako (EP1) M3643 | 31 | 97 |
| Dako (EP1) RTU Auto Plus IS084 | 3 | 100 |
| Dako (EP1) RTU FLEX IR084 | 23 | 87 |
| Dako FLEX (1D5) IR/IS657 | 1 | 0 |
| Dako IR151 Autostainer Link (SP1) | 1 | 0 |
| Dako M3634 (SP1) | 2 | 50 |
| Dako M7047 ER (1D5) | 7 | 86 |
| Dako RTU IR151 (SP1) | 1 | 0 |
| Dako SK310 pharmDX kit (Link) (1D5 + ER-2-123) | 1 | 100 |
| Leica Bond PA0151 (6F11) | 2 | 50 |
| Leica/Novocastra NCL-ER-6F11 (6F11) | 6 | 50 |
| Leica/Novocastra NCL-ER-6F11/2 | 13 | 69 |
| Leica/Novocastra NCL-L-ER- 6F11 | 35 | 66 |
| Leica/Novocastra RTU-ER-6F11 | 5 | 60 |
| Menapath MP-093-CM1 | 1 | 100 |
| Other | 1 | 100 |
| Thermo Fisher/ Neomarkers RM 9101-S (SP1) | 14 | 71 |
| Vector VP-E613/4 (6F11) | 7 | 57 |
| Vector VP-RM05 (SP1) | 1 | 100 |
| Ventana 760-2132 (6F11) | 1 | 100 |
| Ventana 790-4324 (SP1) | 69 | 99 |
| Ventana 790-4325 (SP1) | 34 | 94 |

**Breast Steroid Hormone Receptor Run: 110**

| Automation | Oestrogen receptors N | % |
|---|---|---|
| Dako Autostainer | 3 | 67 |
| Dako Autostainer Link 48 | 33 | 76 |
| Dako Autostainer plus | 2 | 50 |
| Dako Autostainer Plus Link | 3 | 67 |
| Dako Omnis | 4 | 100 |
| LabVision Autostainer | 3 | 33 |
| Leica Bond Max | 34 | 74 |
| Leica Bond-III | 38 | 79 |
| Menarini - Intellipath FLX | 2 | 100 |
| None (Manual) | 4 | 50 |
| Other | 2 | 100 |
| Shandon Sequenza | 3 | 33 |
| Ventana Benchmark GX | 9 | 100 |
| Ventana Benchmark ULTRA | 70 | 94 |
| Ventana Benchmark XT | 59 | 93 |

**Breast Steroid Hormone Receptor Run: 110**

| Heat Mediated Retrieval | Oestrogen receptors N | % |
|---|---|---|
| Biocare Decloaking Chamber | 2 | 100 |
| Dako Omnis | 4 | 100 |
| Dako Pascal | 2 | 0 |
| Dako PTLink | 35 | 74 |
| Lab vision PT Module | 4 | 50 |
| Leica ER1 20 mins | 10 | 60 |
| Leica ER1 30 mins | 14 | 64 |
| Leica ER1 40 mins | 6 | 83 |
| Leica ER2 10 mins | 4 | 75 |
| Leica ER2 20 mins | 27 | 89 |
| Leica ER2 30 mins | 8 | 75 |
| Leica ER2 40 mins | 2 | 100 |
| Microwave | 1 | 0 |
| Other | 2 | 50 |
| Pressure Cooker | 8 | 88 |
| Ventana CC1 16mins | 4 | 75 |
| Ventana CC1 20mins | 1 | 100 |
| Ventana CC1 24mins | 1 | 100 |
| Ventana CC1 32mins | 6 | 100 |
| Ventana CC1 36mins | 16 | 100 |
| Ventana CC1 40mins | 1 | 100 |
| Ventana CC1 48mins | 1 | 100 |
| Ventana CC1 52mins | 5 | 100 |
| Ventana CC1 56mins | 1 | 100 |
| Ventana CC1 64mins | 28 | 96 |
| Ventana CC1 76mins | 1 | 0 |
| Ventana CC1 88mins | 1 | 100 |
| Ventana CC1 8mins | 2 | 100 |
| Ventana CC1 extended | 5 | 80 |
| Ventana CC1 mild | 22 | 95 |
| Ventana CC1 standard | 38 | 92 |
| Ventana CC2 76mins | 1 | 100 |
| Ventana CC2 mild | 1 | 100 |
| Water bath 95-98 OC | 3 | 33 |

**Breast Steroid Hormone Receptor Run: 110**

| Enzyme Mediated Retrieval | Oestrogen receptors N | % |
|---|---|---|
| AS PER KIT | 4 | 50 |
| NOT APPLICABLE | 156 | 85 |
| Ventana Protease 1 (760-2018) | 1 | 100 |

**Breast Steroid Hormone Receptor Run: 110**

| Detection | Oestrogen receptors | |
|---|---|---|
| | N | % |
| AS PER KIT | 10 | 90 |
| Dako EnVision FLEX ( K8000/10) | 7 | 86 |
| Dako EnVision FLEX+ ( K8002/12) | 22 | 73 |
| Dako Envision HRP/DAB ( K5007) | 5 | 80 |
| Dako REAL HRP/DAB (K5001 ) | 1 | 0 |
| LabVision UltraVision ONE Polymer ( TL-012/5-HDJ/T | 1 | 0 |
| Leica Bond Polymer Refine (DS9800) | 69 | 75 |
| MenaPath X-Cell Plus (MP-XCP) | 1 | 100 |
| None | 2 | 100 |
| NOT APPLICABLE | 1 | 0 |
| Novocastra Novolink PDS (RE7-140/150/280/290-K) | 2 | 0 |
| Other | 8 | 63 |
| Vector Elite Universal ABC (PK-6200) | 1 | 100 |
| Vector ImmPRESS Universal (MP-7500) | 1 | 100 |
| Ventana iView system (760-091) | 8 | 100 |
| Ventana OptiView Kit (760-700) | 14 | 93 |
| Ventana UltraView Kit (760-500) | 113 | 94 |

**Breast Steroid Hormone Receptor Run: 110**

| Chromogen | Oestrogen receptors | |
|---|---|---|
| | N | % |
| AS PER KIT | 19 | 89 |
| Dako DAB K3468 | 1 | 0 |
| DAKO DAB+ | 2 | 100 |
| Dako DAB+ Liquid (K3468) | 2 | 50 |
| Dako DAB+ REAL Detection (K5001) | 1 | 100 |
| Dako EnVision Plus kits | 4 | 50 |
| Dako FLEX DAB | 29 | 76 |
| Dako REAL EnVision K5007 DAB | 4 | 75 |
| Dako REAL K5001 DAB | 1 | 0 |
| Leica Bond Polymer Refine kit (DS9800) | 67 | 75 |
| menapath xcell kit DAB (MP-860) | 1 | 100 |
| Other | 7 | 57 |
| Sigma DAB (D5905) | 1 | 100 |
| Ventana DAB | 7 | 100 |
| Ventana Enhanced Alk. Phos. Red Detection Kit | 1 | 100 |
| Ventana iview | 8 | 100 |
| Ventana Ultraview DAB | 113 | 94 |
| Vision BioSystems Bond X DAB | 1 | 100 |

## BEST METHODS

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

**Oestrogen receptors - Method 1**

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

**Primary Antibody:** Dako (EP1) RTU FLEX IR084 , 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

**Oestrogen receptors - Method 2**

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

**Primary Antibody:** Dako (EP1) M3643 , 15 Mins, 22 ºC Dilution 1: 100
**Automation:** Leica Bond-III
**Method:** Leica BondMAx Refine KIT
**Main Buffer:** Bond Wash Buffer (AR9590), PH: 7.6
**HMAR:** Leica ER2 20 mins
**EAR:**
**Chromogen:** Leica Bond Polymer Refine kit (DS9800), 22 ºC., Time 1: 10 Mins
**Detection:** Leica Bond Polymer Refine (DS9800) , 8 Mins, 22 ºC Prediluted

**Oestrogen receptors - Method 3**

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

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

**Oestrogen receptors - Method 4**

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

| | |
|---|---|
| **Primary Antibody:** | Leica/Novocastra NCL-L-ER- 6F11 , 20 Mins, 23 ºC Dilution 1: 100 |
| **Automation:** | Leica Bond-III |
| **Method:** | Leica BondMAx Refine KIT |
| **Main Buffer:** | Bond Wash Buffer (AR9590), PH: 7.6 |
| **HMAR:** | Leica ER1 30 mins |
| **EAR:** | |
| **Chromogen:** | Leica Bond Polymer Refine kit (DS9800), 23 ºC., Time 1: 10 Mins |
| **Detection:** | |

# The Breast HER2 ICC Module Run 110

**Suzanne Parry and Merdol Ibrahim**

| | |
|---|---|
| **Antigen Assessed:** | HER2 |
| **Sections Circulated:** | Composite slide consisting of 4 breast carcinoma cell lines (see table below) |
| **Number of Registered Participants:** | 368 |
| **Number of Participants this Run** | 201 (55%) |

## 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*<br>*The ASCO/CAP guidelines by Wolff et al., (2013) are not currently used for scoring in-house controls |
|---|---|---|
| **A: 3+** | Strong complete cell membrane staining | Strong complete cell membrane staining in >30% of tumour cells |
| **B: 2+** | Weak to moderate complete cell membrane staining | Weak to moderate complete cell membrane staining in >10% of tumour cells |
| **C: 1+** | Faint barely perceptible incomplete membrane staining | Faint barely perceptible incomplete membrane in >10% of cells staining |
| **D: Negative** | No staining in the '0' control cell line | No staining in the '0' control cell line |

## Validation of Distributed UK NEQAS ICC & ISH Cell Lines

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

## Updated Assessment and Scoring Procedure

## UK NEQAS Specific Membrane Scoring Algorithm:

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

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

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

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

**Numerical Scoring Criteria**

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

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

## Introduction

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

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

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

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

## In-House Control Tissue Recommendations

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

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

## Assessment Summary:

Pass rates for the assessment are shown in the subsequent summary graphs. The most popular antibody was the Ventana 4B5, used by 61% (N=123) of participants. 23 labs (11%) are using the Dako HercepTest, and 20 labs (10%) are using the Leica Oracle kit. The rest of the participants are using lab devised methods with a variety of antigen retrieval methods and platforms.

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

**References**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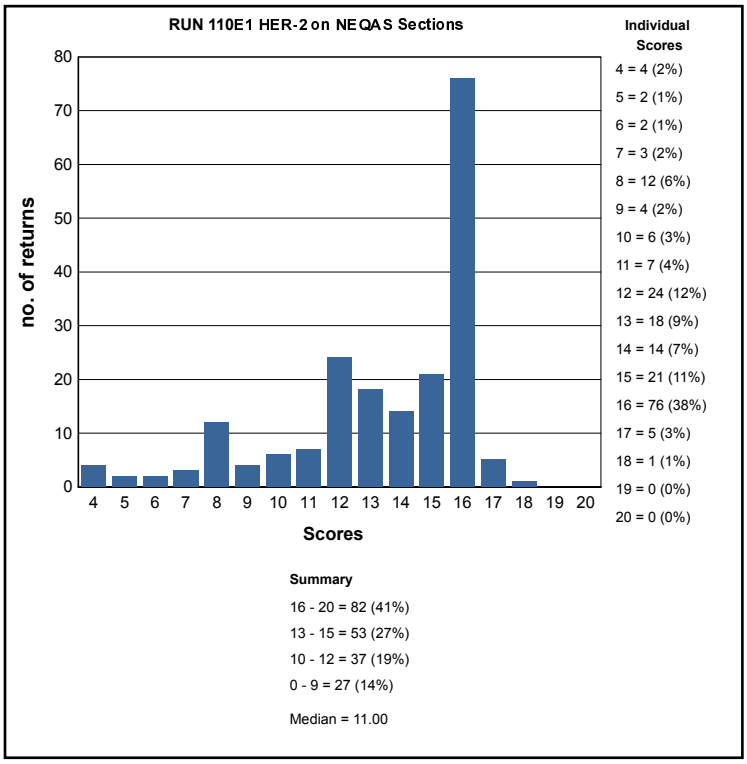
7. Rakha EA, et al. Updated UK rRecommendations for HER2 assessment in breast cancer. J Clin Pathol. 2015;68:93-99.

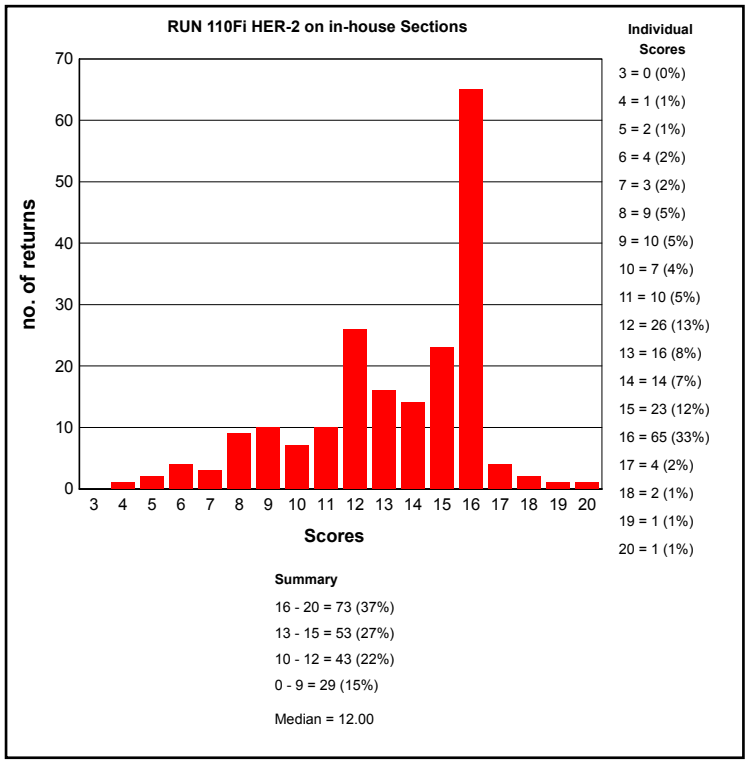


**Acknowledgments**

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

## 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: 110**

| Primary Antibody | N | % |
|---|---|---|
| Cell Marque 237R (SP3) | 2 | 0 |
| Cell Marque CMA 601 (CB11) | 1 | 0 |
| Dako A0485 C-erB-2 (poly) | 11 | 45 |
| Dako HercepTest K5204 (poly) | 5 | 20 |
| Dako HercepTest K5207 (poly) | 5 | 60 |
| Dako Link HercepTest SK001 (poly) | 13 | 54 |
| Labvision / Neomarkers RM-9103 (SP3) | 3 | 33 |
| Leica Oracle HER2 Bond IHC (CB11) | 20 | 55 |
| Novocastra NCL-L-CB11 (CB11) | 5 | 20 |
| Novocastra NCL-L-CBE356 (10A7) | 2 | 50 |
| Novocastra RTU-CB11 (CB11) | 1 | 0 |
| Novocastra RTU-CBE-356 (10A7) | 1 | 0 |
| Other | 4 | 0 |
| Ventana Confirm 790-4493 (4B5) | 24 | 96 |
| Ventana Pathway 790-100 (4B5) | 4 | 50 |
| Ventana Pathway 790-2991 (4B5) | 94 | 84 |

**Breast HER2 ICC Run: 110**

| Automation | N | % |
|---|---|---|
| Dako Autostainer | 2 | 0 |
| Dako Autostainer Link 48 | 19 | 53 |
| Dako Autostainer plus | 2 | 50 |
| Dako Autostainer Plus Link | 4 | 50 |
| LabVision Autostainer | 1 | 0 |
| Leica Bond Max | 14 | 36 |
| Leica Bond-III | 18 | 50 |
| None (Manual) | 8 | 13 |
| Shandon Sequenza | 2 | 100 |
| Ventana Benchmark GX | 7 | 71 |
| Ventana Benchmark ULTRA | 67 | 82 |
| Ventana Benchmark XT | 50 | 88 |

**Breast HER2 ICC Run: 110**

| Heat Mediated Retrieval | N | % |
|---|---|---|
| Biocare Decloaking Chamber | 1 | 0 |
| Dako PTLink | 23 | 52 |
| Lab vision PT Module | 2 | 0 |
| Leica ER1 10 mins | 3 | 33 |
| Leica ER1 20 mins | 6 | 33 |
| Leica ER1 25 mins | 18 | 56 |
| Leica ER1 30 mins | 1 | 0 |
| Microwave | 1 | 0 |
| None | 2 | 50 |
| Other | 4 | 75 |
| Pressure Cooker | 2 | 0 |
| Steamer | 1 | 0 |
| Ventana CC1 16mins | 2 | 100 |
| Ventana CC1 20mins | 4 | 75 |
| Ventana CC1 24mins | 1 | 0 |
| Ventana CC1 32mins | 5 | 60 |
| Ventana CC1 36mins | 34 | 82 |
| Ventana CC1 40mins | 1 | 100 |
| Ventana CC1 48mins | 1 | 0 |
| Ventana CC1 52mins | 5 | 80 |
| Ventana CC1 56mins | 3 | 100 |
| Ventana CC1 64mins | 6 | 83 |
| Ventana CC1 76mins | 1 | 100 |
| Ventana CC1 mild | 41 | 85 |
| Ventana CC1 standard | 16 | 94 |
| Ventana CC2 16mins | 1 | 100 |
| Water bath 95-98 OC | 8 | 38 |

**Breast HER2 ICC Run: 110**

| Detection | N | % |
|---|---|---|
| AS PER KIT | 26 | 62 |
| Dako HerCep Test (K5204) | 3 | 0 |
| Dako EnVision FLEX ( K8000/10) | 7 | 43 |
| Dako EnVision FLEX+ ( K8002/12) | 3 | 67 |
| Dako Envision HRP/DAB ( K5007) | 2 | 50 |
| Dako Envision+ HRP rabbit K4008/9/10/11 | 1 | 0 |
| Dako HerCep Test Autor (K5207) | 3 | 33 |
| Dako HerCep Test Autor (SK001) | 7 | 71 |
| ID Labs SS System HRP (IDST1007) | 1 | 100 |
| LabVision UltraVision ONE Polymer ( TL-012/5-HDJ/T | 1 | 0 |
| Leica Bond Polymer Refine (DS9800) | 14 | 43 |
| None | 1 | 100 |
| Other | 4 | 0 |
| Ventana iView system (760-091) | 8 | 88 |
| Ventana OptiView Kit (760-700) | 9 | 67 |
| Ventana UltraView Kit (760-500) | 96 | 83 |

**Breast HER2 ICC Run: 110**

| Enzyme Retrieval | N | % |
|---|---|---|
| AS PER KIT | 5 | 40 |
| NOT APPLICABLE | 86 | 69 |
| Ventana Protease 1 (760-2018) | 1 | 100 |

**Breast HER2 ICC Run: 110**

| Chromogen | N | % |
|---|---|---|
| AS PER KIT | 43 | 65 |
| DAKO DAB+ | 1 | 100 |
| Dako DAB+ Liquid (K3468) | 2 | 50 |
| Dako EnVision Plus kits | 2 | 50 |
| Dako FLEX DAB | 16 | 44 |
| Dako REAL EnVision K5007 DAB | 1 | 0 |
| Leica Bond Polymer Refine kit (DS9800) | 16 | 44 |
| Other | 9 | 33 |
| Sigma DAB (D5905) | 1 | 0 |
| Ventana DAB | 4 | 50 |
| Ventana iview | 6 | 100 |
| Ventana Ultraview DAB | 93 | 83 |

## BEST METHODS

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

### HER-2 - Method 1

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

| | |
|---|---|
| **Primary Antibody:** | Ventana Pathway 790-2991 (4B5) , 12 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:** | AS PER KIT |
| **Detection:** | AS PER KIT |

### HER-2 - Method 2

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

| | |
|---|---|
| **Primary Antibody:** | Dako Link HercepTest SK001 (poly) , 30 Mins, 25 ºC Prediluted |
| **Automation:** | Dako Autostainer Plus Link |
| **Method:** | Dako FLEX kit |
| **Main Buffer:** | Dako FLEX wash buffer |
| **HMAR:** | Dako PTLink, Buffer: citrate buffer |
| **EAR:** | |
| **Chromogen:** | Dako FLEX DAB, Time 1: 5 Mins, Time 2: 5 Mins |
| **Detection:** | Dako HerCep Test Autor (SK001) , 30 Mins, 30 ºC Prediluted |

### HER-2 - Method 3

Participant scored 16/20 (UK NEQAS Slide) and 16/20 (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:** | |
| **Chromogen:** | Ventana Ultraview DAB |
| **Detection:** | Ventana UltraView Kit (760-500) |

### HER-2 - Method 4

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

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

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# The Gastric HER2 ICC Module Run 110

**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:<br>**A.** 3+<br>**B**. 2+<br>**C**. 1+ or 2+ depending on the serial section received<br>**D**. 0 |
| **Number of Registered Participants** | 103 |
| **Number of Participants this Run** | 50 (49%) |

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

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

## Validation of Distributed Samples

### IHC Validation of Distributed Samples

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

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

**Table 2: HER2 IHC staining and & ISH results**

| Section Label From slide Label End | Staining Pattern with IHC | HER2 status by ISH |
|---|---|---|
| **A** | **3+** | Amplified |
| **B** | **2+** | Amplified |
| **C** | **1+ or 2+** | Non-Amplified<br>**Note:** Some serial sections also showed areas of amplification |
| **D** | **0** | Non-Amplified |

## Assessment Procedure

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

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

| Expected membrane staining | Scoring criteria used by UK NEQAS ICC & ISH |
|---|---|
| **3+** | i) 3+: as expected<br>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+).<br>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.<br>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.<br>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.<br>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:<br>• False positive / negative membrane staining<br>• Excessive cytoplasmic staining<br>• Excessive morphological damage<br>• 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:<br>• Weaker / stronger than expected membrane staining<br>• Some cytoplasmic staining<br>• Morphological damage |
| **4 & 5** | **13-15 = Acceptable**<br>**16-20 = Acceptable/ Excellent Standard** | **Overall the samples show acceptable membrane staining and are suitable for interpretation.** |
| **Further comments are also provided on individual participant reports.** Other factors which may lead to a low score include: excessive/ insufficient haematoxylin staining; poor quality of in-house control tissue; poor/inadequate choice of control tissue; poor/inadequate fixation; excessive antigen retrieval etc. | | |

## Introduction

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

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

## Assessment Results

**Features Of Acceptable Staining: (Figs 1– 4)**

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

**Features Of Suboptimal or Unacceptable Staining:**

- Weaker or stronger staining than the expected expression level of membrane staining in the invasive tumour **(Fig 5)**
- 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 very good and significantly higher than the previous assessment run: 88% of participating labs received an acceptable pass, compared to 63% in run (109), and a further 4 labs (8%) achieved a borderline pass. Only 2 labs failed the assessment; both due to weak membrane staining, and therefore resulting in a lower HER2 expression than the expected level. The reason for one of the failures was most probably due to insufficient antigen retrieval (a retrieval time of 8 minutes on the Ventana platform). Labs that received a borderline score were mostly marked down due to either a slightly higher or lower level of HER2 membrane staining.

49 participants also submitted their in-house controls. The overall pass rate was the same as that seen on the Neqas samples, however, fewer labs received the acceptable level of staining (scores of 13-20/20), and more labs received a borderline pass (scores of 10-12): The acceptable pass rate was 63% (N=31) and the borderline pass rate was 33% (N=16). Several laboratories that received a borderline pass did not submit the required composite control material, consisting of a 3+, 2+ and 1+/0 expressing gastric/breast control tumour sample, and therefore these labs were given a maximum score of 12/20, i.e. 3/5 from each assessor. Other labs were marked down for poor tissue quality or fixation. Similarly to the Neqas section, only 2 labs failed the assessment: One of these labs showed excessive background and cytoplasmic staining, making it difficult to read any membranous staining. Poor tissue quality was the reason for the other failed lab.

## Methodologies

Most labs are using the Ventana 4B5 standardised kit for their gastric HER2 testing: In this particular assessment run the Ventana assay was used by 80% and showed an acceptable pass rate of 92% on the UK NEQAS distributed section. All 6 labs that are using the standardised Dako HercepTest also received acceptable passes.

## 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. Laboratories were therefore not penalised if they submitted breast carcinomas instead of gastric tissue. Whether gastric or breast tissue is used, laboratories should still submit in-house controls showing 3+, 2+ and 1+/0 levels of membrane expression. Due to the heterogenic nature of many gastric tumours, it is acceptable for labs to submit a heterogeneous in-house control with areas of both 3+ and 2+ membrane expression for example, as long as the participant clearly indicates the areas where the assessment should be carried out.

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

**References:**

1. Hofmann M, Stoss O, Shi D et al. Assessment of a HER2 scoring system for gastric cancer: results from a validation study. Histopathology. 2008 52 (7):797-805.
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**Acknowledgments**

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

## Selected Images showing Optimal and Sub-optimal Immunostaining

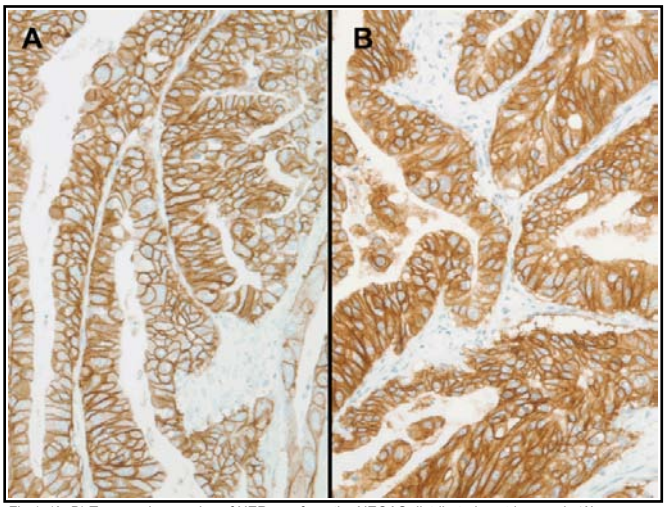



Fig 1. (A, B) Two good examples of HER2 3+ from the NEQAS distributed gastric sample 'A', both showing intense complete membrane staining. (A) Stained on a Ventana Benchmark XT with the Ventana 4B5 (12 mins), with CC1 mild retrieval and (B) Dako Link HercepTest SK001 (30 mins) with Dako PT link retrieval.

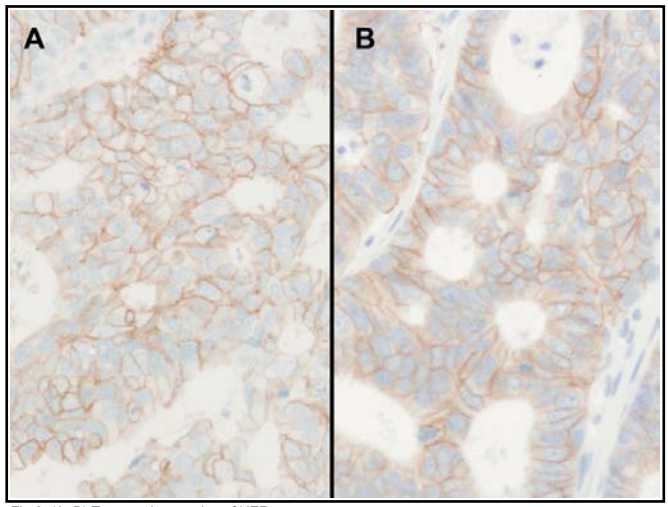



Fig 2. (A, B) Two good examples of HER2 2+ from the NEQAS distributed gastric sample 'B', both showing complete membrane staining but lower intensity than seen in fig 1 (A) Stained on a Ventana Benchmark XT with the Ventana 4B5 (12 mins), with CC1 mild retrieval and (B) Dako Link HercepTest SK001 (30 mins) with Dako PT link retrieval.

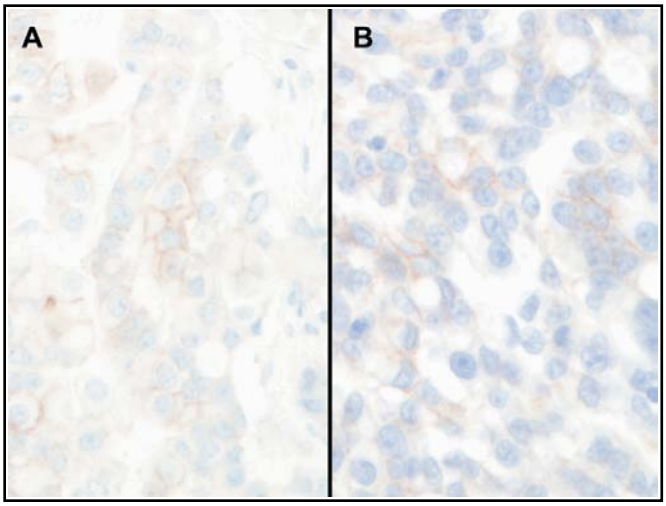



Fig 3. (A, B) Two good examples of HER2 1+ incomplete membrane staining, from the NEQAS distributed gastric sample 'C'. (A) Stained on a Ventana Benchmark XT with the Ventana 4B5 (12 mins), with CC1 mild retrieval and (B) Dako Link HercepTest SK001 (30 mins) with Dako PT link retrieval.

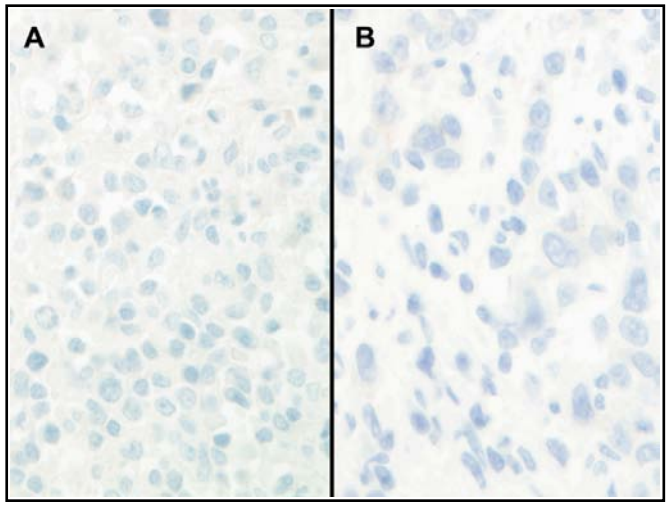



Fig 4. (A, B) Two good examples of negative HER2 IHC gastric samples from the NEQAS distributed sample 'D'. (A) Stained on a Ventana Benchmark XT with the Ventana 4B5 (12 mins), with CC1 mild retrieval and (B) Dako Link HercepTest SK001 (30 mins) with Dako PT link retrieval.

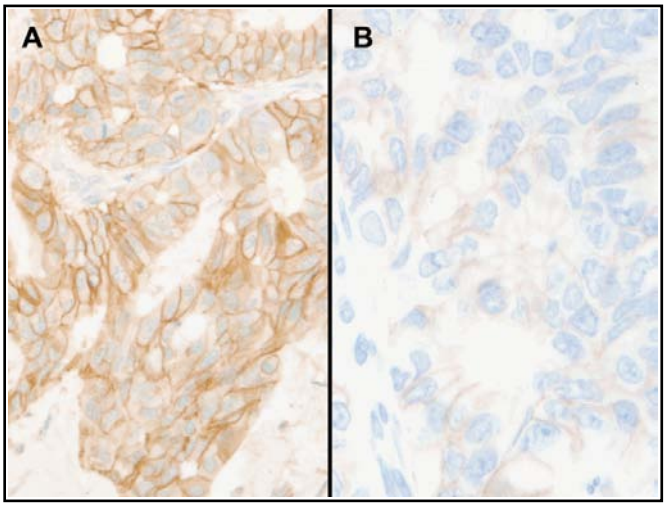



Fig 5. Two examples of unacceptable HER2 2+ staining on the NEQAS distributed sample 'B'. (A) Staining is stronger than expected and resembles 3+ staining. (B) Staining is much weaker than expected and resembles 1+ staining. Both stained using the Ventana 4B5 assay. (B) Incubation was for only 8mins which could account for the very low weak membrane staining.

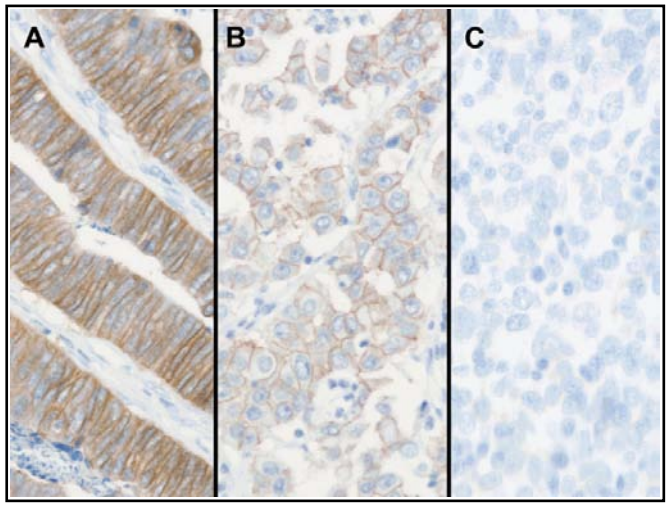
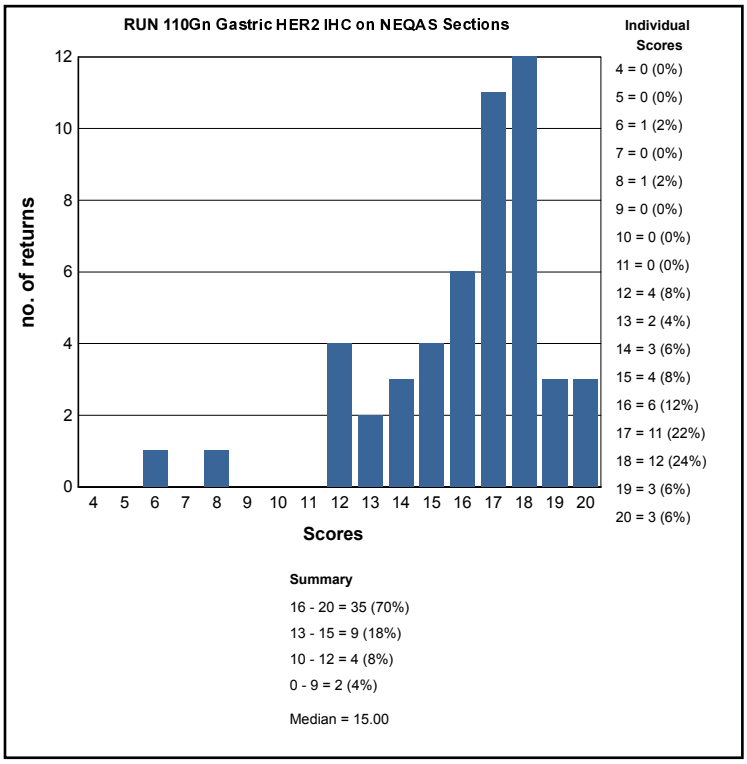
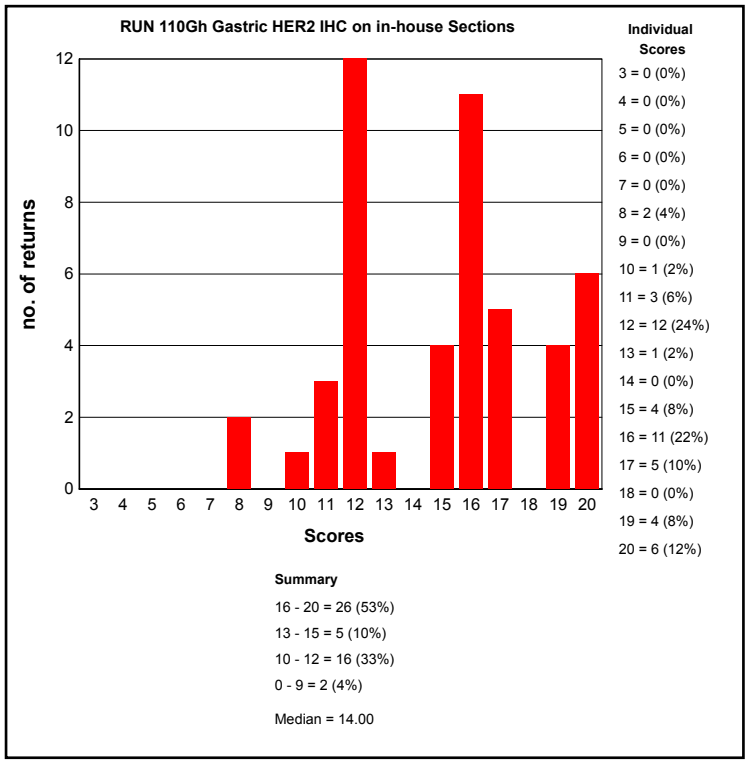



Fig 6. Excellent example of an in-house control and HER2 staining showing (A) 3+, (B) 2+ and (C) 1+ staining. Stained using Dako Link HercepTest SK001 as per manufacturer's instructions.

## 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: 110**

| Primary Antibody | N | % |
|---|---|---|
| Dako Link HercepTest SK001 (poly) | 6 | 100 |
| Leica Oracle HER2 Bond IHC (CB11) | 1 | 0 |
| Ventana Confirm 790-4493 (4B5) | 7 | 71 |
| Ventana Pathway 790-2991 (4B5) | 35 | 94 |

**Gastric HER2 ICC Run: 110**

| Automation | N | % |
|---|---|---|
| Dako Autostainer Link 48 | 4 | 100 |
| Dako Autostainer Plus Link | 2 | 100 |
| Leica Bond Max | 1 | 0 |
| Ventana Benchmark GX | 3 | 33 |
| Ventana Benchmark ULTRA | 20 | 95 |
| Ventana Benchmark XT | 19 | 95 |

**Gastric HER2 ICC Run: 110**

| Heat Mediated Retrieval | N | % |
|---|---|---|
| Dako PTLink | 4 | 100 |
| Leica ER1 25 mins | 2 | 50 |
| Other | 1 | 100 |
| Ventana CC1 16mins | 1 | 100 |
| Ventana CC1 20mins | 1 | 100 |
| Ventana CC1 24mins | 1 | 100 |
| Ventana CC1 32mins | 4 | 75 |
| Ventana CC1 36mins | 11 | 91 |
| Ventana CC1 52mins | 1 | 100 |
| Ventana CC1 56mins | 1 | 100 |
| Ventana CC1 64mins | 3 | 100 |
| Ventana CC1 mild | 13 | 100 |
| Ventana CC1 standard | 4 | 50 |

**Gastric HER2 ICC Run: 110**

| Detection | N | % |
|---|---|---|
| AS PER KIT | 8 | 88 |
| Dako HerCep Test Autor (SK001) | 3 | 100 |
| Ventana iView system (760-091) | 2 | 100 |
| Ventana OptiView Kit (760-700) | 3 | 67 |
| Ventana UltraView Kit (760-500) | 31 | 90 |

**Gastric HER2 ICC Run: 110**

| Enzyme Retrieval | N | % |
|---|---|---|
| NOT APPLICABLE | 21 | 86 |

**Gastric HER2 ICC Run: 110**

| Chromogen | N | % |
|---|---|---|
| AS PER KIT | 9 | 78 |
| DAKO DAB+ | 1 | 100 |
| Dako FLEX DAB | 2 | 100 |
| Ventana DAB | 2 | 100 |
| Ventana iview | 1 | 100 |
| Ventana Ultraview DAB | 34 | 91 |

## BEST METHODS

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

### Gastric HER2 IHC - Method 1

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

| | |
|---|---|
| **Primary Antibody:** | Dako Link HercepTest SK001 (poly) , 30 Mins, 25 ºC Prediluted |
| **Automation:** | Dako Autostainer Plus Link |
| **Method:** | Dako FLEX kit |
| **Main Buffer:** | Dako FLEX wash buffer |
| **HMAR:** | Dako PTLink, Buffer: Citrate buffer |
| **EAR:** | |
| **Chromogen:** | Dako FLEX DAB, 25 ºC., Time 1: 10 Mins, Time 2: 10 Mins |
| **Detection:** | Dako HerCep Test Autor (SK001) , 30 Mins, 25 ºC Prediluted |

### Gastric HER2 IHC - Method 2

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

| | |
|---|---|
| **Primary Antibody:** | Dako Link HercepTest SK001 (poly) Prediluted |
| **Automation:** | Dako Autostainer Link 48 |
| **Method:** | Dako FLEX kit |
| **Main Buffer:** | AS PER KIT |
| **HMAR:** | Dako PTLink, Buffer: as per kit, PH: 6 |
| **EAR:** | |
| **Chromogen:** | AS PER KIT, Time 1: 5 Mins, Time 2: 5 Mins |
| **Detection:** | AS PER KIT |

### Gastric HER2 IHC - Method 3

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

| | |
|---|---|
| **Primary Antibody:** | Ventana Pathway 790-2991 (4B5) , 20 Mins, 37 ºC Prediluted |
| **Automation:** | Ventana Benchmark XT |
| **Method:** | Ventana UltraView DAB |
| **Main Buffer:** | Ventana reaction buffer (950-300) |
| **HMAR:** | Ventana CC1 mild, Buffer: CC1 |
| **EAR:** | |
| **Chromogen:** | Ventana Ultraview DAB, 37 ºC., Time 1: 8 Mins |
| **Detection:** | Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC Prediluted |

### Gastric HER2 IHC - Method 4

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

| | |
|---|---|
| **Primary Antibody:** | Ventana Pathway 790-2991 (4B5) , 16 Mins |
| **Automation:** | Ventana Benchmark XT |
| **Method:** | Ventana UltraView DAB |
| **Main Buffer:** | AS PER KIT |
| **HMAR:** | Ventana CC1 32mins |
| **EAR:** | |
| **Chromogen:** | AS PER KIT |
| **Detection:** | |

David Blythe and Suzanne Parry

| | Gold Standard | Second Antibody |
|---|---|---|
| **Antigens Assessed:** | Cyclin D1 | CD8 |
| **Tissue Sections circulated:** | Reactive Tonsil and Mantle Cell Lymphoma | Normal Tonsil |
| **Number of Registered Participants:** | 228 | |
| **Number of Participants this Run** | 209 (92%) | |

# Introduction

## Gold Standard: Cyclin D1

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

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

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

**Features of Sub-Optimal Immunostaining (Figs 2, 3 & 5):**

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

**References:**

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

## Second Antigen: CD8

CD8 is a transmembrane glycoprotein co-receptor for the T-cell receptor (TCR) which is specific for the class I major histocompatibility complex proteins. The CD8 antigen is found in approximately 80% of thymocytes and 20-35% of peripheral blood lymphocytes. It is also found on Natural Killer cells, T-cells of the tonsil and spleen, 80% of intraepithelial cells and splenic sinusoidal lining cells (Mason et al.). While being useful in the identification of cytotoxic suppressor T-cells and their neoplastic counterparts, The CD8 antibody is helpful in the differential diagnosis between B- and T-cell lymphomas and the sub-typing of T-cell lymphomas. The CD8 antibody is also known to demonstrate a higher proportion of cytotoxic suppressor T-cells in the affected areas of the colon of patients with Crohn's disease and ulcerative colitis (Yamagata et al.)

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

- Strong cytoplasmic and membranous staining in the T-cells of the tonsil.
- Minimal background staining.

**Features of Suboptimal Immunostaining (Figs 8 & 10)**

- Very little or weak staining in the tonsil.
- Background staining in the tonsil.

**References**

1. Mason DY, Cordell JL, Gaulard P, Tse AGD, Brown MH. Immunohistological detection of human cytotoxic/suppressor T cells using antibodies to a CD8 peptide sequence. J Clin Pathol 1992:45:1084-8.
2. Yamagata K, Tanaka M, Kudo H. A quantitative immunohistochemical evaluation of inflammatory cells at the affected and unaffected sites of inflammatory bowel disease. J Gastroenterol 1998;13:801-8.

# Assessment Summary:

Overall the results from the **Cyclin D1** assessment were fairly good and showed similar pass rates to the last time that this antibody was assessed: 85% of participants achieved an acceptable pass and a further 13% received a borderline score (10-12/20). 5 labs failed the assessment, and this was due to weak staining or inappropriate staining, mostly caused by an inappropriate antibody dilution or antigen retrieval protocol. For many labs the scores on the Neqas material was higher than that on their in-house controls. Weak or diffuse staining was the main reason for the lower scores on the participants' in house tissue, but also poor tissue quality, and in some instances the labs did not provide an appropriate control, with 1 lab submitting a non-Hodgkins cell lymphoma but not of Mantle cell type. There was an increase in the number of antibodies using the Dako EP12 clone compared to the last time this marker was assessed. However, overall the most popular clone used was the SP4 from various different sources, but mostly the Ventana SP4-R antibody, used by 35 labs and showed a pass rate of 97%.

The **CD8** antibody showed very similar pass rates to the Cyclin D1 assessment, with 84% receiving an acceptable pass and a further 14% achieved a borderline. 4 labs (2%) failed the assessment, and again the main reason for failure or a borderline pass was due to very weak staining of the T cells. This was predominantly caused by insufficient antigen retrieval methods. The participants' in-house controls also showed similar pass rates to the Neqas slides, with again weak staining being the reason for the lower marks. Tonsil was used by most labs as their in-house control. Several labs do not stock CD8, and were therefore given the opportunity to stain with CD3 as an alternative. It became apparent that some labs had problems with their in house sections lifting. This was taken into consideration during the assessment and labs were not penalised for this, and labs are still allowed to send in a separate in-house slide to be assessed should this problem of lifting occur.

## Selected Images showing Optimal and Sub-optimal Immunostaining

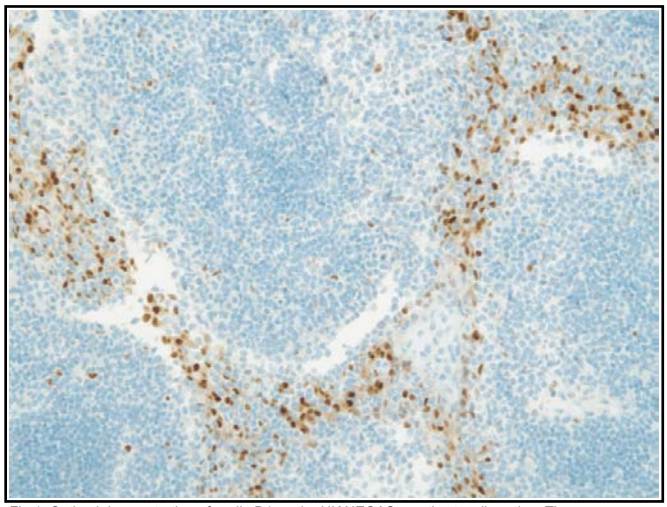


Fig 1. Optimal demonstration of cyclin D1 on the UK NEQAS reactive tonsil section. The squamous epithelial cells show moderate to strong nuclear staining. Section stained with the Thermo/ LabVision SP4 antibody, 1:40 on the Leica Bond with ER2 antigen retrieval for 20 minutes.

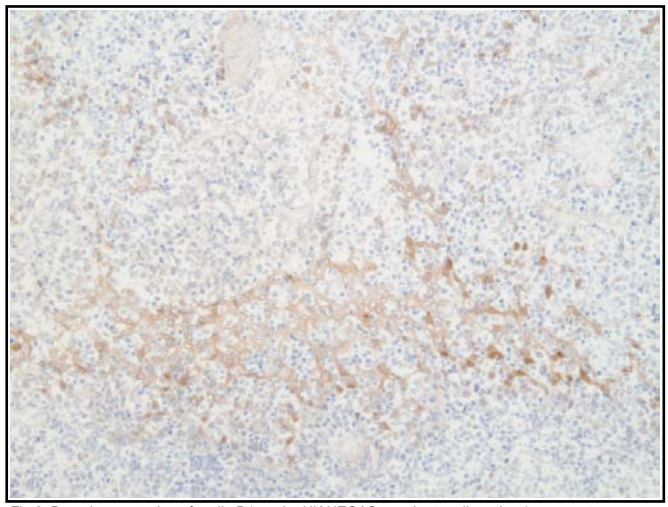


Fig 2. Poor demonstration of cyclin D1 on the UK NEQAS reactive tonsil section (compare to Fig 1). The staining is diffuse and appears over digested. Section stained with the Thermo/LabVision SP4 antibody, 1:40 on the dako Autosatiner with pre-treatment in the Biocare Decloaking Chamber with high pH buffer.

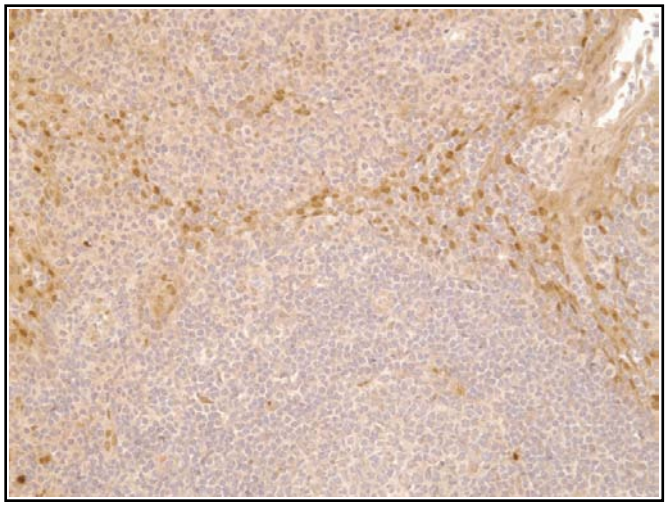


Fig 3. Sub-optimal demonstration of cyclin D1 on the UK NEQAS reactive tonsil. The section shows excessive background staining, most likely caused by over antigen retrieval. The section was stained using the Leica RTU D1-GM antibody on the Shandon Sequenza and microwave pre-treatment.

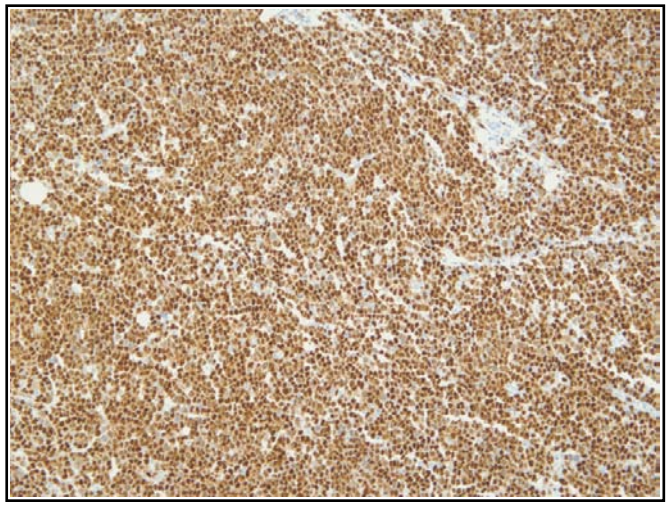


Fig 4. Optimal demonstration of cyclin D1 on the UK NEQAS distributed mantel cell lymphoma (MCL): Virtually all of the tumour cells show strong nuclear staining. Section stained with the Dako RTU SP4 antibody on the Omnis platform, with high pH antigen retrieval.

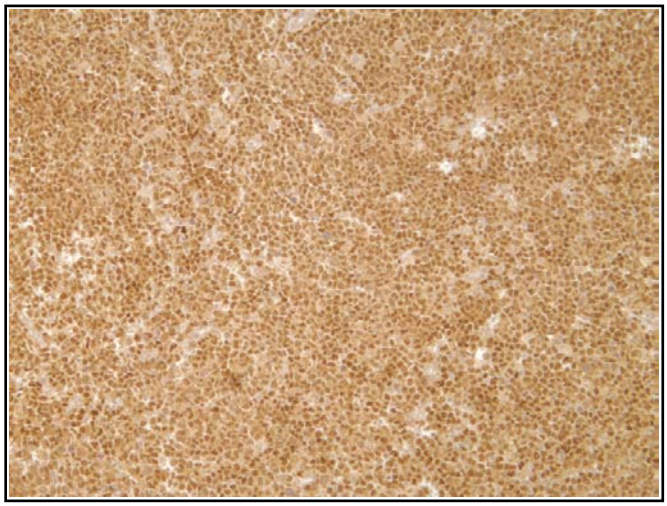


Fig 5. Sub-optimal demonstration of cyclin D1 on the UK NEQAS distributed mantel cell lymphoma (compare to Fig 4). Although the tumour cells are showing strong nuclear staining as expected, the section also has excessive background staining (same protocol used as Fig 3).

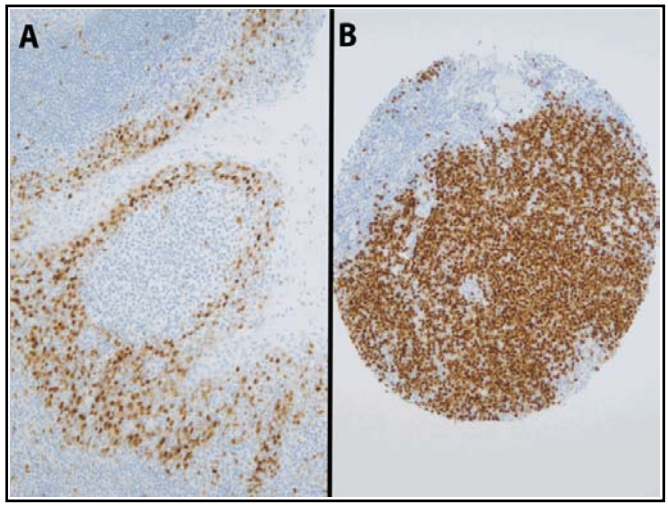



Fig 6. Good example of an in house control stained with cyclin D1. The composite control contained both (A) tonsil and (B) MCL TMA core, and both sections stained optimally. Stained with the Dako RTU EP12 antibody on the Autostainer and pre-treatment in the PT link with high pH buffer.

## Selected Images showing Optimal and Sub-optimal Immunostaining

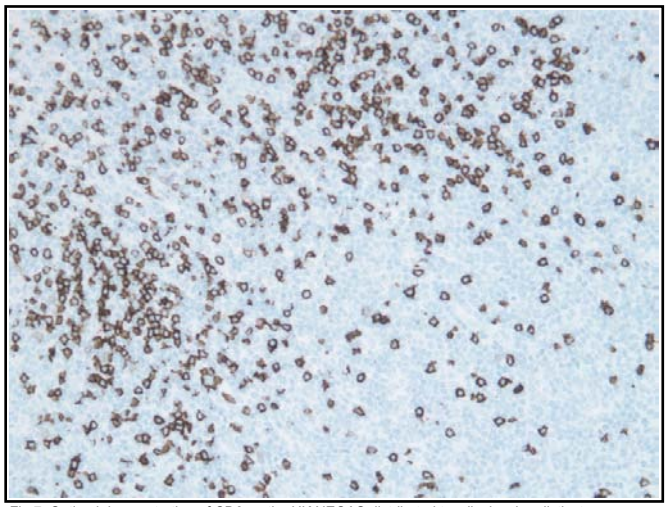


Fig 7. Optimal demonstration of CD8 on the UK NEQAS distributed tonsil, showing distinct staining of the T-cells. Section stained with the Dako C8/144B antibody, 1:200 on the Leica BondMax and ER2 antigen retrieval for 20 minutes.

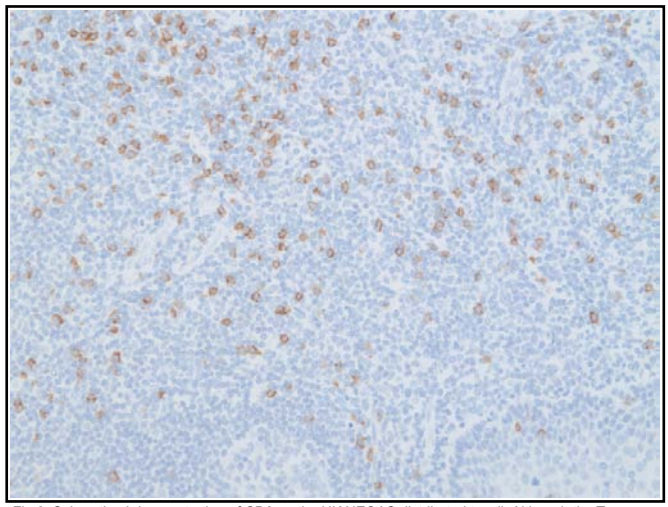


Fig 8. Sub-optimal demonstration of CD8 on the UK NEQAS distributed tonsil. Although the T cells are demonstrated, the staining is weaker than expected (compare to Fig 7). Stained using the Dako C8/144B antibody (no dilution provided) on the Leica BondMax and ER1 antigen retrieval for 30 minutes.

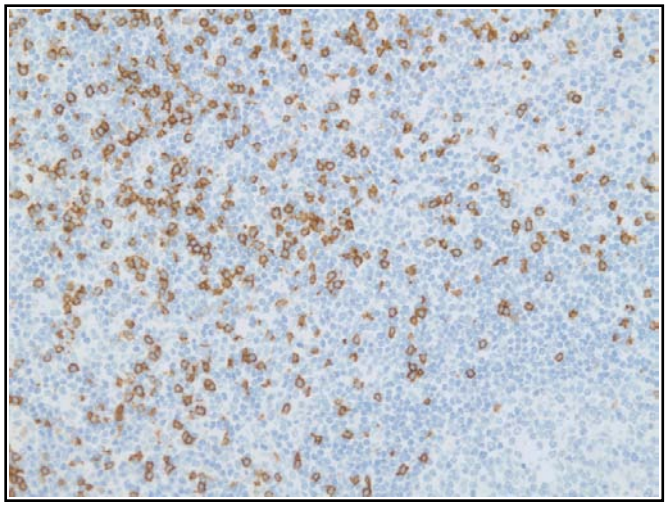


Fig 9. Optimal demonstration of CD8 on the UK NEQAS distributed tonsil: The section shows good membranous staining in the T-cells with a clean background. The section was stained using the Leica RTU 4B11 antibody on the Leica Bond with ER2 retrieval buffer for 20 minutes.

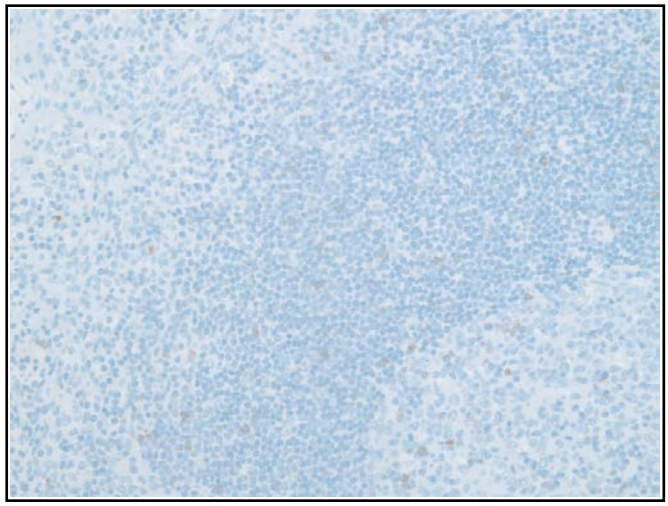


Fig 10. Poor demonstration of CD8 on the UK NEQAS distributed tonsil section. Very weak near false-negative staining (compare to Figs 7&9). This is most likely due to insufficient antigen retrieval. Section stained with the Leica 4B11 and antibody, 1:300 with ER1 antigen retrieval for 20 minutes on the Bond III.

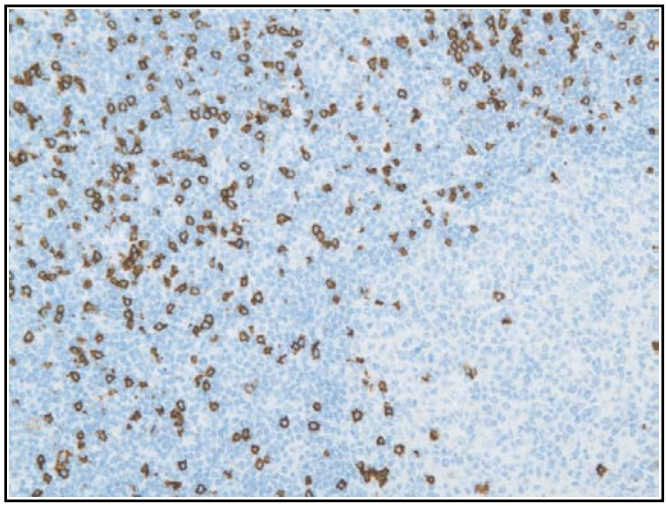


Fig 11. Optimal staining on the UK NEQAS distributed tonsil, showing strong and crisp membranous staining in the T-cells. Section stained with the Dako C8/144B antibody, 1:100 on the Dako Omnis platform with FLEX detection kit and 30 minutes antigen retrieval.

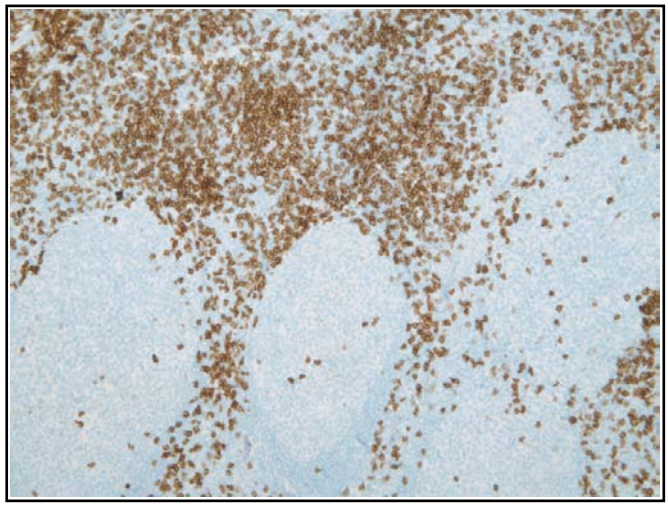
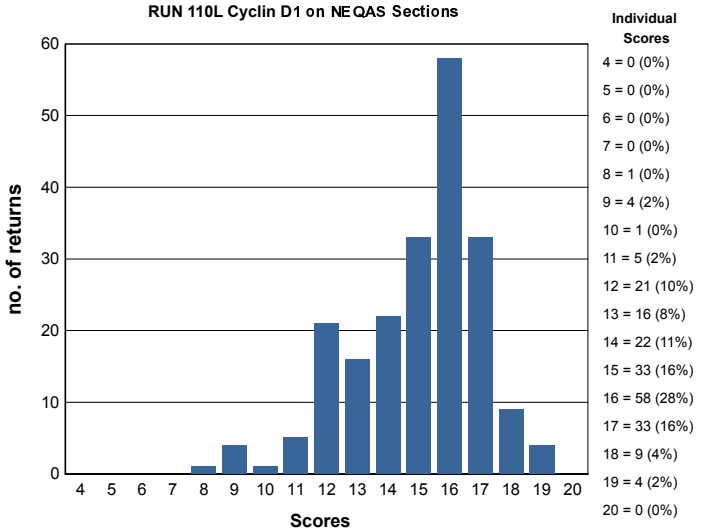
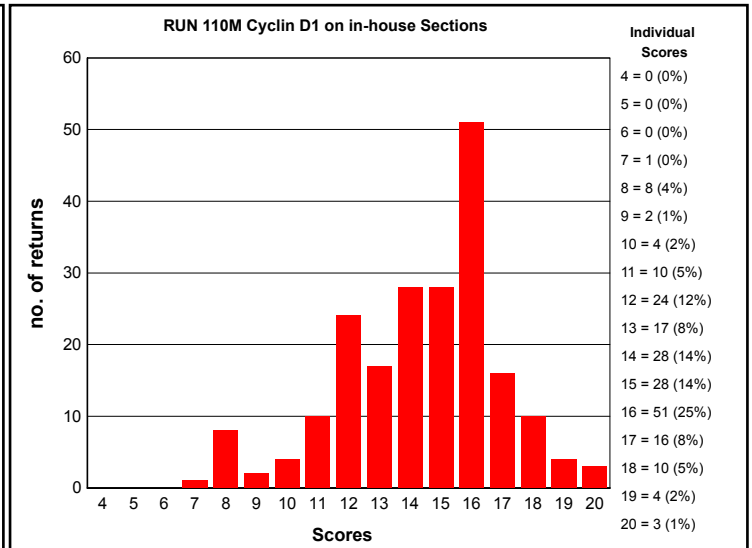
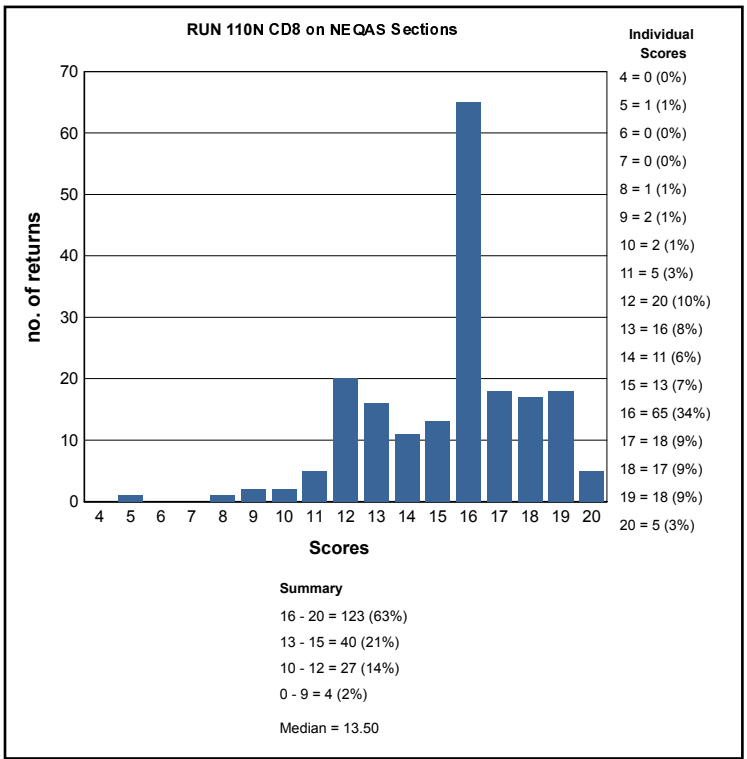


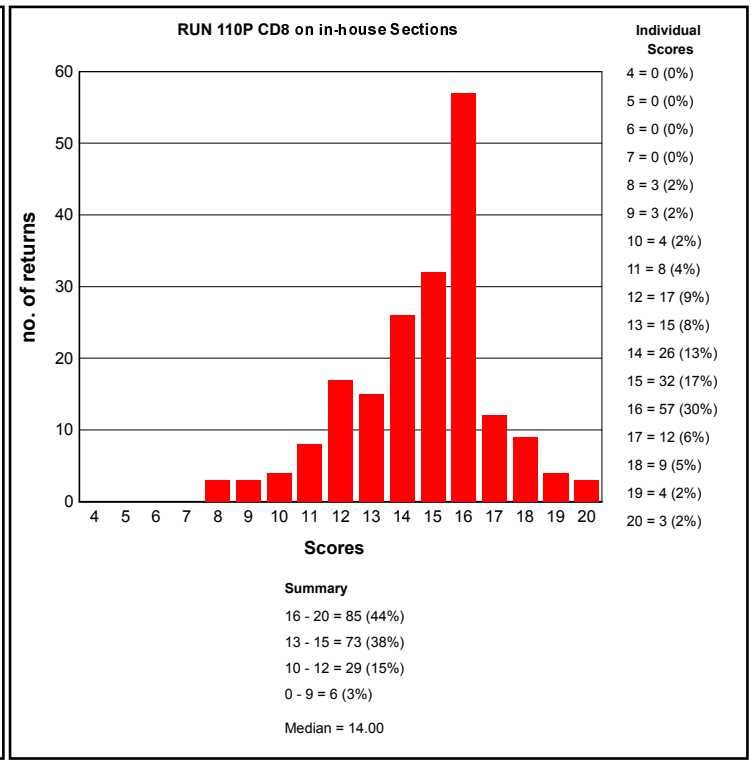
Fig 12. Good example of an in-house tonsil control stained with CD8, demonstrating strong staining of the interfollicular T-cells. The section was stained with the Leica 4B11 antibody on the Ventana Benchmark XT, CC1 standard retrieval and UltraView detection.

## GRAPHICAL REPRESENTATION OF PASS RATES



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

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

**Lymphoma Run: 110**

**Primary Antibody :** Cyclin D1

| Antibody Details | N | % |
|---|---|---|
| Biocare Medical P236B | 1 | 100 |
| Cell Marque (RBT14) CRC011 | 1 | 100 |
| Cell Marque (SP4) 241-R | 9 | 78 |
| Dako (DCS-6) M7155 | 1 | 0 |
| Dako (EP12) M3642 | 17 | 76 |
| Dako (SP4) M3635 | 3 | 100 |
| Dako FLEX RTU (SP4) IR152 | 2 | 100 |
| Dako RTU (EP12) IR083/IS083 | 15 | 87 |
| Lab Vision/Thermo Sci MS 210P (DCS-6) | 1 | 100 |
| LabVision/Thermo Sci (SP4) RM-9104 | 59 | 81 |
| Leica/Novo (P2D11F11) NCL-Cyclin D1-GM-CE | 7 | 86 |
| Leica/Novo RTU (P2D11F11) NCL-RTU-CyclinD1 | 1 | 0 |
| Menapath (SP4) MP-307 | 2 | 100 |
| NeoMarkers/Thermo Sci (EPR2241IHC) RM-2113 | 12 | 92 |
| Neomarkers/Thermo Sci MS 210 PO (DC5-6) | 2 | 100 |
| Other | 24 | 71 |
| Vector rbm VP-RM03 (SP4) | 4 | 100 |
| Ventana (SP4-R) 790-4508 | 34 | 97 |
| Ventana 250-2723 (P2D11F11) | 2 | 100 |
| Ventana rbm 760-4282 (SP4) | 6 | 100 |
| Zymed 18-0220 (AM29) | 1 | 0 |

**Lymphoma Run: 110**

**Primary Antibody :** CD8

| Antibody Details | N | % |
|---|---|---|
| Cell Marque C8/144B 108M | 2 | 100 |
| Dako C8/144B IR623 RTU Autostainer Link | 13 | 85 |
| Dako C8/144B IS623 RTU Autostainer Plus | 3 | 100 |
| Dako C8/144B M7103 | 61 | 79 |
| Leica/Novocastra 1A5 NCL-CD8-295 | 13 | 77 |
| Leica/Novocastra 1A5 RTU CD8-295-R-7-CE | 1 | 100 |
| Leica/Novocastra 4B11 NCL-CD8-4B11 | 17 | 76 |
| Leica/Novocastra 4B11 NCL-L-CD8-4B11 | 6 | 100 |
| Leica/Novocastra 4B11 RTU Bond PA0183 | 21 | 95 |
| Neomarkers/Thermo Sci Ab-1 CD8/144B MS-457-R/S | 2 | 100 |
| Other | 18 | 78 |
| Spring Bioscience SP16 M3160/1/2/4 | 1 | 100 |
| Ventana SP57 790-4460 | 24 | 100 |

**Lymphoma Run: 110**

| Heat Mediated Retrieval | CD8 N | CD8 % | Cyclin D1 N | Cyclin D1 % |
|---|---|---|---|---|
| Biocare Decloaking Chamber | 0 | 0 | 1 | 0 |
| Dako Omnis | 5 | 80 | 4 | 100 |
| Dako Pascal | 1 | 0 | 1 | 100 |
| Dako PTLink | 24 | 92 | 23 | 78 |
| Lab vision PT Module | 2 | 50 | 2 | 100 |
| Leica ER1 10 mins | 1 | 0 | 0 | 0 |
| Leica ER1 20 mins | 6 | 33 | 1 | 0 |
| Leica ER1 30 mins | 6 | 67 | 1 | 0 |
| Leica ER1 40 mins | 0 | 0 | 1 | 0 |
| Leica ER2 10 mins | 1 | 0 | 2 | 0 |
| Leica ER2 20 mins | 46 | 96 | 25 | 88 |
| Leica ER2 30 mins | 7 | 86 | 27 | 78 |
| Leica ER2 40 mins | 0 | 0 | 10 | 70 |
| Microwave | 2 | 100 | 3 | 67 |
| None | 0 | 0 | 1 | 100 |
| Other | 0 | 0 | 1 | 100 |
| Pressure Cooker | 5 | 80 | 2 | 50 |
| Pressure Cooker in Microwave Oven | 1 | 100 | 1 | 100 |
| Steamer | 1 | 100 | 1 | 100 |
| Ventana CC1 16mins | 1 | 100 | 0 | 0 |
| Ventana CC1 20mins | 1 | 0 | 1 | 100 |
| Ventana CC1 24mins | 1 | 100 | 1 | 0 |
| Ventana CC1 32mins | 6 | 100 | 10 | 100 |
| Ventana CC1 36mins | 4 | 75 | 1 | 100 |
| Ventana CC1 40mins | 4 | 100 | 6 | 100 |
| Ventana CC1 44mins | 1 | 100 | 0 | 0 |
| Ventana CC1 48mins | 3 | 100 | 1 | 100 |
| Ventana CC1 52mins | 1 | 0 | 0 | 0 |
| Ventana CC1 56mins | 1 | 100 | 1 | 100 |
| Ventana CC1 64mins | 15 | 87 | 22 | 91 |
| Ventana CC1 76mins | 2 | 50 | 2 | 100 |
| Ventana CC1 8mins | 1 | 100 | 0 | 0 |
| Ventana CC1 92mins | 1 | 100 | 2 | 100 |
| Ventana CC1 extended | 0 | 0 | 5 | 80 |
| Ventana CC1 mild | 12 | 67 | 12 | 92 |
| Ventana CC1 standard | 20 | 95 | 25 | 96 |
| Ventana CC2 64mins | 0 | 0 | 2 | 100 |
| Ventana CC2 standard | 0 | 0 | 1 | 0 |
| Water bath 95-98 OC | 1 | 100 | 2 | 100 |

**Lymphoma Run: 110**

| Enzyme Mediated Retrieval | CD8 N | CD8 % | Cyclin D1 N | Cyclin D1 % |
|---|---|---|---|---|
| NOT APPLICABLE | 64 | 84 | 92 | 83 |
| Ventana Protease 1 (760-2018) | 1 | 100 | 1 | 100 |

| Lymphoma Run: 110 | CD8 | | Cyclin D1 | |
|---|---|---|---|---|
| **Detection** | **N** | **%** | **N** | **%** |
| AS PER KIT | 15 | 93 | 13 | 85 |
| 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) | 6 | 100 | 7 | 86 |
| Dako EnVision FLEX+ ( K8002/12) | 14 | 93 | 13 | 85 |
| Dako Envision HRP/DAB ( K5007) | 4 | 75 | 4 | 75 |
| Dako Envision+ HRP rabbit K4008/9/10/11 | 0 | 0 | 2 | 50 |
| LabVision UltraVision ONE Polymer ( TL-012/5-HDJ/T | 1 | 100 | 0 | 0 |
| Leica Bond Polymer Define (DS9713) | 2 | 100 | 1 | 100 |
| Leica Bond Polymer Refine (DS9800) | 53 | 85 | 63 | 75 |
| None | 1 | 100 | 1 | 100 |
| NOT APPLICABLE | 0 | 0 | 1 | 100 |
| Other | 9 | 56 | 9 | 78 |
| Vector ImmPRESS Universal (MP-7500) | 0 | 0 | 1 | 0 |
| Ventana iView system (760-091) | 2 | 100 | 5 | 100 |
| Ventana OptiView Kit (760-700) | 24 | 92 | 31 | 97 |
| Ventana UltraView Kit (760-500) | 38 | 79 | 52 | 88 |

| Lymphoma Run: 110 | CD8 | | Cyclin D1 | |
|---|---|---|---|---|
| **Chromogen** | **N** | **%** | **N** | **%** |
| AS PER KIT | 28 | 82 | 22 | 82 |
| BioGenex liquid DBA (HK-124-7K) | 1 | 100 | 0 | 0 |
| Dako DAB K3468 | 0 | 0 | 1 | 0 |
| DAKO DAB+ | 1 | 100 | 2 | 100 |
| Dako EnVision Plus kits | 3 | 100 | 3 | 33 |
| Dako FLEX DAB | 15 | 93 | 19 | 89 |
| Dako REAL EnVision K5007 DAB | 4 | 75 | 4 | 75 |
| LabVision DAB | 1 | 100 | 1 | 100 |
| Leica Bond Polymer Refine kit (DS9800) | 56 | 86 | 63 | 76 |
| NOT APPLICABLE | 0 | 0 | 1 | 100 |
| Other | 12 | 83 | 13 | 92 |
| Sigma DAB (D5637) | 0 | 0 | 1 | 100 |
| Sigma DAB (D5905) | 1 | 0 | 1 | 100 |
| Ventana DAB | 13 | 85 | 20 | 90 |
| Ventana Enhanced Alk. Phos. Red Detection Kit | 1 | 100 | 0 | 0 |
| Ventana iview | 2 | 100 | 2 | 100 |
| Ventana Ultraview DAB | 45 | 82 | 52 | 90 |
| Vision BioSystems Bond X DAB | 0 | 0 | 1 | 100 |

| Lymphoma Run: 110 | CD8 | | Cyclin D1 | |
|---|---|---|---|---|
| **Automation** | **N** | **%** | **N** | **%** |
| BioGenex GenoMX 6000i | 1 | 100 | 1 | 100 |
| Dako Autostainer | 0 | 0 | 1 | 0 |
| Dako Autostainer Link 48 | 23 | 96 | 21 | 81 |
| Dako Autostainer plus | 3 | 33 | 3 | 100 |
| Dako Autostainer Plus Link | 2 | 50 | 3 | 67 |
| Dako Omnis | 5 | 80 | 4 | 100 |
| LabVision Autostainer | 3 | 100 | 3 | 100 |
| Leica Bond Max | 28 | 82 | 33 | 70 |
| Leica Bond-III | 39 | 85 | 39 | 79 |
| Menarini - Intellipath FLX | 1 | 100 | 0 | 0 |
| None (Manual) | 3 | 67 | 2 | 50 |
| Other | 0 | 0 | 1 | 100 |
| Shandon Sequenza | 1 | 100 | 1 | 0 |
| Ventana Benchmark GX | 2 | 100 | 3 | 100 |
| Ventana Benchmark ULTRA | 43 | 84 | 54 | 94 |
| Ventana Benchmark XT | 29 | 86 | 36 | 89 |

## BEST METHODS - Gold Standard Antibody

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

### Cyclin D1 - Method 1

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

**Primary Antibody:** Dako FLEX RTU (SP4) IR152 , 20 Mins Prediluted
**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: 10 Mins, Time 2: 10 Mins
**Detection:** Other , 20 Mins Prediluted

### Cyclin D1 - Method 2

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

**Primary Antibody:** LabVision/Thermo Sci (SP4) RM-9104 , 30 Mins, RT ºC Dilution 1: 30
**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

**Cyclin D1 - Method 3**

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

| | |
|---|---|
| **Primary Antibody:** | LabVision/Thermo Sci (SP4) RM-9104 , 32 Mins, RT ºC Dilution 1: 50 |
| **Automation:** | Ventana Benchmark ULTRA |
| **Method:** | Ventana Optiview |
| **Main Buffer:** | Ventana reaction buffer (950-300), PH: 8 |
| **HMAR:** | Ventana CC1 mild, Buffer: Ventana Reaction Buffer, PH: 8 |
| **EAR:** | NOT APPLICABLE |
| **Chromogen:** | Ventana DAB, RT ºC., Time 1: 8 Mins |
| **Detection:** | Ventana OptiView Kit (760-700) , 8 Mins, RT ºC Prediluted |

**Cyclin D1 - Method 4**

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

| | |
|---|---|
| **Primary Antibody:** | Ventana (SP4-R) 790-4508 , 16 Mins, 36 ºC Prediluted |
| **Automation:** | Ventana Benchmark ULTRA |
| **Method:** | Ventana Optiview |
| **Main Buffer:** | Ventana reaction buffer (950-300) |
| **HMAR:** | Ventana CC1 40mins |
| **EAR:** | |
| **Chromogen:** | Ventana DAB |
| **Detection:** | Ventana OptiView Kit (760-700) Prediluted |

## BEST METHODS - Secondary Antibody

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

**CD8 - Method 1**

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

| | |
|---|---|
| **Primary Antibody:** | Dako C8/144B M7103 , 32 Mins, 37 ºC Dilution 1: 1/200 |
| **Automation:** | Leica Bond Max |
| **Method:** | Leica BondMAx Refine KIT |
| **Main Buffer:** | Bond Wash Buffer (AR9590) |
| **HMAR:** | Leica ER2 20 mins |
| **EAR:** | NOT APPLICABLE |
| **Chromogen:** | AS PER KIT |
| **Detection:** | AS PER KIT |

**CD8 - Method 2**

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

| | |
|---|---|
| **Primary Antibody:** | Leica/Novocastra 4B11 RTU Bond PA0183 , 30 Mins, Ambient 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), 25 ºC., Time 1: 10 Mins |
| **Detection:** | Leica Bond Polymer Refine (DS9800) , 8 Mins, Ambient ºC Prediluted |

### CD8 - Method 3

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

| | |
|---|---|
| **Primary Antibody:** | Ventana SP57 790-4460 , 32 Mins, 37 ºC Prediluted |
| **Automation:** | Ventana Benchmark XT |
| **Method:** | Ventana Optiview |
| **Main Buffer:** | Ventana reaction buffer (950-300) |
| **HMAR:** | Ventana CC1 32mins, Buffer: CC1 |
| **EAR:** | |
| **Chromogen:** | Other, 47 ºC., Time 1: 8 Mins, Time 2: 8 Mins |
| **Detection:** | Ventana OptiView Kit (760-700) , 8 Mins, 37 ºC Prediluted |

### CD8 - Method 4

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

| | |
|---|---|
| **Primary Antibody:** | Dako C8/144B M7103 , 30 Mins, 20 ºC Dilution 1: 100 |
| **Automation:** | Dako Autostainer Link 48 |
| **Method:** | Dako FLEX+ kit |
| **Main Buffer:** | Dako FLEX wash buffer, PH: 7.6 |
| **HMAR:** | Dako PTLink, Buffer: Dako target retrieval - High pH, PH: 9 |
| **EAR:** | |
| **Chromogen:** | Dako FLEX DAB, 20 ºC., Time 1: 5 Mins, Time 2: 5 Mins |
| **Detection:** | Dako EnVision FLEX+ ( K8002/12) , 15 Mins, 20 ºC Prediluted |

# The Neuropathology Module Run 110

**Neil Bilbe**

| | Gold Standard | Second Antibody |
|---|---|---|
| **Antigens Assessed:** | Synaptophysin | Neurofilament protein |
| **Tissue Sections circulated:** | Cerebellum and GBM (oligo) | Anaplastic ganglioglioma |
| **Number of Registered Participants:** | 65 | |
| **Number of Participants this Run** | 65 (100%) | |

## Introduction

## Gold Standard: Synaptophysin

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

**Features of Optimal Immunostaining:**

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

**Features of Sub-optimal Immunostaining:**

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

**References**

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

## Second Antigen: Neurofilament protein (NFP)

Neurofilaments (NFs) belong to the family of intermediate filaments (IFs) and are structural elements of the neuronal cytoskeleton in an interconnection with actin microfilaments, microtubules and other IFs. NFs are composed of three different subunits that are different, but related proteins: NF-L (70 kDa), NF -M (150-160 kDa) and NF-H (200 kDa). The antigenic determinants of each of the subunits may be unique or shared and each NF subunit is a separate gene product. During embryonic neurogenesis, the NF-L and NF-M subunits are coexpressed, whereas the activation of the NF-H subunit is delayed to the postnatal period. NF-M and NF-H subunits are unable to self-assemble and, typically, form co-polymers with NF-L (4, 5). Cells labelled by the antibody display a cytoplasmic staining pattern.

Normal tissues: In sections of normal colon, the antibody labels some axons in the axon bundles of the plexus of Auerbach and Meissner, whereas the perikarya of the ganglion cells are not stained.

Abnormal tissues: In cases of Hirschsprung´s disease, the antibody strongly labels axons in the plexus of Auerbach and Meissner in the aganglionic bowel segments. In gangliogliomas, the antibody stains neuronal processes, with occasional staining in the neuronal perikarya. Merkel cell tumours are usually positive in virtually all tumours.

**References**

1. Diepholder HM, Schwechheimer K, Mohadjer M, Knoth R, Volk B. A clinicopathologic and immunomorphologic study of 13 cases of ganglioglioma. Cancer 1991;68:2192-2201.
2. Luider TM, van Dommelen MW, Tibboel D, Meijers JH, Ten Kate FJ,Trojanowski JQ, et al. Differences in phosphorylation state of neurofilament proteins in ganglionic and aganglionic bowel segments of children with Hirschsprung's disease. J Pediatr Surg 1992; 27: 815-9.
3. Van Muijen GNP, Ruiter DJ, Warnaar SO. Intermediate filaments in Merkel cell tumors. Hum Pathol 1985;16:590-

**Assessment Summary:**

There were 65 active labs at the start of the assessment, with 249/260 slide assessed. Two labs who did not stock NFP submitted CD68, as advised by NEQAS*

This was the first run where the single slide NEQAS/In-House system was used. There were some issues with the NEQAS sections being too high up the slide for participants using certain platforms, resulting in a 'tidemark' effect when viewed microscopically. Assessors were made aware of this, and no labs were marked down if this was seen. A few labs reported problems with section adhesion in the bottom half of the slide, below the dividing line, where the in-house section was to be placed prior to staining, others had difficulties as their in-house section was too large. This meant that 23 *separate* in-house control slides were returned, approximately 19%.

In summary: 202 slides (81%) passed, 34 (14%) were borderline, and 13 (5%) failed the assessment.

The failed slides were **G**=3, **H**=0, **J**=7, and **K**=3.

The 3 slides that failed the **Synaptophysin NEQAS (G) section** all had negative staining of the cerebellum, with poor or very weak staining in the tumour sample (see Fig 3). Interestingly, all had used a different primary antibody source: Biogenex, Dako, and Novocastra, and different platforms: a Manual method, Dako, and Ventana.

**Neurofilament protein (J):** When analysing the failed slides, three used the Dako 2F11, two a Novocastra antibody and one lab employed the Progen 2F11 clone. Included in the failed NFP slides was one participant who had stained using CD68, *but had submitted protocols for a NFP primary, the Novocastra RTU N52.1.7 clone! So effectively there were 6 failed NFP slides.

For the Dako 2F11 (used by 51% of labs) one was a RTU solution, one was diluted 1:200, and the other had a stated dilution of **1:16,000**; whether this is a mistake or genuine, we do not know.

For the 2 Novocastra users, one employed the RTU N52.1.7, and the other the DA2 clone diluted 1:100.

The user of the Progen 2F11 marker diluted this antibody to 1:500, but the datasheet has a recommended dilution of only 1:10; whether this had any significant bearing on the outcome is difficult to say.

When looking at the retrieval and platforms used; two used the Ventana Benchmark ULTRA and the CC1; two a Bond Max with ER1 and ER2; two a Bond–III with a ER1 and ER2.

Summary table:

| | G | H | J | K | Average |
|---|---|---|---|---|---|
| Pass | 69% | 98% | 66% | 92% | 81% |
| Borderline | 27% | 2% | 23% | 3% | 14% |
| Fail | 5% | 0% | 11% | 5% | 5% |

In-house control material:

The number of labs who submitted an in-house control considered to be 'suitable' and/or of neurological origin was 52% (**H**) and 74% **(K**).

N.B. As with previous assessments, the Neurofilament slides proved far more challenging than the other or Gold antigen submissions.

## Selected Images showing Optimal and Sub-optimal Immunostaining

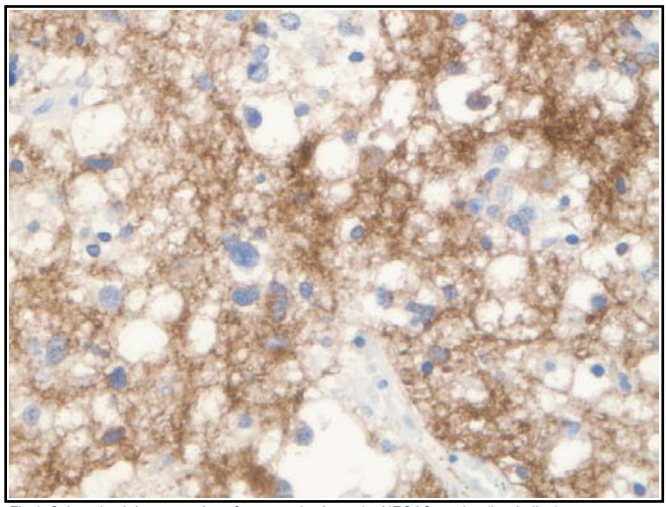


Fig 1. Sub-optimal demonstration of synaptophysin on the NEQAS section (borderline). Although even, the staining could be stronger, but with a nice counterstain intensity. Novocastra antibody at 1:100 for 20 mins, Leica ER1 20 mins, with the Leica Bond Polymer Refine for 8 mins on a Leica Bond-III machine.

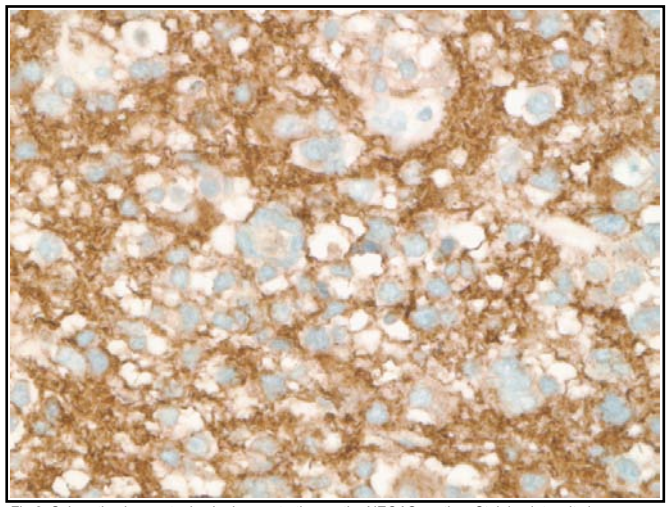


Fig 2. Sub-optimal synaptophysin demonstration on the NEQAS section. Staining intensity is good, but there is evidence of excess pretreatment: morphological damage and section lifting. Cell Marque RTU, with a Ventana UltraView Kit( no times given), Ventana CC1 for 76mins, on the Ventana Benchmark ULTRA.

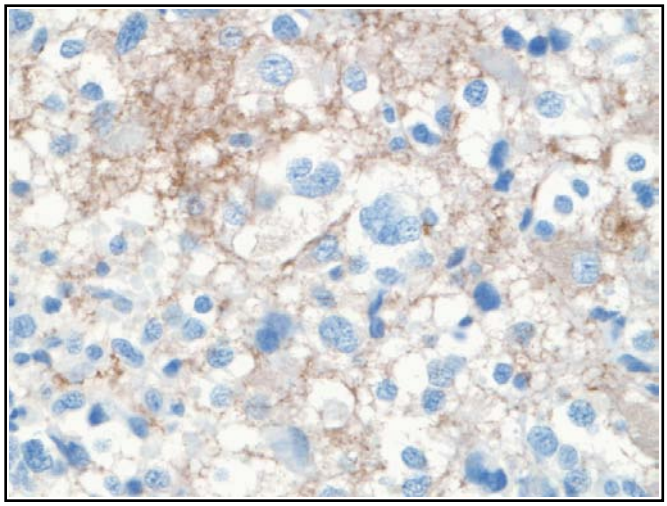


Fig 3. Poor demonstration of synaptophysin, staining is far too weak to be used for diagnostic purposes; therefore this slide failed the assessment. Novocastra (27G12), 1:100, 32 mins, Ventana CC1 32mins, with the Ventana UltraView Kit (no time given), on the Ventana Benchmark XT.

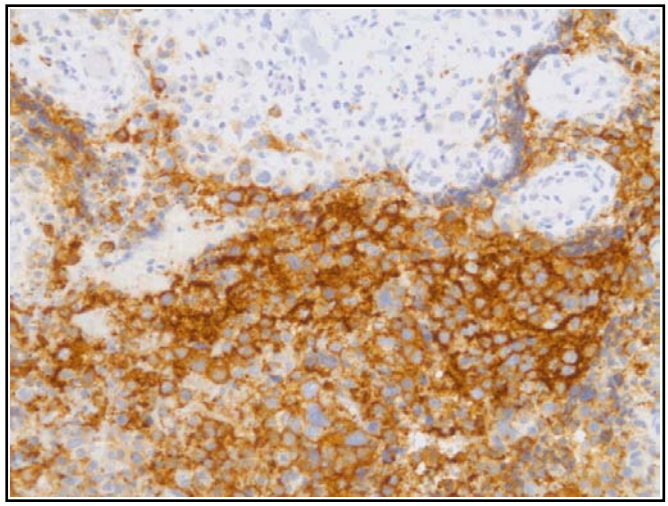


Fig 4. Sub-optimal synaptophysin demonstration on the NEQAS section; staining was uneven: weak/poor in the middle of the section, but stronger at the edges (see image). Borderline score allocated. Dako RTU Dak-Synap clone, 20 mins, with water bath PT at 95 C, Dako EnVision FLEX for 20 mins, and Dako Autostainer Link 48.

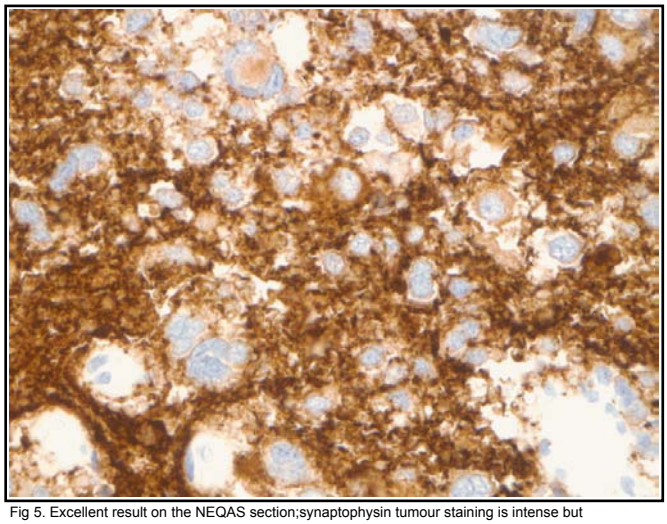


Fig 5. Excellent result on the NEQAS section;synaptophysin tumour staining is intense but even, and the background is clean. Dak-Synap clone, diluted 1:50 (recommended) 30 mins, with a Dako PTLink at high pH for 20 mins, Dako EnVision FLEX+ 30 mins, on a Dako Autostainer Link 48.

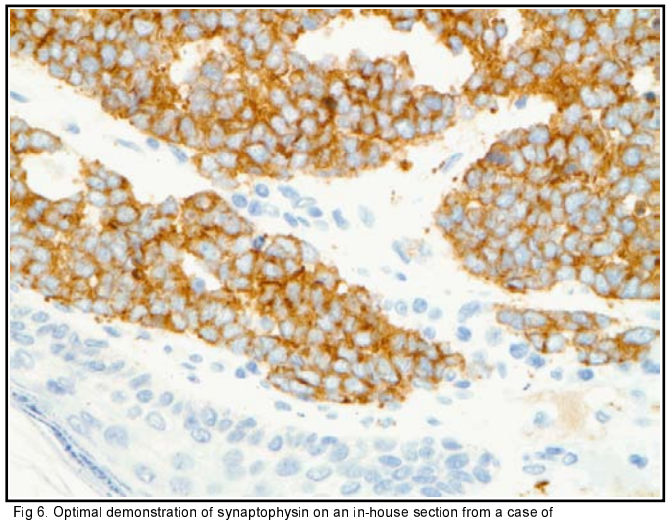


Fig 6. Optimal demonstration of synaptophysin on an in-house section from a case of (neuroendocrine) tumour in skin. Staining is crisp and precise, the background is clean. This is the same participant as figure 4 (uneven staining). The difference in staining patterns could be due to tissue preparation, or the use of a water bath (uncommon today).

## Selected Images showing Optimal and Sub-optimal Immunostaining

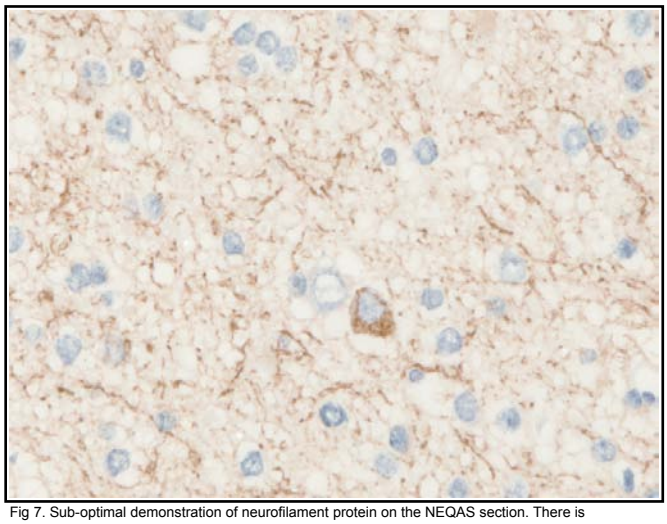


Fig 7. Sub-optimal demonstration of neurofilament protein on the NEQAS section. There is some inappropriate staining (low borderline pass). Dako 2F11, 1:200 for 50 mins, in a pressure cooker, with a Vector low pH unmasking solution, and a RTU Vector Elite ABC Kit 30 mins, manual method.

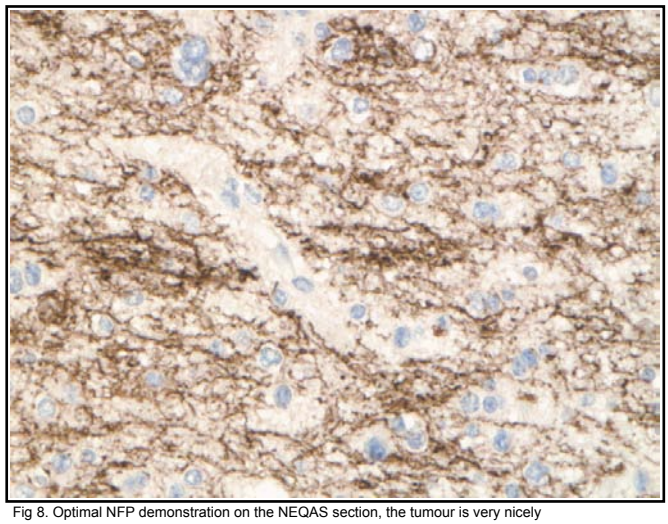


Fig 8. Optimal NFP demonstration on the NEQAS section, the tumour is very nicely demonstrated, counterstain is crisp and the background clean. Dako 2F11, 1:400 for 15 mins; Leica ER1 10 mins, and a RTU Leica Bond Polymer Refine for 8 mins, on a Leica Bond-III.

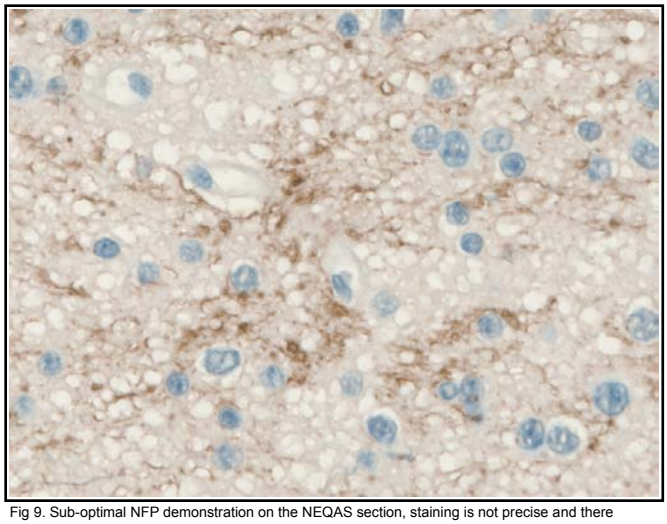


Fig 9. Sub-optimal NFP demonstration on the NEQAS section, staining is not precise and there is background staining (borderline score). Dako 2F11, 1:800 15 mins (compare fig 8), Leica ER1 20 mins, the RTU Leica Bond Polymer Refine for 8 mins, on a Leica Bond Max.

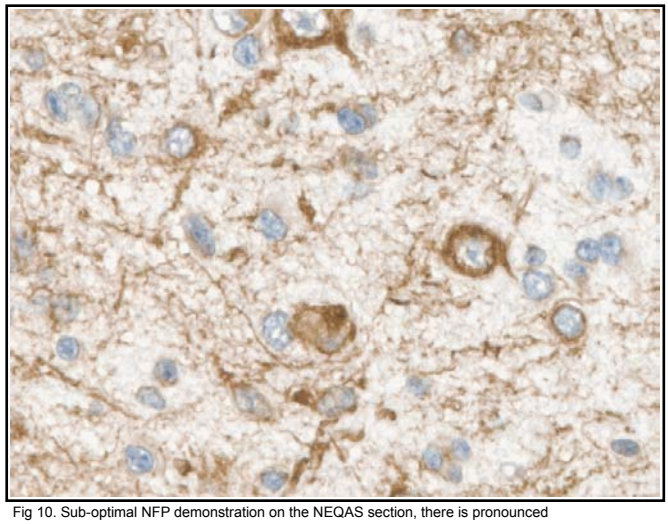


Fig 10. Sub-optimal NFP demonstration on the NEQAS section, there is pronounced non-specific staining, but considered just suitable for diagnostic use. Invitrogen/Zymed antibody, using a microwave oven, Dako DAB+ REAL Detection kit, manual method. No other details given.

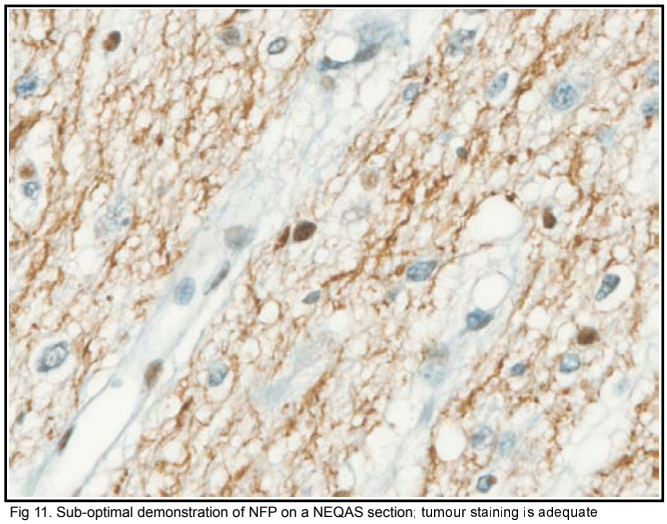


Fig 11. Sub-optimal demonstration of NFP on a NEQAS section; tumour staining is adequate but there is some inappropriate demonstration of other cells. Biogenex (NE14), 1:1000, 32 mins, Ventana CC1 36mins, Ventana UltraView Kit for 8 mins, on a Ventana Benchmark XT.

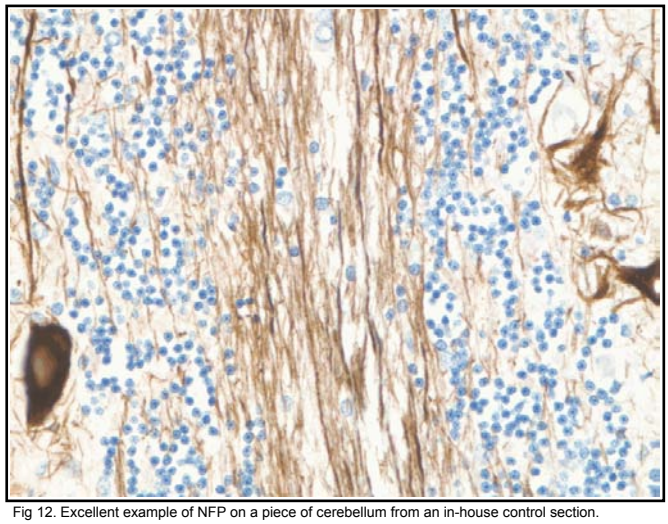
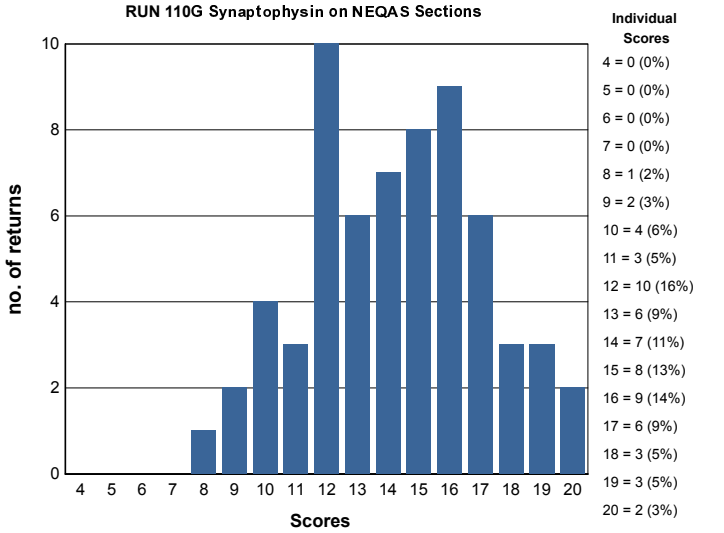
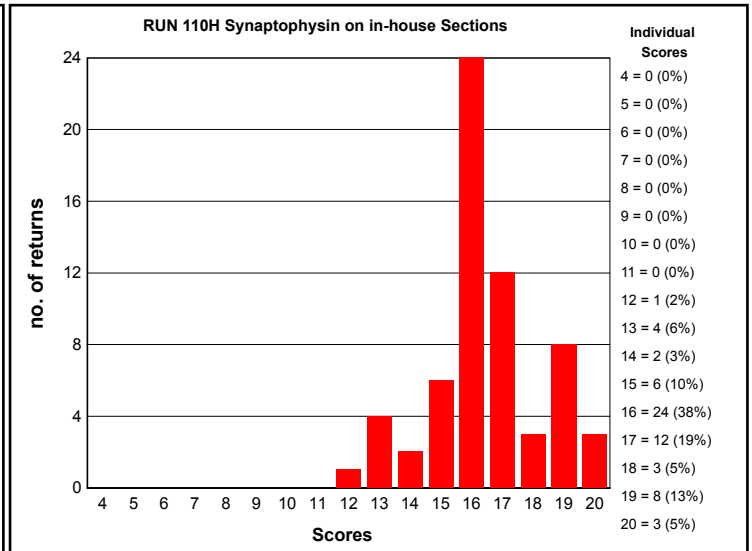
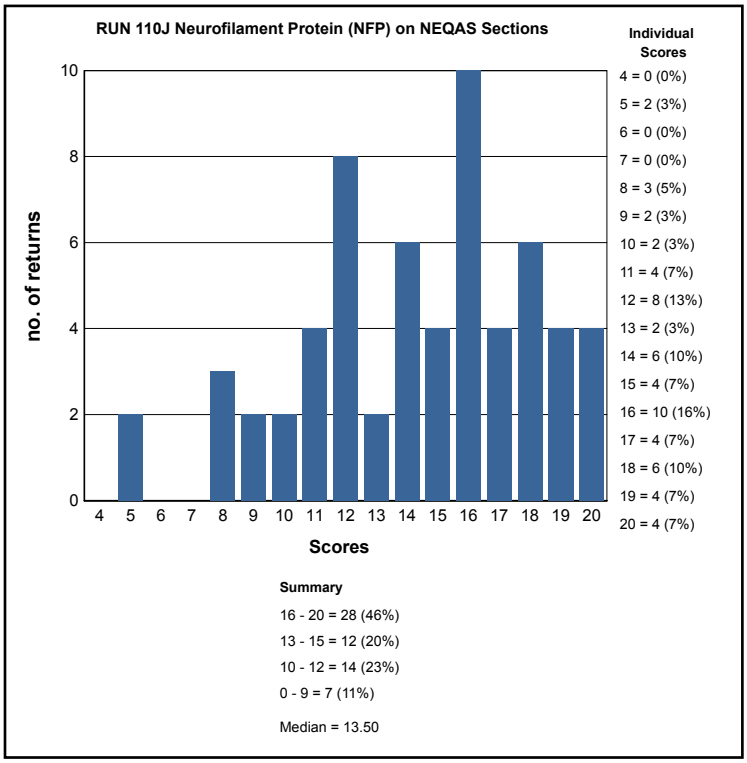


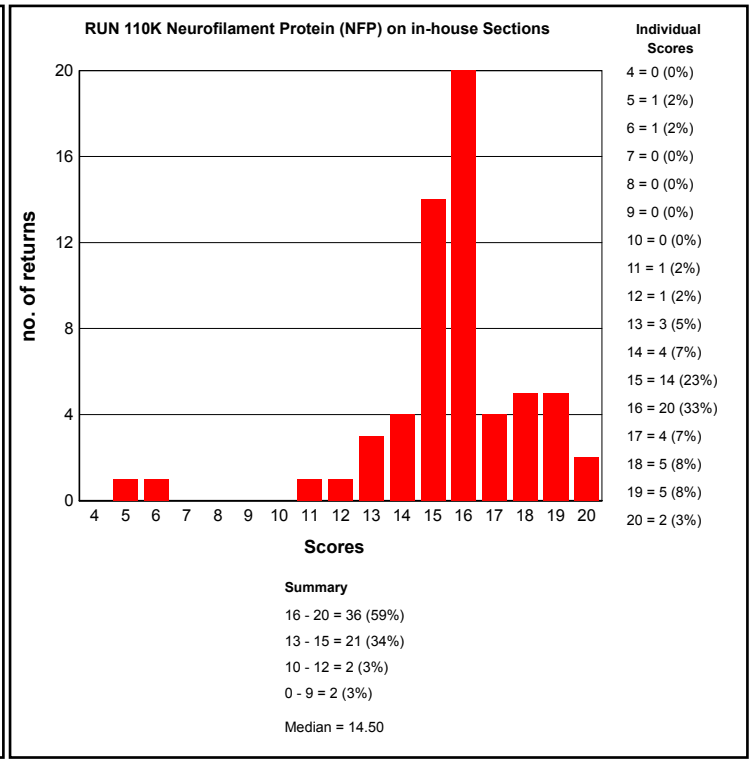
Fig 12. Excellent example of NFP on a piece of cerebellum from an in-house control section. Nerve processes and Purkinje cells are beautifully demonstrated. Sigma N0142 (N52), diluted to 1:3000, Leica ER2 20 mins; Leica Bond Polymer Refine kit (as per kit), on a Leica Bond-III.

## 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: 110**

**Primary Antibody :** Synaptophysin

| Antibody Details | N | % |
|---|---|---|
| BioGenex AM 6 (clone SNP ) | 1 | 0 |
| Biogenex MU 363 UC | 1 | 0 |
| DAKO FLEX IR776 (SY38) | 2 | 100 |
| Dako M0776 (clone SY38 ) | 7 | 71 |
| NeoMarkers SP11 | 1 | 0 |
| Novocastra Bond RTU PA0299 (rb poly) | 2 | 100 |
| Novocastra NCL-L-SYNAP-299 (27G12) | 12 | 58 |
| Novocastra NCL-SYNAP-299 (27G12) | 10 | 90 |
| Other | 15 | 60 |
| Ventana 760-4595 (MRQ-40) | 5 | 80 |
| Ventana CONFIRM 790-4407 (SP-11) | 7 | 86 |

**Neuropathology Run: 110**

**Primary Antibody :** Neurofilament Protein (NFP)

| Antibody Details | N | % |
|---|---|---|
| Biogenex AM073-5M/10M/MU073-UC (NE14) | 2 | 0 |
| Dako FLEX RTU IR607 (2F11) | 5 | 100 |
| Dako M0762 (2F11) | 31 | 71 |
| Invitrogen/Zymed 0 -0171 (clones FNP7/DA2/RMd020/1 | 2 | 0 |
| Leica/Novocastra NCL-NF200-N52 (N52.1.7) | 1 | 0 |
| Leica/Novocastra NCL-NF68-DA2 (DA2) | 1 | 0 |
| Leica/Novocastra PA0371 RTU (N52.1.7) | 4 | 25 |
| Other | 1 | 0 |
| Sigma N0142 (N52) | 3 | 33 |
| Ventana 250-2793 (2F11) | 1 | 100 |
| Ventana RTU 760-2661 (2F11) | 8 | 100 |

**Neuropathology Run: 110**

| Heat Mediated Retrieval | Neurofilament Protein (NFP) N | Neurofilament Protein (NFP) % | Synaptophysin N | Synaptophysin % |
|---|---|---|---|---|
| _Leica BondMax ER1 | 1 | 0 | 0 | 0 |
| _Leica BondMax ER2 | 0 | 0 | 1 | 100 |
| _Microwave Oven | 1 | 0 | 0 | 0 |
| Biocare Decloaking Chamber | 1 | 0 | 1 | 100 |
| Dako Omnis | 0 | 0 | 1 | 100 |
| Dako PTLink | 8 | 100 | 11 | 73 |
| Leica ER1 10 mins | 3 | 67 | 0 | 0 |
| Leica ER1 20 mins | 4 | 25 | 3 | 67 |
| Leica ER1 30 mins | 3 | 100 | 5 | 60 |
| Leica ER2 20 mins | 6 | 33 | 9 | 44 |
| Leica ER2 30 mins | 2 | 50 | 3 | 33 |
| Microwave | 1 | 0 | 2 | 50 |
| None | 2 | 50 | 0 | 0 |
| Other | 1 | 100 | 0 | 0 |
| Pressure Cooker | 1 | 0 | 0 | 0 |
| Ventana CC1 24mins | 1 | 100 | 1 | 100 |
| Ventana CC1 32mins | 1 | 0 | 4 | 75 |
| Ventana CC1 36mins | 2 | 50 | 1 | 0 |
| Ventana CC1 40mins | 3 | 100 | 0 | 0 |
| Ventana CC1 52mins | 0 | 0 | 2 | 50 |
| Ventana CC1 56mins | 0 | 0 | 1 | 100 |
| Ventana CC1 64mins | 2 | 100 | 6 | 100 |
| Ventana CC1 76mins | 0 | 0 | 1 | 0 |
| Ventana CC1 8mins | 2 | 50 | 1 | 100 |
| Ventana CC1 mild | 2 | 100 | 3 | 100 |
| Ventana CC1 standard | 6 | 67 | 6 | 100 |
| Ventana CC2 32mins | 1 | 100 | 0 | 0 |
| Ventana CC2 36mins | 1 | 100 | 0 | 0 |
| Water bath 95-98 OC | 1 | 100 | 1 | 0 |

**Neuropathology Run: 110**

| Enzyme Mediated Retrieval | Neurofilament Protein (NFP) N | Neurofilament Protein (NFP) % | Synaptophysin N | Synaptophysin % |
|---|---|---|---|---|
| NOT APPLICABLE | 28 | 75 | 37 | 70 |
| Other | 1 | 100 | 0 | 0 |
| Ventana Protease 1 (760-2018) | 1 | 0 | 0 | 0 |

| Neuropathology Run: 110 | Neurofilament Protein (NFP) | | Synaptophysin | |
|---|---|---|---|---|
| **Detection** | **N** | **%** | **N** | **%** |
| AS PER KIT | 5 | 60 | 7 | 100 |
| Biocare polymer (M4U534) | 1 | 0 | 1 | 100 |
| Dako EnVision FLEX ( K8000/10) | 1 | 100 | 4 | 50 |
| Dako EnVision FLEX+ ( K8002/12) | 6 | 100 | 5 | 60 |
| Dako Envision HRP/DAB ( K5007) | 2 | 50 | 1 | 0 |
| Leica Bond Polymer Refine (DS9800) | 20 | 45 | 20 | 45 |
| NOT APPLICABLE | 0 | 0 | 1 | 100 |
| Other | 0 | 0 | 1 | 100 |
| Vector Elite ABC Kit (PK-7200) | 1 | 0 | 0 | 0 |
| Ventana iView system (760-091) | 1 | 0 | 2 | 100 |
| Ventana OptiView Kit (760-700) | 6 | 100 | 7 | 100 |
| Ventana UltraView Kit (760-500) | 15 | 80 | 15 | 73 |

| Neuropathology Run: 110 | Neurofilament Protein (NFP) | | Synaptophysin | |
|---|---|---|---|---|
| **Automation** | **N** | **%** | **N** | **%** |
| Dako Autostainer Link 48 | 10 | 90 | 12 | 67 |
| Dako Omnis | 0 | 0 | 1 | 100 |
| Leica Bond Max | 11 | 36 | 11 | 45 |
| Leica Bond-III | 11 | 55 | 11 | 55 |
| Menarini - Intellipath FLX | 1 | 0 | 1 | 100 |
| None (Manual) | 3 | 33 | 2 | 50 |
| Ventana Benchmark GX | 0 | 0 | 1 | 100 |
| Ventana Benchmark ULTRA | 13 | 69 | 14 | 86 |
| Ventana Benchmark XT | 10 | 90 | 11 | 82 |

| Neuropathology Run: 110 | Neurofilament Protein (NFP) | | Synaptophysin | |
|---|---|---|---|---|
| **Chromogen** | **N** | **%** | **N** | **%** |
| AS PER KIT | 8 | 75 | 13 | 85 |
| Dako DAB+ REAL Detection (K5001) | 2 | 50 | 1 | 100 |
| Dako EnVision Plus kits | 2 | 100 | 1 | 100 |
| Dako FLEX DAB | 4 | 75 | 6 | 67 |
| Dako REAL EnVision K5007 DAB | 1 | 0 | 1 | 0 |
| Leica Bond Polymer Refine kit (DS9800) | 20 | 40 | 20 | 45 |
| Other | 3 | 100 | 3 | 100 |
| Ventana DAB | 2 | 100 | 3 | 100 |
| Ventana iview | 1 | 0 | 1 | 100 |
| Ventana Ultraview DAB | 15 | 80 | 15 | 73 |
| 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

### Synaptophysin - Method 1

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

**Primary Antibody:** Novocastra NCL-L-SYNAP-299 (27G12) , 15 Mins, RT ºC Dilution 1: 100
**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

### Synaptophysin - Method 2

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

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

**Synaptophysin - Method 3**

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

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

**Synaptophysin - Method 4**

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

| | |
|---|---|
| **Primary Antibody:** | Dako M0776 (clone SY38 ) |
| **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) |

## BEST METHODS - Secondary Antibody

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

**Neurofilament Protein (NFP) - Method 1**

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

| | |
|---|---|
| **Primary Antibody:** | Dako M0762 (2F11) , 15 Mins Dilution 1: 1:400 |
| **Automation:** | Leica Bond-III |
| **Method:** | Leica BondMAx Refine KIT |
| **Main Buffer:** | Bond Wash Buffer (AR9590) |
| **HMAR:** | Leica ER1 10 mins |
| **EAR:** | |
| **Chromogen:** | Leica Bond Polymer Refine kit (DS9800), Time 1: 10 Mins |
| **Detection:** | Leica Bond Polymer Refine (DS9800) , 8 Mins Prediluted |

**Neurofilament Protein (NFP) - Method 2**

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

| | |
|---|---|
| **Primary Antibody:** | Dako FLEX RTU IR607 (2F11) Prediluted |
| **Automation:** | Dako Autostainer Link 48 |
| **Method:** | Dako FLEX kit |
| **Main Buffer:** | AS PER KIT |
| **HMAR:** | Dako PTLink, PH: 9 |
| **EAR:** | |
| **Chromogen:** | AS PER KIT |
| **Detection:** | Dako EnVision FLEX ( K8000/10) Prediluted |

**Neurofilament Protein (NFP) - Method 3**

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

| | |
|---|---|
| **Primary Antibody:** | Dako M0762 (2F11) , 32 Mins, 36 ºC Dilution 1: 100 |
| **Automation:** | Ventana Benchmark ULTRA |
| **Method:** | Ventana UltraView DAB |
| **Main Buffer:** | Ventana reaction buffer (950-300) |
| **HMAR:** | Ventana CC1 64mins |
| **EAR:** | NOT APPLICABLE |
| **Chromogen:** | Ventana Ultraview DAB, 36 ºC., Time 1: 8 Mins |
| **Detection:** | Ventana UltraView Kit (760-500) , 8 Mins Prediluted |

**Neurofilament Protein (NFP) - Method 4**

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

| | |
|---|---|
| **Primary Antibody:** | Ventana RTU 760-2661 (2F11) , 32 Mins, 36 ºC Prediluted |
| **Automation:** | Ventana Benchmark ULTRA |
| **Method:** | Ventana Optiview |
| **Main Buffer:** | Ventana reaction buffer (950-300) |
| **HMAR:** | Ventana CC2 36mins |
| **EAR:** | |
| **Chromogen:** | Other, 36 ºC., Time 1: 8 Mins, Time 2: 8 Mins |
| **Detection:** | Ventana OptiView Kit (760-700) , 8 Mins, 36 ºC Prediluted |

**Neil Bilbe and Irena Srebotnik Kirbis**

| | Gold Standard | Second Antibody |
|---|---|---|
| **Antigens Assessed:** | Melanoma markers | Ki67 |
| **Sample circulated; cytospins and cell block sections:** | Human melanoma, cervical cancer, and bladder cancer cell lines, plus an effusion with lymphocytes and epithelial cells. | Human melanoma, cervical cancer, and bladder cancer cell lines, plus an effusion with lymphocytes and epithelial cells. |
| **Number of Registered Participants:** | 82 | |
| **Number of Participants this Run** | 81 (99%) | |

## Introduction

**Gold Standard: Melanoma markers**

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

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

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

**Features of Optimal Immunostaining:**
- Intense cytoplasmic staining of tumour cells.
- Clean background.
- No non-specific staining of other cell types
- Adequate nuclear counterstain.

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

**References**

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

## Second Antigen: Ki67

Antigen Ki-67 is a nuclear protein that is associated with and may be necessary for cellular proliferation. Furthermore it is associated with ribosomal RNA transcription. Inactivation of antigen Ki-67 leads to inhibition of ribosomal RNA synthesis.

Ki-67 is an excellent marker to determine the growth fraction of a given cell population. The fraction of Ki-67-positive tumour cells (the *Ki-67 labelling index*) is often correlated with the clinical course of cancer. The best-studied examples in this context are carcinomas of the prostate, brain and the breast and nephroblastoma . For these types of tumours, the prognostic value for survival and tumour recurrence have repeatedly been proven in uni- and multivariate analysis.

Ki-67 and MIB-1 monoclonal antibodies are directed against different epitopes of the same proliferation related antigen. MIB-1 is used in clinical applications to determine the Ki-67 labelling index. Both Ki-67 and MIB-1 may be used on fixed sections.

**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. D C Brown, et al. Proliferation in non-Hodgkin's lymphoma: a comparison of Ki67 staining on fine needle aspiration and cryostat sections. J Clin Pathol 1990;43:325-328

**Features of Optimal Immunostaining:**
- Intense and well-localised nuclear staining of tumour cells
- Clean background
- No non-specific staining
- Adequate counter-stain

**Features of Suboptimal Immunostaining:**
- Weak, uneven, or lower than expected level of staining in the tumour cells
- Diffuse staining
- High background or non-specific staining of cell types not expected to stain
- Excessive or very weak counterstain

**References (cell blocks in cytology)**

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

## Assessment Summary:

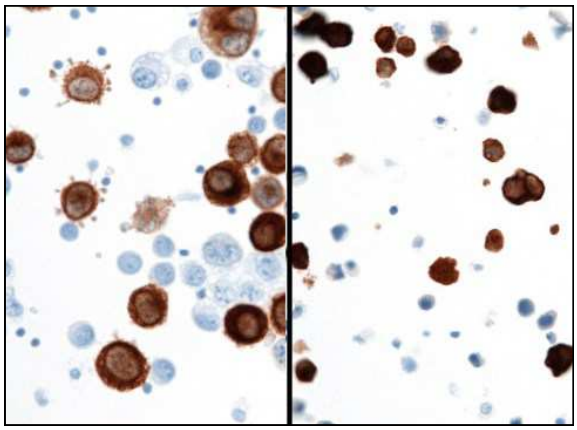
82 active labs were registered with the scheme at the start of the run; a single lab did not submit any slides at all. Several labs did not return an in-house sample (**S**=7, and **U**=5), something which is common in the cytology module. A few (**R**= 2 and **T**=1) did not send in their NEQAS samples. This left a total of 313 slides for the assessors to score.

NEQAS samples: cytospin v cell block

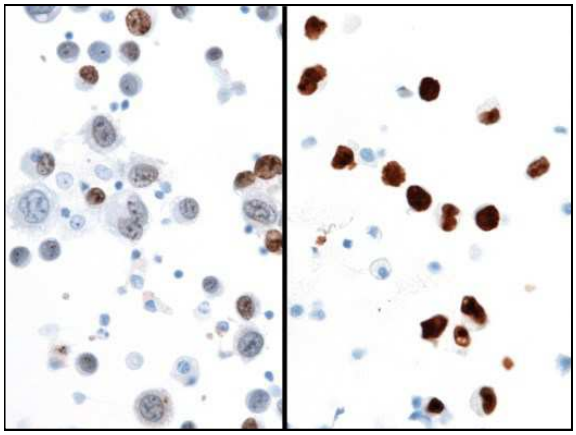
Of the 82 labs that were sent slides for this run, 57 (70%) requested cell block sections (CB) and 25 (30%) had requested cytospins (CS). This represented another increase in the number of participants choosing CB from Run 109: 63% . Part of this is due to some labs leaving the scheme and others joining the cytology module, rather than a change to a lab's existing requirements from previous run/s.

**Examples of the validation samples sent with the slides:**

Below: Melan A staining on the NEQAS CS (left) and CB (right) sample showing strong staining of melanoma cells.



Below: Ki67 staining on the NEQAS CS (left) and CB (right) sample. Some staining should be seen in approximately 60% of cells in the sample.



**Assessment outcome**

There were **8** (2.5%) **failed** slides out of the 313 assessed. Five of these were on a NEQAS samples **(R** = 3 **& T** = 2**),** and three were on the in-house Ki67 slide **(U).** No in-house Melanoma slides (**S**) failed the assessment.

**31** (9.9%) of all slides were assessed as being **borderline**. The spread for the four slide types, (**R, S, T, U**) can be gleaned from the table below :

| | Pass | Bord | Fail | | | Pass | Bord | Fail |
|---|---|---|---|---|---|---|---|---|
| R | 70 | 7 | 3 | 80 | R | 88% | 9% | 4% |
| S | 67 | 8 | 0 | 75 | S | 89% | 11% | 0% |
| T | 67 | 12 | 2 | 81 | T | 83% | 15% | 2% |
| U | 70 | 4 | 3 | 77 | U | 91% | 5% | 4% |
| Total | 274 | 31 | 8 | 313 | Ave | 87.6% | 9.9% | 2.5% |

**Cell Block v Cytospin:**
Of the five NEQAS slides that failed the assessment, three were on cytospins and two were on the cell block sections.
NB. Patchy (weaker) staining was noticed in one small area of some of the CB sections; assessors scored these accordingly.

For the Melanoma (**R**) slides this was split 2:1 CB to CS. As a percentage this equates to 4% of slides for each sample type: (2/55 for CS and 1/25 for CS). Two out of the three labs had employed the Dako Melan A (25% overall usage), at 1:100 (CS no retrieval) and 1:200 (CB with ER2 for 20 min). The other failed slide applied a RTU Ventana S100 (sole user) with *no retrieval* on a CB section.
The failed Ki67 (**T**) slides were both on cytospins, which equates to 8% of all cytospins (2/25). Interestingly, the two labs that failed their NEQAS Ki67 (**T**) assessment, also failed with their in-house Ki67 (**U**) controls (a smear and a FFPE section). Each had used a Dako MIB-1 primary (used by 56% of participants), diluted 1:100, on the Leica Bond (Max and III) platforms (see Fig 9). Both had entered details in the HMAR section (ER1 and ER2) but only one had indicated that this was used on the NEQAS cytospin sample (**T**).

Type of in-house samples submitted (**S**: n=75,& **U**: n=77)
As with previous runs, the sample type submitted for the in-house controls recorded at the assessment, are as follows:

Summary: difference between Run 109 and Run 110

| S (Mel) | No | % | U (Ki67) | No | % | Total | Total % |
|---|---|---|---|---|---|---|---|
| FFPE | 42 | 56% | FFPE | 41 | 53% | 83 | 55% |
| CS | 10 | 13% | CS | 11 | 14% | 21 | 14% |
| CB | 17 | 23% | CB | 18 | 23% | 35 | 23% |
| Smear | 3 | 4% | Smear | 3 | 4% | 6 | 4% |
| Thin Prep | 3 | 4% | Thin Prep | 4 | 5% | 7 | 5% |
| | 75 | 100% | | 77 | 100% | 152 | 100% |

- FFPE ↑ 10% / Cytospins ↓ 2% / Cell blocks ↓5%
- Thin preps ↓ 2% / Smears unchanged

As this is the first run of the new EQA year a direct comparison with the previous Gold in-house samples (**S**) is not possible. But needless to say there is a significant increase in the number of participants submitting a tissue section. This may be due to two factors:

a) more labs requesting a CB for their NEQAS sample and therefore a corresponding FFPE is more likely be used
b) the antigens being requested; some markers; e.g. Ki67 lend themselves better to a tissue section (e.g. tonsil) than others

**Laboratory self-assessment**
It is always of interest to the assessors and UK NEQAS ICC & ISH to compare the score recorded by the *laboratory* (Tech and Path), with those allocated by the assessors (NEQAS).

Comparative scores for the 5 failed NEQAS slides:

1. Melanoma (R): 8 (NEQAS ) v 12 (Tech) - Fig 4
2. Melanoma (R): 8 (NEQAS ) v 12 (Tech) 15 (Path)
3. Melanoma (R): 8 (NEQAS ) v 16 (Self) 16 (Path)*
4. Ki67 (T): 8 (NEQAS) v 10 (Tech) 11 (Path)
5. Ki67 (T): 9 (NEQAS) v 18 (Tech) 18 (Path)

*This slide was heavily contaminated (see below) and therefore failed; the self-assessment marks were 16/20.

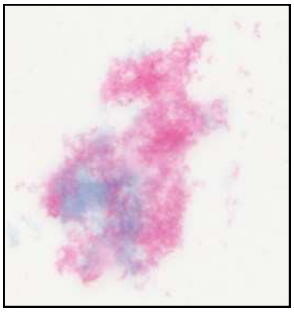


Fig shows: NEQAS Melanoma (R) cell block slide: Melan A, ER2, Bond Max with an APAAP chromogen step.

## Selected Images showing Optimal and Sub-optimal Immunostaining

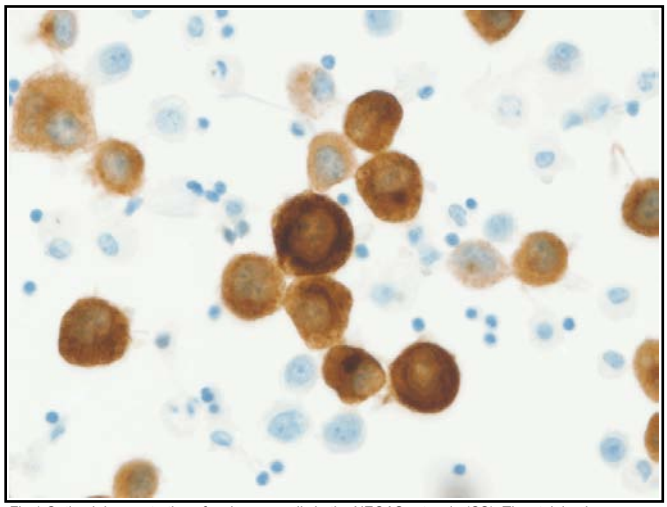


Fig 1.Optimal demonstration of melanoma cells in the NEQAS cytospin (CS). The staining is crisp and the background and other components are clean. Novocastra Melan A, 1:10 for 40 mins, with the CC1 for 64mins, and a prediluted UltraView Kit for 8 mins, on a Ventana Benchmark ULTRA.

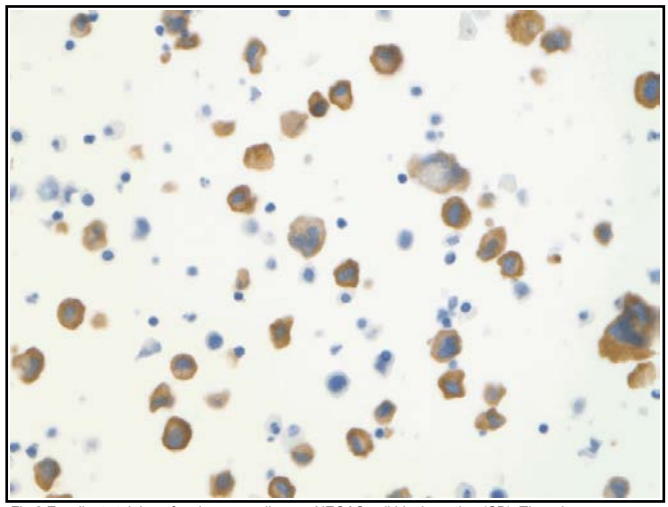


Fig 2.Excellent staining of melanoma cells on a NEQAS cell block section (CB). There is no non-specific staining either of non-melanoma cells or the background. RTU Novocastra Melan A for 15 mins, Leica ER2 20 mins, on a Leica Bond-III and using the Leica Bond Polymer Refine kit.

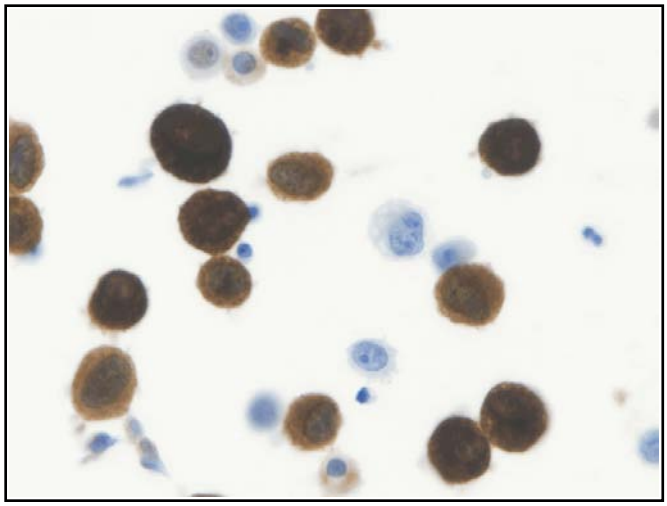


Fig 3.Sub-optimal staining in the NEQAS CS; melanoma cells are clearly demonstrated, but there is some inappropriate staining and the slide appeared slightly 'dirty' but still assessed as a pass. Dako Melan A, 1:50, no time stated; no HMAR, using Leica Bond Polymer Refine kit, on a Leica Bond-III.

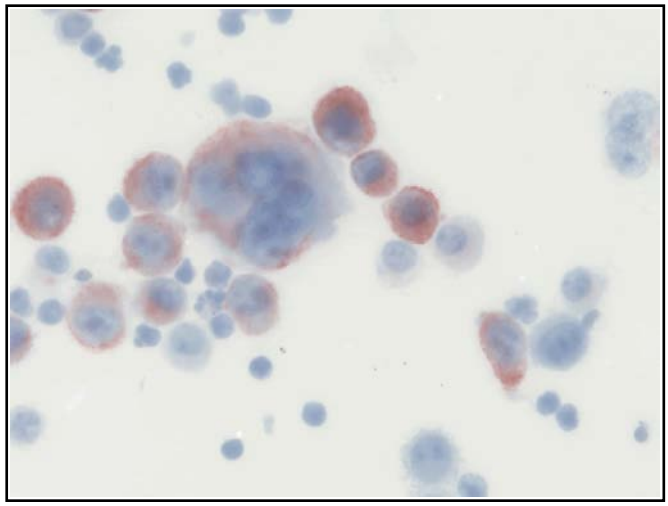


Fig 4.Poor demonstration on a NEQAS CS, this slide failed the assessment, most cells were either unstained or very weakly so. Dako Melan A, 1:100, for 30 mins, no retrieval, a RTU Dako Envision HRP/DAB kit 30 mins, and the Dako AEC+ K3469 chromogen/substrate Dako Autostainer Link 48.

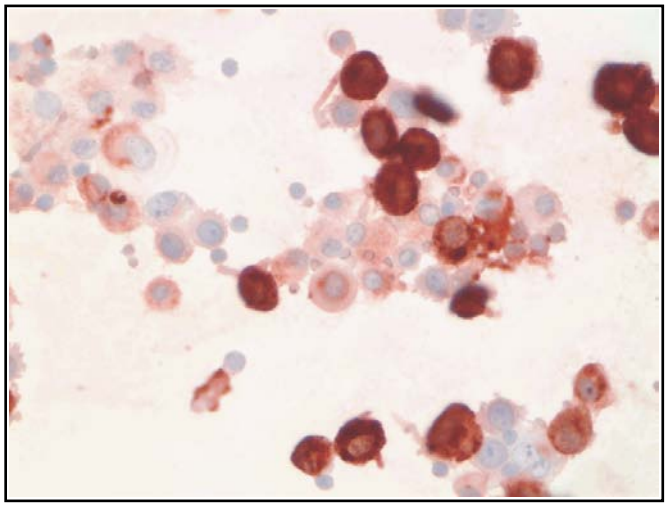


Fig 5.Sub-optimal demonstration on a NEQAS CS; there is excessive non-specific staining of other cellular components, albeit melanoma cells are distinguishable. Slide was assessed as low borderline. Dako HMB45, 1:100, 30 mins, no RT, RTU Leica Bond Polymer Refine 8 mins, on a Leica Bond Max.

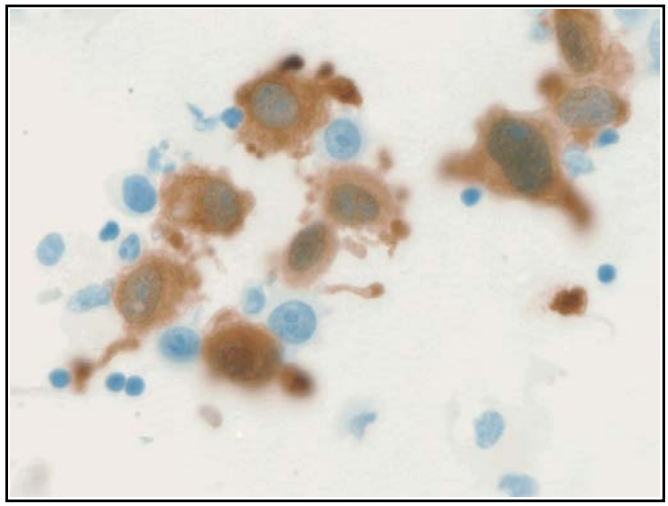


Fig 6.Excellent demonstration on a NEQAS CS using an S100 antibody. It is not common to see an S100 of such good quality on cytology samples. Dako polyclonal, 1:2400, 15 mins, no retrieval, RTU Leica Bond Polymer Refine for 8 mins, pH 7.6 buffer, on the Leica Bond-III.

## Selected Images showing Optimal and Sub-optimal Immunostaining

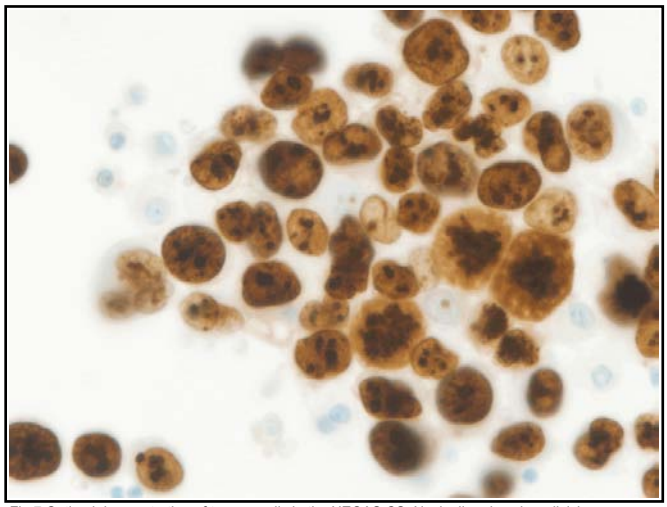


Fig 7.Optimal demonstration of tumour cells in the NEQAS CS. Nucleoli and nuclear division are nicely shown. Dako MIB-1, 1:100, 32 mins, with no retrieval, and the RTU Ventana UltraView Kit for 8 mins, with the Ventana Ultraview DAB kit on the Ventana Benchmark ULTRA.

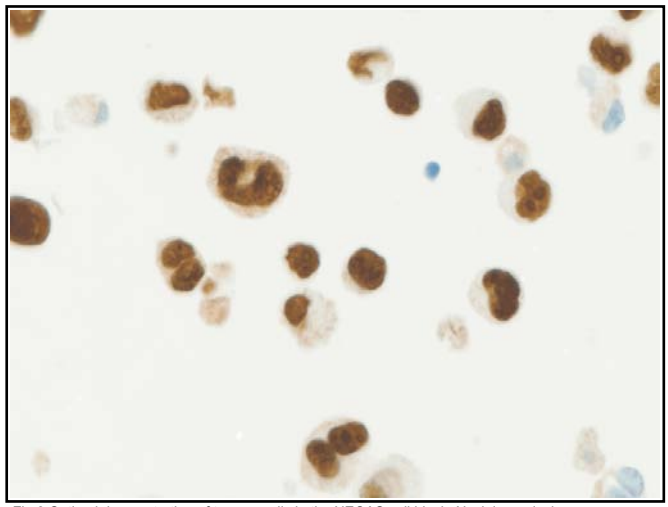


Fig 8.Optimal demonstration of tumour cells in the NEQAS cell block. Nuclei are nicely demonstrated and there is minimal background staining. This lab also employed the Dako MIB-1, but diluted 1:200, with a PT link, a RTU Dako EnVision FLEX+ 15 mins, on a Dako Autostainer Link 48.

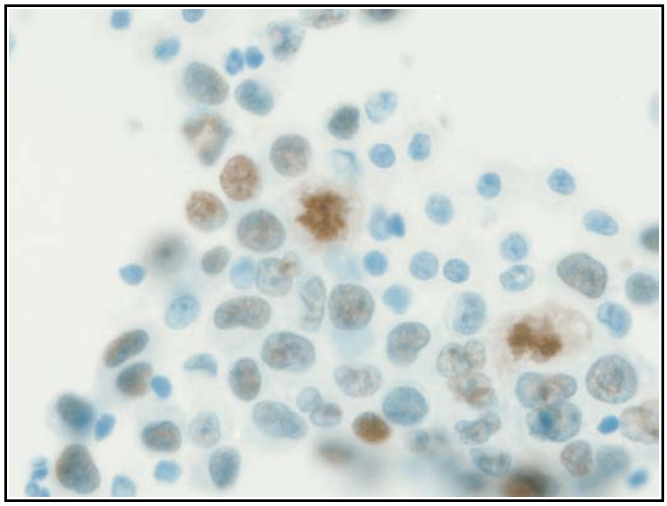


Fig 9.Failed assessment outcome on a NEQAS CS. Only the occasional cell was (weakly) stained (compare with fig 7). Also using the Dako MIB-1, 1:100, 15 mins, no retrieval, RTU Leica Bond Polymer Refine for 8 mins, on a Leica Bond-III.

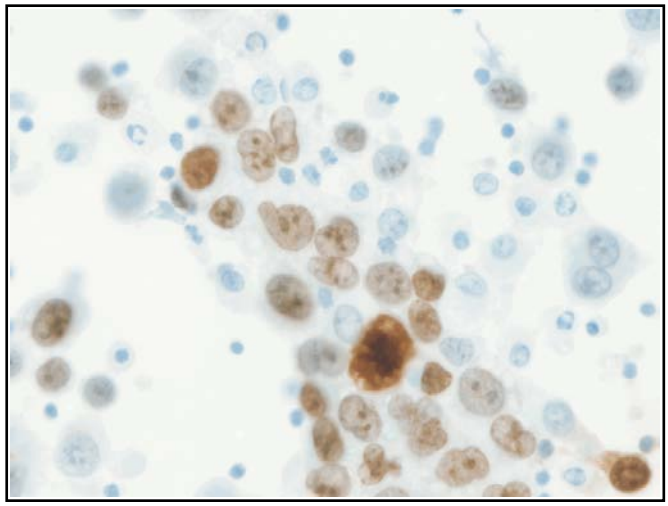


Fig 10.Sub-optimal demonstration on a NEQAS CS. Staining is weak, but considered adequate for diagnostic purposes and therefore was assessed as borderline. Dako MIB-1, 1:100, 32 mins, no retrieval, using the Ventana iView Kit and stained on a Ventana Benchmark XT.

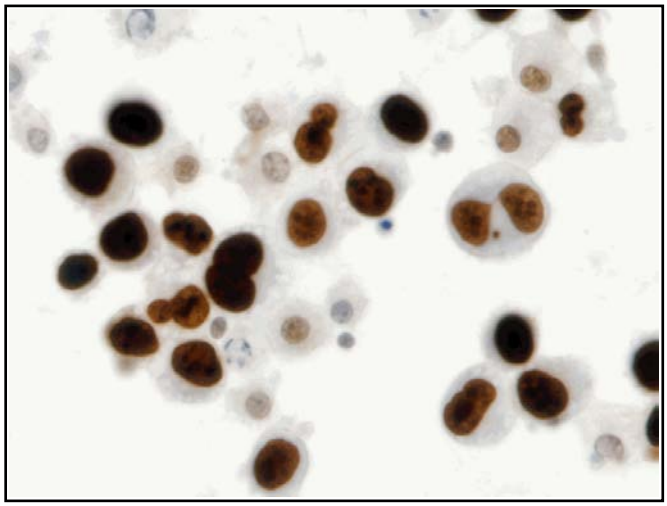


Fig 11.Sub-optimal demonstration on a NEQAS CS; the lack of an adequate counterstain and some non-specific staining, meant the slide was marked down to a low pass. Cell Marque 275R-16 antibody, 1:50, for 15 mins, no retrieval, RTU Leica Bond Polymer Refine, on a Leica Bond Max.

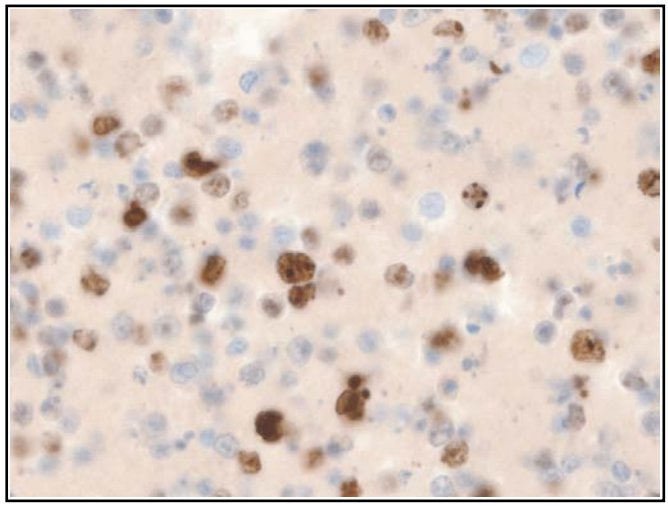
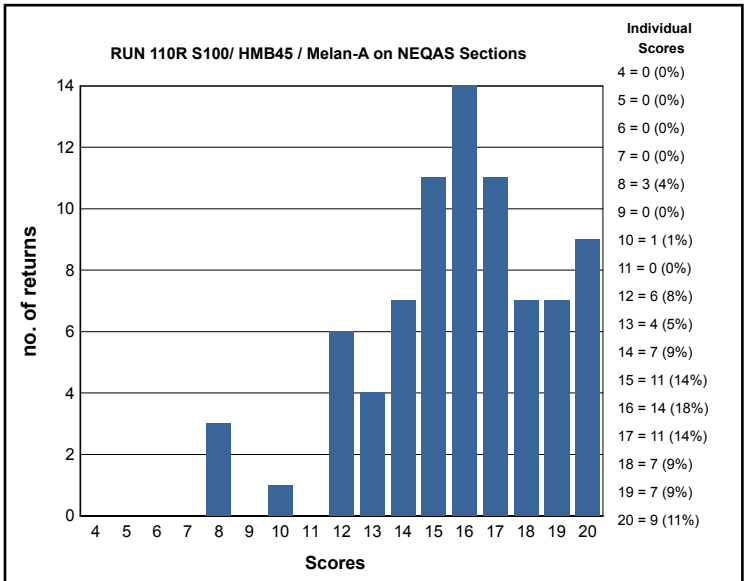


Fig 12.Sub-optimal outcome on an in-house cell block section. These preparations often suffer from background staining of the gel, even though there is little or no inappropriate staining of the cellular components. Dako MIB-1, 1:80, 44 mins, CC1 std, RTU UltraView Kit 8 mins, on Benchmark XT.

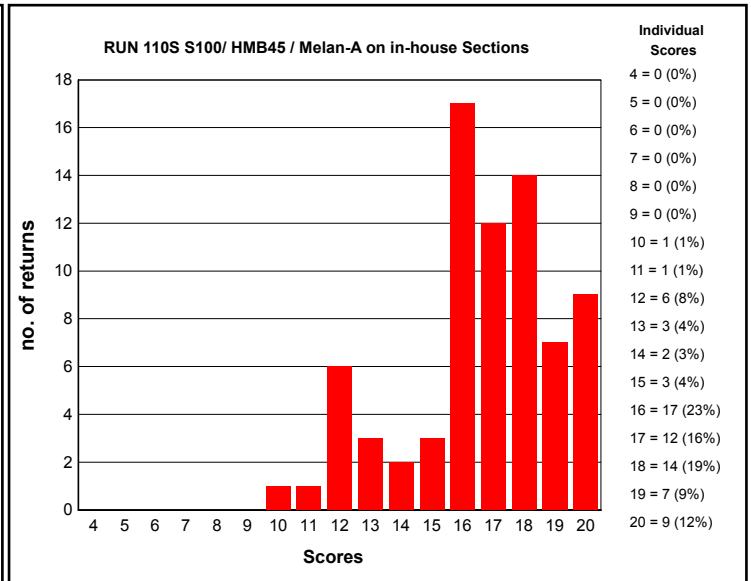
## GRAPHICAL REPRESENTATION OF PASS RATES




**Summary**

16 - 20 = 48 (60%)
13 - 15 = 22 (28%)
10 - 12 = 7 (9%)
0 - 9 = 3 (4%)

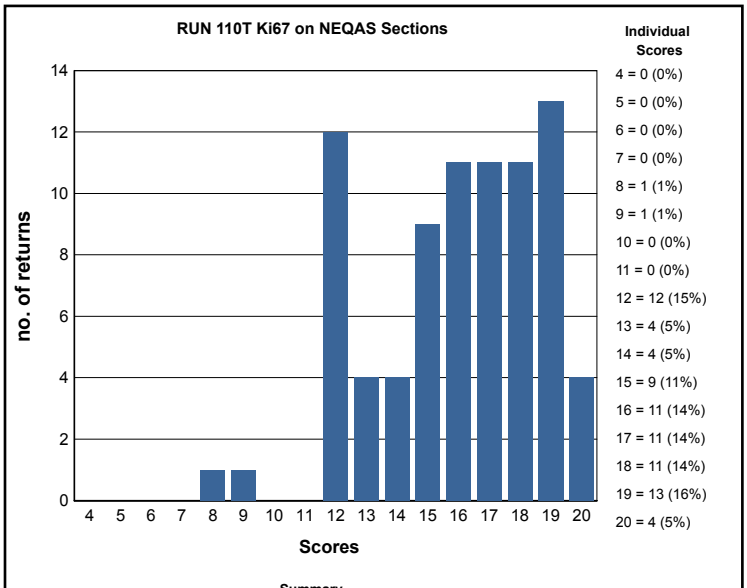
Median = 15.00




**Summary**

16 - 20 = 59 (79%)
13 - 15 = 8 (11%)
10 - 12 = 8 (11%)
0 - 9 = 0 (0%)

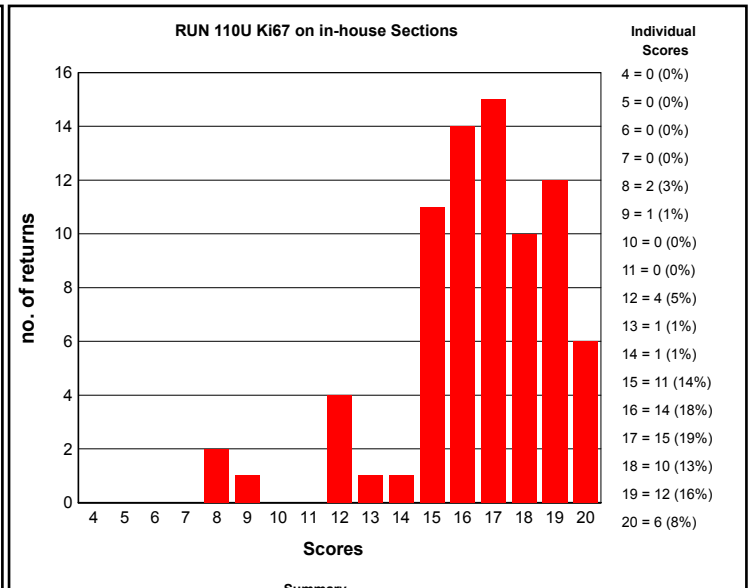
Median = 15.00




**Summary**

16 - 20 = 50 (62%)
13 - 15 = 17 (21%)
10 - 12 = 12 (15%)
0 - 9 = 2 (2%)

Median = 15.00




**Summary**

16 - 20 = 57 (74%)
13 - 15 = 13 (17%)
10 - 12 = 4 (5%)
0 - 9 = 3 (4%)

Median = 15.00

## 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: 110**

**Primary Antibody : S100/ HMB45 / Melan-A**

| Antibody Details | N | % |
|---|---|---|
| A. Menarini MU 001 UC (HMB45) | 1 | 100 |
| Biogenex Melan A (MART-1) (A103) AM361/MU361 | 2 | 100 |
| Cell Marque CMA710 (HMB45) | 1 | 100 |
| Dako FLEX RTU Melan A (A103) IR633 | 5 | 100 |
| Dako M0634 (HMB45) | 10 | 80 |
| Dako M7196 (A103 Melan A) | 20 | 85 |
| Dako U7025 (clone HMB45) | 1 | 100 |
| Dako Z0311 (S100) | 3 | 100 |
| Novocastra/Leica NCL-MELAN A (Melan A) | 15 | 80 |
| Novocastra/Leica NCL-RTU-MelanA(A103) PA0233 | 4 | 100 |
| Other | 6 | 100 |
| Ventana HMB45 790-2523 | 1 | 100 |
| Ventana Melan A (MART-1) 790-2990 | 9 | 89 |
| Ventana S100 (4C4.9) 790-2914 | 1 | 0 |

**Cytology Run: 110**

**Primary Antibody : Ki67**

| Antibody Details | N | % |
|---|---|---|
| Dako 7240 (MIB-1) | 45 | 80 |
| Dako FLEX RTU IR626 (MIB-1) | 5 | 80 |
| Dako M7187 (Ki-67) | 5 | 60 |
| Leica/Novocastra (MM1) NCL-Ki67-CE | 2 | 100 |
| Leica/Novocastra RTU (K2) PA0230 | 5 | 100 |
| Leica/Novocastra RTU (MM1) PA0118 | 1 | 100 |
| Other | 5 | 80 |
| Ventana RTU (30-9) 790-4286 | 12 | 92 |

**Cytology Run: 110**

**Primary Antibody : S100/ HMB45 / Melan-A**

| Antigen Retrieval | N | % |
|---|---|---|
| YES | 29 | 36 |
| NO | 52 | 64 |
| **Breakdown of participants reporting YES** | **N** | |
| Heat Mediated | 0 | |
| Enzyme | 0 | |
| Both | 29 | |
| Not Specified | 0 | |

**Cytology Run: 110**

**Primary Antibody : Ki67**

| Antigen Retrieval | N | % |
|---|---|---|
| YES | 32 | 40 |
| NO | 49 | 60 |
| **Breakdown of participants reporting YES** | **N** | |
| Heat Mediated | 0 | |
| Enzyme | 0 | |
| Both | 32 | |
| Not Specified | 0 | |

**Cytology Run: 110**

**Heat Mediated Retrieval**

**Cytology Run: 110**

**Heat Mediated Retrieval**

**Cytology Run: 110**

**Enzyme Mediated Retrieval**

**Cytology Run: 110**

**Enzyme Mediated Retrieval**

**Cytology Run: 110**

| Detection | Ki67 | | S100/ HMB45 / Melan-A | |
|---|---|---|---|---|
| | N | % | N | % |
| AS PER KIT | 9 | 78 | 8 | 100 |
| Dako EnVision FLEX ( K8000/10) | 1 | 100 | 0 | 0 |
| Dako EnVision FLEX+ ( K8002/12) | 9 | 78 | 10 | 90 |
| Dako Envision HRP/DAB ( K5007) | 1 | 100 | 1 | 0 |
| Dako Envision+ HRP mouse K4004/5/6/7 | 1 | 100 | 1 | 100 |
| Leica Bond Intense R Detection (DS9263) | 1 | 100 | 0 | 0 |
| Leica Bond Polymer Refine (DS9800) | 19 | 89 | 20 | 85 |
| None | 0 | 0 | 1 | 100 |
| Other | 4 | 50 | 6 | 50 |
| Power Vision DPVB999 HRP | 1 | 100 | 0 | 0 |
| Ventana iView system (760-091) | 4 | 75 | 4 | 100 |
| Ventana OptiView Kit (760-700) | 9 | 89 | 9 | 100 |
| Ventana UltraView Kit (760-500) | 18 | 83 | 18 | 83 |

**Cytology Run: 110**

| Automation | Ki67 | | S100/ HMB45 / | |
|---|---|---|---|---|
| | N | % | N | % |
| Dako Autostainer | 1 | 100 | 2 | 100 |
| Dako Autostainer Link 48 | 12 | 92 | 13 | 85 |
| Dako Autostainer Plus Link | 3 | 67 | 1 | 100 |
| Dako Omnis | 2 | 50 | 2 | 50 |
| Leica Bond Max | 9 | 78 | 11 | 73 |
| Leica Bond-III | 12 | 83 | 14 | 93 |
| None (Manual) | 1 | 100 | 0 | 0 |
| Ventana Benchmark GX | 3 | 100 | 2 | 100 |
| Ventana Benchmark ULTRA | 18 | 83 | 18 | 83 |
| Ventana Benchmark XT | 18 | 78 | 17 | 94 |

**Cytology Run: 110**

| Chromogen | Ki67 | | S100/ HMB45 / | |
|---|---|---|---|---|
| | N | % | N | % |
| AS PER KIT | 10 | 70 | 9 | 78 |
| DAKO DAB+ | 1 | 100 | 1 | 100 |
| Dako DAB+ Liquid (K3468) | 0 | 0 | 1 | 100 |
| Dako EnVision Plus kits | 1 | 100 | 0 | 0 |
| Dako FLEX DAB | 11 | 73 | 11 | 82 |
| Dako REAL EnVision K5007 DAB | 2 | 100 | 1 | 100 |
| Leica Bond Polymer Refine kit (DS9800) | 19 | 89 | 20 | 85 |
| Other | 5 | 100 | 7 | 86 |
| Sigma DAB (D5637) | 1 | 100 | 0 | 0 |
| Ventana DAB | 5 | 80 | 5 | 100 |
| Ventana iview | 4 | 50 | 5 | 100 |
| Ventana Ultraview DAB | 21 | 86 | 20 | 85 |

## BEST METHODS - Gold Standard Antibody

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

### S100/ HMB45 / Melan-A - Method 1

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

| | |
|---|---|
| **Primary Antibody:** | Novocastra/Leica NCL-MELAN A (Melan A) , 40 Mins, 36 ºC Dilution 1: 10 |
| **Automation:** | Ventana Benchmark ULTRA |
| **Method:** | Ventana UltraView DAB |
| **Main Buffer:** | Ventana reaction buffer (950-300) |
| **HMAR:** | Ventana CC1 64mins |
| **EAR:** | NOT APPLICABLE |
| **Chromogen:** | Ventana Ultraview DAB, 37 ºC., Time 1: 12 Mins, Time 2: 12 Mins |
| **Detection:** | Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC Prediluted |

### S100/ HMB45 / Melan-A - Method 2

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

| | |
|---|---|
| **Primary Antibody:** | Novocastra/Leica NCL-RTU-MelanA(A103) PA0233 , 15 Mins, 20 ºC Prediluted |
| **Automation:** | Leica Bond-III |
| **Method:** | Leica BondMAx Refine KIT |
| **Main Buffer:** | Bond Wash Buffer (AR9590) |
| **HMAR:** | Leica ER2 20 mins |
| **EAR:** | NOT APPLICABLE |
| **Chromogen:** | Leica Bond Polymer Refine kit (DS9800) |
| **Detection:** | Leica Bond Polymer Refine (DS9800) |

### S100/ HMB45 / Melan-A - Method 3

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

| | |
|---|---|
| **Primary Antibody:** | Dako FLEX RTU Melan A (A103) IR633 , 20 Mins, RT ºC Prediluted |
| **Automation:** | Dako Omnis |
| **Method:** | Dako FLEX+ kit |
| **Main Buffer:** | Dako FLEX wash buffer |
| **HMAR:** | Dako Omnis, Buffer: EnVFLEX TRS HIGH PH |
| **EAR:** | NOT APPLICABLE |
| **Chromogen:** | DAKO DAB+, RT ºC., Time 1: 3 Mins |
| **Detection:** | Dako EnVision FLEX+ ( K8002/12) , 20 Mins, RT ºC Prediluted |

### S100/ HMB45 / Melan-A - Method 4

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

| | |
|---|---|
| **Primary Antibody:** | Dako M0634 (HMB45) , 32 Mins, 37 ºC Dilution 1: 400 |
| **Automation:** | Ventana Benchmark GX |
| **Method:** | Ventana Optiview |
| **Main Buffer:** | Ventana reaction buffer (950-300) |
| **HMAR:** | |
| **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

### Ki67 - Method 1

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

| | |
|---|---|
| **Primary Antibody:** | Leica/Novocastra RTU (K2) PA0230 , 15 Mins, 25 ºC Prediluted |
| **Automation:** | Leica Bond Max |
| **Method:** | Leica BondMAx Refine KIT |
| **Main Buffer:** | Bond Wash Buffer (AR9590) |
| **HMAR:** | Leica ER2 20 mins |
| **EAR:** | NOT APPLICABLE |
| **Chromogen:** | Leica Bond Polymer Refine kit (DS9800), Time 1: 10 Mins |
| **Detection:** | Leica Bond Polymer Refine (DS9800) , 8 Mins, 25 ºC Prediluted |

### Ki67 - Method 2

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

| | |
|---|---|
| **Primary Antibody:** | Dako 7240 (MIB-1) , 24 Mins Dilution 1: 50 |
| **Automation:** | Ventana Benchmark XT |
| **Method:** | Ventana iView Kit |
| **Main Buffer:** | Ventana reaction buffer (950-300) |
| **HMAR:** | Ventana CC1 standard |
| **EAR:** | NOT APPLICABLE |
| **Chromogen:** | Ventana DAB |
| **Detection:** | Ventana iView system (760-091) |

### Ki67 - Method 3

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

| | |
|---|---|
| **Primary Antibody:** | Dako FLEX RTU IR626 (MIB-1) |
| **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 |

### Ki67 - Method 4

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

| | |
|---|---|
| **Primary Antibody:** | Dako 7240 (MIB-1) , 30 Mins, 21 ºC Dilution 1: 200 |
| **Automation:** | Dako Autostainer Link 48 |
| **Method:** | Dako FLEX+ kit |
| **Main Buffer:** | Dako FLEX wash buffer |
| **HMAR:** | Dako PTLink, Buffer: DAKO High pH, PH: 9.5 |
| **EAR:** | |
| **Chromogen:** | Dako FLEX DAB, 21 ºC., Time 1: 5 Mins, Time 2: 5 Mins |
| **Detection:** | Dako EnVision FLEX+ ( K8002/12) , 20 Mins, 21 ºC Prediluted |

# The Alimentary Tract Module: GIST Run 110

**Suzanne Parry**

| | First Antibody | Second Antibody |
|---|---|---|
| **Antigens Assessed:** | CD117 | DOG-1 |
| **Tissue Sections circulated:** | GIST, Appendix & Desmoid | GIST, Appendix & Desmoid |
| **Number of Registered Participants:** | 117 | |
| **Number of Participants this Run** | CD117 112 (96%) and DOG-1 101 (86%) | |

## Introduction

### Gold Standard: CD117

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

**Features of Optimal Immunostaining**: **(See Figs 1, 2, 3 & 5)**

- Good localisation of CD117 to mast cells in the appendix and desmoid sections (Fig 1—desmoid not shown)
- Good localisation of CD117 to interstitial cells of Cajal (Fig 2)
- Good localisation of CD117 to cells of the GIST (Fig 3)
- No staining of the desmoid tumour

**Features of Suboptimal Immunostaining**: **(See Figs 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 (Fig 5)
- 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, 11 & 12)**

- Good localisation of DOG-1 to cells of the GIST (Fig 7)
- Good localisation of DOG-1- to the interstitial cells of Cajal (Fig 11)
- No staining of desmoid tumour

**Features of Suboptimal Immunostaining (See Figs 8 & 9)**

- Weak and/or patchy staining of the tumour cells of the GIST (Fig 8)
- Excessive background or non specific staining (Fig 9)
- 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 DOG-1) 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 were slightly lower than the previous assessment run (109), and showed an acceptable pass rate of 82%. A further 15% of labs (N=17) received a borderline pass (scores of 10-12), and 3 labs (3%) failed the assessment on the Neqas distributed sample. Weak staining, particularly noticeable in the GIST, was the reason for failure. This was either due to an inappropriate antibody dilution factor being used, or little/no antigen retrieval. Borderline scores were also given for weak staining or inappropriate or background staining, although these sections were still deemed clinically diagnostic. The Dako polyclonal antibody was again the most popular, used by 95 participants and showed a pass rate of 83%. The Dako datasheet recommends using a high pH antigen retrieval buffer solution in the pre-treatment protocol for this antibody, which most labs did carry out in their assays.

There was a higher acceptable pass rate of 91% for the **DOG-1** assessment results. A further 7 labs (7%) received a borderline result, and 2 labs failed the assessment. One of these labs was given an alternative antibody (SMA), but the section showed excessive background staining. Borderline scores were either due to weak or inappropriate non-specific staining. The most popular antibody used for the DOG-1 stain was the Leica K9 clone, which worked well on all the automated platforms and showed a pass rate of 97%.

Most labs are now using composite sections for their own in-house controls, and the standard of staining on the in-house was similar to that seen on the UK NEQAS distributed tissues.

**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. West RB, Corless CL, Chen X et al. The novel marker, DOG1, is expressed ubiquitously in gastrointestinal stromal tumors irrespective of KIT or PDGFRA mutation status. Am. J. Pathol. 2004; 65; 107–113.
5. Espinosa I, Lee CH, Kim MK et al. A novel monoclonal antibody against DOG1 is a sensitive and specific marker for gastrointestinal stromal tumors. Am. J. Surg. Pathol. 2008; 32; 210– 218.
6. Novelli M, Rossi S, Rodriguez-Justo M, Taniere P, Seddon B, Toffolatti L, Sartor C, Hogendoorn PC, Sciot R, Van Glabbeke M, Verweij J, Blay JY, Hohenberger P, Flanagan A, Dei Tos AP. DOG1 and CD117 are the antibodies of choice in the diagnosis of gastrointestinal stromal tumours. Histopathology 2010, 57 (2):259-270.
7. Parfitt JR, Rodriguez-Justo M, Feakins R, Novelli MR. Gastrointestinal Kaposi's sarcoma: CD117 expression and the potential for misdiagnosis as gastrointestinal stromal tumour. Histopathology 2008; 52; 816–823.

## Selected Images showing Optimal and Sub-optimal Immunostaining

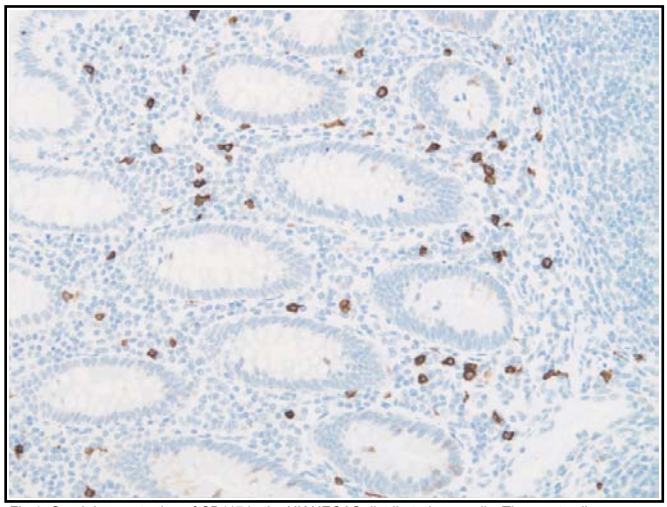


Fig 1. Good demonstration of CD117 in the UK NEQAS distributed appendix. The mast cells show distinct membranous staining, while the background remains clean. Stained with the Dako polyclonal antibody, 1:200, Leica Bond Max, ER2 pre-treatment for 20 minutes.

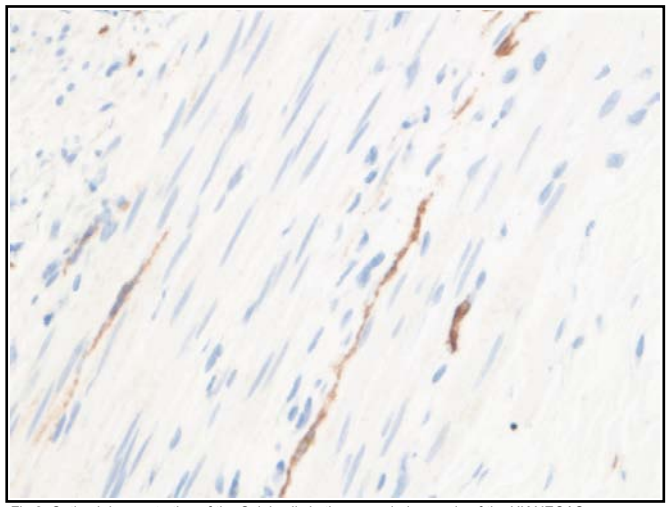


Fig 2. Optimal demonstration of the Cajal cells in the muscularis propria of the UK NEQAS distributed appendix. The staining of the Cajal cells is distinct while the smooth muscle cells remain unstained. The section was stained with the Dako polyclonal antibody, 1:50 on the Dako Autostainer with no antigen retrieval.

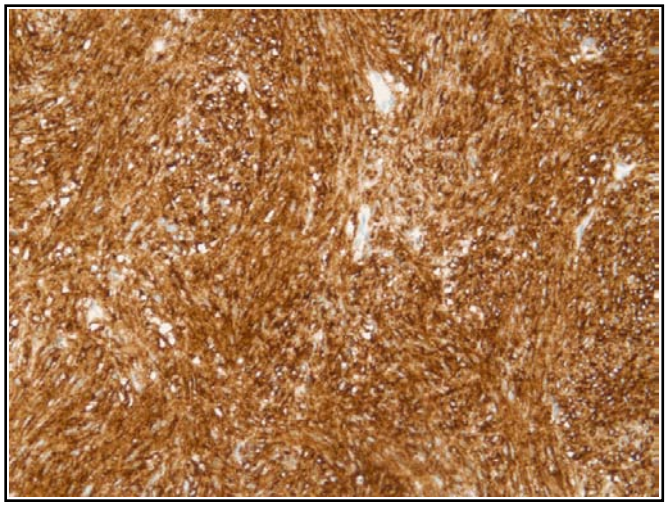


Fig 3. Good example of CD117 staining of the UK NEQAS distributed GIST, showing strong cytoplasmic and membranous staining in the tumour cells. Section stained with the Ventana pre-diluted 9.7 clone antibody on the Ventana Benchmark XT with CC1 pre-treatment for 32 minutes and Optiview detection kit

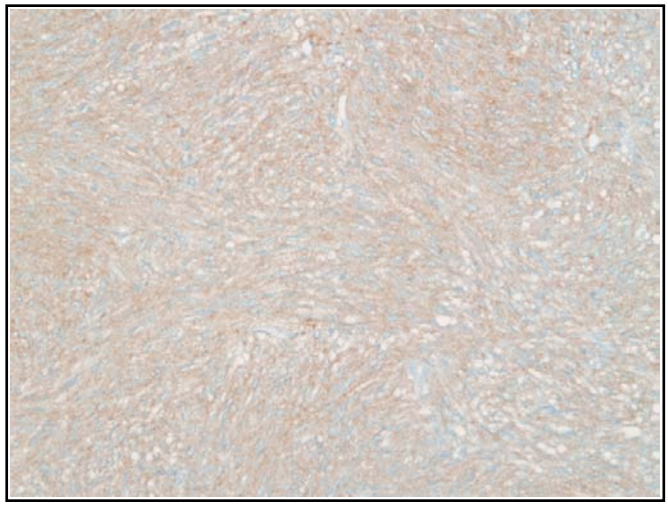


Fig 4. Poor CD117 staining in the UK NEQAS distributed GIST section (compare to Fig 3). The staining is very weak: Stained with the Ventana pre-diluted 9.7 clone antibody on the Ventana ULTRA with no antigen retrieval.

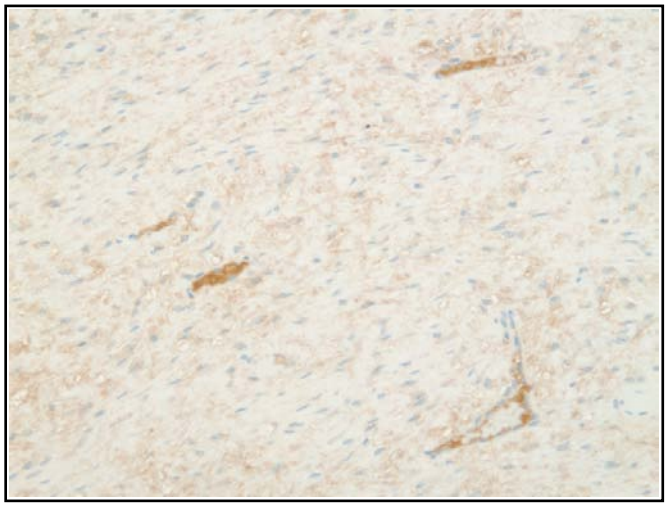


Fig 5. Sub-optimal CD117 staining in the UK NEQAS distributed desmoid tumour. The example shows excessive background and non-specific staining. The slide was stained using the Dako polyclonal antibody, 1:300 on the Dako Auotstainer and pre-treatment in high retrieval buffer for 20 minutes.

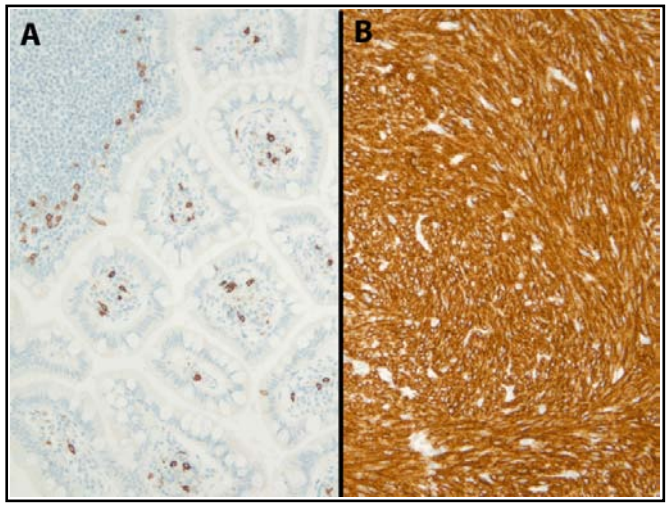



Fig 6. Good example of an in house control submitted for assessment of CD117: The control included a GIST along with normal gastric epithelium, showing positive staining in the mast cells which act as an ideal internal control. Section stained with the Dako polyclonal antibody, 1:100 on the Autostainer with no antigen retrieval.

## Selected Images showing Optimal and Sub-optimal Immunostaining

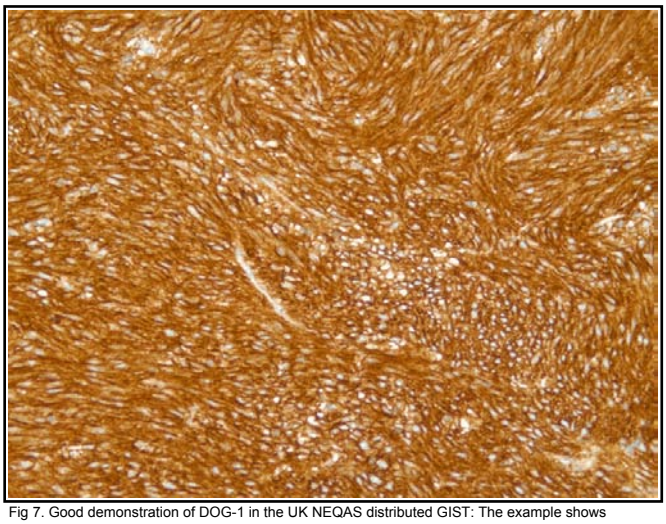


Fig 7. Good demonstration of DOG-1 in the UK NEQAS distributed GIST: The example shows strong crisp staining in the tumour cells. Section stained with the Leica K9 antibody, 1:50 and stained on the Ventana Benchmark XT with CC1 standard antigen retrieval.

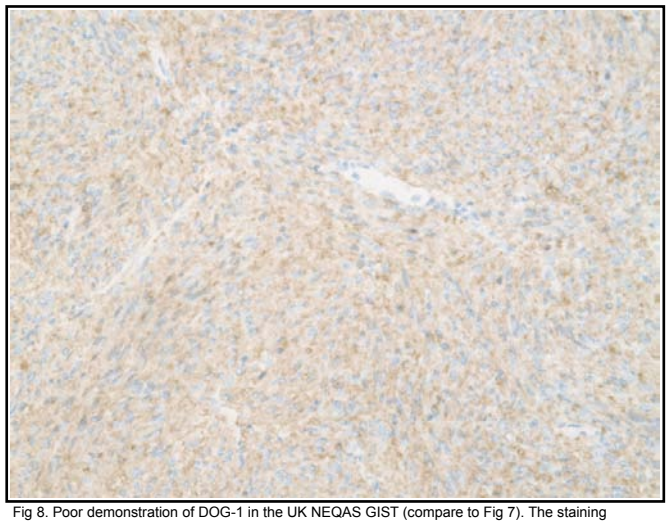


Fig 8. Poor demonstration of DOG-1 in the UK NEQAS GIST (compare to Fig 7). The staining is much weaker than expected, most likely due to insufficient antigen retrieval. Section stained with the Cell Marque SP31 antibody, and pre-treatment in the Labvision PT module for 16 minutes with high pH buffer, stained on the Dako Autostainer.

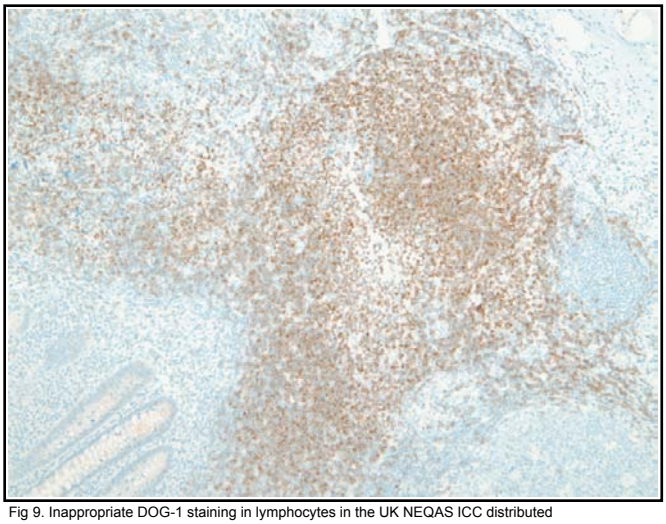


Fig 9. Inappropriate DOG-1 staining in lymphocytes in the UK NEQAS ICC distributed appendix. The lymphocytes should be negative for DOG-1. The section was stained using the Ventana pre-diluted antibody on the Ventana Benchmark XT with CC1 pre-treatment for 40 minutes.

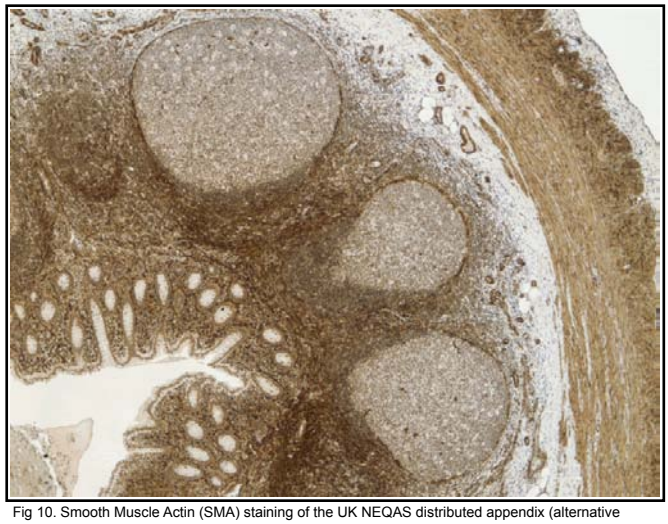


Fig 10. Smooth Muscle Actin (SMA) staining of the UK NEQAS distributed appendix (alternative to DOG-1). However, the staining is excessive with a high level of background. Stained on the Leica Bond platform with ER2 antigen retrieval for 20 minutes.

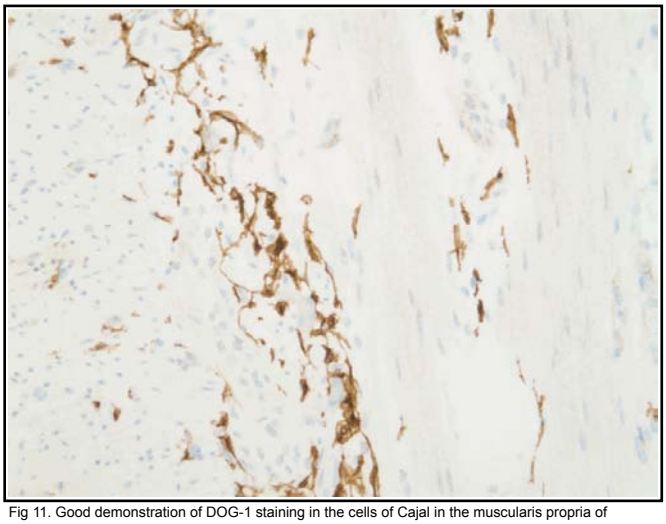


Fig 11. Good demonstration of DOG-1 staining in the cells of Cajal in the muscularis propria of the UK NEQAS distributed appendix. The section was stained with the Dako polyclonal antibody, 1:100 on the Dako Autostainer with no antigen retrieval.

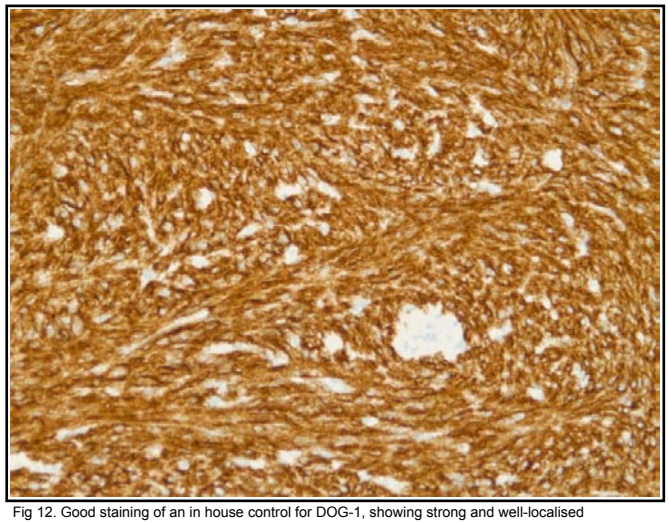
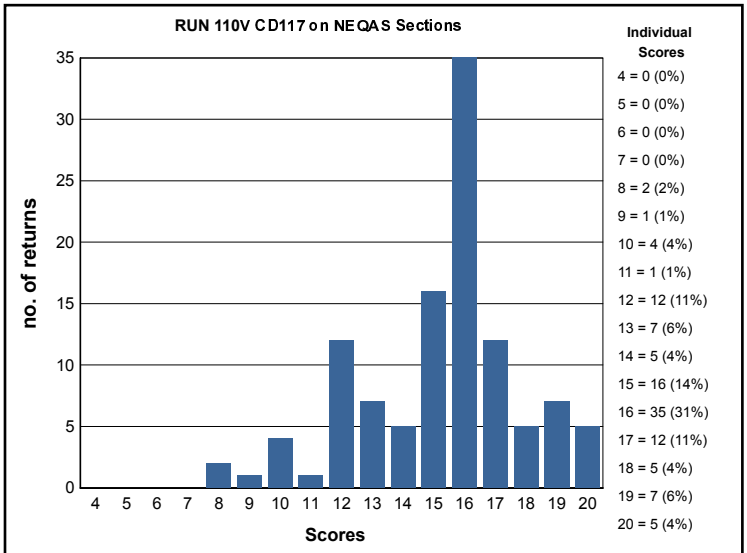
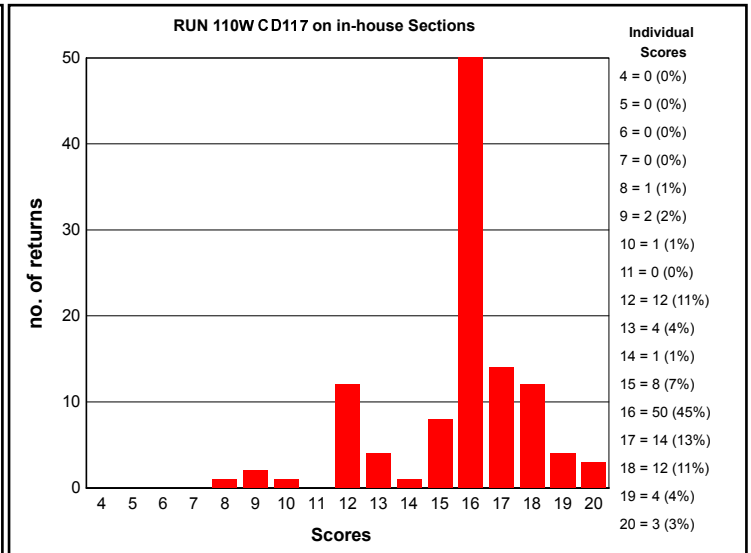
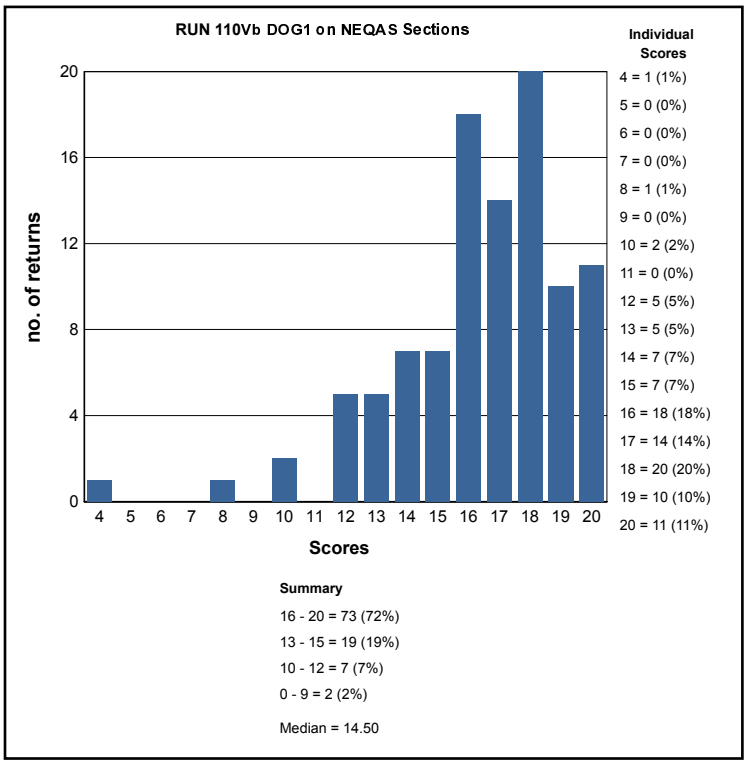


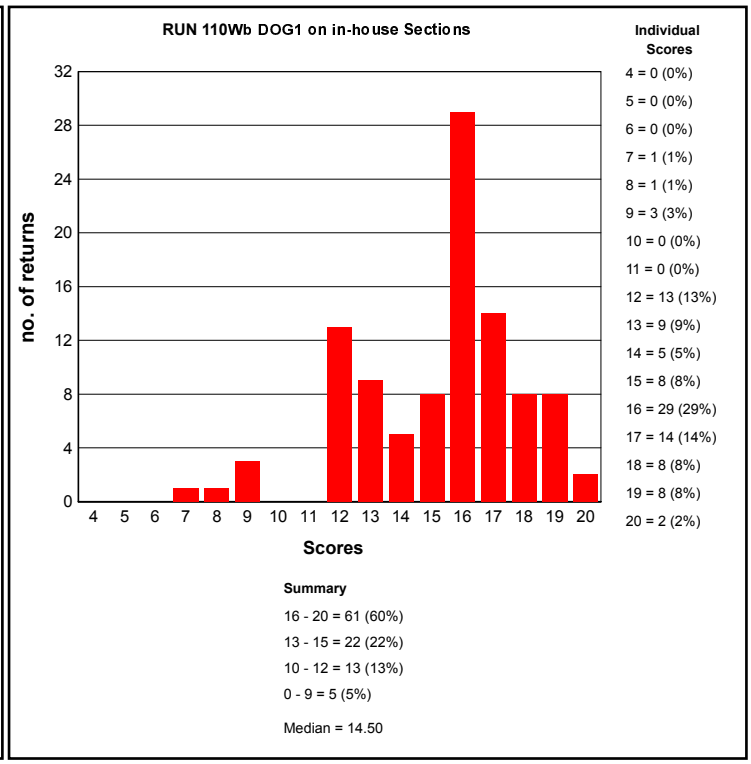
Fig 12. Good staining of an in house control for DOG-1, showing strong and well-localised cytoplasmic and membranous staining.

## 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: 110**

**Primary Antibody :** CD117

| Antibody Details | N | % |
|---|---|---|
| Cell Marque 117R/S-xx (YR145) | 3 | 67 |
| Dako A4502 (rb poly) | 95 | 83 |
| Epitomics AC-0029 (EP10) | 1 | 100 |
| Leica/Novocastra NCL-CD117 (T595) | 1 | 100 |
| Other | 2 | 100 |
| Ventana 790-2951 (9.7) | 10 | 70 |

**Alimentary Tract Pathology Run: 110**

**Primary Antibody :** DOG1

| Antibody Details | N | % |
|---|---|---|
| Abcam TMEM16A (ab53212) | 1 | 0 |
| Biocare CM 385 (1.1) | 1 | 100 |
| Cell Marque 244R-14/15/16 (SP31) | 3 | 67 |
| Cell Marque 244R-17/18 (SP31) | 3 | 100 |
| Diagnostic Biosystems Mob466 (DOG1.1) | 1 | 0 |
| Leica NCL-L-DOG-1 (K9) | 53 | 94 |
| Leica PA0219 (K9) | 18 | 100 |
| Menarini MP-385-CM01/1 | 1 | 0 |
| Other | 3 | 67 |
| Spring Biosciences M3311 (SP31) | 1 | 100 |
| Thermo RM-9132-R7 (SP31) | 1 | 100 |
| Ventana (SP31) 760-4590 | 14 | 93 |

**Alimentary Tract Pathology Run: 110**

| Heat Mediated Retrieval | CD117 N | CD117 % | DOG1 N | DOG1 % |
|---|---|---|---|---|
| Dako Omnis | 0 | 0 | 1 | 100 |
| Dako PTLink | 14 | 86 | 13 | 77 |
| Lab vision PT Module | 1 | 0 | 1 | 0 |
| Leica ER1 10 mins | 1 | 100 | 0 | 0 |
| Leica ER1 20 mins | 4 | 100 | 8 | 88 |
| Leica ER1 30 mins | 4 | 100 | 0 | 0 |
| Leica ER2 10 mins | 1 | 100 | 0 | 0 |
| Leica ER2 20 mins | 19 | 84 | 22 | 95 |
| Leica ER2 30 mins | 8 | 63 | 6 | 100 |
| Microwave | 0 | 0 | 1 | 100 |
| None | 5 | 60 | 1 | 100 |
| Pressure Cooker | 1 | 100 | 1 | 100 |
| Steamer | 0 | 0 | 1 | 100 |
| Ventana CC1 16mins | 2 | 50 | 2 | 100 |
| Ventana CC1 24mins | 0 | 0 | 2 | 100 |
| Ventana CC1 32mins | 5 | 60 | 5 | 100 |
| Ventana CC1 36mins | 6 | 67 | 3 | 100 |
| Ventana CC1 40mins | 1 | 0 | 1 | 0 |
| Ventana CC1 48mins | 0 | 0 | 1 | 100 |
| Ventana CC1 52mins | 2 | 100 | 1 | 100 |
| Ventana CC1 56mins | 4 | 100 | 1 | 100 |
| Ventana CC1 64mins | 8 | 63 | 9 | 89 |
| Ventana CC1 88mins | 1 | 100 | 1 | 100 |
| Ventana CC1 8mins | 0 | 0 | 1 | 100 |
| Ventana CC1 mild | 9 | 89 | 5 | 100 |
| Ventana CC1 standard | 16 | 100 | 12 | 92 |
| Ventana CC2 44mins | 0 | 0 | 1 | 100 |
| Ventana CC2 48mins | 1 | 100 | 0 | 0 |
| Ventana CC2 standard | 0 | 0 | 1 | 0 |

**Alimentary Tract Pathology Run: 110**

| Enzyme Mediated Retrieval | CD117 N | CD117 % | DOG1 N | DOG1 % |
|---|---|---|---|---|
| AS PER KIT | 0 | 0 | 1 | 100 |
| NOT APPLICABLE | 71 | 83 | 59 | 90 |
| Ventana Protease 1 (760-2018) | 1 | 100 | 1 | 100 |

| Alimentary Tract Pathology Run: 110 | CD117 | | DOG1 | |
|---|---|---|---|---|
| **Detection** | **N** | **%** | **N** | **%** |
| AS PER KIT | 7 | 57 | 8 | 88 |
| BioGenex SS Polymer (QD 420-YIKE) | 0 | 0 | 1 | 100 |
| BioGenex SS Polymer (QD 430-XAKE) | 1 | 100 | 0 | 0 |
| Dako EnVision FLEX ( K8000/10) | 1 | 100 | 1 | 100 |
| Dako EnVision FLEX+ ( K8002/12) | 7 | 100 | 7 | 86 |
| Dako Envision HRP/DAB ( K5007) | 1 | 100 | 1 | 100 |
| Dako Envision+ HRP mouse K4004/5/6/7 | 0 | 0 | 1 | 100 |
| Dako Envision+ HRP rabbit K4008/9/10/11 | 1 | 100 | 0 | 0 |
| Leica Bond Intense R Detection (DS9263) | 0 | 0 | 1 | 100 |
| Leica Bond Polymer Refine (DS9800) | 36 | 83 | 33 | 94 |
| None | 0 | 0 | 1 | 100 |
| Other | 5 | 80 | 4 | 50 |
| Ventana iView system (760-091) | 3 | 67 | 1 | 100 |
| Ventana OptiView Kit (760-700) | 18 | 72 | 13 | 92 |
| Ventana UltraView Kit (760-500) | 32 | 84 | 27 | 93 |

| Alimentary Tract Pathology Run: 110 | CD117 | | DOG1 | |
|---|---|---|---|---|
| **Automation** | **N** | **%** | **N** | **%** |
| BioGenex GenoMX 6000i | 1 | 100 | 1 | 100 |
| Dako Autostainer Link 48 | 13 | 85 | 11 | 82 |
| Dako Autostainer plus | 2 | 50 | 2 | 50 |
| Dako Autostainer Plus Link | 2 | 100 | 2 | 50 |
| Dako Omnis | 0 | 0 | 1 | 100 |
| LabVision Autostainer | 0 | 0 | 1 | 100 |
| Leica Bond Max | 15 | 93 | 14 | 100 |
| Leica Bond-III | 24 | 79 | 23 | 91 |
| Ventana Benchmark GX | 2 | 100 | 2 | 100 |
| Ventana Benchmark ULTRA | 26 | 69 | 23 | 96 |
| Ventana Benchmark XT | 28 | 86 | 20 | 85 |

| Alimentary Tract Pathology Run: 110 | CD117 | | DOG1 | |
|---|---|---|---|---|
| **Chromogen** | **N** | **%** | **N** | **%** |
| A. Menarini Liquid Stable DAB kit | 1 | 100 | 0 | 0 |
| AS PER KIT | 16 | 81 | 15 | 93 |
| BioGenex liquid DBA (HK-124-7K) | 0 | 0 | 1 | 100 |
| Dako EnVision Plus kits | 2 | 100 | 2 | 100 |
| Dako FLEX DAB | 9 | 100 | 9 | 78 |
| Dako REAL EnVision K5007 DAB | 1 | 100 | 1 | 100 |
| Leica Bond Polymer Refine kit (DS9800) | 33 | 82 | 34 | 94 |
| Other | 4 | 25 | 3 | 33 |
| Ventana DAB | 8 | 75 | 5 | 100 |
| Ventana iview | 2 | 50 | 1 | 100 |
| Ventana Ultraview DAB | 37 | 84 | 30 | 90 |

## BEST METHODS - Gold Standard Antibody

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

### CD117 - Method 1

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

**Primary Antibody:** Dako A4502 (rb poly) Dilution 1: 200
**Automation:** Leica Bond Max
**Method:** Leica BondMAx Refine KIT
**Main Buffer:** Bond Wash Buffer (AR9590)
**HMAR:** Leica ER2 20 mins
**EAR:**
**Chromogen:** AS PER KIT
**Detection:** Leica Bond Polymer Refine (DS9800)

### CD117 - Method 2

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

**Primary Antibody:** Dako A4502 (rb poly) , 32 Mins, 42 ºC Dilution 1: 1/100
**Automation:** Ventana Benchmark XT
**Method:** Ventana iView Kit
**Main Buffer:** Ventana reaction buffer (950-300), PH: 7.5
**HMAR:** Ventana CC1 standard
**EAR:** NOT APPLICABLE
**Chromogen:** Ventana iview, 37 ºC., Time 1: 8 Mins
**Detection:** Ventana iView system (760-091) , 8 Mins, 37 ºC Prediluted

**CD117 - Method 3**

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

| | |
|---|---|
| **Primary Antibody:** | Dako A4502 (rb poly) , 20 Mins, 21 ºC Dilution 1: 100 |
| **Automation:** | Dako Autostainer Link 48 |
| **Method:** | Dako FLEX+ kit |
| **Main Buffer:** | Dako FLEX wash buffer |
| **HMAR:** | Dako PTLink, Buffer: high flex solution |
| **EAR:** | |
| **Chromogen:** | Dako FLEX DAB, 21 ºC. |
| **Detection:** | Dako Envision+ HRP rabbit K4008/9/10/11 , 20 Mins, 21 ºC Prediluted |

**CD117 - Method 4**

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

| | |
|---|---|
| **Primary Antibody:** | Ventana 790-2951 (9.7) , 32 Mins |
| **Automation:** | Ventana Benchmark XT |
| **Method:** | Ventana Optiview |
| **Main Buffer:** | AS PER KIT |
| **HMAR:** | Ventana CC1 64mins |
| **EAR:** | |
| **Chromogen:** | AS PER KIT |
| **Detection:** | |

## BEST METHODS - Secondary Antibody

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

**DOG1 - Method 1**

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

| | |
|---|---|
| **Primary Antibody:** | Leica NCL-L-DOG-1 (K9) , 20 Mins Dilution 1: 100 |
| **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), Time 1: 10 Mins |
| **Detection:** | Leica Bond Polymer Refine (DS9800) , 8 Mins |

**DOG1 - Method 2**

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

| | |
|---|---|
| **Primary Antibody:** | Leica PA0219 (K9) , 15 Mins, 22 ºC Dilution 1: RTU |
| **Automation:** | Leica Bond-III |
| **Method:** | Leica BondMAx Refine KIT |
| **Main Buffer:** | Bond Wash Buffer (AR9590) |
| **HMAR:** | Leica ER2 20 mins |
| **EAR:** | NOT APPLICABLE |
| **Chromogen:** | Leica Bond Polymer Refine kit (DS9800), Time 1: 10 Mins |
| **Detection:** | Leica Bond Polymer Refine (DS9800) , 8 Mins |

**DOG1 - Method 3**

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

| | |
|---|---|
| **Primary Antibody:** | Leica NCL-L-DOG-1 (K9) , 60 Mins, 37 ºC Dilution 1: 1/50 |
| **Automation:** | Ventana Benchmark XT |
| **Method:** | Ventana UltraView DAB |
| **Main Buffer:** | Ventana reaction buffer (950-300), PH: 7.6 |
| **HMAR:** | Ventana CC1 standard |
| **EAR:** | |
| **Chromogen:** | Ventana Ultraview DAB, 37 ºC., Time 1: 8 Mins, Time 2: 8 Mins |
| **Detection:** | Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC |

**DOG1 - Method 4**

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

| | |
|---|---|
| **Primary Antibody:** | Thermo RM-9132-R7 (SP31) , 30 Mins, 22 ºC Dilution 1: 100 |
| **Automation:** | Dako Autostainer Link 48 |
| **Method:** | Dako FLEX+ kit |
| **Main Buffer:** | AS PER KIT |
| **HMAR:** | Dako PTLink, Buffer: TRS High pH |
| **EAR:** | NOT APPLICABLE |
| **Chromogen:** | AS PER KIT, 22 ºC., Time 1: 5 Mins, Time 2: 5 Mins |
| **Detection:** | AS PER KIT , 20 Mins, 22 ºC Prediluted |

# Lynch Syndrome/HNPCC Run 110

**Suzanne Parry**

| | Gold Standard | Second Antibody |
|---|---|---|
| **Antigens Assessed:** | MLH1 | PMS2 |
| **Tissue Sections circulated:** | Normal Appendix & Colonic Tumours | Normal Appendix, & Colonic Tumours |
| **Number of Registered Participants:** | 84 | |
| **Number of Participants This Run:** | 75 (89%) | |

## 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 **MSH1** (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, 2, 4, 6, 7, 9, 11 &12)**

**Appendix: (Figs 1, 2 & 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 3, 5, 8 & 10)**

**Appendix: (Figs 3 & 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 **MSH1** assessment were lower than the previous time this antibody was assessed, with 56% 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 77%. There was a fail rate of 23%, which was higher than the previous assessment for MLH1 (Run 108. 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 76%. The Leica/Novocastra ES05 clone was also popular , used by 21 labs and showed a pass rate of 71%.

The **PMS2** assessment showed a lower pass rate to that of the M1H1 antibody: Only 36% of participants received an acceptable pass, and a further 37% received a borderline pass. Similarly to the MLH1 assessment, the fail rate was quite high with 20 laboratories (27%) receiving a score of under 10. The scores overall were lower than the last time PMS2 was assessed. Again, 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 27 labs, but only showed a pass rate of 15%. The BDPharmingen A16-4 and the DakoEP51 clones were also commonly used, and they showed pass rates of 31% 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**


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

## Selected Images showing Optimal and Sub-optimal Immunostaining

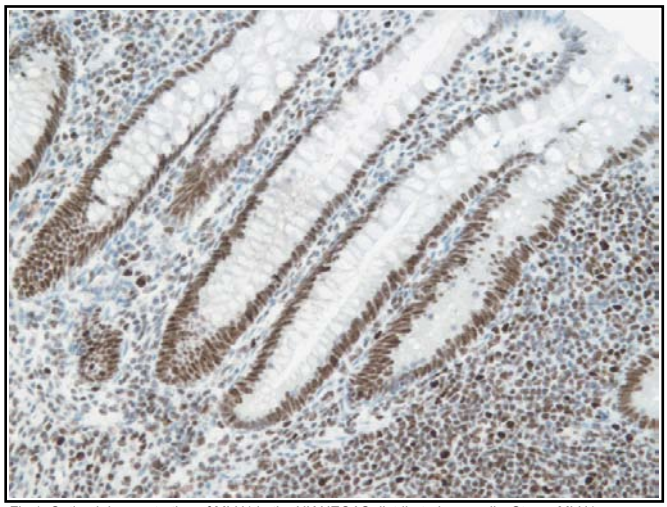


Fig 1. Optimal demonstration of MLH1 in the UK NEQAS distributed appendix. Strong MLH1 staining of the epithelial and stromal cells, with fading of staining intensity towards the luminal surface of the epithelial crypts. Stained with Ventana M1 clone (12 mins) on the Ventana Benchmark XT with Ventana CC1 for 64mins and the Ventana OptiView detection.

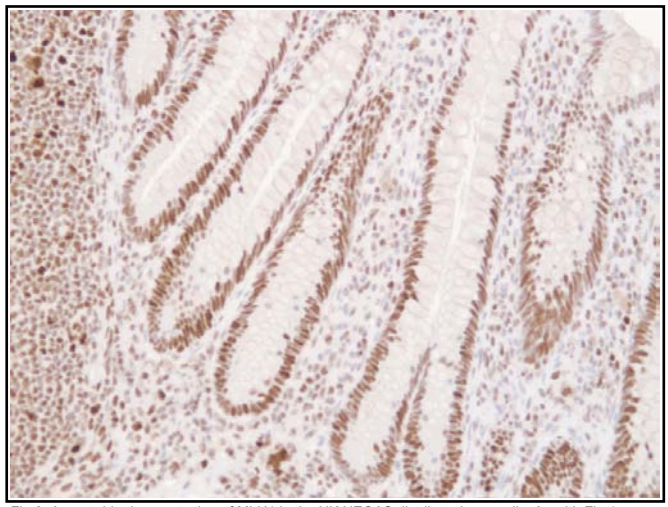


Fig 2. Acceptable demonstration of MLH1 in the UK NEQAS distributed appendix. As with Fig 1 there is strong MLH1 staining of the epithelial and stromal cells, with fading of staining intensity towards the luminal surface of the epithelial crypts. Stained with BD Pharmingen G168-15 clone (1:30 for 15 mins) on a Leica Bond-III, ER2 40mins and Bond Refine detection.

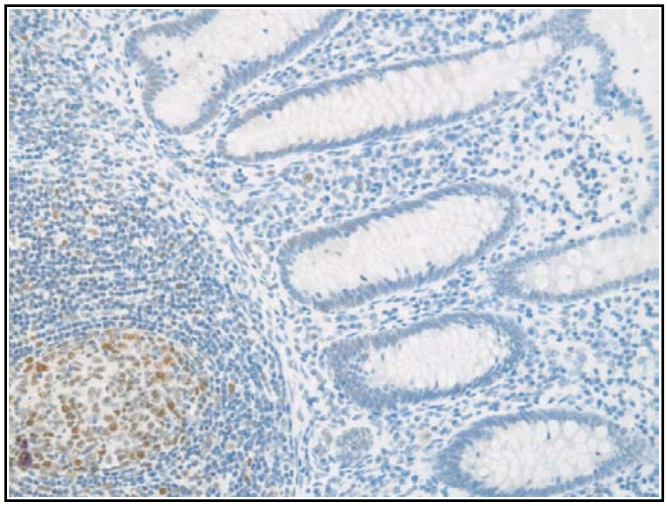


Fig 3. Weak demonstration of MLH1 on the UK NEQAS distributed appendix. Although some lymphocytes are stained the epithelial crypts are very weakly stained, making this example unacceptable. Stained with the Novocastra NCL-L-MLH1 ES05 clone (1:100 for 30 mins) on a Leica Bond Max using Leica ER1 retrieval for 20mins and Bond Polymer Refine detection.

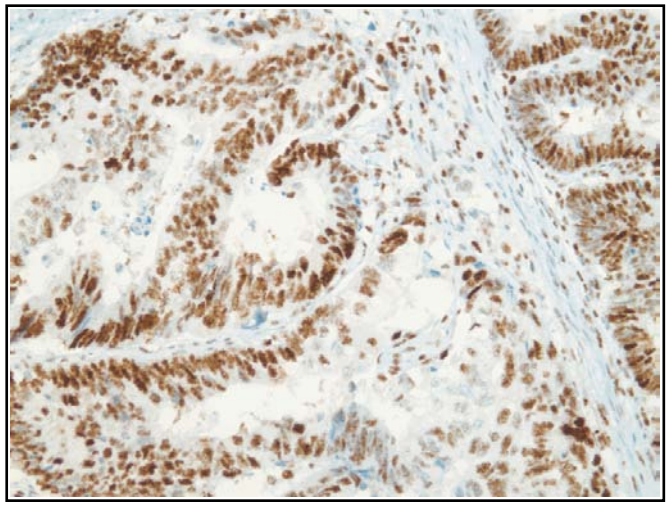


Fig 4. Optimal demonstration of MLH1 on the UK NEQAS distributed colonic tumour demonstrating strong nuclear staining of virtually all tumour cells, with staining in lymphocytes and stromal cells. Stained with Ventana M1 clone (12 mins) on the Ventana Benchmark XT with Ventana CC1 for 64mins and the Ventana OptiView detection.

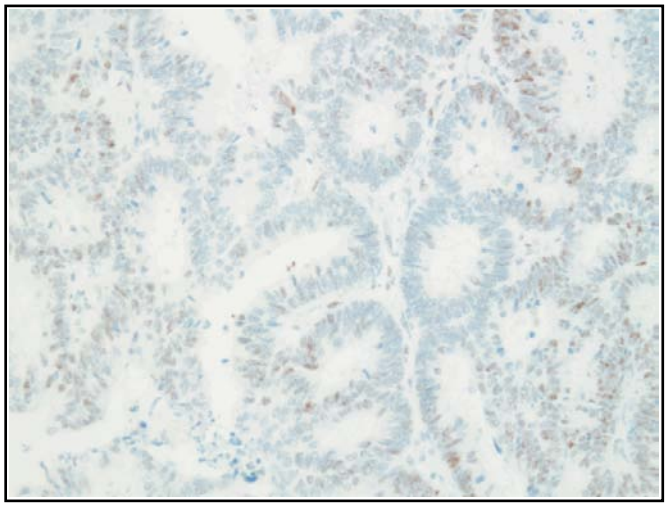


Fig 5. Unacceptable demonstration of MLH1 on the UK NEQAS distributed colonic tumour. Staining is very sporadic and weak in both the tumoural cells and surrounding lymphocytes. Stained with Dako ES05 clone (1:50 for 15 mins) on a Leica Bond-III using ER2 retrieval for 20mins and Bond Polymer Refine Detection.

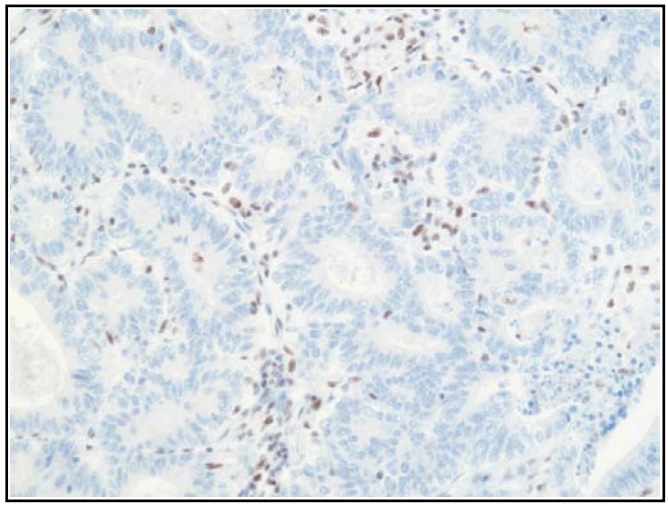


Fig 6. Optimal demonstration of MLH1 on the UK NEQAS distributed MLH1 negative colonic tumour showing only the intratumoral lymphocytes and stromal cells are staining positive. Section stained with the Dako FLEX RTU ES05 clone, incubated for 20mins with Dako PT Link retrieval for 20mins on a Dako Autostainer Link 48 with Dako EnVision detection.

## Selected Images showing Optimal and Sub-optimal Immunostaining

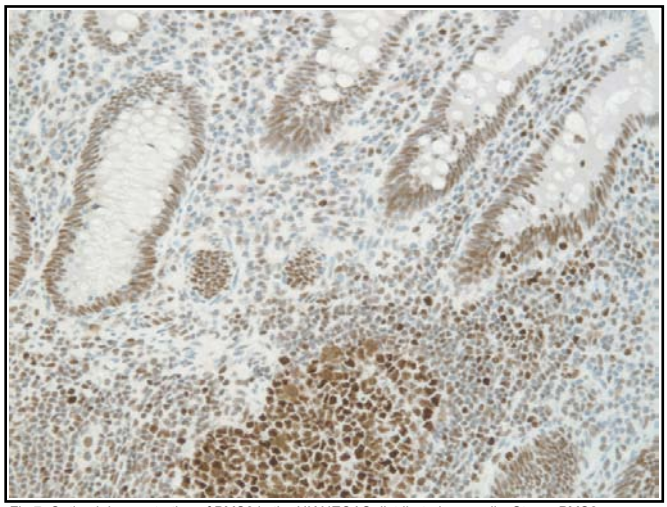


Fig 7. Optimal demonstration of PMS2 in the UK NEQAS distributed appendix. Strong PMS2 staining of the epithelial and stromal cells, with fading of staining intensity towards the luminal surface of the epithelial crypts. Stained with the Epitomics EP51 (1:20 for 60mins) on a Leica Bond Max using Leica ER2 retrieval for 20mins and Bond Polymer Refine Detection.

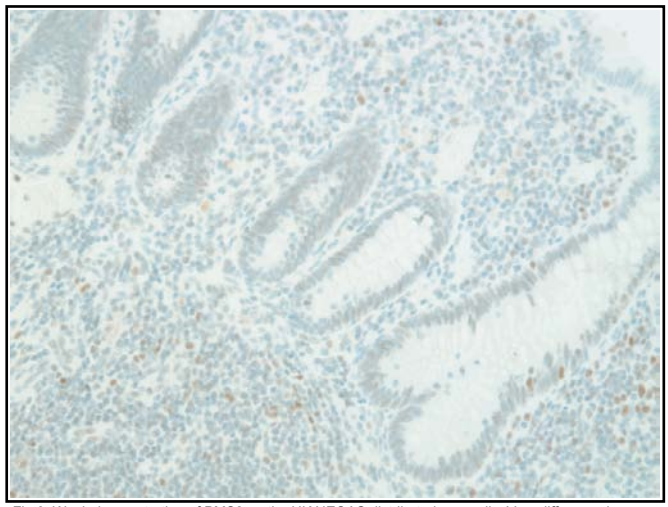


Fig 8. Weak demonstration of PMS2 on the UK NEQAS distributed appendix. Very diffuse and weak staining of the tumour cells and surrounding intratumoral lymphocytes and stromal cells. Stained with the Ventana PR3947 clone (60mins) on a Ventana Benchmark XT using CC1 retrieval for 64mins with Ventana Optiview detection.

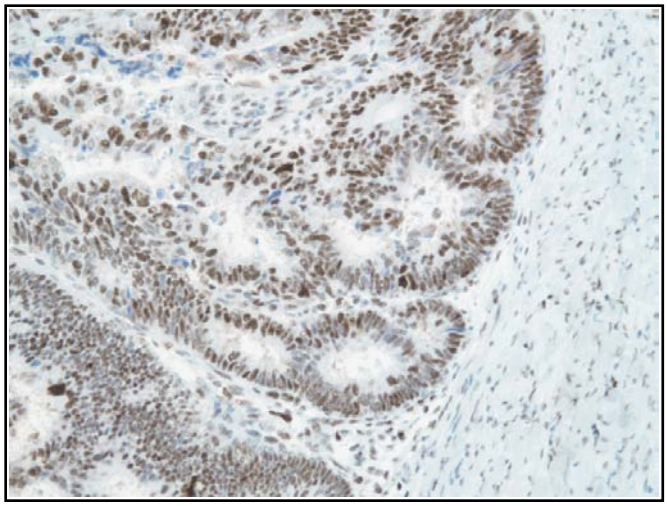


Fig 9. Optimal demonstration of PMS2 on the UK NEQAS distributed PMS2 positive colonic tumour showing nice crisp staining of tumour cells and intratumoral lymphocytes and stromal cells. Stained with the Dako RTU FLEX EP51 clone (30mins) on a Dako Autostainer Link 48 with Dako PTLink retrieval for 20mins and Dako FLEX+ detection.

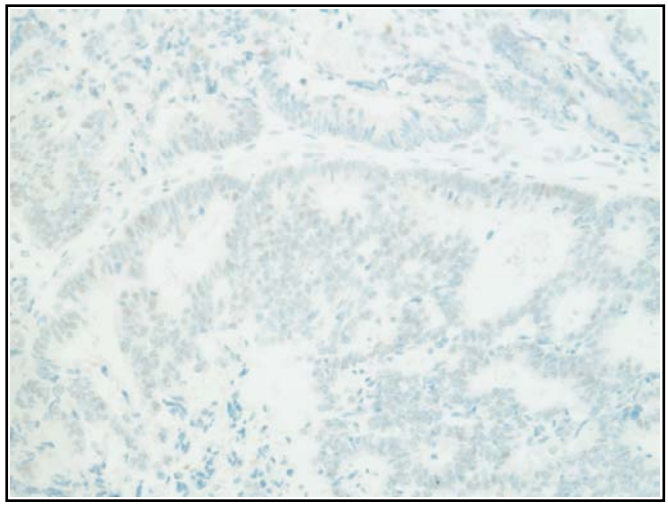


Fig 10. Weak demonstration of PMS2 on the UK NEQAS distributed PMS2 positive colonic tumour (compare with Fig 9). The tumoural cells are very weakly stained with the majority of tumour cells not showing any staining. Stained with the Ventana EPR3947 clone (60mins) on a Ventana Benchmark XT with CC1 for 64mins and Ventana Optiview detection.

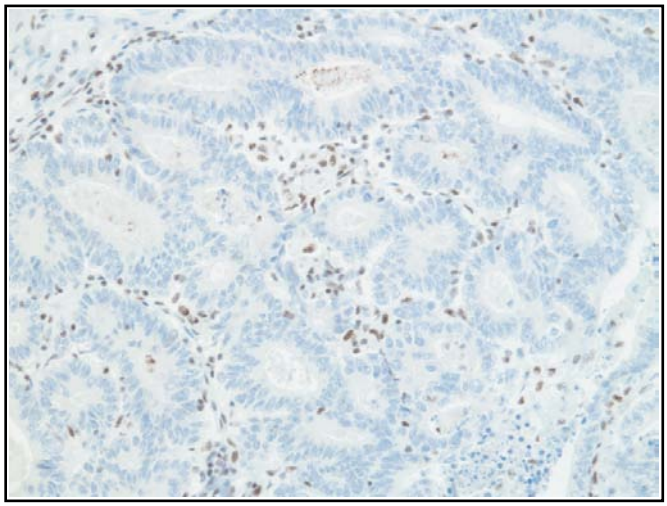


Fig 11. Optimal demonstration of PMS2 on the UK NEQAS distributed PMS2 negative colonic tumour showing intratumoral lymphocytes and stromal cell staining. Stained with the Dako RTU FLEX EP51 clone (30 mins) on a Dako Autostainer Link 48 with Dako PTLink retrieval for 20mins and Dako FLEX+ detection.

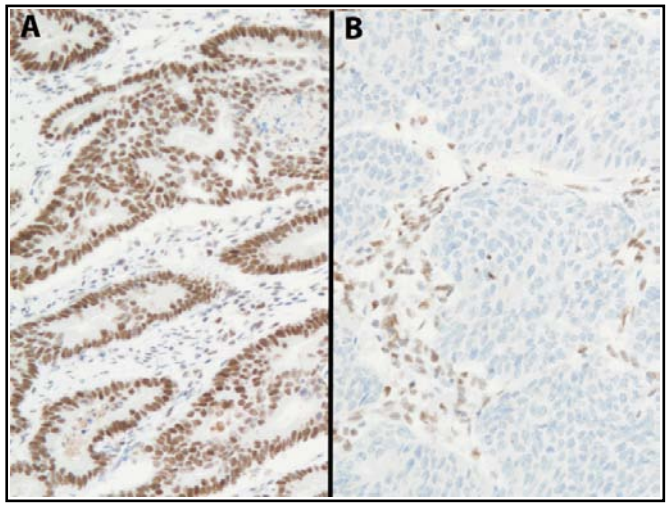
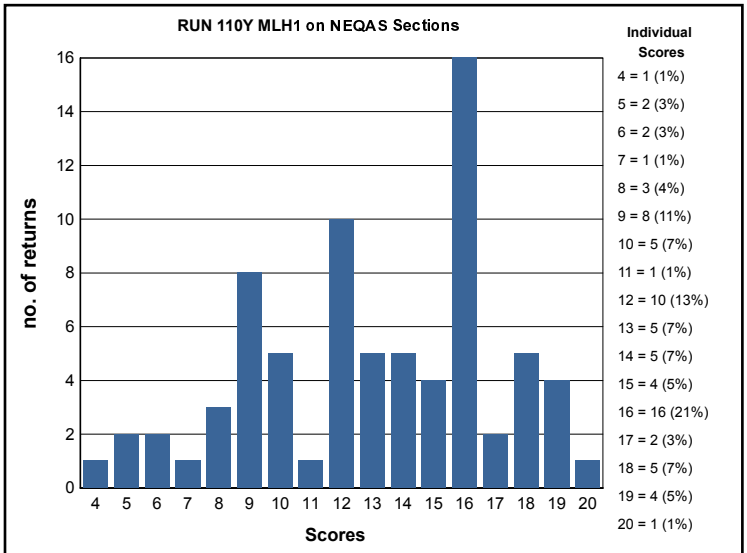
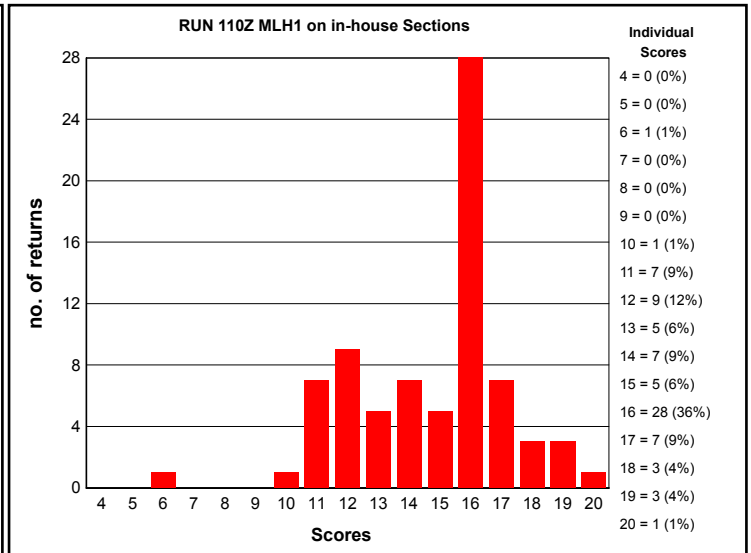
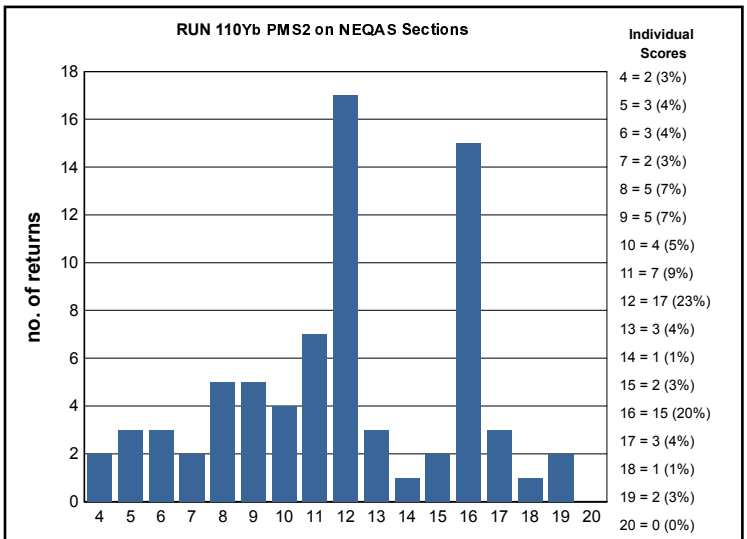


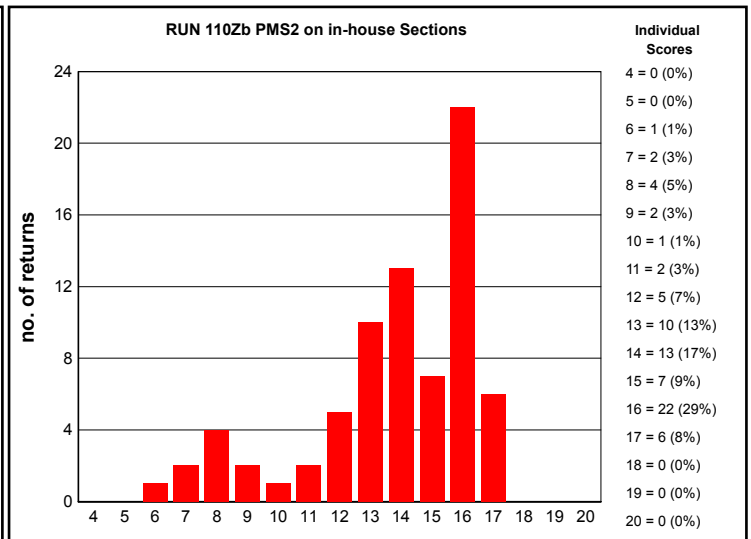

Fig 12. Good demonstration of an in-house PMS2 (A) positive and (B) negative colon tumours. Stained with the Dako EP51 clone (1:20 for 15 mins) on a Leica Bond-III with ER2 retrieval for 40mins and Bond Polymer Refine Detection.

## 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: 110**

**Primary Antibody :** MLH1

| Antibody Details | N | % |
|---|---|---|
| BD Pharmingen (G168-15) | 9 | 22 |
| BD Pharmingen (G168-728) | 1 | 0 |
| Biocare medical CM/PM 220 (G168-15) | 1 | 0 |
| Dako Flex RTU IR079/IS079 (ES05) | 9 | 89 |
| Dako M3640 (ES05) | 6 | 33 |
| Leica Bond RTU PA0610 (ES05) | 2 | 100 |
| Novocastra NCL-L-MLH1 (ES05) | 19 | 42 |
| Other | 2 | 50 |
| Ventana 760-4264 (G168-728) | 1 | 0 |
| Ventana 790-4535 (M1) | 25 | 76 |

**Primary Antibody :** PMS2

| Antibody Details | N | % |
|---|---|---|
| BD Bio/Pharmingen 556415 (A16-4) | 16 | 31 |
| Cell Marque 288M -16 (MRQ28) | 2 | 50 |
| Cell Marque 288R -17/18 (EPR3947) | 2 | 50 |
| Dako M3647 (EP51) | 15 | 60 |
| Dako RTU FLEX IR087 (EP51) | 8 | 75 |
| Leica/Novoca NCL-L-PMS2 (MOR4G) | 2 | 0 |
| Other | 3 | 33 |
| Ventana 760-4531 (EPR3947) | 27 | 15 |

**HNPCC Run: 110**

| Heat Mediated Retrieval | MLH1 N | MLH1 % | PMS2 N | PMS2 % |
|---|---|---|---|---|
| Biocare Decloaking Chamber | 1 | 100 | 1 | 0 |
| Dako PTLink | 9 | 89 | 9 | 89 |
| Lab vision PT Module | 1 | 0 | 1 | 100 |
| Leica ER1 20 mins | 4 | 25 | 0 | 0 |
| Leica ER1 30 mins | 1 | 0 | 0 | 0 |
| Leica ER2 20 mins | 8 | 38 | 12 | 25 |
| Leica ER2 30 mins | 7 | 43 | 8 | 38 |
| Leica ER2 40 mins | 8 | 50 | 8 | 50 |
| Pressure Cooker in Microwave Oven | 0 | 0 | 1 | 0 |
| Steamer | 0 | 0 | 1 | 0 |
| Ventana CC1 20mins | 0 | 0 | 1 | 0 |
| Ventana CC1 32mins | 4 | 75 | 0 | 0 |
| Ventana CC1 40mins | 2 | 100 | 1 | 0 |
| Ventana CC1 44mins | 1 | 100 | 0 | 0 |
| Ventana CC1 48mins | 2 | 50 | 3 | 33 |
| Ventana CC1 56mins | 3 | 33 | 0 | 0 |
| Ventana CC1 64mins | 14 | 57 | 11 | 27 |
| Ventana CC1 72mins | 0 | 0 | 1 | 0 |
| Ventana CC1 76mins | 0 | 0 | 1 | 0 |
| Ventana CC1 80mins | 1 | 0 | 2 | 0 |
| Ventana CC1 88mins | 2 | 0 | 1 | 0 |
| Ventana CC1 92mins | 2 | 50 | 8 | 38 |
| Ventana CC1 extended | 0 | 0 | 1 | 100 |
| Ventana CC1 standard | 6 | 83 | 4 | 0 |
| Ventana CC2 64mins | 0 | 0 | 1 | 0 |

**HNPCC Run: 110**

| Enzyme Mediated Retrieval | MLH1 N | MLH1 % | PMS2 N | PMS2 % |
|---|---|---|---|---|
| AS PER KIT | 2 | 0 | 1 | 100 |
| NOT APPLICABLE | 34 | 59 | 34 | 32 |

| HNPCC Run: 110 | MLH1 | | PMS2 | |
|---|---|---|---|---|
| **Detection** | **N** | **%** | **N** | **%** |
| AS PER KIT | 2 | 100 | 3 | 33 |
| Biocare polymer (M4U534) | 1 | 100 | 1 | 0 |
| Dako EnVision FLEX ( K8000/10) | 0 | 0 | 1 | 0 |
| Dako EnVision FLEX+ ( K8002/12) | 7 | 100 | 4 | 100 |
| Dako Envision HRP/DAB ( K5007) | 0 | 0 | 1 | 0 |
| Dako Envision+ HRP mouse K4004/5/6/7 | 1 | 0 | 1 | 100 |
| Leica Bond Polymer Define (DS9713) | 0 | 0 | 1 | 0 |
| Leica Bond Polymer Refine (DS9800) | 26 | 35 | 25 | 40 |
| None | 0 | 0 | 2 | 100 |
| Other | 3 | 67 | 3 | 33 |
| Ventana OptiView Kit (760-700) | 26 | 54 | 25 | 32 |
| Ventana UltraView Kit (760-500) | 9 | 67 | 7 | 0 |

| HNPCC Run: 110 | MLH1 | | PMS2 | |
|---|---|---|---|---|
| **Automation** | **N** | **%** | **N** | **%** |
| BioGenex GenoMX 6000i | 0 | 0 | 1 | 0 |
| Dako Autostainer Link 48 | 7 | 100 | 6 | 83 |
| Dako Autostainer plus | 2 | 0 | 2 | 100 |
| Dako Autostainer Plus Link | 1 | 100 | 2 | 100 |
| LabVision Autostainer | 0 | 0 | 2 | 0 |
| Leica Bond Max | 11 | 36 | 10 | 30 |
| Leica Bond-III | 18 | 44 | 18 | 39 |
| Menarini - Intellipath FLX | 1 | 100 | 1 | 0 |
| Ventana Benchmark GX | 1 | 0 | 1 | 0 |
| Ventana Benchmark ULTRA | 21 | 67 | 21 | 29 |
| Ventana Benchmark XT | 14 | 50 | 12 | 17 |

| HNPCC Run: 110 | MLH1 | | PMS2 | |
|---|---|---|---|---|
| **Chromogen** | **N** | **%** | **N** | **%** |
| AS PER KIT | 14 | 43 | 13 | 15 |
| Dako EnVision Plus kits | 2 | 100 | 2 | 50 |
| Dako FLEX DAB | 5 | 100 | 6 | 83 |
| Dako REAL EnVision K5007 DAB | 0 | 0 | 1 | 0 |
| Leica Bond Polymer Refine kit (DS9800) | 26 | 38 | 25 | 40 |
| Other | 7 | 71 | 9 | 56 |
| Ventana DAB | 10 | 50 | 11 | 18 |
| Ventana Ultraview DAB | 12 | 75 | 9 | 22 |

## BEST METHODS - Gold Standard Antibody

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

### MLH1 - Method 1

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

**Primary Antibody:** Leica Bond RTU PA0610 (ES05) , 25 Mins, 20 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)
**Detection:** Leica Bond Polymer Refine (DS9800) Prediluted

### MLH1 - Method 2

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

**Primary Antibody:** Dako Flex RTU IR079/IS079 (ES05) , 30 Mins, 23 ºC Prediluted
**Automation:** Dako Autostainer Plus Link
**Method:** Dako FLEX+ kit
**Main Buffer:** Dako FLEX wash buffer
**HMAR:** Dako PTLink, Buffer: high pH target retrieval solution
**EAR:**
**Chromogen:** Dako FLEX DAB, 23 ºC., Time 1: 10 Mins, Time 2: 10 Mins
**Detection:** Dako EnVision FLEX+ ( K8002/12) , 30 Mins, 23 ºC Prediluted

**MLH1 - Method 3**

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

| | |
|---|---|
| **Primary Antibody:** | BD Pharmingen (G168-15) , 15 Mins, RT ºC Dilution 1: 30 |
| **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), RT ºC., Time 1: 10 Mins, Time 2: 10 Mins |
| **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:** | Ventana 790-4535 (M1) , 12 Mins, 37 ºC Prediluted |
| **Automation:** | Ventana Benchmark XT |
| **Method:** | Ventana Optiview |
| **Main Buffer:** | Ventana reaction buffer (950-300) |
| **HMAR:** | Ventana CC1 64mins, Buffer: CC1 |
| **EAR:** | NOT APPLICABLE |
| **Chromogen:** | Other, 37 ºC., Time 1: 8 Mins, Time 2: 8 Mins |
| **Detection:** | Ventana OptiView Kit (760-700) , 8 Mins, 37 ºC Prediluted |

## BEST METHODS - Secondary Antibody

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

**PMS2 - Method 1**

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

| | |
|---|---|
| **Primary Antibody:** | Dako M3647 (EP51) , 15 Mins Dilution 1: 40 |
| **Automation:** | Leica Bond-III |
| **Method:** | AS PER KIT |
| **Main Buffer:** | Bond Wash Buffer (AR9590) |
| **HMAR:** | Leica ER2 40 mins |
| **EAR:** | |
| **Chromogen:** | Leica Bond Polymer Refine kit (DS9800) |
| **Detection:** | Leica Bond Polymer Refine (DS9800) |

**PMS2 - Method 2**

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

| | |
|---|---|
| **Primary Antibody:** | BD Bio/Pharmingen 556415 (A16-4) , 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 pH TRS, 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 |

**PMS2 - Method 3**

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

| | |
|---|---|
| **Primary Antibody:** | Dako RTU FLEX IR087 (EP51) , 12 Mins, 37 ºC Prediluted |
| **Automation:** | Ventana Benchmark XT |
| **Method:** | Ventana Optiview |
| **Main Buffer:** | Ventana reaction buffer (950-300) |
| **HMAR:** | Ventana CC1 64mins, Buffer: CC1 |
| **EAR:** | NOT APPLICABLE |
| **Chromogen:** | Other, 37 ºC., Time 1: 8 Mins, Time 2: 8 Mins |
| **Detection:** | Ventana OptiView Kit (760-700) , 8 Mins, 37 ºC Prediluted |

**PMS2 - Method 4**

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

| | |
|---|---|
| **Primary Antibody:** | Dako RTU FLEX IR087 (EP51) , 30 Mins, 23 ºC Prediluted |
| **Automation:** | Dako Autostainer Link 48 |
| **Method:** | Dako FLEX kit |
| **Main Buffer:** | Dako Wash Buffer (S3006), PH: 7.7 |
| **HMAR:** | Dako PTLink, Buffer: EnVision FLEX High pH, PH: 9 |
| **EAR:** | NOT APPLICABLE |
| **Chromogen:** | Dako FLEX DAB, PH: 7.5, 23 ºC., Time 1: 5 Mins, Time 2: 5 Mins |
| **Detection:** | None |

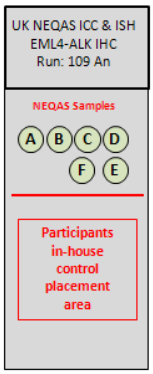
# Non-Small Cell Lung Carcinoma (NSCLC) ALK IHC EQA
# Run 110 Assessment Results

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

## Sample and Slide Distribution

| | |
|---|---|
| Antibody Assessed | ALK |
| Samples Circulated | Composite slide (see table below) |
| Number Participants | 40 |

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




| Sample code | Sample | FISH status (Vysis) | IHC status (Roche D5F3) |
|---|---|---|---|
| A | **Cell line**: 50% **knock in** + 50% adenocarcinoma | -ve | Approx. 50% +ve & 50% -ve |
| B | **Cell line**: 100% adenocarcinoma | -ve | 100% -ve |
| C | **Cell line**: 50% isogenic + 50% adenocarcinoma | +ve (Break apart: inversion) | Approx. 50% +ve & 50% -ve |
| D | **Cell line**: 50% isogenic + 50% adenocarcinoma | +ve (Break apart: inversion) | Approx. 50% +ve & 50% -ve |
| E | NSCLC adenocarcinoma | +ve (Break apart: inversion + deletion) | +ve |
| F | NSCLC adenocarcinoma | -ve | -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 guidlines

- 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:<br>- False positive/negative ALK IHC membrane staining<br>- Excessive cytoplasmic staining<br>- 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. | |

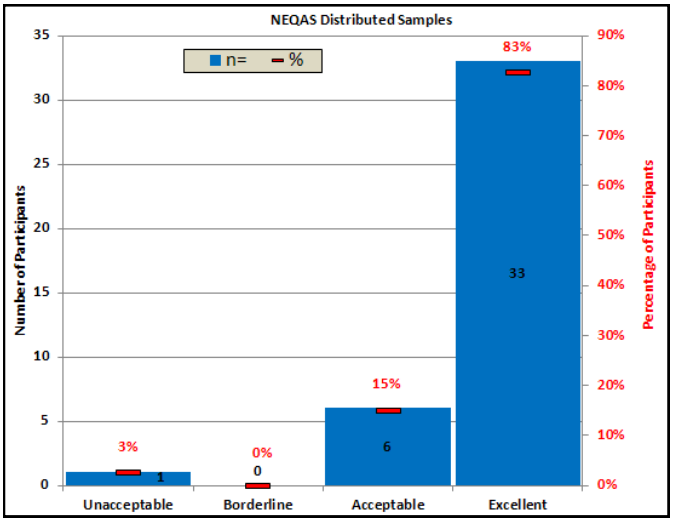
## Results & Discussion

### Distributed NEQAS Sample Results

There was a decrease in the number of participants in this assessment round, down from 53 to 40.

Figure 1 shows the distribution of pass rates for the NEQAS distributed samples, with 98% (n=39) of participants achieving an acceptable/excellent level of staining, with only 1 participant (3%) exhibiting an unacceptable level of staining, due to very weak ALK expression in all expected ALK positive samples.

**Fig 2. Pass rates on NEQAS distributed samples**




A breakdown of the submitted methods and detection systems (see table 4 & 5) showed that the main antibody clones used include D5F3, ALK1 and 5A4. The following points highlight the main findings:

- The Roche D5F3 (See Table 4 and fig 4) was the main antibody used accounting for 68% (n=38) of users, with 100% demonstrating either excellent (92%) or acceptable (8%) results. there were not labs who had either borderline or unacceptable results during this assessment.
- The Leica Biosystems 5A4 clone (See Table 4 and fig 5) clone was used by 8 participants in either a concentrate (n=7) or pre-diluted (n=1). The concentrate 5A4 clone was used on numerous staining platforms (see previous report for examples) and had a an overall Excellent/Acceptable pass rate of 86%. Only one participant (14%) had an unacceptable results due to staining being much weaker than excepted. The one participant using the pre-diluted 5A4 clone showed an acceptable level of staining.
- The cell signalling D5F3 clone (see Table 4), used by 2 participants and showed a 100% pass rate.
- The Dako ALK1 (See Table 4 and fig 6) although not recommended by Dako for NSCLC was used by 2 participants with a 100% pass rate. However, all the assessors did note that the staining intensity was much lower than the other platforms and was detection system related.
- Further evidence that the choice of detection system does influence the staining intensity can be seen in Fig 7 A-C, where the Novocastra clone was used on a Ventana platform with Ventana OptiView detection (table 5).

**Features of Acceptable Staining**

- Each of the ALK IHC positive cell line samples (samples A, C & D) should demonstrate granular cytoplasmic staining in at least 50% of tumour cells. See figs 4A, C & D; 5A, C & D; 6A, C & D and 7A
- ALK IHC positive tumour sample (sample E) should demonstrate granular cytoplasmic staining in the majority of the tumour cells. See figs 4E, 5E, 6E & 7D
- No attaining should be observed in either the ALK IHC negative cell lines (figs 4B, 5B, 6B & 7B) or negative tumour sample (figs 4F, 5F and 6F).
- Macrophages may be stained positive
- Appendix (submitted by participants) (fig 7I) should show ALK IHC staining of the ganglion cells and axons.

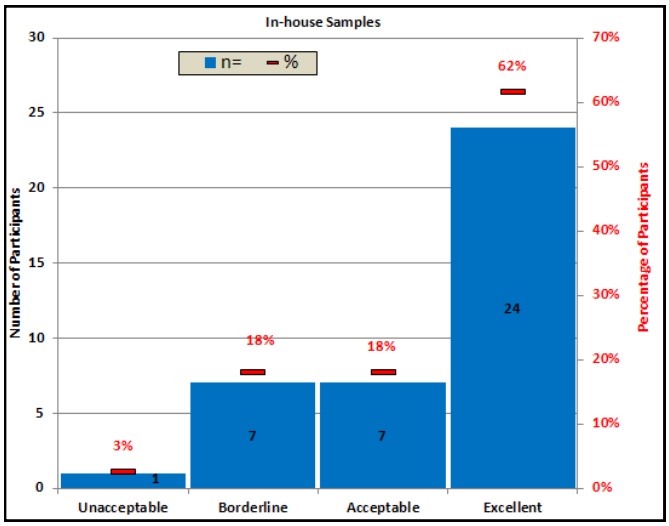
**Features of Unacceptable Staining**

- False negative or absence of ALK staining where tumour cells should be staining positive (fig 7E)
- Non-specific / Excessive Tyramide staining
- Absence of staining in appendix (Fig 7F)

### In-house Control Results

Of the 40 participants taking part in this assessment, 39 (98%) also submitted in-house controls with the majority placing their samples alongside the NEQAS sample (see fig 1). As illustrated in figure 3, 80% (n=31) of participants were assessed as having an acceptable/excellent level of staining and good choice of in-house control, with 18% (n=7) attaining a borderline score and only 3% (n=1) having an unacceptable result.

**Fig 3. Pass rates on in-house samples**




**Note that the assessor were lenient in their scoring of in-house controls during this assessment and in future assessments more stringent scoring criteria for in-house controls will be used (see below: 'Recommended ALK IHC controls)**

There were numerous numbers and tissue types submitted for in-house controls and a breakdown of the data is shown in Table 5. In summary:

- 2 (5%) of participants did not provide an in-house sample for evaluation.
- 17 (41%) of participants submitted only a single tissue control which mainly consisted of an ALK positive lung adenocarcinoma (69%). A single ALK positive sample is not able to provide information on possible false-positive results.
- 14 (34%) of participants submitted controls consisting of two tissue samples, with just less that half (43%) submitting both a positive and negative NSCLC control. Further tissue types are shown in Table 5.

- 6 (15%) participants submitted 3 samples as an in-house control, with 3 (50%) submitting 2 x NSCLC (+ve) + NSCLC (-ve) controls.

### Lymphoma control

Lymphoma is not recommended as a control in the lung setting and as shown in table 5 there were a total of 6 (15%) of participants who included lymphoma as part of their in-house control. Lymphoma control can lead to potential false-negative result in the lung setting as illustrated in fig 7 D-F.

### Appendix control

Appendix controls were used by 6 (15%) of participants who either submitted it as a single in-house control or as part of a composite block. The forthcoming NEQAS ALK assessments will also include an appendix sample to test whether such a control is suitable in ALK IHC testing.

## 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.

- 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.

**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).

## Scoring Systems Employed by Participants

A straightforward '+ve/-ve' interpretation scoring system was used by 87% of participants, with 13% using an intensity based method (3+,2+,1+ and Neg.) (see Table 3). As previously indicated this appears to be detection system related. In summary:

- **Roche:** 100% (n=26) indicated that they used a simple '+ve/-ve' scoring criteria.
- **Novocastra:** Of the 5A4 clone, users 5 out of 8 (63%) participants used a '+ve/-ve' method.
- **Dako:** 100% (n=2) used a +ve/-ve scoring criteria
- **Cell Signalling Technologies:** 100% (n=2) used a an intensity based method (3+,2+,1+ and Neg.)

As indicated in the introduction, although there is evidence that ALK IHC along with intensity could be used as an affective screening tool,[13,14] there is also further evidence that the variability in intensity is due to the choice of detection system (Table 5), with for example the Roche tyramide detection providing a more clear cut distinction between a positive or negative tumour.

**Table 3: Scoring systems submitted by participants**

| | | Scoring Methods Used | |
|---|---|---|---|
| **Antibody (clone)** | **n =** | **+ve / -ve** | **3+,2+,1+, neg.** |
| Ventana/Roche (D5F3) | 26 | 100% | - |
| Novocastra NCL-ALK (5A4) | 7 | 57% | 43% |
| Cell Signalling Tech. (D5F3) | 2 | - | 100% |
| Dako M7195 (ALK1) | 2 | 100% | - |
| Novocastra PA0306 (5A4) | 1 | 100% | - |
| | **Overall** | **87%** | **13%** |

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**Acknowledgements**

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**Table 4**: Antibodies submitted by participants along with their respective assessment pass rates . A total of 40 participants submitted completed methodology data.

| Antibody | n= | % of total methods submitted | Excellent | Acceptable | Borderline | Unacceptable |
|---|---|---|---|---|---|---|
| Ventana/Roche (D5F3) | 26 | 68% | 24 (92%) | 2 (8%) | - | - |
| Novocastra NCL-ALK (5A4) | 7 | 18% | 3 (43%) | 3 (43%) | - | 1 (14%) |
| Cell Signalling Tech. D5F3 | 2 | 5% | 2 (100%) | - | - | - |
| Dako M7195 (ALK1) | 2 | 5% | 2 (100%) | - | - | - |
| Novocastra PA0306 (5A4) | 1 | 3% | 0 (0%) | 1 (100%) | - | - |

**Table 5**: In-house controls and tissue type submitted

| No. of in-house controls submitted per participant | No. of participants (%) | In-house tissue composition |
|---|---|---|
| **0** | 2 (5%) | - |
| **1** | 17 (41%) | 69%: NSCLC (+ve),<br>13%: Appendix<br>6%: Lymphoma (+ve)<br>6%: NSCLC cell block (+ve)<br>6%: Cell line (+ve) |
| **2** | 14 (34%) | 43%: NSCLC (+ve) + NSCLC (-ve)<br>14%: NSCLC (+ve) + Lymphoma (+ve)<br>7%: NSCLC (+ve) + Appendix<br>7%: Other (+ve) + Other (-ve)<br>7%: 2 x NSCLC (+ve)<br>7%: Lymphoma (+ve) + Appendix<br>7%: 2 x Appendix<br>7%: NSCLC (+ve) + Other (-ve) |
| **3** | 6 (15%) | 50%: 2 x NSCLC (+ve) + NSCLC (-ve)<br>17%: 2 x NSCLC (+ve) + Lymphoma + Thymus<br>17%: NSCLC (+ve) + 2 x Appendix<br>17%: 2 x Lymphoma (+ve) + Appendix |
| **>3** | 2 (5%) | - |

**Table 6**: Pass rates showing associated antibody, automated platform and detection systems employed.

| Primary antibody | Automation Instrument | Detection kit | Excellent | Acceptable | Borderline | Unacceptable |
|---|---|---|---|---|---|---|
| Cell Signalling Tech. D5F3 | LabVision Autostainer | Dako Envision HRP/DAB ( K5007) | 1 (100%) | - | - | - |
| | Ventana Benchmark ULTRA | Ventana OptiView Kit (760-700) | 1 (100%) | - | - | - |
| Dako M7195 (ALK1) | Dako Autostainer Link 48 | DAKO Envision FLEX+ mouse Linker | 1 (100%) | - | - | - |
| | Ventana Benchmark XT | Ventana OptiView Kit (760-700) | 1 (100%) | - | - | - |
| Novocastra PA0306 (5A4) | Dako Autostainer Link 48 | Dako EnVision FLEX+ ( K8002/12) | 1 (100%) | - | - | - |
| | | DAKO Envision FLEX+ mouse Linker | - | 1 (100%) | - | - |
| | Leica Bond Max | Leica Bond Polymer Refine (DS9800) | - | 1 (100%) | - | - |
| | Leica Bond-III | Leica Bond Polymer Refine (DS9800) | - | - | - | 1 (100%) |
| | Ventana Benchmark ULTRA | Ventana OptiView Kit (760-700) | - | 1 (100%) | - | - |
| | Ventana Benchmark XT | Ventana OptiView Kit (760-700) | 2 (100%) | - | - | - |
| Novocastra PA0306 (5A4) | Leica Bond Max | Leica Bond Polymer Refine (DS9800) | - | 1 (100%) | - | - |
| Ventana/Roche (D5F3) | Ventana Benchmark | Ventana OptiView Kit (760-700) | 1 (100%) | - | - | - |
| | Ventana Benchmark ULTRA | Ventana OptiView Kit (760-700) | 2 (67%) | 1 (33%) | - | - |
| | Ventana Benchmark XT | OptiView amplification kit 760-099 | 1 (100%) | - | - | - |
| | | Ventana OptiView Kit (760-700) | 19 (95%) | 1 (5%) | - | - |
| | | Ventana UltraView Kit (760-500) | 1 (100%) | - | - | - |

**Fig 4:** Samples stained with the Roche D5F3 assay

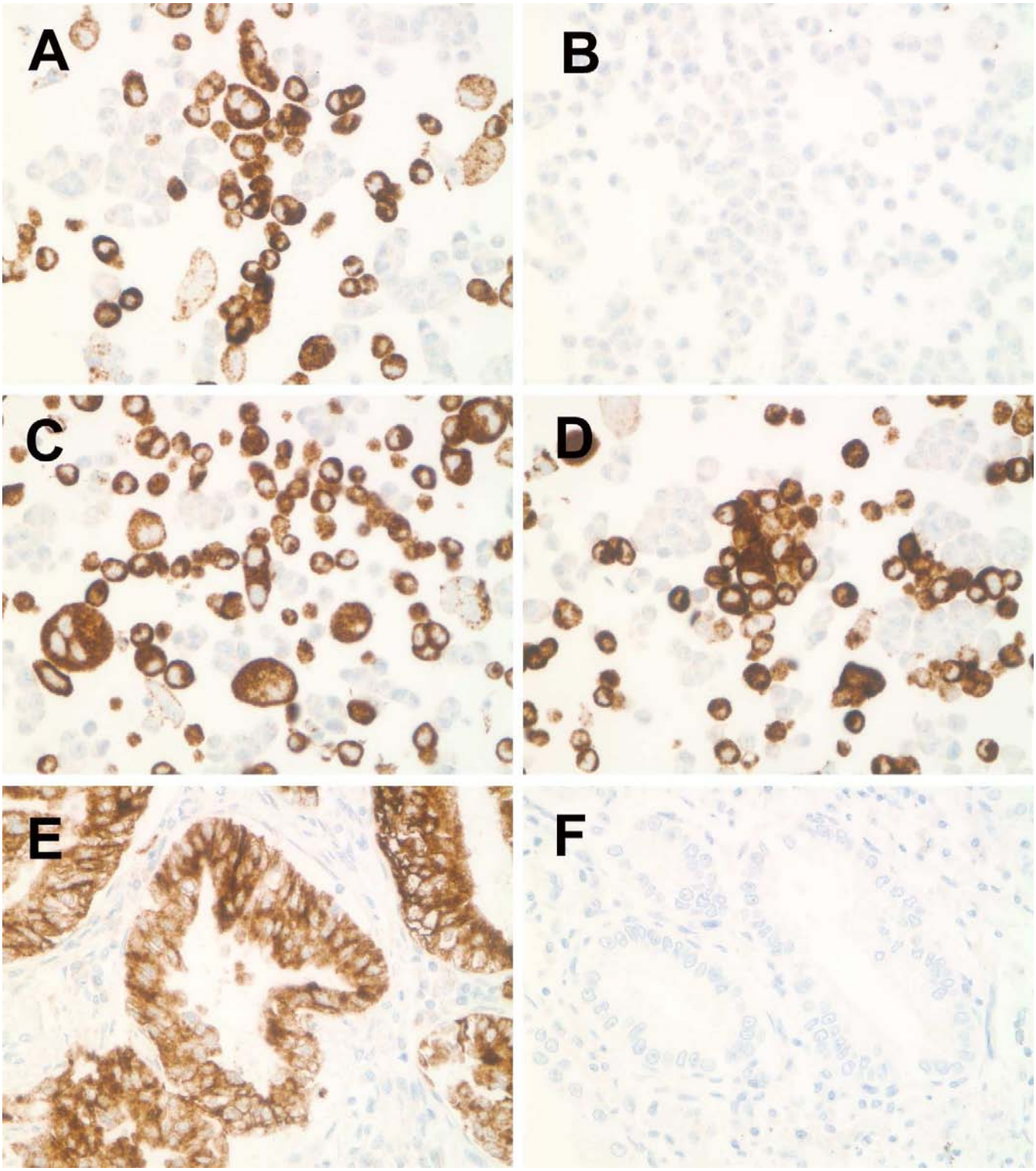



**Fig 4**. Examples from participants who showed good demonstration of ALK IHC in the distributed Cell lines (A-D) and Tumour samples (E & F) using the Roche D5F3 assay. (A,C,D & E) showing expected ALK IHC positive staining and (B & F) ALK IHC negative staining. Stained with the Roche D5F3 assay on a Ventana Benchmark XT using CC1 92 minutes antigen retrieval and Optiview detection.

**Fig 5:** Samples stained with the Leica Biosystems ALK 5A4

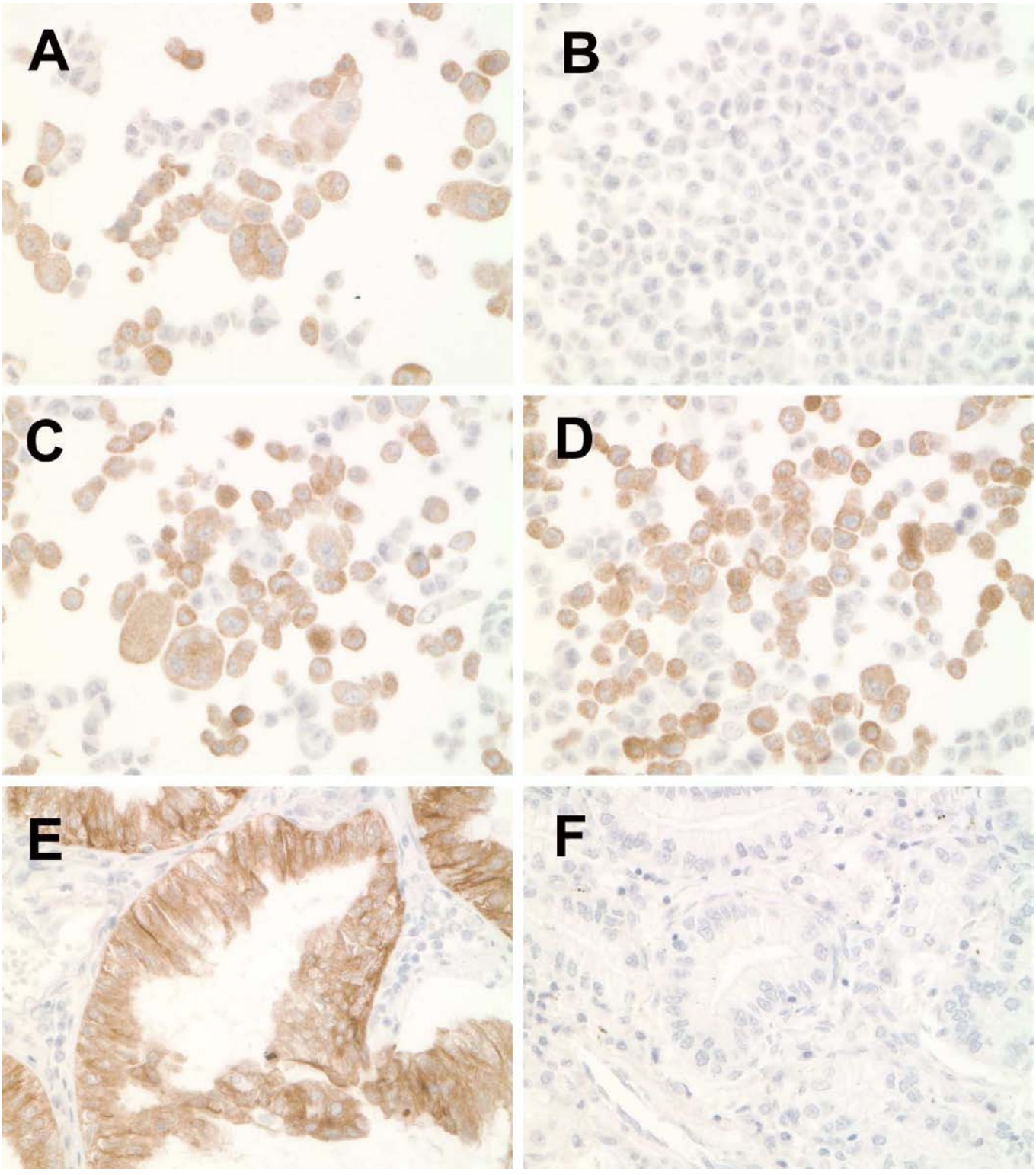



**Fig 5**. Acceptable demonstration of ALK IHC on the NEQAS distributed (A-D) cell lines and (E &F) Tumour samples using the Leica Biosystems RTU ALK 5A4 clone. Although the intensity of staining is lower than that seen using the Roche D5F3 assay (see Fig 2), the assessment panel found the sensitivity of the method to be acceptable in discerning positivity of staining. Samples stained with the Leica Biosystems ALK 5A4 diluted 1:100 on a Bond III using ER2 for 30 minutes and Leica Bond Polymer Refine detection

**Fig 6:** Samples stained with the Dako ALK1 antibody

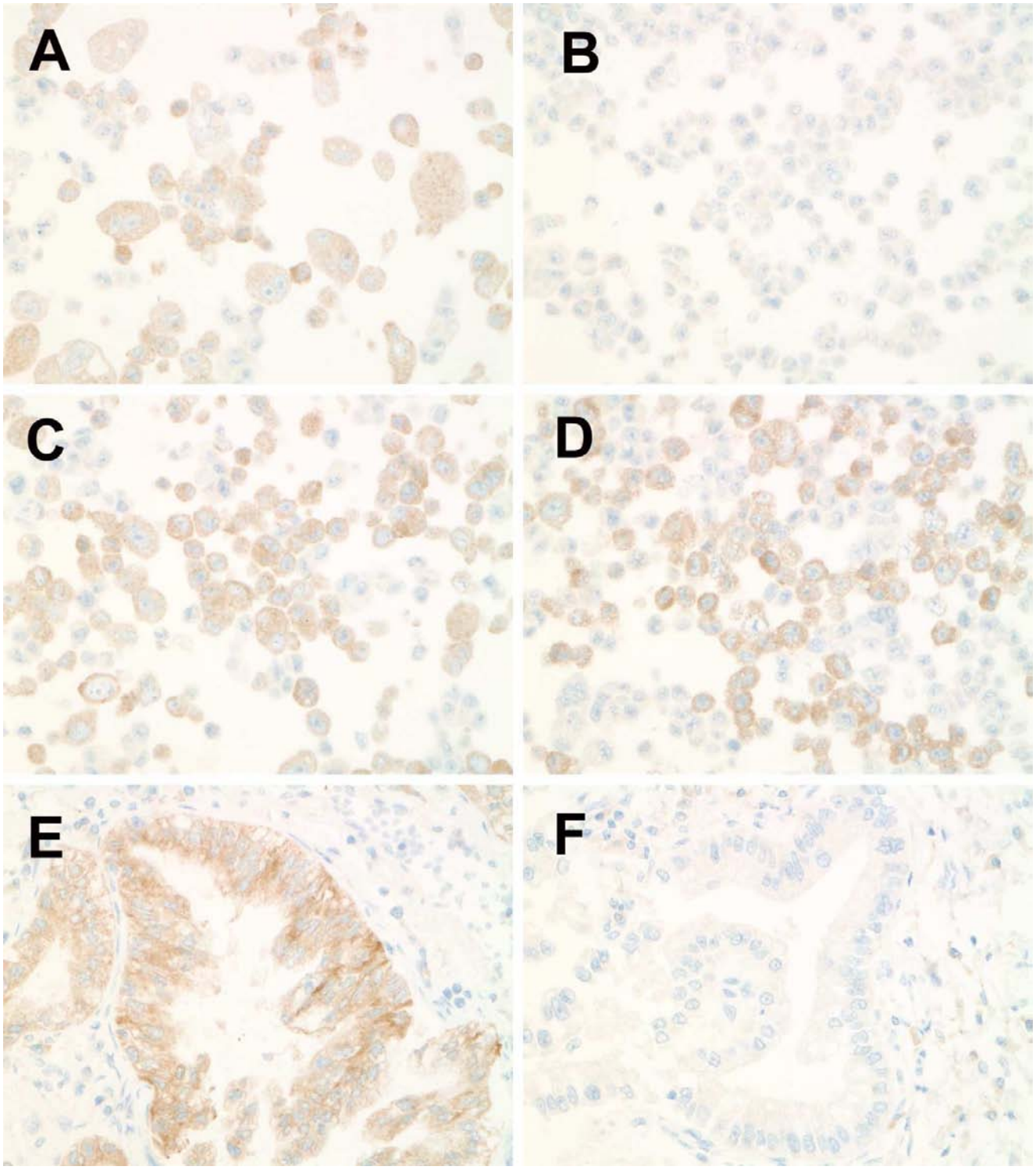



**Fig 6**. Acceptable demonstration of ALK IHC on the NEQAS distributed (A-D) cell lines and (E &F) tumour samples using the Dako ALK1 antibody. The assessors found the staining intensity although weaker than either the Roche or the Novocastra clone, this did no impact on the overall interpretation.
Samples stained using Dako PT link retrieval, Dako Autostainer Link 48 using Dako Envision Flex detection.
**Important: The Dako ALK1 antibody is not recommended by Dako for the detection of ALK in NSCLC**

**Fig 7.** Acceptable and unacceptable examples. See figure legends below

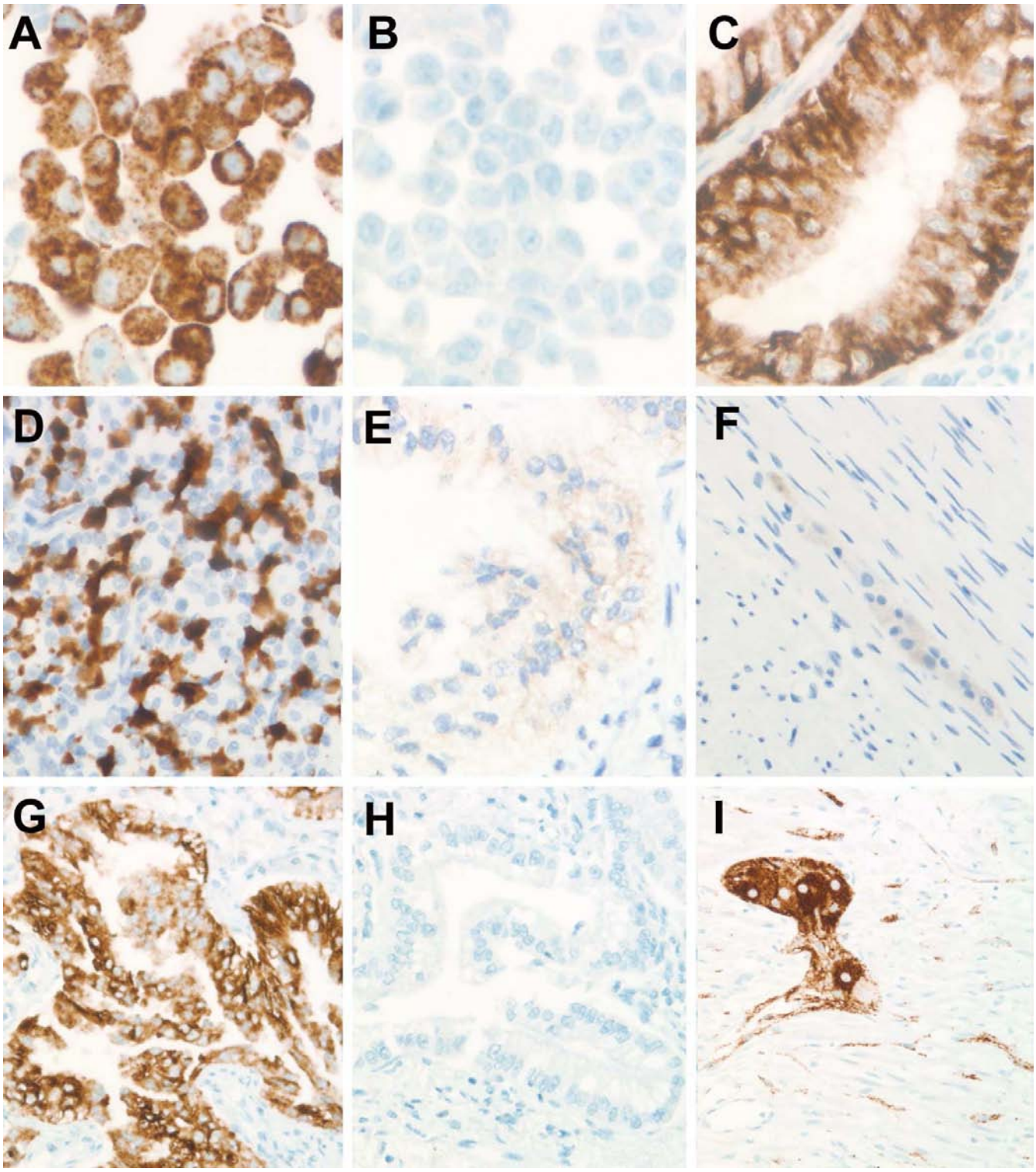



**Fig 7**
(A-C) Good demonstration of NEQAS samples stained with the Leica Biosystems RTU ALK 5A4 (1:20) used on a Ventana Benchmark CT and Ventana Optiview detection. (A) Sample 'A' ALK IHC +ve. (B) Sample 'B' AL:K IHC negative. (C) Sample 'E' ALK IHC positive

(D & F) Participants' in-house (D) lymphoma and (F) appendix control placed alongside (E) NEQAS distributed sample 'E'. (D) Lymphoma control shows ALK IHC positivity but the corresponding (E) NEQAS sample 'E' shows very little ALK staining. (F) the drop in sensitivity id further see in the appendix control. Sample stained with the Novocastra 5A4 clone (1:50) on a Dako Autostainer and Dako flex+ detection.

(G-I) Good choice of control tissue for NSLC ALK IHC. (G) ALK positive NSCLC (H) ALK negtiave NSCLC and (I) Appendix showing ALK positivity in ganglion cells and axons. Stained with the Roche D5F3 assay on a Ventana Benchmark XT using CC1 92 minutes antigen retrieval and Optiview detection.

# The HER2 ISH Module - Interpretive Run 39

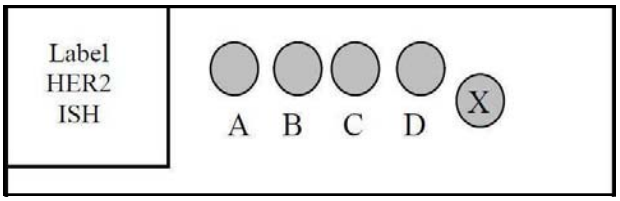
**Merdol Ibrahim and Suzanne Parry**

| | |
|---|---|
| **Gene Assessed:** | HER2 |
| **Sections Circulated:** | Composite slide consisting of 4 breast carcinoma sections (see table below) |
| **Number of Registered Participants:** | 188 |
| **Number of Participants This Run** | 118 (63%) |

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

| Sample | HER2 status by IHC | HER2 status by FISH |
|---|---|---|
| **A** | **2+** | Non-amplified |
| **B** | **2+** | Amplified |
| **C*** | **1+** | Non-amplified |
| **D** | **1+** | Non-Amplified |
| **X**** | **3+** | Amplified |

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




* 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

## 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.

## Updated 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

Similarly to the previous assessments, all participants taking part in the module are using a dual probe (ratio) method. 60% of labs are using the fluorescence ISH technique, which is similar to the previous Run (38). The Pathvysion Vysis kit still remains the most popular FISH method, and this was used by 38 labs. The Ventana DDISH was the most favoured chromogenic ISH technique, which was used by 40 labs (80% of CISH method users).

Overall the acceptable pass rate for all participants was 78% (≥30/36). A further 17% received a borderline pass (24-29/36), and 5 labs (4%) failed the assessment (scores of <24/36). The acceptable pass rate for the FISH users was 74% compared to 48% for those labs using the chromogenic method. The Ventana DDISH method alone showed a pass rate of 71%, which was higher than the previous run where only 51% of participants using this kit achieved an acceptable pass in the assessment. In comparison the FISH Pathvysion Vysis kit showed a 84% acceptable pass rate. The Dako IQFISH Pharm DX (n=9) and the Leica FISH (N=9) both showed good pass rates 100% . Please see graphical representation and methods used for a detailed breakdown of results.

## 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.

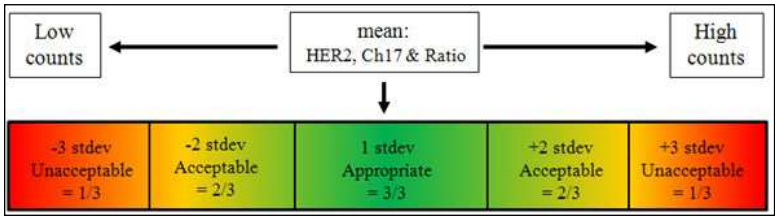
**Note: Sample 'C' has been voided in the interpretive ISH procedure and all participants have been awarded a default 'pass' score. This sample distributed as a non-amplified sample; however some of the distributed slides showed areas of high amplification only. It was therefore not possible to accurately determine a mean value from the collated HER2 or Cen17 results.**

## 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.

**Table: Statistical Approach to the Interpretive ISH Scoring System**

1. All participant data is initially evaluated to exclude '**outliers**'. An outlier is defined as those scores that are 1.5 times the interquartile range (IQR) outside of the lower and upper quartiles.
2. A mean score is then generated for the submitted HER2 copy, chromosome 17 and ratios.
3. Counts for HER2 copy, Cen17 and ratio are scored individually such that results within +/- 1 standard deviation (stdev) of the mean are score 3/3, +/- 2 stdev = 2/3 and ≥ +/- 3 stdev = 1/3. A non submission is scored 0/3.
4. For each of the samples there is therefore a possible score out of 9 (those using a ratio method) and 3 (those using a single copy method). As NEQAS ICC & ISH distributes 4 samples there is a possible score out of 36 (those using a ratio method) and 12 (those using a single copy method). (score = 3).




**Dual Probe: Ratio Scoring Method**

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

**Single Probe: HER2 Copy Scoring Method**

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

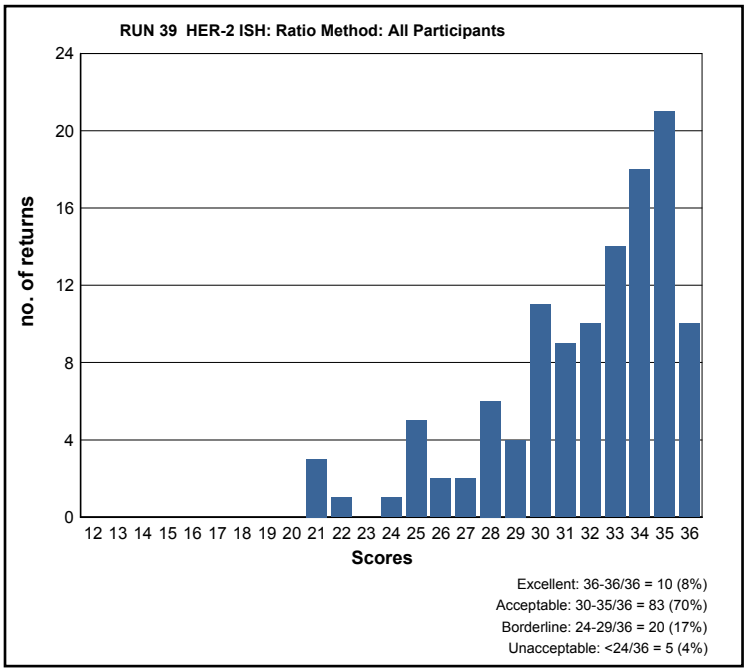
**References**

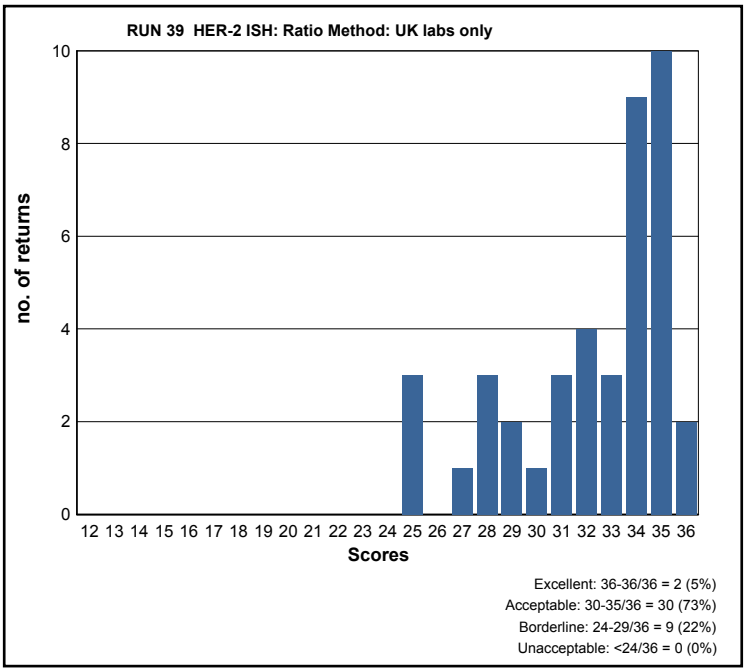
1. Walker RA, Bartlett JM, Dowsett M, Ellis IO, Hanby AM, Jasani B, Miller K. Pinder SE. (2008) HER2 testing in the UK: further update recommendations. J Clin Pathol;61818-824.
2. Bartlett JM, Starczynski J, Atkey N, Kay E, O'Grady A, Gandy M, Ibrahim M, Jasani B, Ellis IO, Pinder SE, Walker RA. (2011)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

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

| | | Sample A: mean (stdev) | | | Sample B: mean (stdev) | | | Sample C Not assessed | | | Sample D: mean (stdev) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| METHOD | n= | Copy No | Cen17 | Ratio | Copy No | Cen17 | Ratio | | | | Copy No | Cen17 | Ratio |
| **Ratio: Pathvysion Vysis Kit** | 38 | 4.07 (1.18) | 2.64 (0.41) | 1.5 (0.33) | 4.24 (1.05) | 1.73 (0.34) | 2.44 (0.67) | | | | 2.85 (0.98) | 2.34 (0.47) | 1.22 (0.42) |
| **Ratio: Ventana DDISH (780/800-4422)** | 38 | 3.97 (1.12) | 2.25 (0.64) | 1.7 (0.62) | 4.25 (1.85) | 1.7 (0.6) | 2.65 (0.71) | | | | 2.89 (1.1) | 2.13 (0.77) | 1.35 (0.48) |
| **Ratio: Leica HER2 FISH TA9217** | 9 | 4.28 (0.32) | 2.6 (0.19) | 1.65 (0.1) | 4.82 (1.1) | 1.72 (0.16) | 2.78 (0.55) | | | | 2.94 (0.29) | 2.25 (0.26) | 1.31 (0.1) |
| **Ratio: Other - CISH** | 1 | 3.23 (n/a) | 1.58 (n/a) | 2.04 (n/a) | 5.15 (n/a) | 1.28 (n/a) | 4.02 (n/a) | | | | 2.68 (n/a) | 1.87 (n/a) | 1.43 (n/a) |
| **Ratio: Dako IQFISH pharmDX** | 9 | 4.4 (0.22) | 2.71 (0.32) | 1.65 (0.19) | 4.55 (0.8) | 1.79 (0.24) | 2.56 (0.45) | | | | 3.02 (0.32) | 2.38 (0.83) | 1.23 (0.07) |
| **Ratio: Dako Pharm Dx** | 3 | 3.18 (2.04) | 2.23 (1.29) | 1.85 (0.09) | 2.57 (1.3) | 1.55 (0.89) | 2.47 (0.66) | | | | 2.3 (0.31) | 2.11 (1.22) | 1.24 (0.08) |
| **Ratio: Kreatech Probes** | 4 | 3.88 (0.31) | 2.27 (0.06) | 1.4 (0.17) | 3.74 (1.26) | 1.7 (0.21) | 2.22 (0.42) | | | | 2.62 (0.1) | 2.06 (0.41) | 1.28 (0.3) |
| **Ratio: Zytovision ZytoLight** | 6 | 4.06 (0.68) | 2.7 (0.37) | 1.5 (0.1) | 4.73 (0.67) | 1.82 (0.75) | 2.49 (0.45) | | | | 2.96 (0.22) | 2.3 (0.26) | 1.27 (0.11) |
| **Ratio: Dako DuoCISH** | 1 | 3.2 (n/a) | 2.3 (n/a) | 1.4 (n/a) | 3.1 (n/a) | 1.9 (n/a) | 1.63 (n/a) | | | | 2.16 (n/a) | 2.05 (n/a) | 1.05 (n/a) |
| **Ratio: Ventana BDISH 800-098/505** | 3 | 4.12 (1.02) | 2.26 (0.16) | 1.66 (1.27) | 3.32 (0.38) | 1.53 (0.23) | 2.18 (0.03) | | | | 2.43 (0.55) | 2.02 (0.22) | 1.23 (0.45) |
| **Ratio: Zytovision ZytoDot 2C** | 1 | 3.16 (n/a) | 2.24 (n/a) | 1.41 (n/a) | 2.5 (n/a) | 2 (n/a) | 1.25 (n/a) | | | | 2.38 (n/a) | 1.6 (n/a) | 1.5 (n/a) |
| **Ratio: Ventana Inform Silver ISH** | 4 | 4.08 (2.5) | 2.2 (0.39) | 1.79 (1.08) | 3.29 (2.2) | 1.77 (0.24) | 1.9 (1.16) | | | | 2.76 (1.97) | 2.07 (1.27) | 1.3 (0.84) |
| **Ratio: Other - FISH** | 1 | 4.35 (n/a) | 2.95 (n/a) | 1.47 (n/a) | 2.18 (n/a) | 1.98 (n/a) | 1.1 (n/a) | | | | Nd (n/a) | 2.67 (n/a) | Nd (n/a) |

## GRAPHICAL REPRESENTATION OF PASS RATES (ratio methods only)




## METHODS USED and PASS RATES

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

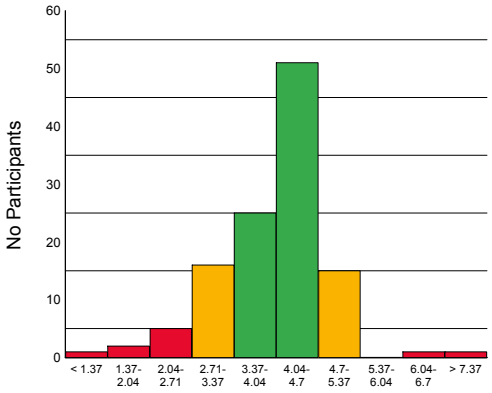
| Ratio Method | N | % Pass (score ≥ 30/36) |
|---|---|---|
| Ratio: Dako DuoCISH | 1 | 100% |
| Ratio: Dako IQFISH pharmDX | 9 | 100% |
| Ratio: Dako Pharm Dx | 3 | 33% |
| Ratio: Kreatech Probes | 4 | 100% |
| Ratio: Leica HER2 FISH TA9217 | 9 | 100% |
| Ratio: Other - CISH | 1 | 0% |
| Ratio: Other - FISH | 1 | 0% |
| Ratio: Pathvysion Vysis Kit | 38 | 84% |
| Ratio: Ventana BDISH 800-098/505 | 3 | 67% |
| Ratio: Ventana DDISH (780/800-4422) | 38 | 71% |
| Ratio: Ventana Inform Silver ISH | 4 | 50% |
| Ratio: Zytovision ZytoDot 2C | 1 | 0% |
| Ratio: Zytovision ZytoLight | 6 | 100% |

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

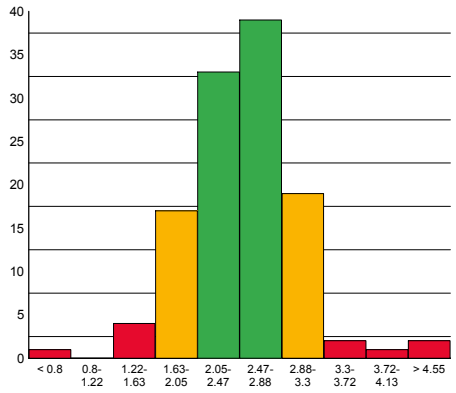
## FREQUENCY HISTOGRAMS

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

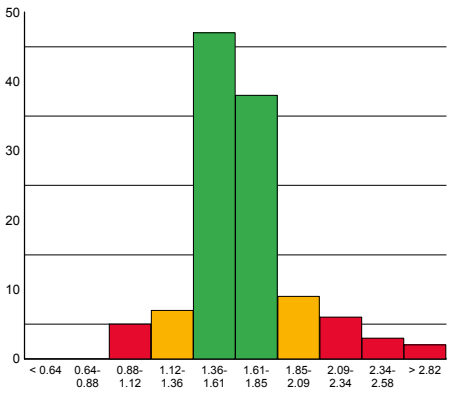
Green = mean +/- 1 Stdev  Amber = mean +/- 2 Stdev  Red = mean ≥ +/- 3 stdev



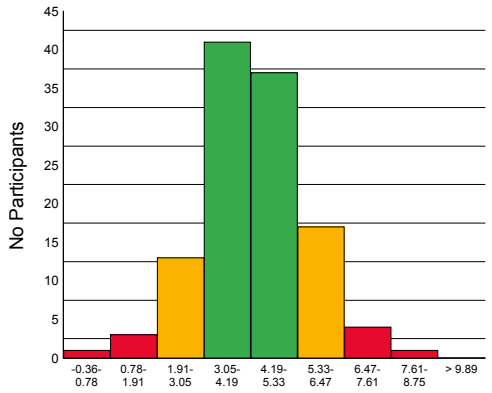

Sample A: Av. HER2 copy



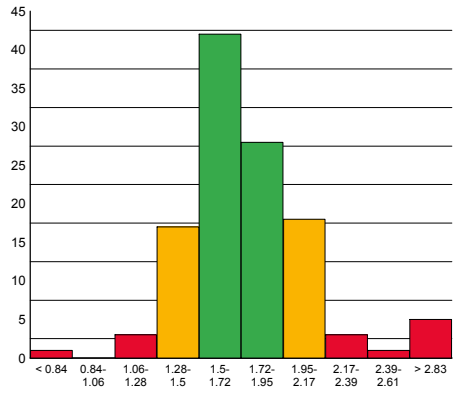

Sample A: Av. Chr17



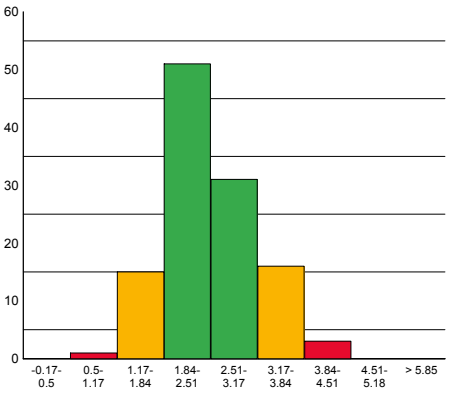

Sample A: Ratio




Sample B: Av. HER2 copy



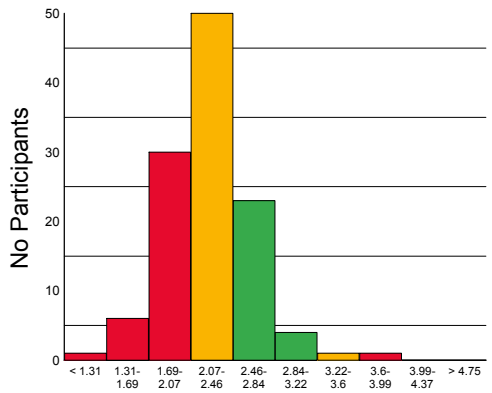

Sample B: Av. Chr17



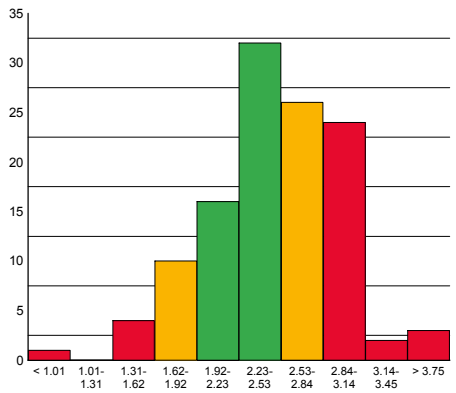

Sample B: Ratio

**Note**

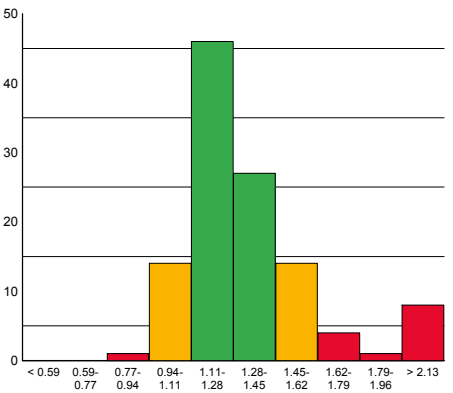
Sample `C' was distributed as a non-amplified sample; however some of the distributed slides showed areas of high amplification only. It was therefore not possible to accurately determine a mean value from the collated HER2 or Chr17 results. Sample `C' has been voided in the interpretive ISH procedure and all participants have been awarded a default `pass' score.




Sample D: Av. HER2 copy




Sample D: Av. Chr17




Sample D: Ratio

# The HER2 ISH Module - Technical (Pilot) Run 39

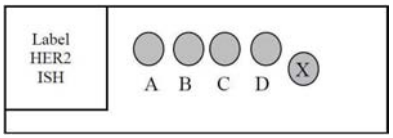
**Merdol Ibrahim and Suzanne Parry**

| | |
|---|---|
| **Gene Assessed:** | HER2 |
| **Sections circulated:** | Composite slide consisting of 4 breast carcinoma sections (see table below) |
| **Number of Registered Participants:** | 188 |
| **Number of Participants Taking Part this Run** | 110 (59%) (60 Fluorescent and 50 Chromogenic) |

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

| Sample | HER2 status by IHC | HER2 status by FISH |
|---|---|---|
| **A** | **2+** | Non-amplified |
| **B** | **2+** | Amplified |
| **C*** | **1+** | Non-amplified |
| **D** | **1+** | Non-Amplified |
| **X**** | **3+** | 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.**

## Results Summary

### CISH Results

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

The overall results from the CISH technical assessment saw an improvement from the previous Run (38): 50% of labs received an acceptable pass, compared to 28% in Run 38, a further 38% achieved a borderline pass in the current run. 6 labs (12%) failed the assessment, but this was a very good improvement on the previous run where 38% 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 over the last year. Another reason for failure was due to excessive signals obscuring the nuclei and signals outside of the nuclei, which made the sections uninterpretable. The borderline passes were mainly marked down due to weak, but still readable signals; again, mostly due to weak Cen17 signals. Some slides showed dust-like silver chromogen signals, but unless obscuring the specific HER2 signals and the slide was deemed still readable, the section did not fail, but was given a borderline pass. These observational results refer mainly to the Ventana BDISH or 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 a few labs, but the data has only collected the results from 1 lab as the other participants using this kit did not submit their methodology details. However, these slides were recognised as being stained with the Dako DuoCISH kit by the assessment team as the staining is very distinct: The HER2 signals are red and the Cen17 signals are blue. The cytoplasm also has a very red blush which is inherent of this method. The data from the 1 lab shown in the report graphs show that this lab achieved an acceptable level of staining. The Zytovision ZytoDot 2C method

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

| **Scoring Interpretation** | |
|---|---|
| **Acceptable**<br><br>Individual Assessor 4-5/5<br>or<br>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**<br><br>Individual Assessor 3/5<br>or<br>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:<br>Excessive / Weak Nuclear staining<br>Level of probe hybridisation<br>Excessive background staining<br>**Also see assessor comments on your report** |
| **Unacceptable**<br><br>Individual Assessor 1-2/5<br>or<br>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:<br>Excessive / Weak Nuclear staining<br>Poor probe hybridisation<br>Missing Her2 copy no. / CEN 17<br>Excessive background staining<br>**Also see assessor comments on your report** |
| **Score = 0** | Slide Not submitted for assessment |

was also used by 1 lab, and this achieved a borderline pass because the signals were weak in some of the cores. This again is another CISH methodology recognised by the assessment team, as the HER2 signals are green and the Cen17 signals are red.

**Important: Whichever CISH methdology 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 7-12.

Overall the FISH results showed slightly lower rates than the previous assessment run (38): 62% of labs received an acceptable pass, compared to 71% in run 38, and a further 13% achieved a borderline, whereas only 9% received a borderline in run 38. The current run showed a fail rate of 25%, slightly higher to that of the previous run, and also much higher than that for the CISH assessment for this run.
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 52% of labs for this assessment. Labs using this method showed an acceptable pass rate of 32%, which was much lower than the previous assessment, where 61% of labs received an acceptable pass. A further 19 % of labs using the Vysis kit received a borderline, and 48% of Vysis users failed the assessment. The Dako IQFISH and the Leica FISH kits were the next popular choices of kits, with 15% of FISH submissions stained with the Dako IQFISH and 13% with the Leica kit. These showed very good acceptable pass rates of 89% and 100% respectively. A variety of other kits were used, including the Dako Pharm Dx, Kreatech Probes and the Zytovision ZytoLight, all of which performed well, although the numbers of users of these kits is 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**<br>The NEQAS samples show a good standard of staining **and** have been interpreted correctly |
| Unacceptable | Acceptable | **Unacceptable**<br>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.<br>**Recommend that scoring/counting criteria is reviewed** |
| Appropriate or Acceptable | Borderline | **Borderline Acceptable**<br>The NEQAS samples are of borderline acceptability for staining quality.<br>**Recommend that technical method (kit/assay) is further optimised.** |
| Unacceptable | Borderline | **Unacceptable**<br>The technical staining can be improved as this may be effecting interpretation.<br>**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**<br>The NEQAS samples are unacceptable for interpretation and caution should be taken when interpreting from these slides.<br>**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**<br>The NEQAS samples are unacceptable for technical staining and interpretation.<br>**Reporting from such cases is very likely to lead to incorrect interpretation of clinical cases.**<br>**If there is persistent underperformance:**<br>• **seek assistance from kit/assay manufacturer**<br>• **seek assistance from UK NEQAS or colleagues**<br>• **re-validate protocol (retrospectively and prospectively)**<br>• **review scoring criteria**<br>• **send clinical cases to a reference centre to confirm your results** |

## Selected Images showing Optimal and Sub-optimal Immunostaining

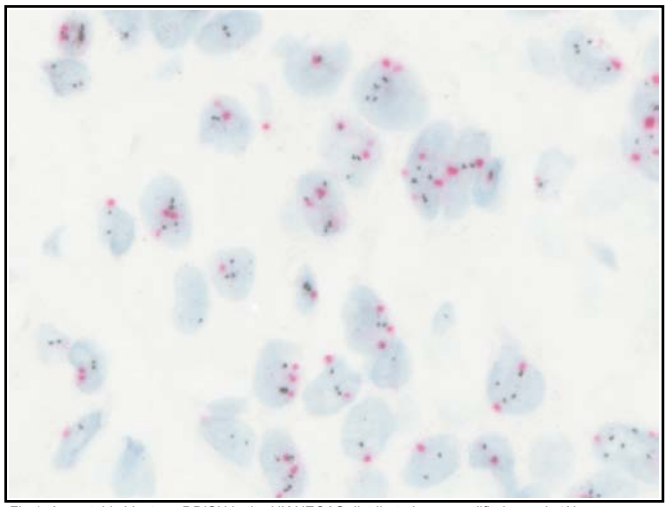


Fig 1. Acceptable Ventana DDISH in the UK NEQAS distributed non-amplified sample 'A' showing distinct HER2 signals (black) and Chr17 signals (red).

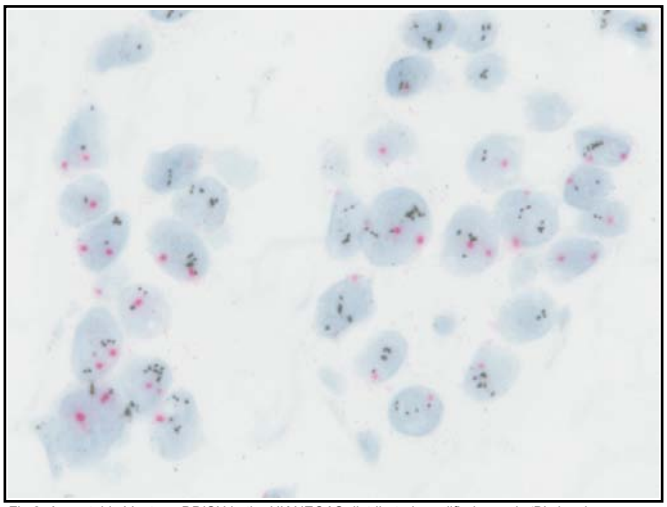


Fig 2. Acceptable Ventana DDISH in the UK NEQAS distributed amplified sample 'B' showing distinct HER2 signals (black) and Chr17 signals (red). This sample has an average ratio of 2.6 but an average copy number of 4.19

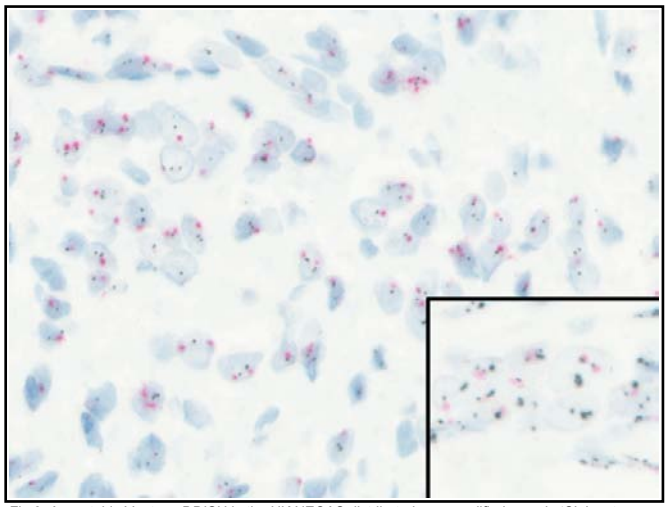


Fig 3. Acceptable Ventana DDISH in the UK NEQAS distributed non-amplified sample 'C'. Inset image shows some amplified nuclei which was noted on some of the distributed samples.

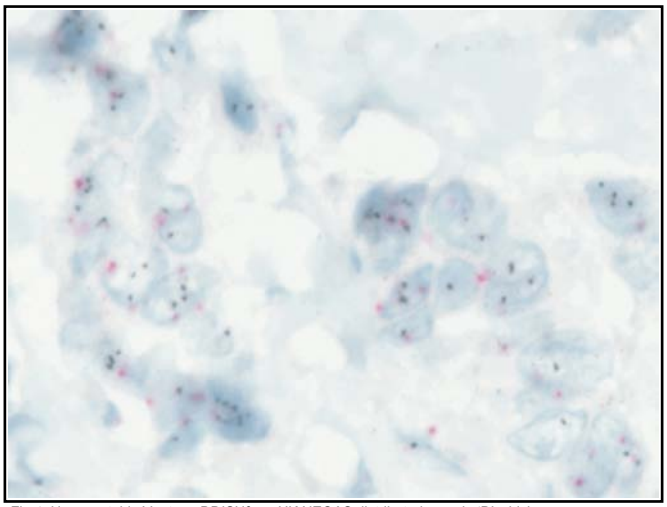


Fig 4. Unacceptable Ventana DDISHfrom UK NEQAS distributed sample 'D' which was a non-amplified case. Although some HER2 and Chr17 signals are present the sample appears to be over digested making the Chr17 unreliable for interpretation.

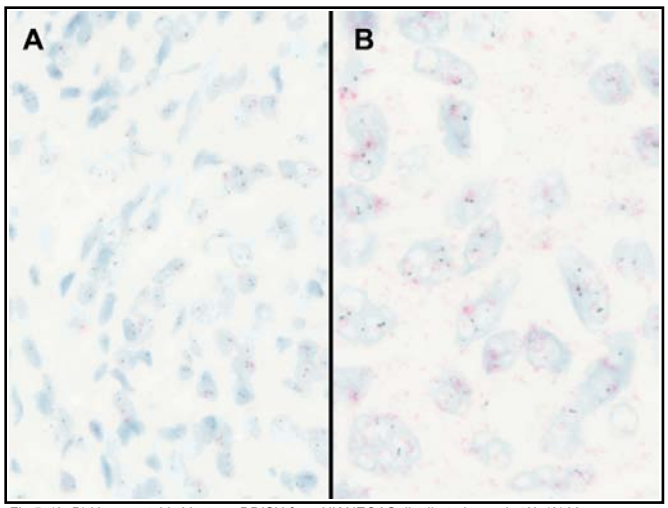



Fig 5. (A, B) Unacceptable Ventana DDISH from UK NEQAS distributed sample 'A'. (A) Very weak barely perceptible Chr17 signal and (B) Leeching of Chr 17 signal. Both examples were deemed unreliable for interpretation.

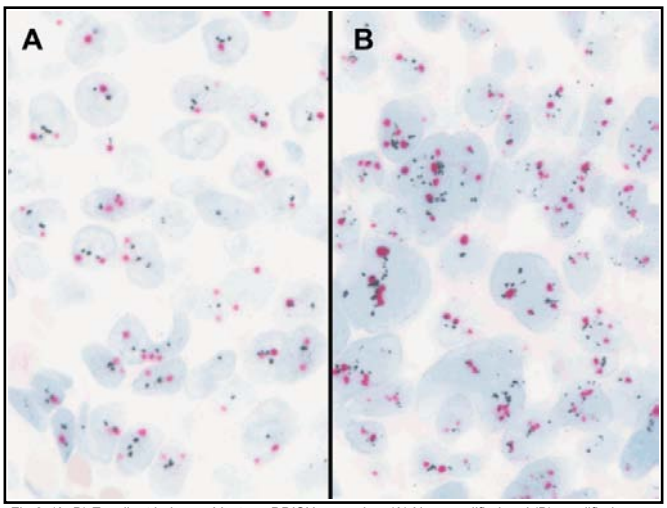



Fig 6. (A, B) Excellent in-house Ventana DDISH examples. (A) Non-amplified and (B) amplified case, with each showing good HER2 (black) and Chr17 (red) signals.

## Selected Images showing Optimal and Sub-optimal Immunostaining

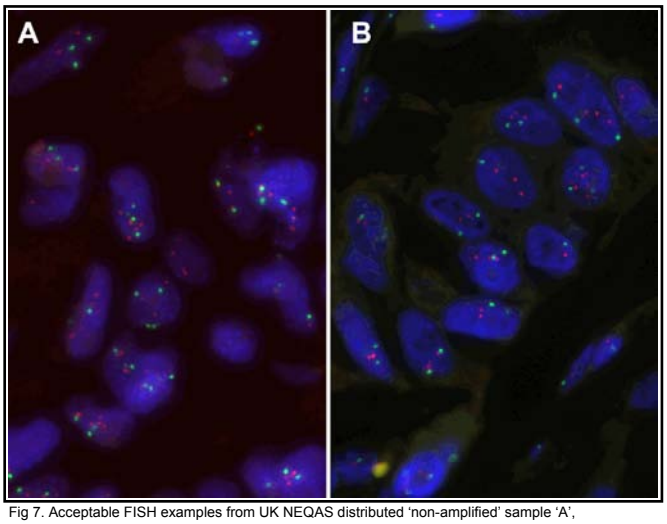



Fig 7. Acceptable FISH examples from UK NEQAS distributed 'non-amplified' sample 'A', stained using, (A) Vysis and (B) Leica probes. Both samples demonstrate distinct HER2 signals (red) and Chr17 signals (green).

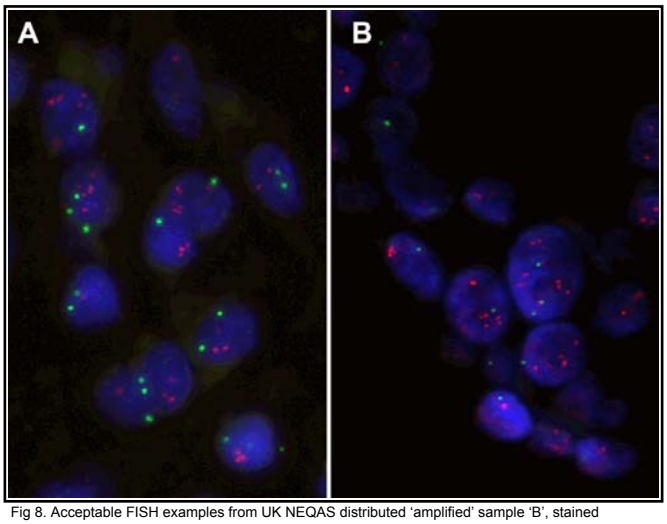



Fig 8. Acceptable FISH examples from UK NEQAS distributed 'amplified' sample 'B', stained using, (A) Vysis and (B) Dako Pharm DX. Both samples demonstrate distinct HER2 signals (red) and Chr17 signals (green).

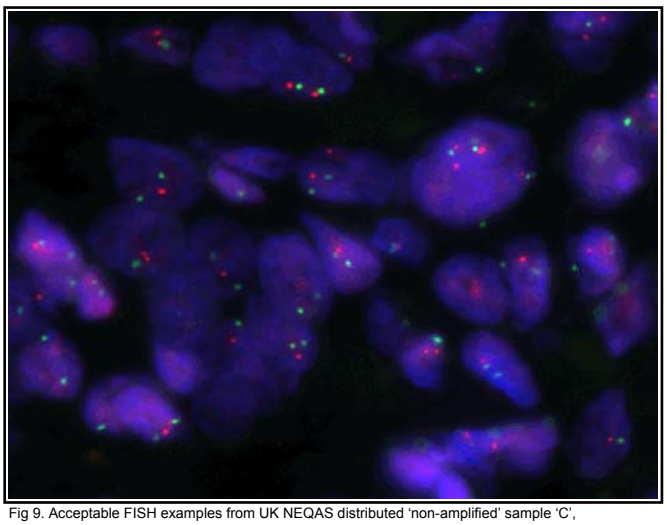


Fig 9. Acceptable FISH examples from UK NEQAS distributed 'non-amplified' sample 'C', stained using the Dako Pharm DX with distinct HER2 signals (red) and Chr17 signals (green).

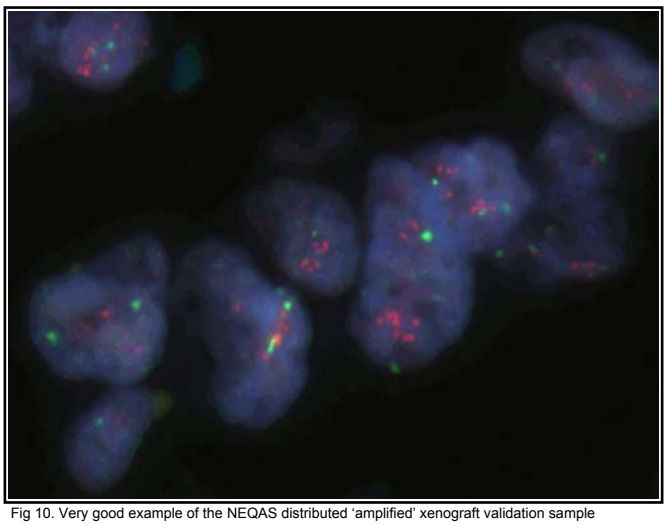


Fig 10. Very good example of the NEQAS distributed 'amplified' xenograft validation sample (sample E) hybridised using the Dako Pharm. HER2 signals (red) and Chr17 signals (green).

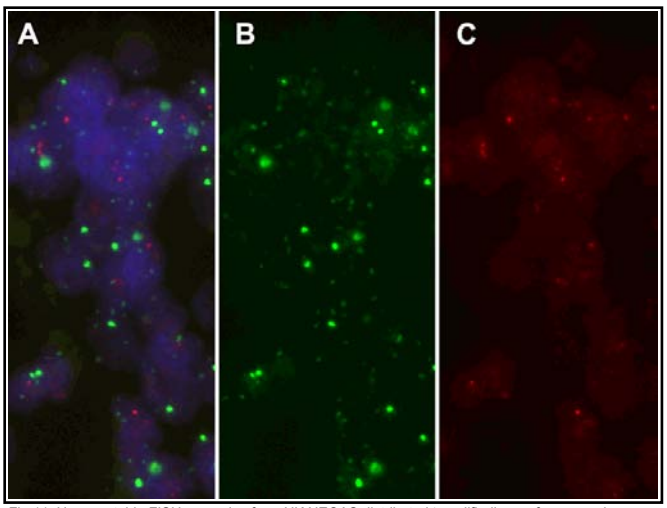



Fig 11. Unacceptable FISH examples from UK NEQAS distributed 'amplified' case from sample 'B', stained using Vysis FISH. (A) Combined triple label. (B) Excessive Chr17 signal within the nuclei making it difficult to identify 'true' green signal (C) Acceptable Her2 signal.

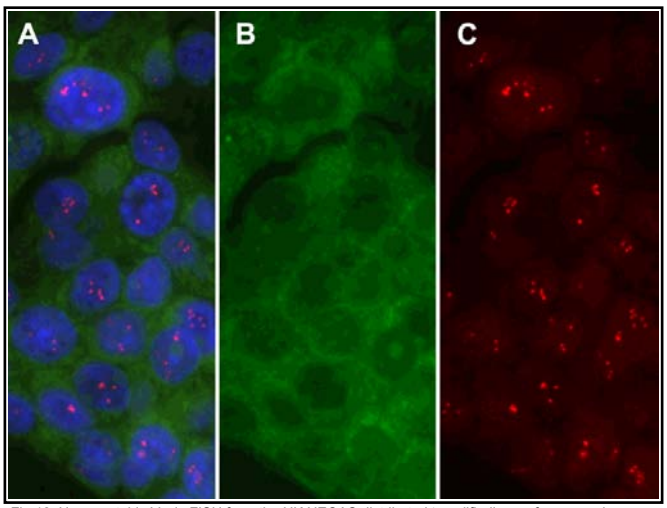
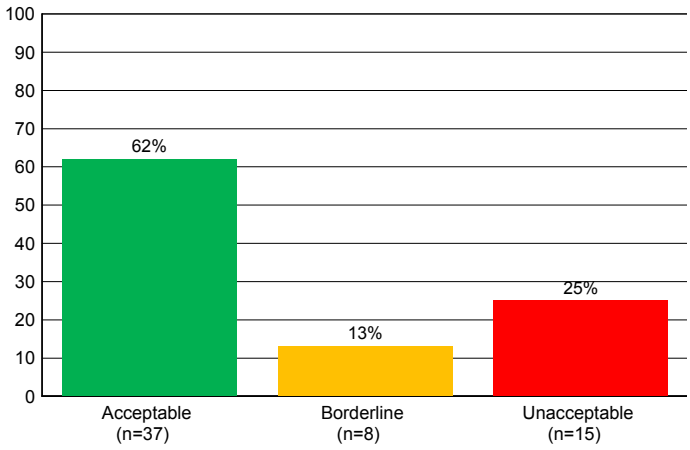



Fig 12. Unacceptable Vysis FISH from the UK NEQAS distributed 'amplified' case from sample 'B'. (A) Combined triple label. (B) Absence of Chr17 signal. (C) Distinct Her2 signal. This sample was deemed unreliable for interpretation.

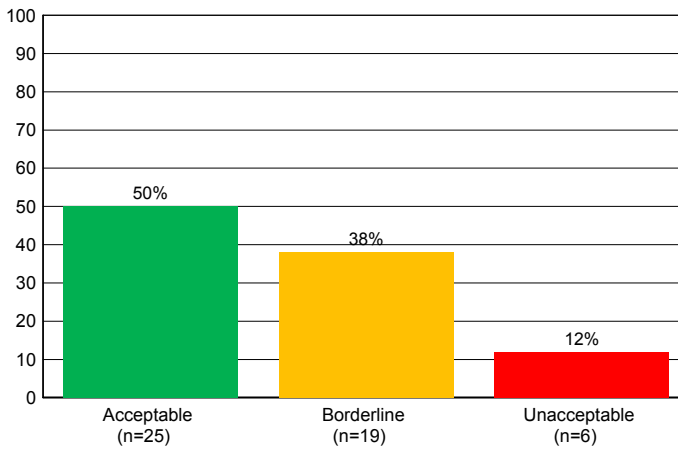
# Technical ISH: Pass Rates and Methods

## Overall Pass Rates

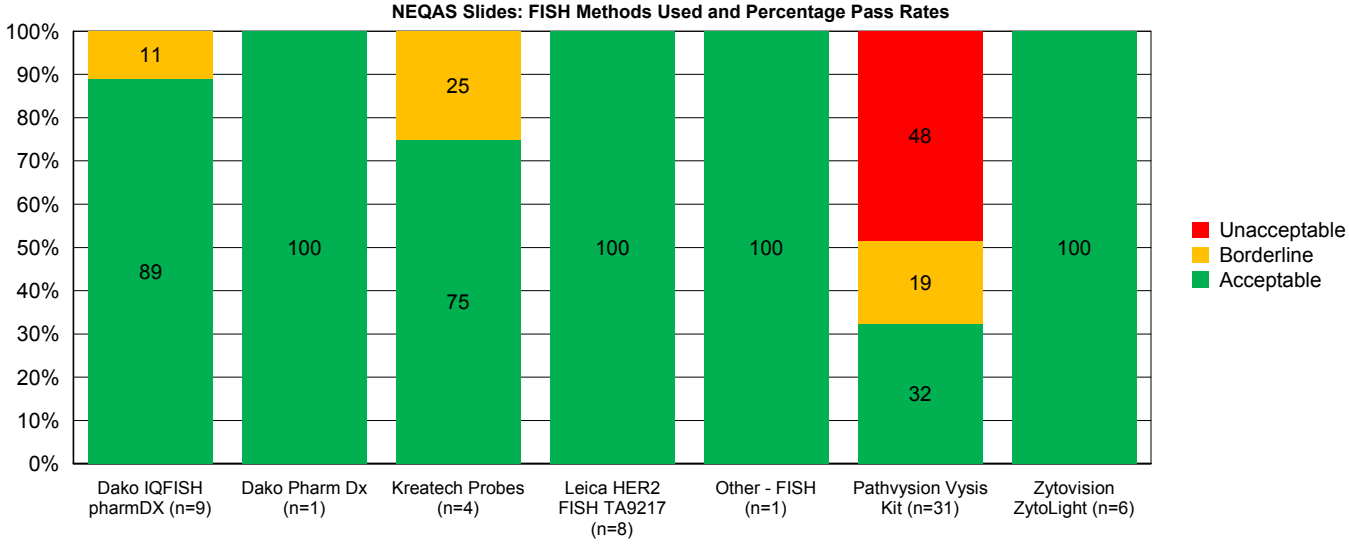
### FISH NEQAS slide (n=60)



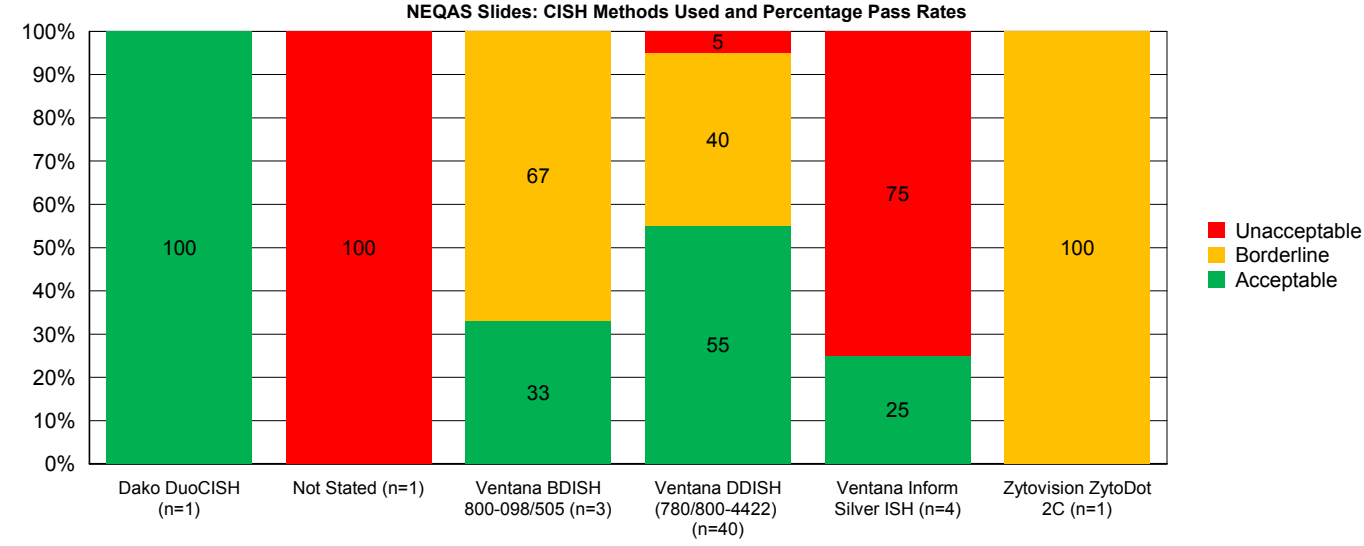

### CISH NEQAS Slide (n=50)




### NEQAS Slides: FISH Methods Used and Percentage Pass Rates




### NEQAS Slides: CISH Methods Used and Percentage Pass Rates



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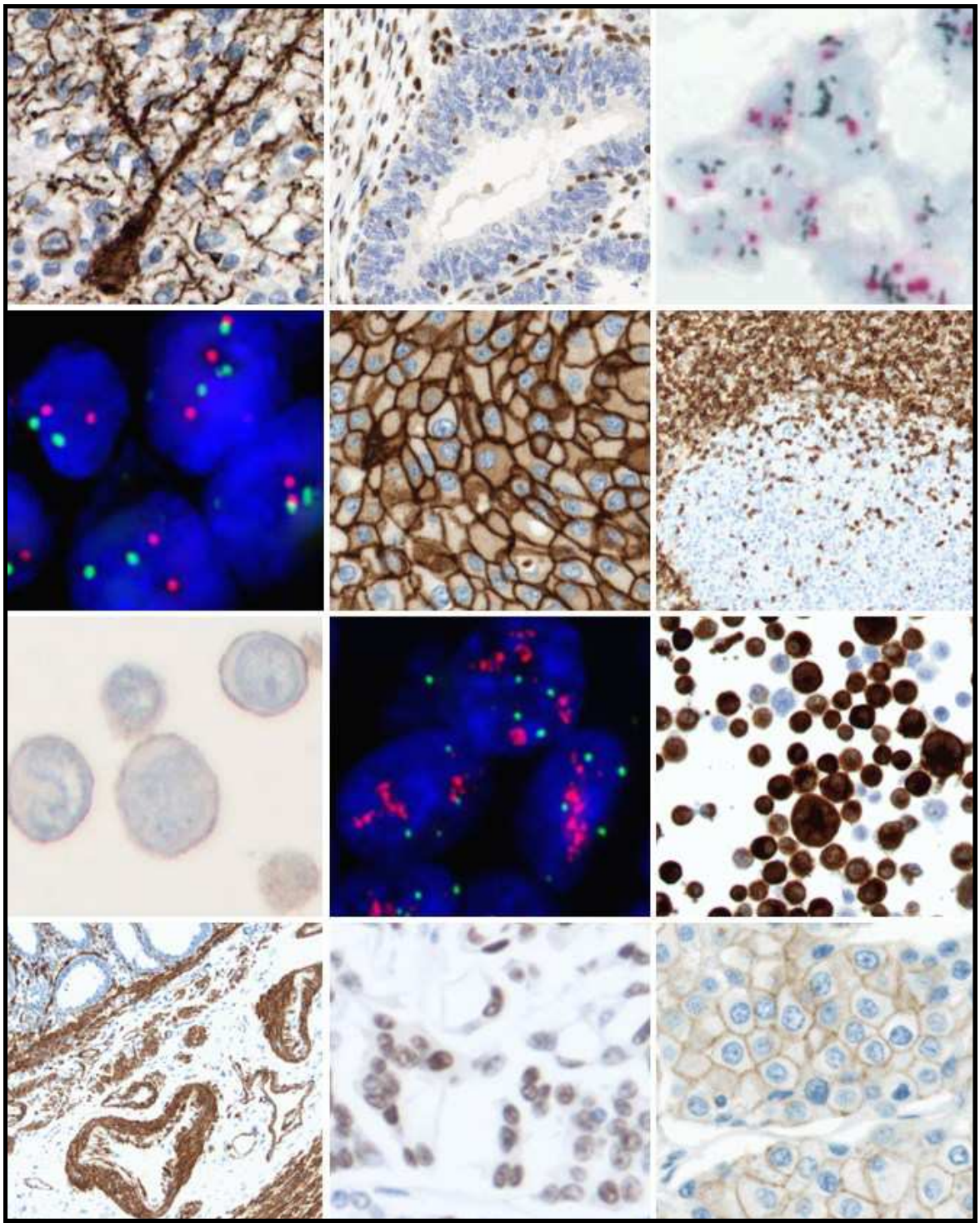

**For advertising opportunities contact:**
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