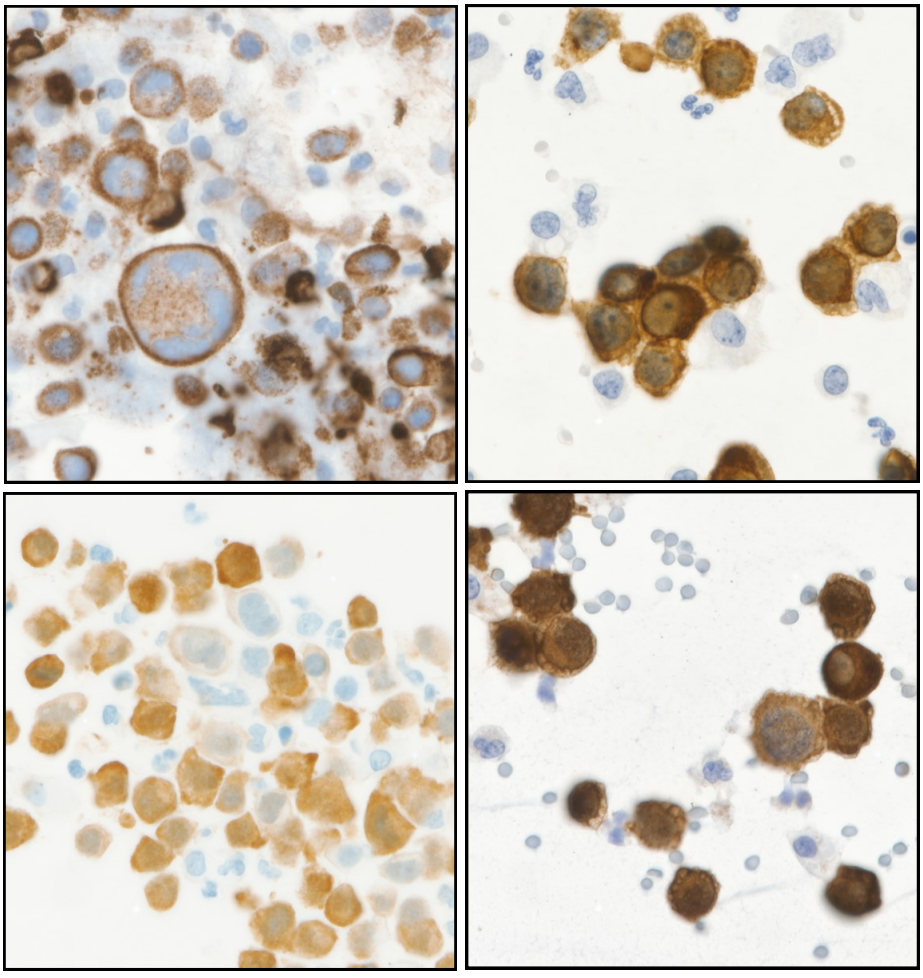
UK NEQAS
International Quality Expertise

Immunocytochemistry

Improving Immunocytochemistry for Over 25 Years

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

Assessment Dates: 27th March — 13th April 2017



Cover Photo: Taken from the : Cytopathology Module

Top Left: Good Cytokeratin demonstration on a NEQAS cell block sample (R)
Top Right: Optimal Cytokeratin demonstration on a NEQAS cytospin sample (R)
Bottom Left: Excellent Calretinin staining on a NEQAS cell block section (T)
Bottom Right: Sub-optimal Calretinin staining on a NEQAS cytospin preparation (T)

Articles / Reports

Immunocytochemistry Modules

In situ Hybridisation Modules

Click on sponsor logos below to go straight to the sponsor webpage

Also In This Issue

General Information

UK NEQAS
International Quality Expertise

7833

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.

UK NEQAS ICC & ISH does not endorse any of the products featured by the commercial sponsors and are placed at the discretion of UK NEQAS ICC & ISH. Furthermore, commercial companies featured do not have any input or influence over the content, including results that are shown.

For further information of the UK NEQAS ICC & ISH scheme, general EQA enquiries, slide returns and advertising opportunities please contact:

Suzanne Parry,
Acting Scheme Manager
UK NEQAS ICC & ISH
Room 127, Finsbury Business Centre
40 Bowling Green Lane
London EC1R 0NE
Tel: +44 (2) 07 415 7065
Fax: +44 (2) 07 415 7048
E-mail: s.parry@ucl.ac.uk

For enquiries concerning training issues, meetings, or courses, please contact:

Mr Keith Miller, Scheme Director
Cancer Diagnostic Quality Assurance Services CIC
Poundbury Cancer Institute
Newborough House, 3 Queen Mother Square
Poundbury, Dorchester
Dorset DT1 3RZ
United Kingdom
Tel: 07548795116
E-mail: k.miller@ucl.ac.uk

ASSESSORS

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

United Kingdom
Mr C Abbott, Bath
Mr D Allen, London
Prof M Arends, Edinburgh
Mr N Bilbe, London
Mr D Blythe, Leeds
Ms A Brown, London
Ms A Clayton, Preston
Dr C Cardozo, Preston
Dr L Carson, Aberdeen
Mr A Dodson, London
Mr I Downie, Glasgow
Mr R Fincham, Cambridge
Ms J Freeman, London
Ms L Govan, Glasgow
Mr J Gregory, Birmingham
Mr N Hand, Nottingham
Ms L Happerfield, Cambridge
Dr C Hawkes, Nottingham
Ms S Jordan, London
Dr J Joseph, Preston
Mr J Linares, London
Ms J MacMillan, Glasgow
Mr C Marsh, Newcastle
Ms H McBride, Belfast
Mr K Miller, London
Ms A Newman, London
Ms S Parry, London
Ms A Patterson, Belfast
Dr M Pitt, Cambridge
Mr G Rock, Birmingham
Mr A Shore, London
Dr J Starczynski, Birmingham
Dr P Taniere, Birmingham
Ms C Thomas, Preston
Dr P Wencyk, Nottingham
Mrs D Wilkinson, London

Germany
Dr I Nagelmeier, Kassel

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

Portugal
Mr R Roque, Lisbon

Slovenia
Mr D Vidovic, Ptuj

Switzerland
Dr P-A Dernier, St. Gallen

Journal layout and design prepared by UK NEQAS ICC & ISH

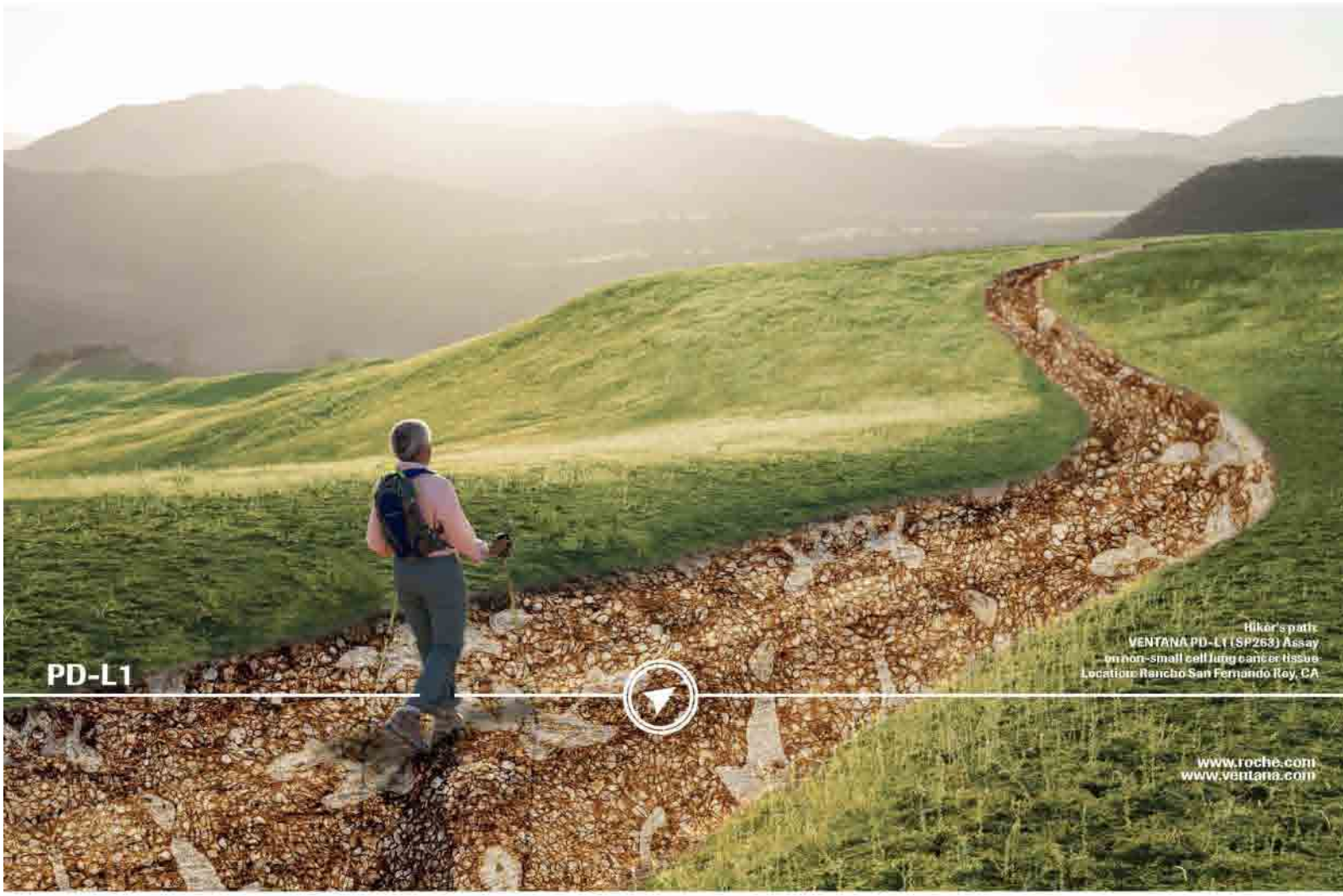
Guiding immunotherapy

VENTANA PD-L1 (SP263) Assay

A new option for identifying NSCLC patients most likely to benefit from immunotherapy.

Empowering pathologists to answer PD-L1 questions by

- Identifying NSCLC patients most likely to respond to immunotherapy
- Providing robust PD-L1 staining in both tumor cells (TC) and tumor-infiltrating immune cells (IC)
- Allowing optimal lung tissue management through PD-L1 testing of archived unstained slides within 12 months



For healthcare professional use only.
Roche Diagnostics Limited. Charles Avenue, Burgess Hill, West Sussex, RH15 9RY, United Kingdom.
Registration number 571546. Date of Preparation: November 2016. Material No: 08176230001

UK NEQAS ICC & ISH User Satisfaction Survey: 2017

Seema Dhanjal, Neil Bilbe and Keith Miller

Overview

At June 2017, approximately **450** active participants were registered with UK NEQAS ICC & ISH, a reduction of around **100** (20%) from the previous year. Excluding any central contacts or agents, and new laboratories (**15)** recently joining the scheme, a total of **441** labs were emailed, with a link to the online survey. The survey was open for 4 weeks – the closing date was chosen as 1st July 2017 to allow time for review prior to the 2017 AMR.

Regional breakdown (441 Labs):
UK & Eire (UK): 209 Labs – 47% of registered participants
Rest of World (OS): 232 Labs – 53% of registrants

At the start of the survey, there were 4 mandatory fields to complete:

- Participant Code
- Region (UK & Eire or ROW)
- Modules participated in Last Year (Runs: 114 -117)
- Cytology sample type if this module was selected

There were 31 questions/sections. Respondents were asked to give a rating of **Very Satisfied, Satisfied, Neutral, Dissatisfied, or Very Dissatisfied** to the first 16 questions; Q.17 asked for the reason for participation in UK NEQAS ICC & ISH, Q.18 how likely they are to continue to use our service; Q.19 to 24, required a **Yes or No** type response.
Q. 25 asked participants to rate the quality of the EQA material by sample type: a) tissue sections and cell lines, b) cell blocks and cytospins (cytology module only)
Q. 26 asked if a change in methods based on EQA results had been made
Q. 27 asked whether any reassessments requests had been made.

The next three questions (Q. 28, 29, 30) asked for the rating of our service; firstly by level of satisfaction, secondly, with an overall score out of 10, and thirdly by individual modules subscribed to, also out of 10. The final question (Q. 31) asked if participants were satisfied with the range of antibodies offered, which was a new question this year.
At the end of each section, or following some individual questions, there were comments sections allowing participants to express their views.

Return details

Over 140 responses were received; any duplicate, unusable, or multiple entries were removed, we contacted any labs submitting incomplete surveys to ask for a completed form. A final total of **136** replies for analysis were received.

The overall response rate to the survey was therefore approximately 31%, a small increase from last year.

74 UK & Eire labs responded (**54%)** out of a total of 209 labs registered in the UK & Eire which represents **35%** of all registered UK & Eire labs.
62 Non-UK & Eire labs replied **(46%)** out of a total of 332 registered with the scheme which represents **27%** of all registered OS labs.

Overall analysis of satisfaction

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

- Very Satisfied (43) 31.6%
- Satisfied (84) 61.8%
- Neutral (8) 5.9%
- Dissatisfied (1) 0.7%

Effectively, over 93% of users were either Very Satisfied or Satisfied with the service; 5.9% responded to being Neutral. This year there was one Dissatisfied response.

Response by region 2017:

2017	UK (N=74)	OS (N=62)
Very Satisfied	23 (31%)	20 (32.2%)
Satisfied	46 (62.1%)	38 (61.2%)
Neutral	4 (5.4%)	4 (6.4%)
Very/Dissatisfied	1 (1.3%)	0 (0%)

Comparisons with data from 2016 Survey

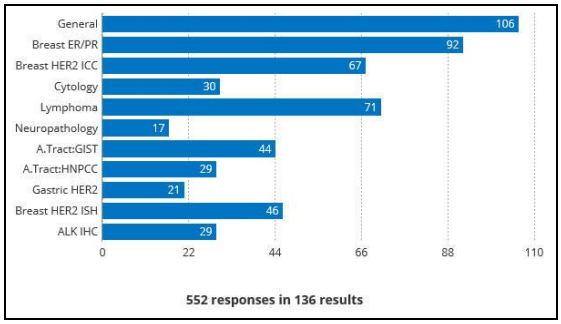
2016	UK (N=79)	OS (N=64)
Very Satisfied	26 (32.9%)	18 (28.1%)
Satisfied	50 (63.3%)	43 (67.2%)
Neutral	3 (3.8%)	3 (4.7%)
Very/Dissatisfied	0 (0%)	0 (0%)

Responses	2016 (%)	2017 (%)
Very Satisfied	30.8	31.6
Satisfied	65	61.8
Neutral	4.2	5.9
Dissatisfied	0	0.7

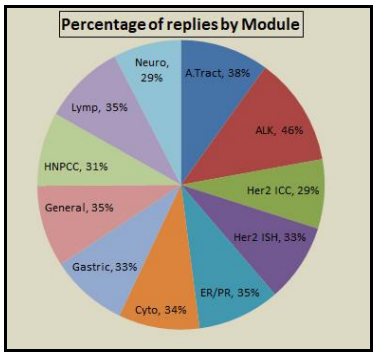
The overall levels of satisfaction remain *relatively* unchanged, but there has been a small increase in the number of Very Satisfied (↑ 0.8%) responses. There was a decrease in the levels of satisfied replies (↓ 3.2%), a small increase in neutral responses (↑ 1.7%) plus the single dissatisfied response.
There appears to have been a drop in the level of UK Very Satisfied responses of 1.9%, but a 4.1% increase from overseas (OS) participants.

Some module related data

The average number of modules that all registered labs participate in is approximately 3.5 The number for those actually responding is 4: The combined number of modules for all labs responding was 552 (136 replies), the number of labs for each module can be seen in the table below:



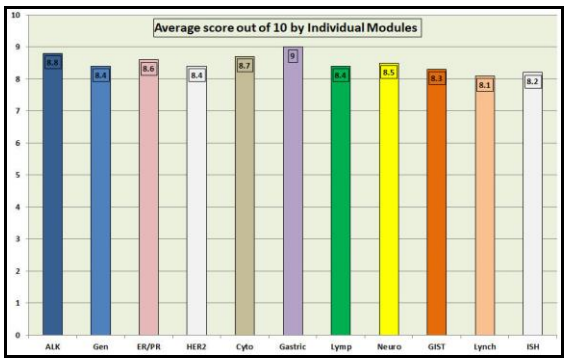
The actual percentage of labs registered for each module who responded was:



Average of 34% of labs registered for a particular module responded, with a median of 34%. Lowest response 29% (Neuro & Her2 ISH), to the highest 46% (ALK).

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

The overall average score (Q.29) was 8.1, (8.2 last year), this was unrelated to any specific modules.



The average the scores from all the *individual* modules (Q. 30) is 8.5, a slight increase from last year (8.3). Interstingly for the Cytology module both the cell block (CB) users and cytospins users (CS) averaged 8.7.
The lowest average score was 8.1 (Lynch Syndrome) and the highest of 9 (Gastric).
The biggest *increases* in score were for the Gastric (1.0), from 8 last year to 9 this year, and the ER/PR from 8.2 (2016) to 8.6 (2017). For all the other modules the changes were minor ($^{+}/_{-}$ 0.1 or 0.2), with more modules increasing their score, albeit marginally, and essentially ratings for the scheme and modules are consistent year on year.

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.

This year the method employed was slightly different from previous surveys, the percentage of non-satisfied responses was derived from Q. 1- 13; the final 3 questions (Q. 14 – 16) relating to UK NEQAS ICC & ISH meetings was filtered to only include those that attended last year's meeting(s). The overall level of dissatisfaction was 3.4% a slight increase (3.2%), but in line with previous years.

Levels of dissatisfaction 2016

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

- Turnaround times: increased to 8.9% from 8.5%
- Assessor comments: decreased again to 8.1% from 11.3%
- Time to stain samples: decreased from 6.3% to 3%
- The labelling of slides: decreased from 3.5% to 0.7%

The other main area where there was a general decreased level of participant satisfaction were the questions relating to: technical help, communication, dealing with enquiries and contacting the scheme. These averaged 1.2% last year but now are 3.1%.
The three questions relating to UK NEQAS ICC & ISH meetings (Q. 13 – 16) only had 38 respondents with 2 (1.8%) dissatisfied replies, both relating to the location of the meetings. There were no adverse comments about meeting content or frequency.

Quality of material provided by UK NEQAS

We asked the participants to give us feedback on the quality of the EQA material/samples provided. This was not module specific, although cell lines are only used on a few modules (ALK and Breast Her2), the two questions relating to cytology material (cytospins and cell blocks) were only visible to those who selected Cytology at the beginning of the survey, and was filtered depending on their sample type: Cell Blocks or Cytospins so that there was no chance of the wrong sample being selected.

2016 Quality of Material

	FFPE (n=141)	Cell Lines (n=93)	Cytospins (n=23)	Cell Blocks (n=22)	Ave
Excellent	7.1%	15.1%	4.3%	9.1%	8.9%
Very Good	41.1%	44.1%	65.2%	59.1%	52.4%
Good/Acceptable	46.8%	36.6%	30.4%	31.8%	36.4%
Poor	5.0%	3.2%	0.0%	0.0%	2.0%
Very Poor	0.0%	1.1%	0.0%	0.0%	0.3%

2017 Quality of Material

	FFPE (n=133)	Cell Lines (n=88)	Cytospins (n=9)	Cell Blocks (n=21)	Ave
Excellent	9.0%	18.2%	8.3%	17.3%	13.2%
Very Good	36.8%	39.8%	58.3%	47.8%	45.7%
Good/Acceptable	50.4%	40.9%	0.0%	26.0%	29.3%
Poor	3.0%	1.1%	8.3%	0.0%	3.1%
Very Poor	0.8%	0.0%	0.0%	0.0%	0.2%

There appears to be a general trend upwards in terms of the overall quality of material, when comparing the 2016 and 2017 results. The one blip is that there was a single cytology cytospin user, who returned a response of Poor. As there were only 9 cytospin users this equates to 8.3%.

When trying to equate quality of material with individual modules, a cross tabulation, or by applying module filters gives a good indication, although this is not in response to a question for each specific module, as most labs subscribe to 3 or 4 different modules, and the number of participants varies:

Rating	General	Breast ER/PR	Breast HER2 ICC	Lymphoma	Neuropathology	A.Tract: GIST	A.Tract: Lynch	Gastric HER2	Breast HER2 ISH	ALK IHC (pilot)
Excellent	7%	7%	10%	4%	6%	5%	8%	14%	11%	14%
Very Good	34%	32%	25%	31%	47%	32%	28%	33%	33%	28%
Good/Acceptable	55%	58%	61%	63%	41%	59%	66%	52%	53%	59%
Poor	4%	3%	3%	0%	0%	2%	3%	0%	2%	0%
Very Poor	1%	1%	0%	1%	6%	2%	0%	0%	0%	0%

Overall, the biomarker type modules (Gastric Her2, ALK, and Breast Her2 ISH) have the highest level of 'Excellent' responses, though these modules are more likely to be subscribed to as a standalone than some of the generic modules: Lymphoma, Neuropathology and Alimentary Tract; this should be taken into account when viewing the table.

Range of antibodies offered by UK NEQAS ICC & ISH

A new question (Q. 31) was introduced to the survey this year: 'Are you satisfied with the range of antibodies offered?' This was in response to the comments and communication from participants following UKAS visits. This was set as a mandatory question.

87.5% of participants said that they were satisfied. When this is filtered by region; 95% of OS labs stated that they were satisfied, as against 81% of UK & Eire participants, but still an overwhelming majority. In terms of any module variance:

Highest satisfaction: Neuro: 94%

Lowest satisfaction: Lynch syndrome: 72%

Comments, suggestions, and feedback about the service

Comments were returned throughout the survey; these were collated and categorized into 11 main areas:

- Sample and results section (24 comments)
- Participant feedback and communication section (28)
- UK NEQAS ICC & ISH meetings section (8)
- Complaints about the service (9)
- Treatment of in-house samples and UK NEQAS ICC & ISH samples (18)
- Assessment of in-house controls (12)
- Quality of the UK NEQAS ICC & ISH EQA material (24)
- Use of EQA results to improve in-house staining (57)
- Reassessment requests (18)
- Range of antibodies offered (18)
- General comments and feedback about the service (27)

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

In total of **240** plus comments were received, a scheme reply and comments on some of these are given further down.

The main themes are summarised below:

- Not enough time between results and next run to tweak protocols
- Assessor comments are contradictory, probably due to stock comments – more free text would be better
- Scores of $^{4}I_{5}$ do not have comments
- More meetings (2) and locations outside of London
- Journal should be produced at the same time as the results
- No reply or late reply to queries and enquiries
- Slides received without any sections on
- Fixation differences between NEQAS and in-house
- Sections have been arriving scratched or damaged
- Many labs use their results to optimise methods
- Range of antibodies: General & Lymphoma
- UKAS comments
- Assessment of antibodies outside of runs
- *Plus lots of complimentary comments about the scheme*

Summary

This year's response level of 31% was encouraging, but may be due to the selective removal of any rogue responses and the fact that a large portion of labs who were perennial non respondents (commercially sponsored laboratories who often only submitted one or two runs per year) have now left the scheme.

There was a slight dip in the overall participant satisfaction level at c. 93%, from a consistent 95% in previous years. This is due to an increase in Neutral replies (↑ 1.7%) and the single Dissatisfied response, but the actual level of Very Satisfied respondents has increased to 31.6%, up from 30.8%. The difference is mainly due to the OS participants showing a shift of around 5% to Very Satisfied from Satisfied.

The results for the scores out of 10 are still of the same magnitude, with an 8.1 overall average for the question 'Please rate (out of 10) UK NEQAS ICC & ISH for overall quality and service to EQA and education? '

A quick survey of the results for individual modules shows slightly more tailored replies rather than allocating the same score for every module, which is encouraging.

The E-Journal again showed a slightly decreased satisfaction level at 81%, with comments about the time taken to publish it the main reason for dissatisfaction.

The sample quality rating shows an improvement from last year; having shown a decline in previous years.

Cross tabulation shows a wide range of results across the modules. Some had no adverse (Poor or Very Poor) responses (ALK, Gastric), the rest ranged from 1% (Lymphoma) to 7% (Cytology) with a median of 3%.
The overall Excellent rating across all samples was 13.2%, again up from last year of 8.9%.

UK v OS levels of dissatisfaction

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

Turnaround times:	10.8% (UK) v 6.4% (OS)
Assessor comments:	9.45 v 6.4%
Communication with scheme:	4% v 4.8%
Time given to stain/return:	1.3% v 4.8%
Information sent with material:	4% v 1.6%
Technical help and advice:	4% v 3.2%

The main findings, when comparing the regional responses is that there appears to be less disparity between UK and OS labs than last year, although Overseas labs appear much more satisfied than their UK counterparts in these particular areas.

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

1. *Communication and advice from the scheme*

We appreciate that on occasion the time taken to respond to participant queries has not been ideal. Due to the recent departure of two key staff members and limited availability of appropriate personnel to respond to some specific queries has meant that there has been some delays. The use of the info@ukneqasiccish.org email address, whilst being a useful way of contacting UK NEQAS ICC & ISH and is to be encouraged, the subject matter should be clearly stated in the message header to aid allocation of the query and therefore speed up the response.

2. *Time taken to produce the E-Journals*

The Journals are essentially a single record of the EQA Runs and all modules. Some parts of the Journal are available for the modules labs are subscribed to; e.g. images, graphs and best methods as part of their results and reports. The additional write ups and interpretations produced by UK NEQAS ICC & ISH technical staff are the time consuming sections. What we are looking to do is to produce a summary Journal within a few weeks of the assessments, which will not contain any in-depth analysis for each run and module. Participants will then have access to the overall results for all modules. A more detailed version will then follow at a later date.

3. *Problems with sections being damaged/scratched*

This is something that has been brought to our attention more over some of the preceding runs. The scheme is trying to pinpoint the main cause of this problem:

- The supplier(s) of the sections as most of these are provided by third parties
- Our packaging and handling methods
- The containers and boxes used

Needless to say UK NEQAS ICC & ISH is aware of this, and no participant is marked down because of any tissue damage of this nature, i.e. not caused by any protocol or staining related issues.

4. *Assessment of antibodies not requested by UK NEQAS ICC & ISH*

There has been a constant 'enquiry' from participants, mainly as a result of labs being assessed by UKAS under ISO: 15189, and also the fact that other EQA schemes offer some sort of standalone 'assessment' as a paid-for service. Unfortunately, at present UK NEQAS ICC & ISH are unable to offer this as an option to laboratories, but is something that will be frequently discussed as a possible future addition to the scheme's repertoire.

5. *Variations in scores between NEQAS and In-house sections*

Another longstanding and frequent comment from participants is the variance in scores between the NEQAS material, and a laboratory's own in-house sections or samples, usually as a result of the in-house sample obtaining higher scores than the NEQAS one. This is a double edged sword: UK NEQAS ICC & ISH has to ensure that control material is treated in exactly the same way as the NEQAS sample. The only fool proof way to do this is to request that the in-house sample be placed on the same slide as the NEQAS one. In doing so of course individual tweaking of samples/sections is not possible. EQA should also be testing that methods and protocols are robust enough to perform adequately on samples other than the lab's own tissues. The UK NEQAS ICC & ISH samples are sourced, tested, and treated (fixation, processing) in a manner not dissimilar or the same as those used by most routine diagnostic departments.

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

The General Pathology Module Run 117

Gavin Rock, Suzanne Parry and Amy Newman

	Gold Standard	Second Antibody
Antigens Assessed:	Ki-67	TTF-1
Tissue Sections circulated:	Breast carcinoma x 2 and normal tonsil	Normal Lung and lung adenocarcinoma
Number of Registered Participants:	314	
Number of Participants this Run:	306 (97%)	

Introduction

Gold Standard: Ki-67

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

Features of Optimal Immunostaining:
Tonsil: (Fig 1 & 6)
- Intense and well-localised nuclear staining of 80-90% of the germinal centre B-cells.
- Intense staining of the basal epithelial cells.
- Clean background with no non-specific staining.

Breast Tumour A: (Fig 3A & 4A)
- Intense and well-localised nuclear staining in approx. 70 % of tumour cells, with varying intensity of expression.
- Clean background with no non-specific staining in the stroma or cell types not expected to stain.

Breast Tumour B: (Fig 3B & 4B)
- Intense and well-localised nuclear staining in approx. 5 % of tumour cells, with varying intensity of expression.
- Clean background with no non-specific staining in the stroma or cell types not expected to stain.

Features of Sub-Optimal Immunostaining: (Fig 2 & 5)
- Weak, uneven, partially missing staining of relevant cells.
- Fewer tumour cells staining than expected in the breast.

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

Second Antigen: TTF-1

Thyroid transcription factor (TTF1) is part of the family of homeodomain (protein structural domain that binds DNA or RNA) transcription factors and is a gene regulatory factor expressed in the follicular epithelial and parafollicular c-cells of the thyroid, epithelial cells of the lung, brain and pituitary gland. TTF1 is essential for the normal development of both the thyroid and lung, and increased TTF1 immunohistochemical expression if seen in both pulmonary adenocarcinomas and thyroid neoplasms of follicular origin (follicular adenoma and follicular and papillary carcinoma). Not all pulmonary adenocarcinomas have been shown to be positive for TTF1, with a positivity range of between 65-80% (Comperat et al 2005), depending on the antibody clone used. TTF1 in small lung carcinomas have been shown in about 96% of cases (Ordonez et al., 2000) and in 75% of non-small cell pulmonary carcinomas, but generally it is not thought to be expressed in typical pulmonary carcinoids, although the literature does have some contrasting views. TTF1 can also be used to differentiate between tumours of primary and metastatic origin e.g. primary lung adenocarcinoma (TTF1+/cdx2-) vs metastatic gastrointestinal adenocarcinomas (TTF1+/cdx2-). Furthermore, TTF1 alongside other antibodies such as CK20 can help to further identify tumour types including Merkel cell carcinoma (TTF1-/CK20+) and metastatic small cell lung carcinoma (TTF1+/CK20-).

Features of Optimal Immunostaining:
Normal Lung: (Fig 8A & 12)
- Strong, nuclear staining of the basal epithelial cells lining the bronchial ducts
- Weak to moderate nuclear staining of bronchial luminal epithelial cells
- Clean background

Lung Adenocarcinoma: (Fig 7, 8B, 9 & 12)
- Strong, nuclear staining in virtually all neoplastic cells
- Clean background

Features of Suboptimal Immunostaining: (Figs 10 & 11)
- Weak staining of the basal epithelial cells lining the bronchial ducts
- Weak staining of neoplastic cells of the tumour
- Non-specific nuclear staining
- Excessive background staining

References
1. Comperat E, et al; Variable sensitivity and specificity of TTF1 antibodies in lung metastatic adenocarcinoma of colorectal origin. Mod Pathol. 2005 18(10):1371-6.
2. Ordonez NG. Value of thyroid transcription factor-1 immunostaining in distinguishing small cell lung cancer.
3. Holzinger A, Dingle S, Bejarano PA, Miller M-A, Weaver TE, Di Lauro R, Whitsett JA. Monoclonal antibody to thyroid transcription factor-1: Production, characterization and usefulness in tumour diagnosis. Hybridoma 1996;15:49 , 53
4. Khoor A, Whitsett JA, Stahlman MT, Stephenson M, Olson SJ, Cagle PT. U Utility of surfactant protein B precursor and thyroid transcription factor differentiating adenocarcinoma of the lung from malignant mesothelioma. Hum Pathol 1999;30(6):695
5. Bohinski RJ, Bejarano PA, Balko G, Warnick RE and Whitsett JA. Determination of lung as the primary site of cerebral metastatic adenocarcinomas using monoclonal antibody to thyroid transcription factor-1. J Neuro-Oncol 1998;40(3):227-31

Gavin Rock, Suzanne Parry and Amy Newman

Assessment Summary

Ki67

306 laboratories submitted slides for the Ki 67 assessment, and all but 2 laboratories submitted their in-house control sections for this run. The results show a noticeable increase in acceptable results from Run 116, as shown in the table below:
Only 5% of participants received an unacceptable result on the NEQAS section. This was mostly due to weak or very weak demonstration of the Ki-67 antibody. This fell even more on the in-house tissue with only 2 (1%) participants failing. The most popular clone used in this run was DAKO M7240 (clone MIB1) used by 135 participants with a pass rate of 79%. Another popular choice is the Ventana 790-429=86 (clone 30-9) used by 70 participants with a 100% acceptable pass rate. The acceptable pass rate of the in-house was 95% compared to the NEQAS of 86%, this is due to the fact that many laboratories are only using an appendix as an in-house positive control, however NEQAS recommend a composite control to be able to measure the sensitivity of your assay. Unlike other modules at this moment in time, we do not penalise for just using a single control, however best practice would be to use a known positive control and a control that measures the sensitivity of you assay .

Ki-67 Pass Rates : NEQAS section		
	Run 116	Run 117
Acceptable	74%(N=230)	86%(N=264)
Borderline	14%(N=43)	8%(N=26)
Unacceptable	11%(N=35)	5%(N=16)

TTF-1

302 laboratories submitted slides for TTF-1 assessment. The results were very impressive with 97% and 95% achieving either an acceptable pass of >13/20 in the NEQAS and in-house components respectively as outlined in the graph below. The last time TTF-1 was assessed was back in 2014, Run 107 with the acceptable pass rate on the NEQAS section being 83% (n=264). So this is a marked improvement.
The most common TTF-1 antibody was the Leica TTF-1 clone SPT24 in either RTU or concentrate form (n=120, 40%), followed by the Dako clone 8G7G3/1 (n=73, 24%) and the Ventana clone SP141 (n=52, 17%).

TTF-1 Pass Rates: Run 117		
	NEQAS	In-House
Acceptable	97% (N=292)	95%(N=285)
Borderline	3% (N=10)	4%(N=13)
Unacceptable	0% (N=0)	0%(N=1)

Selected Images showing Optimal and Sub-optimal Immunostaining

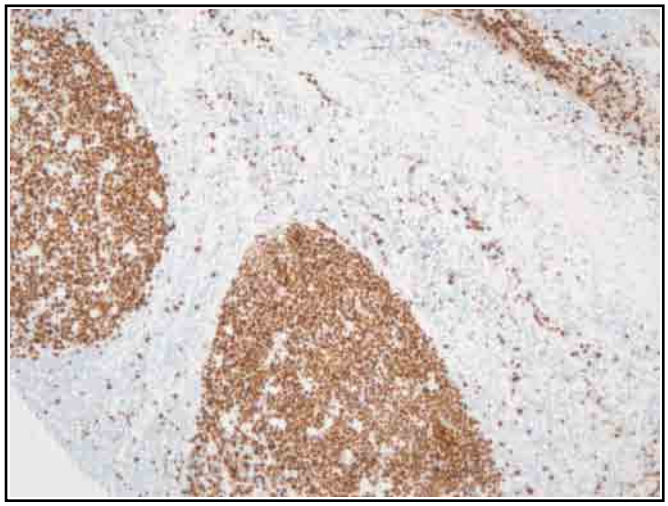


Fig 1. Good demonstration of Ki67 in the UK NEQAS tonsil. Even at low power it is clear to see the strong and well-localised staining in the germinal centre B-cells. The supra-basal squamous epithelium (top right) also shows the expected level of staining. The Leica K2 pre-diluted antibody was used on the Ventana ULTRA, iView detection with CC1 for 64'.

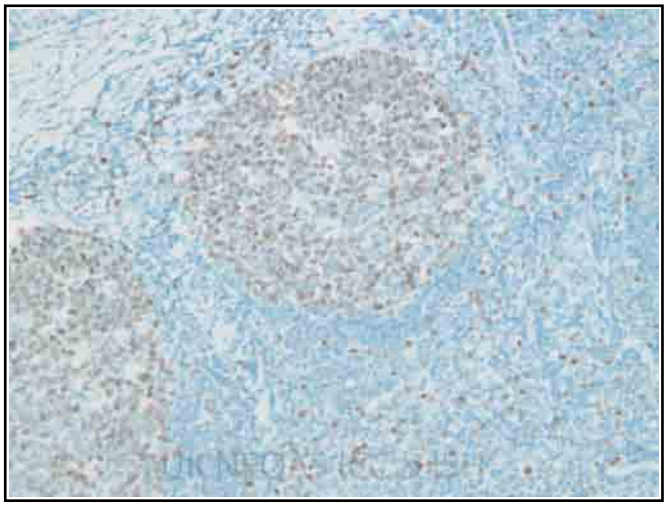


Fig 2. Poor demonstration of Ki67 in the UK NEQAS tonsil (compare to Fig 1). The staining is weak with fewer cells demonstrated than expected. No staining was observed in the breast tumour samples also present on the UK NEQAS distributed slides. Stained with the Dako MIB1 clone (no dilution provided) on the Dako autostainer, with pre-treatment in the PT Link.

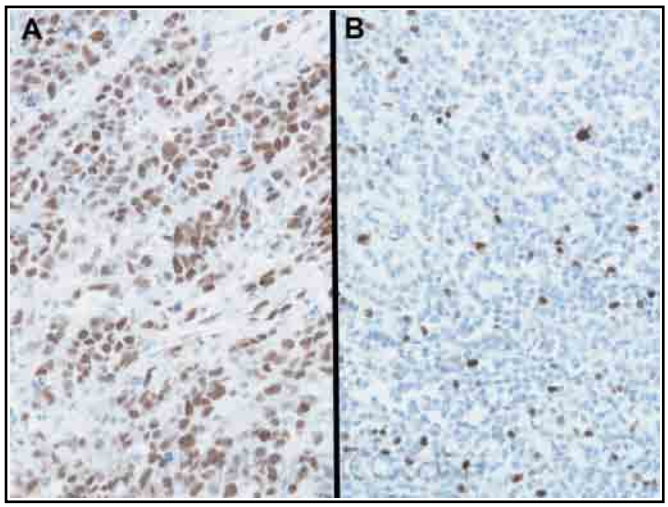


Fig 3. Optimal staining of Ki67 in the UK NEQAS high and low-expressing breast tumour samples: (A); high expressor shows moderate nuclear staining in 70% of neoplastic cells. (B); low expressor shows moderate intensity staining in approx. 5% of neoplastic cells. Stained with the Dako MIB-1 antibody, 1:100, on the Leica Bond III, ER2 retrieval for 30'.

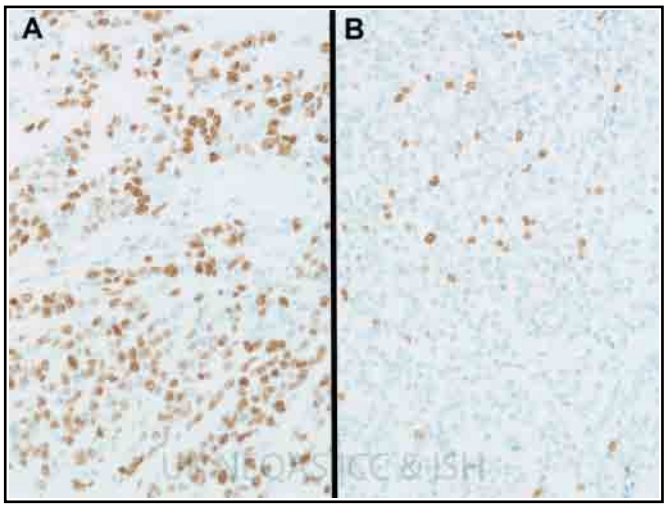


Fig 4. Good demonstration of Ki67 in the UK NEQAS high and low-expressing breast tumour samples. Both the high expressor (A) and the low expressor (B) show the expected level of staining while the background remains clean. (Same protocol as Fig 1).

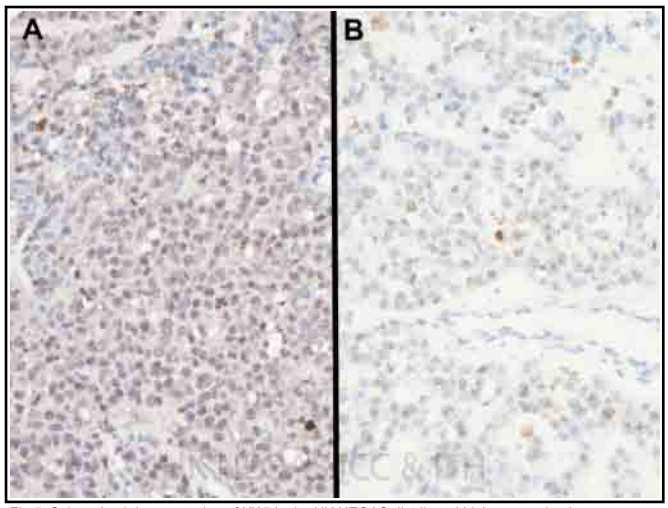


Fig 5. Sub-optimal demonstration of Ki67 in the UK NEQAS distributed high-expressing breast tumour samples (compare to Figs 3A & 4A). Example A shows excessive background staining, while the staining in example B is weak and diffuse; the expression level is much weaker than expected.

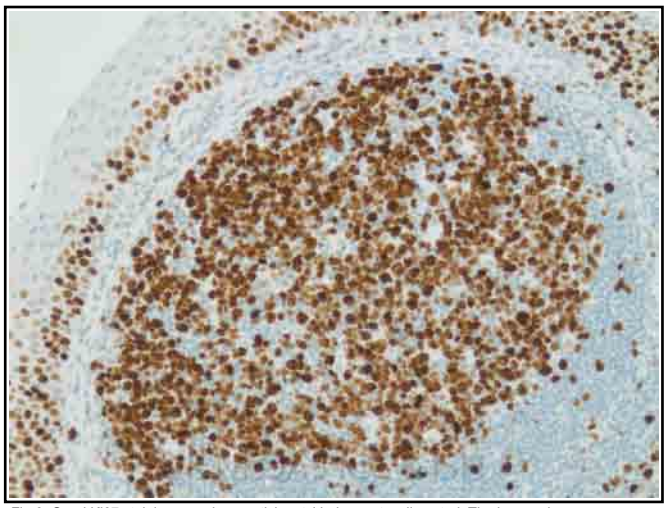


Fig 6. Good Ki67 staining seen in a participants' in-house tonsil control. The image shows intense and well-localised staining in the germinal centre B-cells and supra-basal squamous epithelium, while the background remains clean. Stained using the Dako MIB1 clone, 1:75, on the Ventana ULTRA, CC1 for 64 minutes.

Selected Images showing Optimal and Sub-optimal Immunostaining

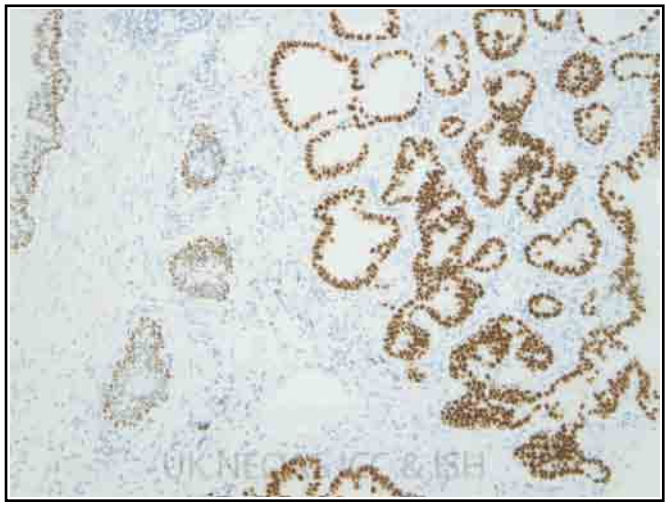


Fig 7.Optimal demonstration of Thyroid Transcription Factor 1 (TTF-1) on the UK NEQAS lung tumour sample. The example shows strong and distinct staining of the tumour cells and the basal epithelial cells lining the bronchial duct, while the staining of the luminal epithelial cells is of moderate intensity. Stained with the NCL/Leica SPT24 antibody on the Dako Omnis.

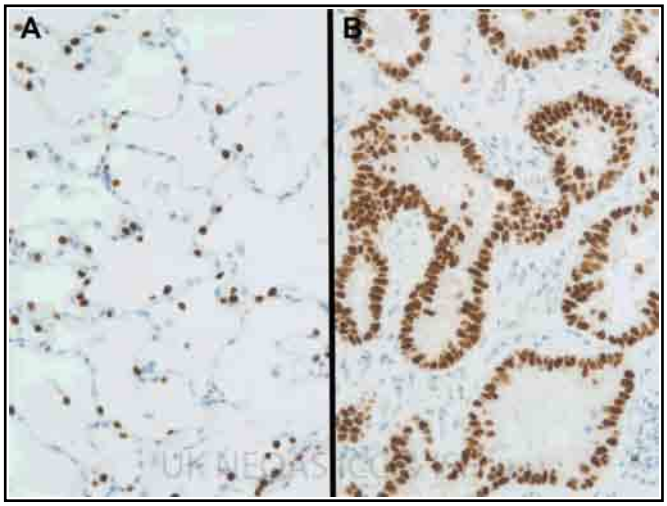


Fig 8. Good demonstration of TTF-1 on the UK NEQAS normal lung sample (A) and the lung adenocarcinoma sample (B). Both images show strong distinct staining while the background remains clean. Stained using the Novocastra/Leica SPT24 antibody, 1:400, on the Leica BondMax, ER2 for 30 minutes.

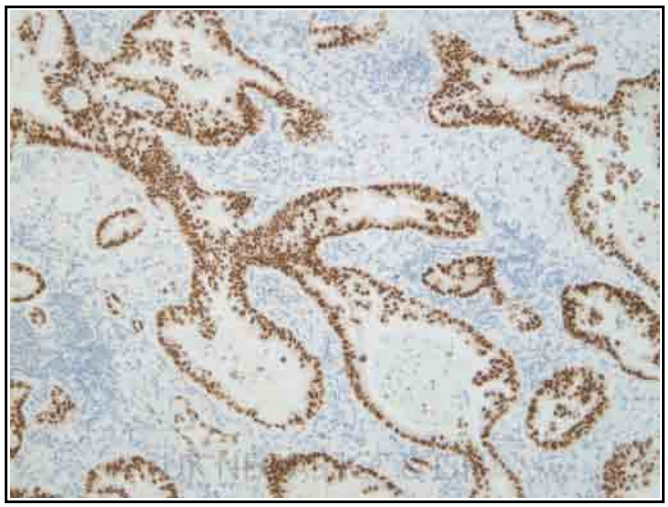


Fig 9. Optimal demonstration of TTF-1 on the UK NEQAS lung adenocarcinoma sample. Even at low power it is clear to see that virtually all of the neoplastic cells show strong and distinct nuclear staining, while the background remains clean. Stained with the Novocastra/Leica SPT24 antibody, 1:200, on the Leica Bond III, ER2 for 30 minutes.

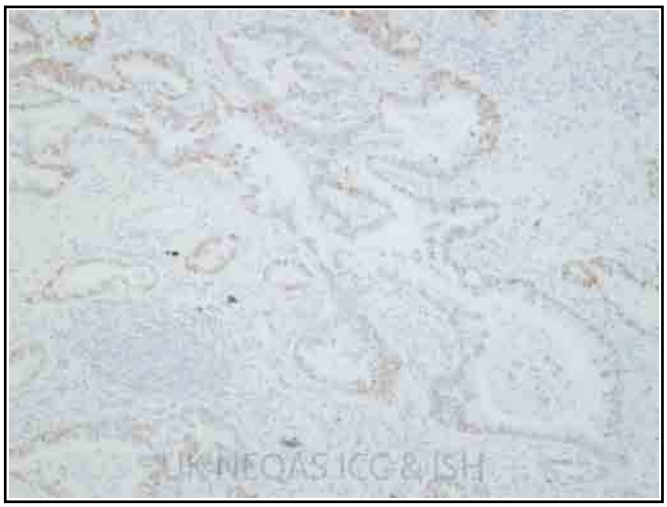


Fig 10. Sub-optimal TTF-1 staining of the UK NEQAS lung adenocarcinoma sample (compare to Fig 9). Not only is the staining weak, but the percentage of cells staining is much lower than expected. Stained with the Zmyed 8G7G3/1 antibody, 1:400, on the Dako autostainer with pre-treatment in the PT Link.

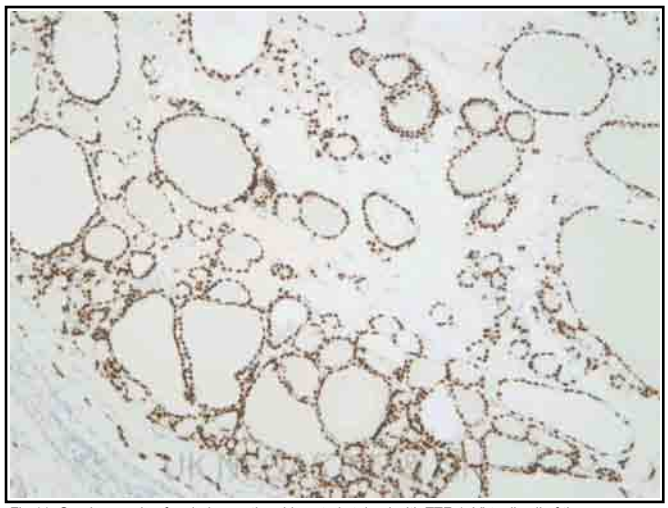


Fig 11. Good example of an in-house thyroid control stained with TTF-1. Virtually all of the follicular epithelial cells show strong nuclear staining, while the background remains clean. (Same protocol as Fig 8).

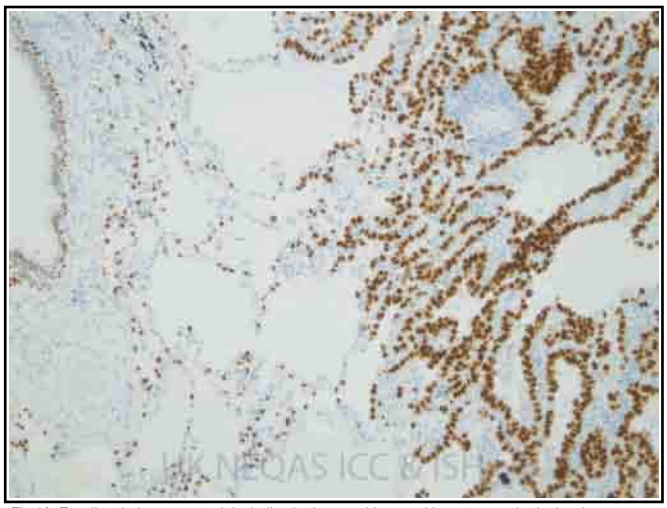
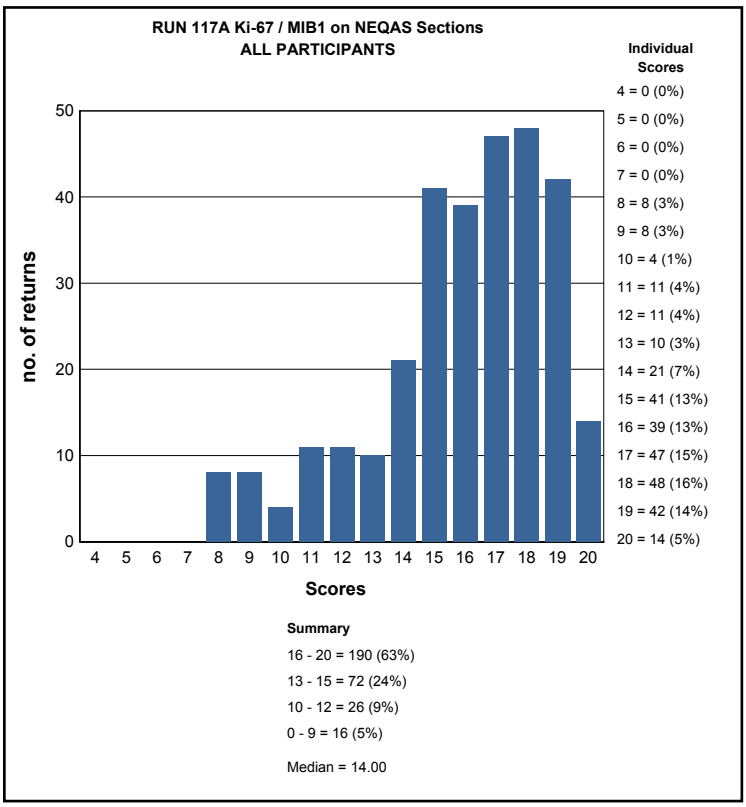
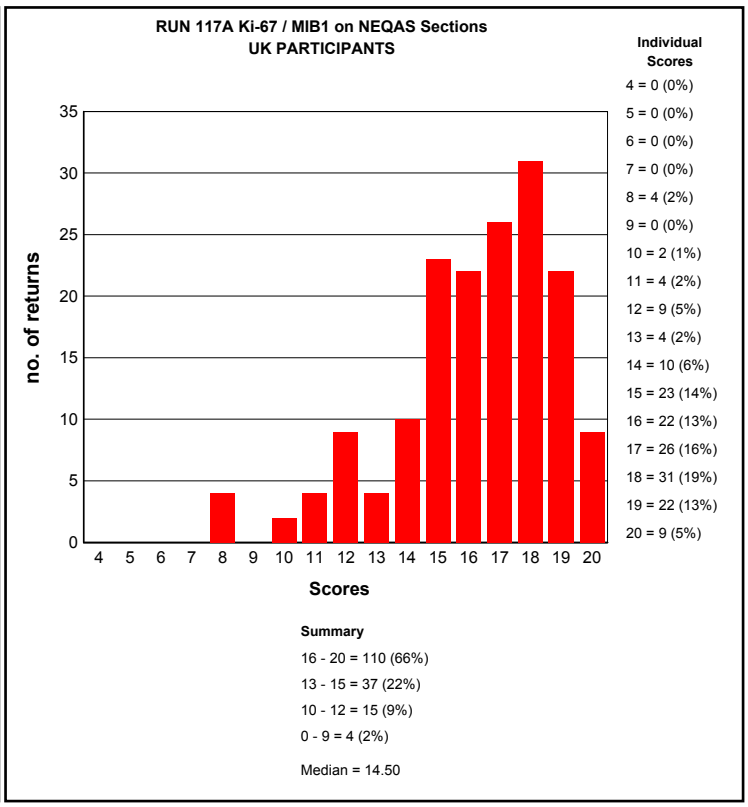
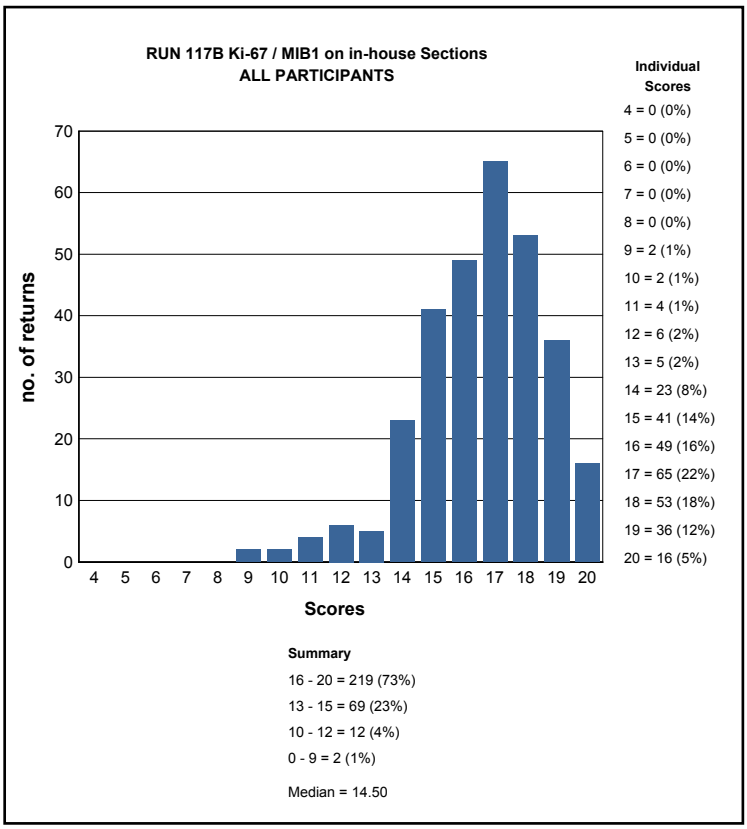


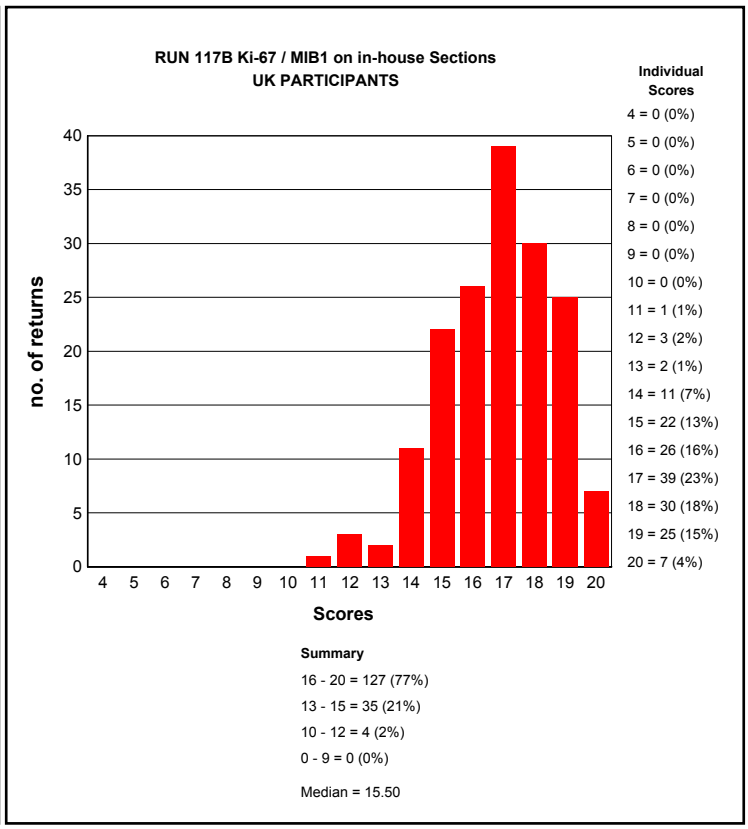
Fig 12. Excellent in-house control, including both normal lung and lung tumour; both showing strong nuclear staining. The bronchial duct also present shows the expected strong distinct nuclear staining, while the staining in the luminal epithelial cells is weak to moderate. (Same protocol as Fig 7).

GRAPHICAL REPRESENTATION OF PASS RATES

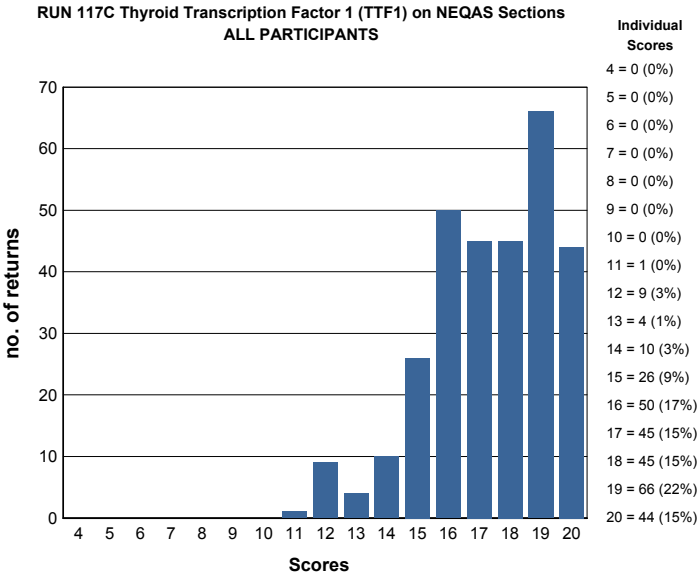


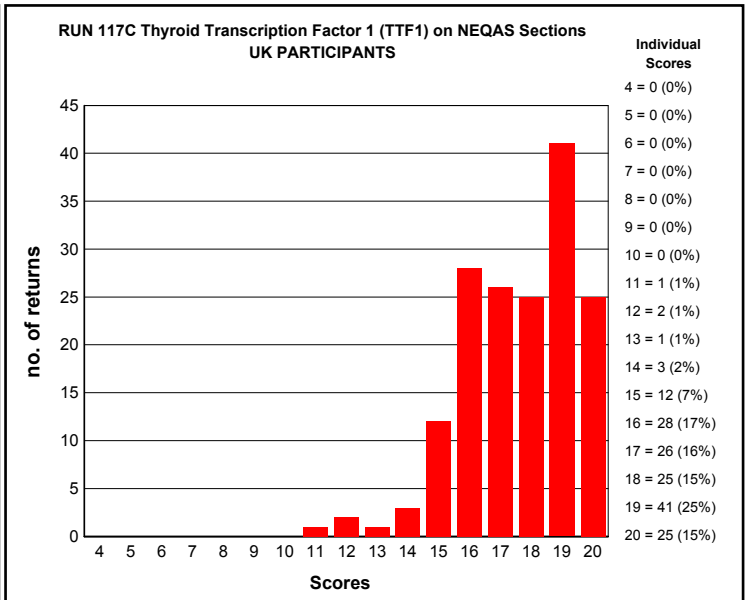


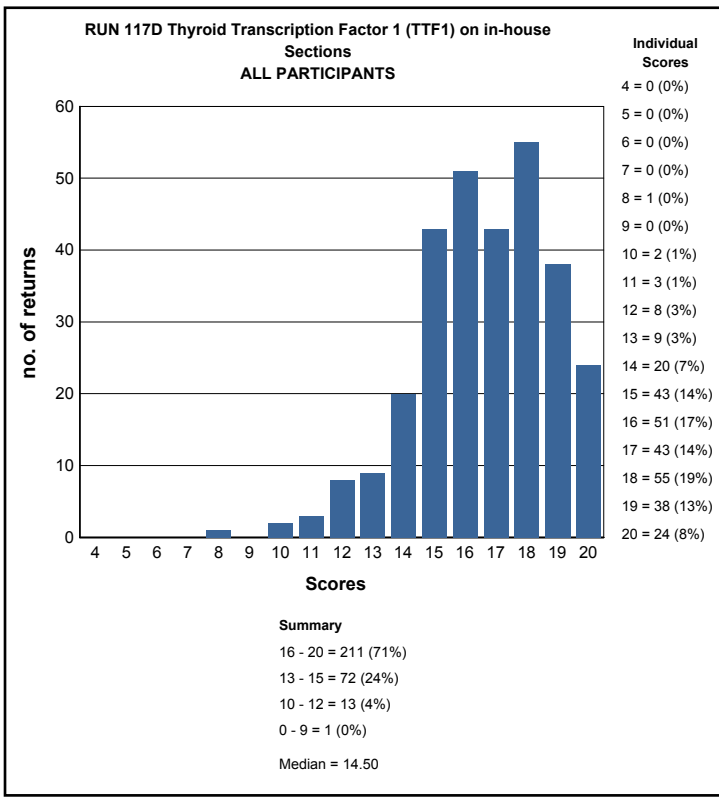


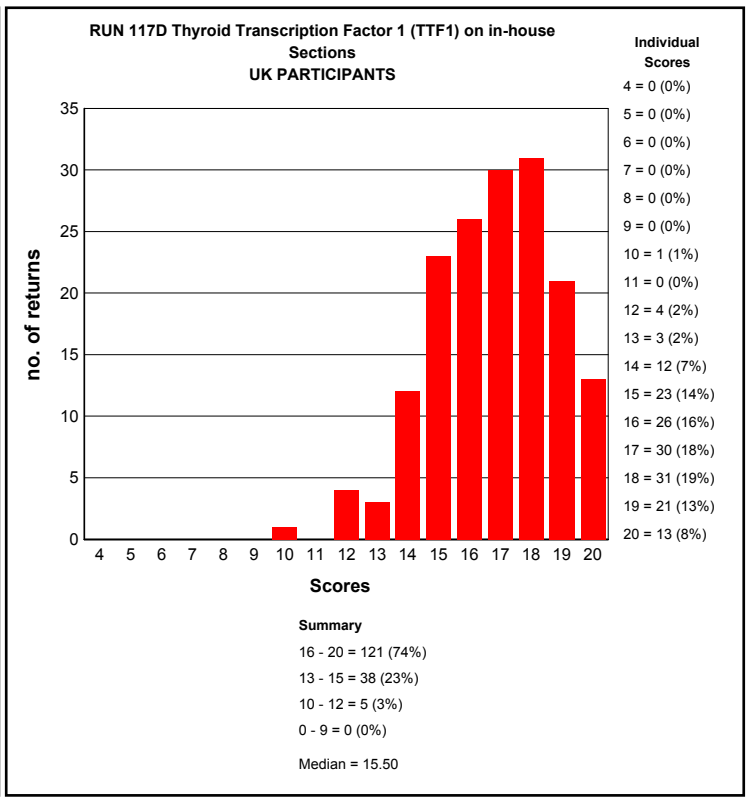


GRAPHICAL REPRESENTATION OF PASS RATES









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: 117

Primary Antibody : Ki-67 / MIB1

Antibody Details	N	%
Dako M7240 (clone MIB1)	135	79
NeoMarkers RM 9106 (clone SP6)	3	100
Novocastra NCL-Ki67 (clone MM1)	3	67
Vector VP K452 (clone MM1)	1	100
Ventana 760-2910	1	100
Other	7	100
Dako IR/IS626 (clone MIB-1)	19	89
Leica NCL-Ki67-MM1 (MM1)	1	100
Leica RTU PA0410 (MM1)	5	40
Leica PA0118 (MM1)	8	63
Leica PA0230 (K2)	29	97
Ventana 790-4286 (clone30-9)	70	100
Cell Marque 275R-14/15/16/17/18 (SP6)	5	100
Dako Omnis GA626 (MIB-1)	13	77

General Pathology Run: 117

Primary Antibody : Thyroid Transcription Factor 1 (TTF1)

Antibody Details	N	%
Dako IR056 (8G7G3/1)	17	100
Dako IS056 (8G7G3/1)	3	100
Dako M3575 (8G7G3/1)	53	94
Invitrogen 081221 (8G7G3/1)	1	100
Invitrogen 180221 (8G7G3/1)	3	100
Neomarkers MS-69-XX (8G7G3/1)	1	100
Novocastra NCL-L-TTF-1 (SPT24)	51	100
Novocastra NCL-TTF-1 (SPT24)	13	100
Novocastra PA0364 (SPT24)	7	100
Ventana 760-2829 (8G7G3/1)	9	89
Other	15	87
Zymed 08-1221 (8G7G3/1)	1	0
BOND RTU TTF-1 (SPT24) PA0364	3	100
Cell Marque 343M-95/96/97 (8G7G3/1)	8	88
Labvision MS-699	1	100
Leica Bond TTF1 SPT24 (RTU) PA0364	23	100
Leica NCL-L-TTF L136446	24	100
THermo-Scientific MS/699P1	1	100
Ventana TTF-1 (SP141) 790-4756	43	98
Vector VP-T483 (SPT24)	1	100
Cell Path MOB 285 (8G7G3/1)	1	100
Ventana TTF-1 (SP141) 790-4398	9	100

General Pathology Run: 117

Heat Mediated Retrieval	Ki-67 / MIB1 N	Ki-67 / MIB1 %	Thyroid Transcription Factor 1 (TTF1) N	Thyroid Transcription Factor 1 (TTF1) %
Biocare Decloaking Chamber	1	100	1	100
Dako Omnis	16	75	13	100
Dako PTLink	32	84	36	94
Lab vision PT Module	2	100	1	100
Leica ER1 20 mins	3	67	22	100
Leica ER1 25 mins	1	0	0	0
Leica ER1 30 mins	1	100	15	100
Leica ER1 40 mins	0	0	2	100
Leica ER2 10 mins	1	100	2	100
Leica ER2 20 mins	59	76	36	97
Leica ER2 30 mins	18	56	10	100
Leica ER2 40 mins	4	100	0	0
Microwave	5	80	3	67
None	1	100	2	100
Pressure Cooker	3	100	2	100
Pressure Cooker in Microwave Oven	0	0	1	100
Steamer	2	100	2	100
Ventana CC1 24mins	6	100	4	100
Ventana CC1 32mins	24	96	7	100
Ventana CC1 36mins	9	89	7	100
Ventana CC1 40mins	4	100	10	100
Ventana CC1 48mins	5	100	7	100
Ventana CC1 52mins	3	100	1	100
Ventana CC1 56mins	7	100	8	100
Ventana CC1 64mins	43	98	54	96
Ventana CC1 72mins	0	0	2	100
Ventana CC1 76mins	0	0	3	67
Ventana CC1 88mins	1	100	0	0
Ventana CC1 92mins	2	100	1	0
Ventana CC1 extended	1	100	2	100
Ventana CC1 mild	10	90	3	100
Ventana CC1 standard	29	100	27	96
Ventana CC2 24mins	1	100	0	0
Ventana CC2 36mins	1	100	0	0
Ventana CC2 44mins	1	0	0	0
Ventana CC2 64mins	1	100	1	100
Water bath 68 OC	1	100	1	100
Water bath 95-98 OC	2	0	1	100

General Pathology Run: 117

Enzyme Mediated Retrieval	Ki-67 / MIB1 N	Ki-67 / MIB1 %	Thyroid Transcription Factor 1 N	Thyroid Transcription Factor 1 %
AS PER KIT	6	100	9	89
NOT APPLICABLE	176	86	145	97
Ventana Protease 1 (760-2018)	1	100	2	100

General Pathology Run: 117

Detection	Ki-67 / MIB1 N	Ki-67 / MIB1 %	Thyroid Transcription Factor 1 N	Thyroid Transcription Factor 1 %
A Menerini Polymer (MP-XCP)	1	100	0	0
AS PER KIT	18	61	22	86
Dako EnVision FLEX (K8000/10)	12	75	8	100
Dako EnVision FLEX+ (K8002/12)	25	92	31	100
Dako Envision HRP/DAB (K5007)	4	75	2	100
Dako Envision+ HRP mouse K4004/5/6/7	2	50	3	67
Dako rb-a-mo Ig (E0354)	1	100	0	0
Dako REAL HRP/DAB (K5001)	1	100	0	0
LabVision UltraVision ONE Polymer (TL-012/5-HDJ/T	1	100	1	100
Leica Bond Polymer Define (DS9713)	1	100	0	0
Leica Bond Polymer Refine (DS9800)	80	75	80	99
MenaPath X-Cell Plus (MP-XCP)	0	0	1	100
None	2	100	0	0
NOT APPLICABLE	0	0	1	0
Novocastra Novolink PDS (RE7-140/150/280/290-K)	1	100	1	100
Other	6	83	8	100
Ventana iView system (760-091)	2	100	6	100
Ventana OptiView (760-700) + Amp. (7/860-099)	4	100	1	100
Ventana OptiView Kit (760-700)	58	100	55	100
Ventana UltraView Kit (760-500)	77	95	66	95

General Pathology Run: 117

Automation	Ki-67 / MIB1 N	Ki-67 / MIB1 %	Thyroid Transcription Factor 1 (TTF1) N	Thyroid Transcription Factor 1 (TTF1) %
BioGenex GenoMX 6000i	0	0	1	100
Dako Autostainer	2	100	2	100
Dako Autostainer Link 48	31	84	30	97
Dako Autostainer plus	0	0	3	100
Dako Autostainer Plus Link	2	100	4	75
Dako Omnis	16	75	12	100
LabVision Autostainer	1	100	2	100
Leica Bond Max	32	59	41	98
Leica Bond-III	54	80	48	100
Menarini - Intellipath FLX	1	100	1	100
None (Manual)	8	75	5	80
Shandon Sequenza	2	50	2	100
Ventana Benchmark GX	6	100	3	100
Ventana Benchmark ULTRA	104	96	94	96
Ventana Benchmark XT	41	98	42	98

General Pathology Run: 117

Chromogen	Ki-67 / MIB1 N	Ki-67 / MIB1 %	Thyroid Transcription Factor 1 (TTF1) N	Thyroid Transcription Factor 1 (TTF1) %
AS PER KIT	32	81	43	95
BioGenex liquid DBA (HK-124-7K)	1	100	1	100
DAKO DAB+	1	100	1	100
Dako DAB+ Liquid (K3468)	4	100	3	100
Dako DAB+ REAL Detection (K5001)	1	100	0	0
Dako EnVision Plus kits	3	67	3	100
Dako FLEX DAB	36	83	34	97
Dako REAL EnVision K5007 DAB	4	75	4	100
Dako REAL K5001 DAB	1	100	0	0
Leica Bond Polymer Refine kit (DS9800)	81	73	78	99
menapath xcell kit DAB (MP-860)	1	100	1	100
Other	16	100	18	94
Sigma DAB (D5637)	1	100	1	100
Sigma DAB (D5905)	1	100	0	0
Ventana DAB	34	100	25	100
Ventana iview	2	100	5	100
Ventana Ultraview DAB	81	94	73	95

BEST METHODS - Gold Standard Antibody

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

Ki-67 / MIB1 - Method 1

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

Primary Antibody: Vector VP K452 (clone MM1) , 30 Mins, rt ºC Dilution 1: 100
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 30 mins
EAR:
Chromogen: Leica Bond Polymer Refine kit (DS9800), rt ºC., Time 1: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins, rt ºC

Ki-67 / MIB1 - Method 2

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

Primary Antibody:	Ventana 790-4286 (clone30-9) , 32 Mins, 24 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Other
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.6
HMAR:	Ventana CC1 64mins
EAR:	NOT APPLICABLE
Chromogen:	Other
Detection:	Ventana OptiView Kit (760-700) , 24 ºC Prediluted

Ki-67 / MIB1 - Method 3

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

Primary Antibody:	Leica PA0230 (K2) , 15 Mins, 21 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), 21 ºC., Time 1: 8 Mins, Time 2: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 5 Mins, 21 ºC Prediluted

Ki-67 / MIB1 - Method 4

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

Primary Antibody:	Dako M7240 (clone MIB1) , 32 Mins, 24 ºC Dilution 1: 100
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	NOT APPLICABLE
Chromogen:	Other
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099)

BEST METHODS - Secondary Antibody

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

Thyroid Transcription Factor 1 (TTF1) - Method 1

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

Primary Antibody:	Novocastra NCL-L-TTF-1 (SPT24) , 15 Mins Dilution 1: 400
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 30 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

Thyroid Transcription Factor 1 (TTF1) - Method 2

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

Primary Antibody:	Novocastra NCL-L-TTF-1 (SPT24) , 20 Mins, 32 ºC Dilution 1: 200
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Dako Omnis, Buffer: TRS HIGH pH
EAR:	
Chromogen:	Ventana Ultraview DAB, 32 ºC., Time 1: 5 Mins
Detection:	Dako EnVision FLEX (K8000/10) , 10 Mins, 32 ºC Prediluted

Thyroid Transcription Factor 1 (TTF1) - Method 3

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

Primary Antibody:	Leica Bond TTF1 SPT24 (RTU) PA0364 , 15 Mins, 21 ºC Prediluted
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER1 20 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800), 21 ºC., Time 1: 8 Mins, Time 2: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 5 Mins, 21 ºC Prediluted

Thyroid Transcription Factor 1 (TTF1) - Method 4

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

Primary Antibody:	Ventana TTF-1 (SP141) 790-4756 , 32 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700) Prediluted

The Breast Hormonal Receptor Module Run 117

Suzanne Parry

Antigen Assessed:	Oestrogen Receptor (ER)
Tissue Sections circulated:	Composite slide consisting of 3 breast carcinomas with different levels of receptor expression and normal tonsil
Number of Registered Participants:	289
Number of Participants This Run:	267 (93%)

Table below shows the expected staining characteristics of the UK Neqas distributed tissue for Run 116: This composed of three infiltrating ductal carcinomas (IDCs) with differing levels of ER expression and a normal tonsil section.

Sections	% Positivity	Expected Staining Intensity	Allred / Quick Score
A. IDC	>95%	High	8
B. IDC	11-33%	Mid	4-6*
C. IDC	0%	Negative	0
E. Tonsil	≤ 3%	Weak to Medium	0 (Negative)

***Please Note:** Multiple tissue blocks are compiled from the same cases, however, there may on occasions be variability in staining from the Allred 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 of in-house material, due to pre-analytics
- Poor choice of in-house material

In-House Tissue Recommendations:

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

The required composite control should consist of the following samples:

1. >80% tumour positivity with high intensity (Allred/ Quick score 7-8)
2. 11-66% tumour positivity with moderate intensity (Allred/Quick score 3-6)
3. Negative tumour, ideally with normal positively stained glands (Allred/Quick score '0')

Participants NOT using a composite control are scored a 'borderline' pass (scores10-12/20).

Introduction

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

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

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

Choice of Tissue for Assessments

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

NEQAS tissue samples were tested by staining every 50th section from the relevant tissue blocks using the Leica (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 and included Leica (clone 6F11), Dako (1D5/ER-2-123 clones), Dako (EP1 clone) and Ventana (SP1 clone).

It should be noted that the tonsil showed staining in up to 3% of cells. This was mainly seen within the germinal centre lymphocytes, but also in the epithelial cells. It has been indicated in the datasheets from the commercial suppliers, such as the Dako (EP1) and Ventana SP1 clones that staining is seen in 1-5% of cells in the tonsil. Although not noted on the datasheet of the Leica 6F11 clone, staining was also seen in the tonsil of slides stained with this antibody.

Features of the Expected ER Immunostaining (Figs 1,2,5,6,7,9,10)

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

Features of Unacceptable ER Immunostaining (Figs 3,4,8,11,12)

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

NEQAS Section Assessment Results

267 laboratories submitted their slides for the ER assessment. The acceptable pass rate was similar to the previous ER run (Run 116). Both results for Runs 116 and 117 were an improvement on the scores achieved in Run 115. Summarised in the table below:

ER NEQAS Pass Rates :			
	Run 115	**Run 116**	**Run 117**
Acceptable	75%(N=197)	84%(N=228)	81%(N=217)
Borderline	18%(N=47)	10%(N=27)	13%(N=35)
Unacceptable	8%(N=20)	6%(N=15)	6%(N=15)

The borderline and failed marks for this assessment were mostly due to weak staining, particularly in the mid-expressing tumour. False positive staining in the tumour known to be negative was also the reason for failure. Excessive background was also noted on several slides. Inappropriate or over antigen retrieval was the main reason for the false positive staining, which was predominantly seen with users of the Leica 6F11 clone.

In-House Tissue Assessment Results

99% of participants also submitted their in-house controls for assessment. Overall these showed a slightly higher acceptable pass rate to the NEQAS sections, and also slightly more borderline passes, but with only one laboratory failing on their in-house material. The borderline passes were mostly due to poor tissue quality or not including the required composite control as recommended by UK NEQAS guidelines.

The in-house material is scored differently to that of the NEQAS material, so participants should be cautious when comparing their UK NEQAS score directly to that of in-house scores. Please note the following points when reviewing the NEQAS and In-House scores:

- 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 clone; 3) re-tested and validated samples by the relevant commercial companies to further verify the expected level of staining.
- The **In-house** samples are scored on the staining quality, readability of the samples, the correct choice of tissue, and good morphological preservation. If the in-house control samples do not comprise of the required composite breast tissue, containing a high (Allred 7-8), mid- (Allred 3-6) and a negative (Allred 0) ER-expressing tumour, participants are given a maximum score of 12/20. The presence of normal staining glands is also encouraged.

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

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.
5. Ciocca DR and Elledge R. Molecular markers for predicting response to tamoxifen in breast cancer patients. Endocrine. 2000;13: 1-10.
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

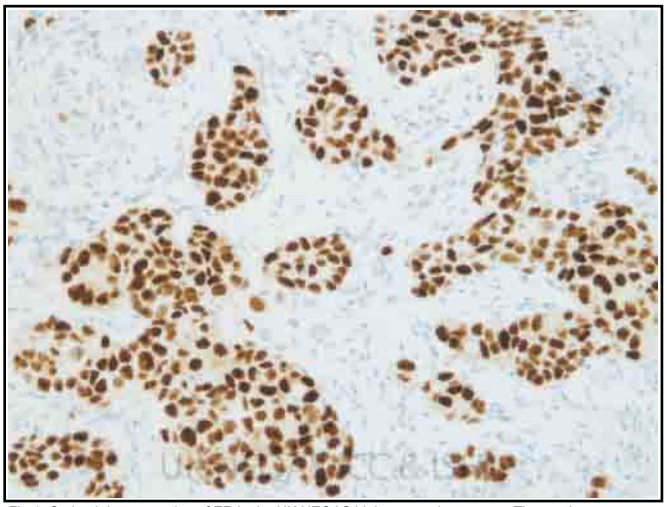


Fig 1. Optimal demonstration of ER in the UK NEQAS high-expressing tumour. The section shows intense staining in over 95% of neoplastic cells. Stained using the Dako RTU EP1 antibody on the autostainer with pre-treatment in the PT link.

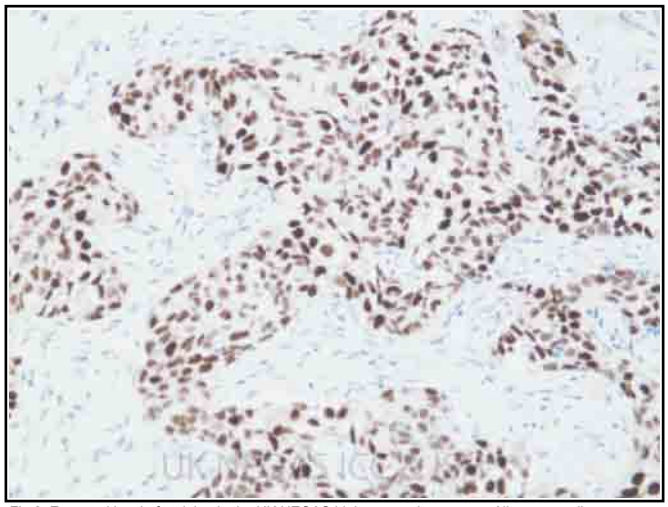


Fig 2. Expected level of staining in the UK NEQAS high-expressing tumour. All tumour cells show strong nuclear staining. Section stained on the Leica BondMax platform with the Leica 6F11 antibody, 1:50, and ER1 retrieval for 30 minutes.

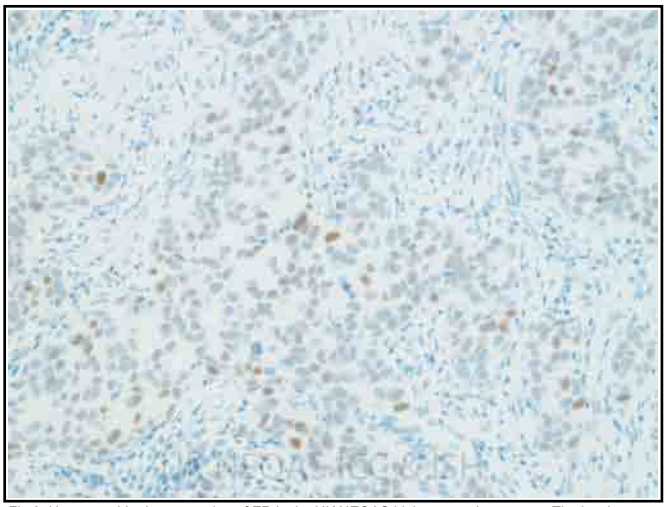


Fig 3. Unacceptable demonstration of ER in the UK NEQAS high-expressing tumour. The level of staining is much weaker than expected and more representative of a mid-expressing tumour. The mid-expressing tumour on the same slide showed a false negative result. Stained with the Leica 6F11 antibody, 1:50, on the Ventana Benchmark XT.

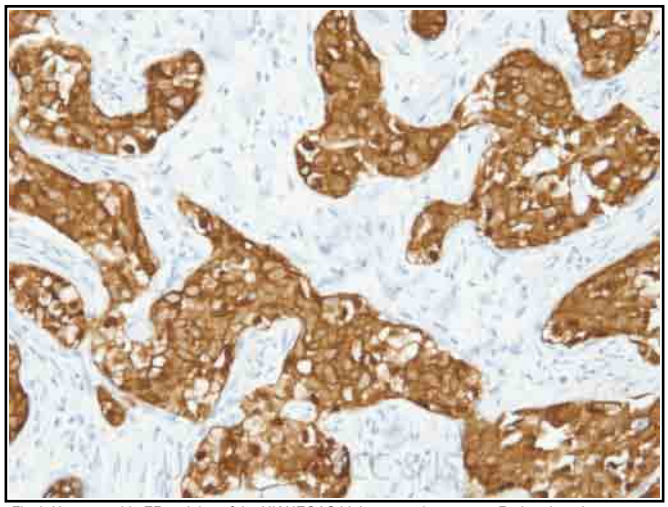


Fig 4. Unacceptable ER staining of the UK NEQAS high-expressing tumour. Rather than the expected nuclear staining, the section shows membranous and cytoplasmic demonstration. Stained using the Dako EP1 clone and the Dako Envision detection manual kit. Pretreatment was carried out in the pressure cooker.

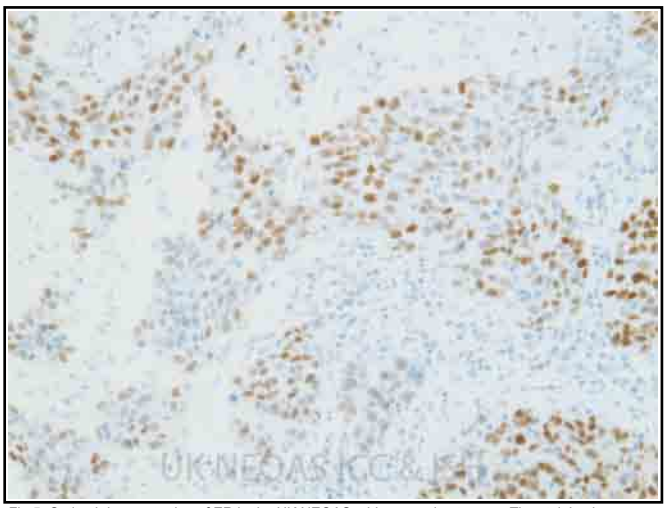


Fig 5. Optimal demonstration of ER in the UK NEQAS mid-expressing tumour. The staining is of varying intensity in over 60% of neoplastic cells. (Same protocol as Fig 1).

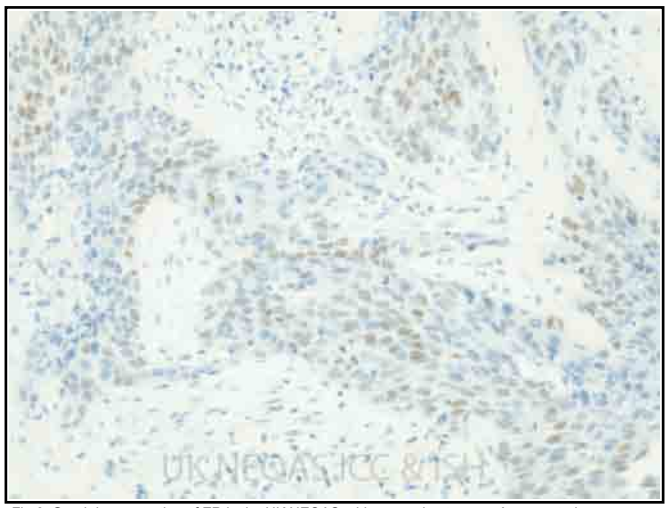


Fig 6. Good demonstration of ER in the UK NEQAS mid-expressing tumour. As expected over 60% of the neoplastic cells show weak to moderate nuclear staining while the background remains clean.

Selected Images showing Optimal and Sub-optimal Immunostaining

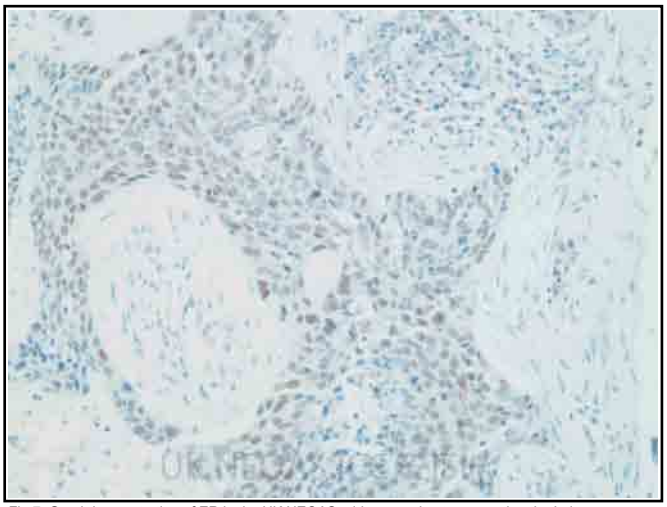


Fig 7. Good demonstration of ER in the UK NEQAS mid-expressing tumour using the Leica 6F11 clone, 1:100 dilution. As expected over 60% of neoplastic cells show a variation of weak, moderate and a few strong intensity staining. Stained on the Leica BondMax with ER1 pre-treatment for 30 minutes.

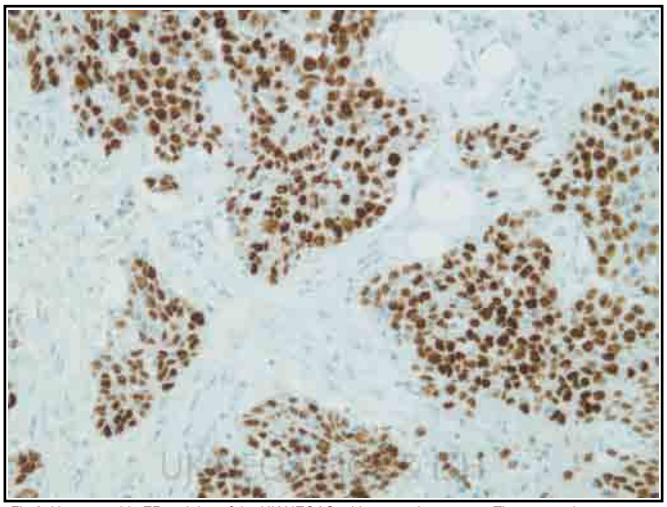


Fig 8. Unacceptable ER staining of the UK NEQAS mid-expressing tumour. The expression level of staining is far higher than expected and more representative of a high-expressing tumour rather than a mid. This is possibly due to a combination of using the Leica 6F11 antibody too concentrated (1:40), with the sensitive OptiView kit. Stained on the Ventana

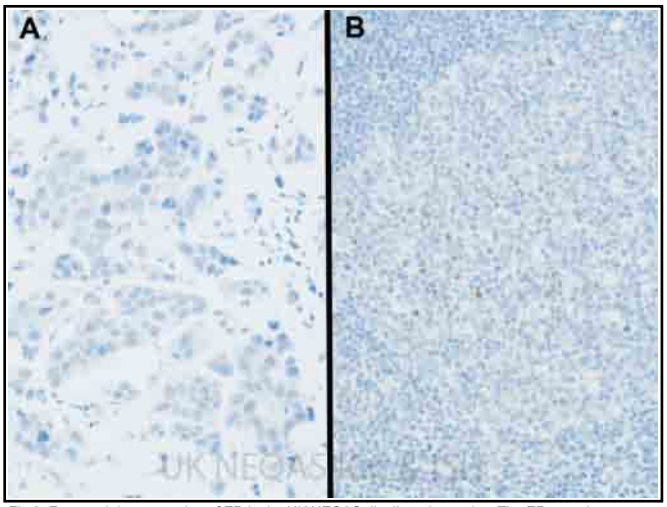


Fig 9. Expected demonstration of ER in the UK NEQAS distributed samples. The ER negative tumour (A) remains unstained, and as expected a small % of lymphocytes are staining positive in the tonsil section (B). Stained with the Leica RTU antibody on the Bond III, ER1 for 20 minutes.

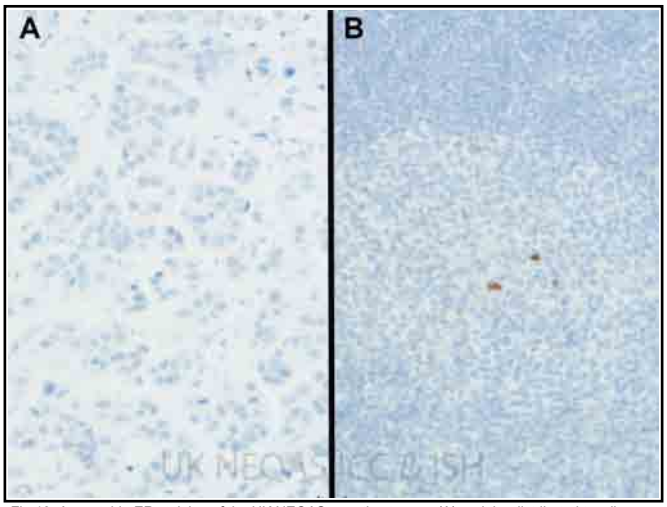


Fig 10. Acceptable ER staining of the UK NEQAS negative tumour (A) and the distributed tonsil (B). Both sections show the expected demonstration. (Same protocol as Fig 1).

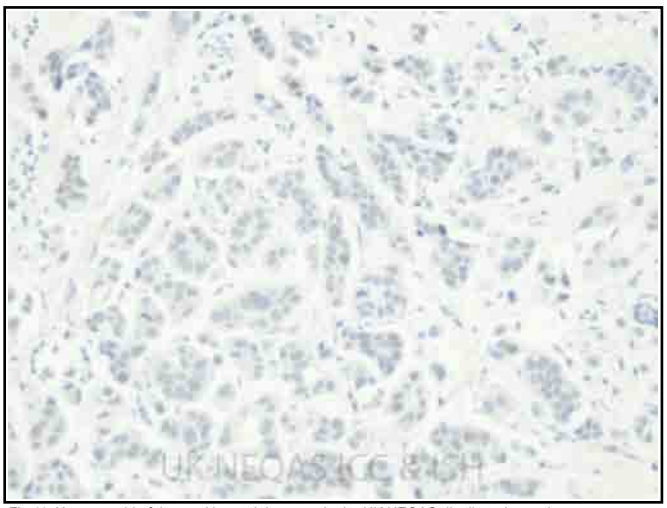


Fig 11. Unacceptable false positive staining seen in the UK NEQAS distributed negative tumour. Although the Gold standard slides did show a few weakly positive cells, staining in the example shown is much higher than expected. Stained using the Leica 6F11 clone, 1:40, on the Bond III with ER1 retrieval for 30 minutes.

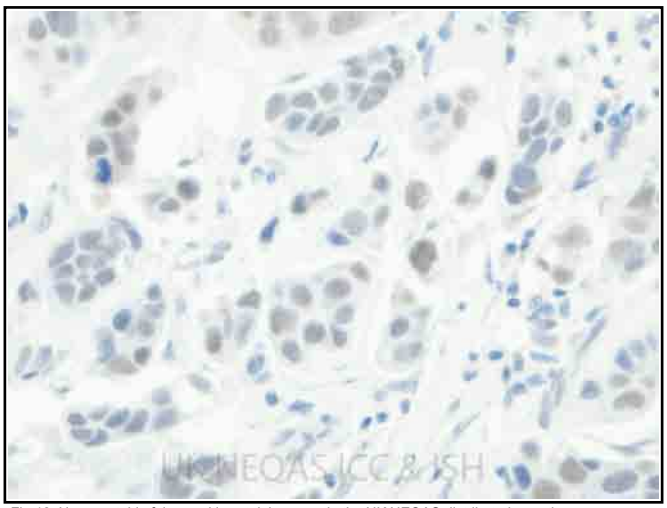
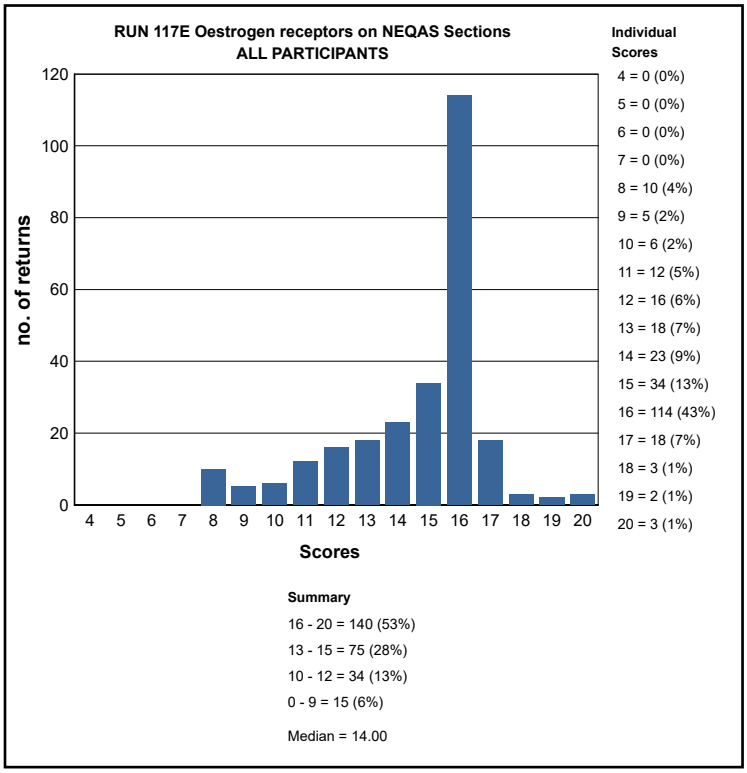
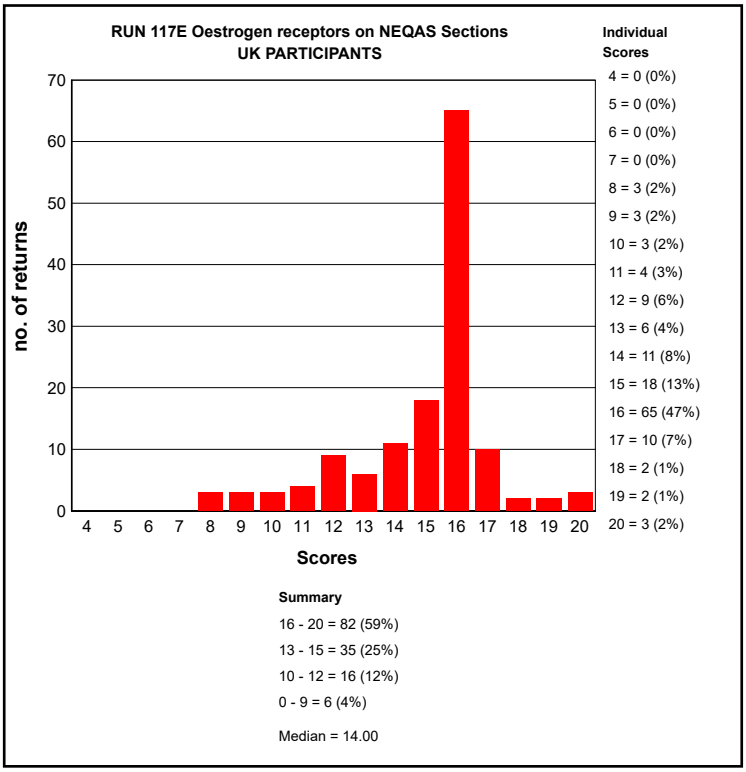
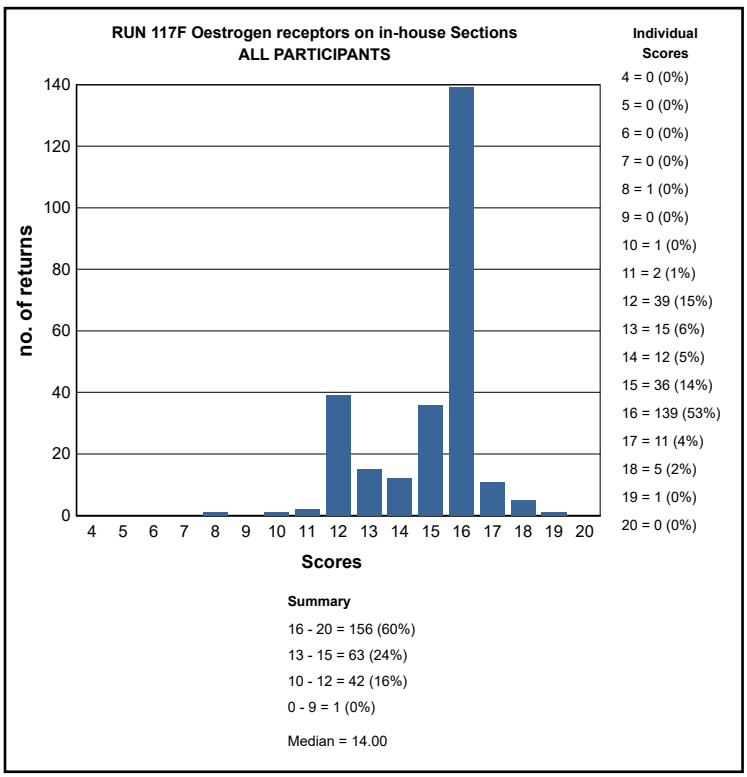


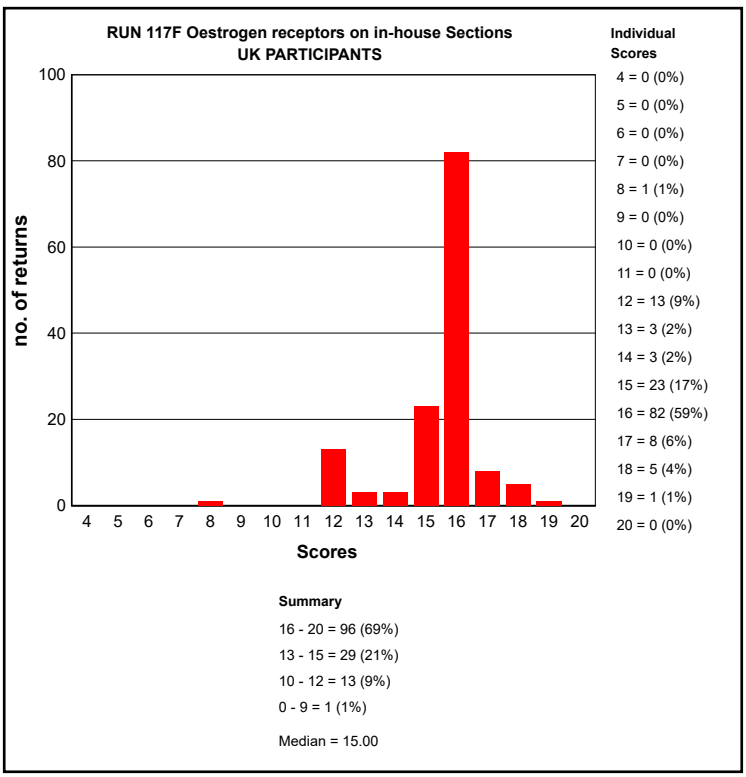
Fig 12. Unacceptable false positive staining seen in the UK NEQAS distributed negative tumour (shown at higher power). This is most likely caused by an excessive antigen retrieval protocol. I Leica recommends antigen retrieval for 20 minutes with ER1 retrieval solution. Whereas, the example was pre-treated for 30 minutes with ER2 solution.

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: 117

Primary Antibody : Oestrogen receptors

Antibody Details	N	%
Dako M7047 ER (1D5)	3	67
Thermo Fisher/ Neomarkers RM 9101-S (SP1)	13	92
Leica/Novocastra NCL-ER-6F11 (6F11)	13	54
Vector VP-E613/4 (6F11)	1	0
Ventana 250- 2596 ER (6F11)	1	100
Ventana 790-4324 (SP1)	73	89
Leica Bond PA0151 (6F11)	6	17
Dako M3634 (SP1)	5	100
Dako RTU IR151 (SP1)	1	100
Ventana 790-4325 (SP1)	42	98
Leica/Novocastra NCL-L-ER- 6F11	21	67
Leica/Novocastra RTU-ER-6F11	3	67
Leica/Novocastra NCL-ER-6F11/2	9	78
Dako SK310 pharmDX kit (Link) (1D5 + ER-2-123)	1	100
Dako IR151 Autostainer Link (SP1)	1	0
Dako (EP1) RTU FLEX IR084	23	96
Dako (EP1) M3643	24	79
Dako FLEX (1D5) IR/IS657	1	0
Other	4	50
Dako (EP1) RTU Auto Plus IS084	2	100
Biocare Medical (SP1+6F11) RTU APA 308	1	100
Cell Marque 249-R (SP1)	3	33

Breast Steroid Hormone Receptor Run: 117

Automation	Oestrogen receptors N	Oestrogen receptors %
Dako Autostainer	1	0
Dako Autostainer Link 48	23	87
Dako Autostainer plus	1	0
Dako Autostainer Plus Link	2	50
Dako Omnis	9	100
LabVision Autostainer	2	50
Leica Bond Max	20	60
Leica Bond-III	45	73
Menarini - Intellipath FLX	1	0
None (Manual)	5	60
Shandon Sequenza	1	0
Ventana Benchmark GX	8	100
Ventana Benchmark ULTRA	99	88
Ventana Benchmark XT	44	89

Breast Steroid Hormone Receptor Run: 117

Heat Mediated Retrieval	Oestrogen receptors N	Oestrogen receptors %
Biocare Decloaking Chamber	2	50
Dako Omnis	9	100
Dako PTLink	24	83
Lab vision PT Module	3	33
Leica ER1 20 mins	12	58
Leica ER1 30 mins	12	50
Leica ER1 40 mins	8	75
Leica ER2 10 mins	3	33
Leica ER2 20 mins	26	85
Leica ER2 30 mins	3	67
Microwave	1	0
Other	2	100
Pressure Cooker	2	50
Ventana CC1 16mins	2	100
Ventana CC1 20mins	1	100
Ventana CC1 24mins	3	100
Ventana CC1 32mins	7	57
Ventana CC1 36mins	25	84
Ventana CC1 40mins	4	75
Ventana CC1 48mins	2	50
Ventana CC1 52mins	7	86
Ventana CC1 56mins	3	100
Ventana CC1 64mins	43	91
Ventana CC1 76mins	2	100
Ventana CC1 88mins	1	100
Ventana CC1 92mins	2	50
Ventana CC1 extended	3	100
Ventana CC1 mild	14	100
Ventana CC1 standard	29	93
Ventana CC2 mild	2	100
Water bath 95-98 OC	4	50

Breast Steroid Hormone Receptor Run: 117

Enzyme Mediated Retrieval	Oestrogen receptors N	Oestrogen receptors %
AS PER KIT	6	67
NOT APPLICABLE	173	83
Ventana Protease 1 (760-2018)	1	100

Breast Steroid Hormone Receptor Run: 117

Detection	Oestrogen receptors N	%
AS PER KIT	11	82
Biocare polymer (M4U534)	1	100
Dako EnVision FLEX (K8000/10)	10	100
Dako EnVision FLEX+ (K8002/12)	16	75
Dako Envision HRP/DAB (K5007)	2	50
Dako REAL HRP/DAB (K5001)	1	0
LabVision UltraVision ONE Polymer (TL-012/5-HDJ/T	1	0
Leica Bond Polymer Refine (DS9800)	61	70
MenaPath X-Cell Plus (MP-XCP)	1	0
None	1	100
NOT APPLICABLE	2	100
Novocastra Novolink PDS (RE7-140/150/280/290-K)	1	0
Other	5	40
Ventana iView system (760-091)	3	100
Ventana OptiView Kit (760-700)	18	78
Ventana UltraView Kit (760-500)	125	90

Breast Steroid Hormone Receptor Run: 117

Chromogen	Oestrogen receptors N	%
AS PER KIT	18	72
BioGenex liquid DBA (HK-124-7K)	1	0
Dako DAB K3468	1	0
DAKO DAB+	2	100
Dako EnVision Plus kits	2	100
Dako FLEX DAB	22	82
Dako REAL EnVision K5007 DAB	4	75
Dako REAL K5001 DAB	1	0
Leica Bond Polymer Refine kit (DS9800)	61	70
menapath xcell kit DAB (MP-860)	1	0
Other	8	88
Ventana DAB	13	92
Ventana Enhanced Alk. Phos. Red Detection Kit	1	100
Ventana iview	3	100
Ventana Ultraview DAB	123	89

BEST METHODS

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

Oestrogen receptors - Method 1

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

Primary Antibody: Leica/Novocastra NCL-ER-6F11/2 , 15 Mins, RT ºC Dilution 1: 50
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER1 30 mins, PH: 6
EAR:
Chromogen: Leica Bond Polymer Refine kit (DS9800), RT ºC., Time 1: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins, RT ºC Prediluted

Oestrogen receptors - Method 2

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

Primary Antibody: Dako (EP1) M3643 , 20 Mins, 22 ºC Dilution 1: 50
Automation: Dako Autostainer Link 48
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako PTLink, Buffer: FLEX TRS HIGH PH
EAR: NOT APPLICABLE
Chromogen: Dako FLEX DAB, 22 ºC., Time 1: 10 Mins
Detection: Dako EnVision FLEX+ (K8002/12) , 20 Mins, 22 ºC Prediluted

Oestrogen receptors - Method 3

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

Primary Antibody: Dako (EP1) M3643 , 32 Mins, 36 ºC Dilution 1: 1/40
Automation: Ventana Benchmark ULTRA
Method: Ventana Optiview
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 56mins, Buffer: CC1
EAR: NOT APPLICABLE
Chromogen: Ventana DAB
Detection: Ventana OptiView Kit (760-700) Prediluted

Oestrogen receptors - Method 4

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

Primary Antibody:	Ventana 790-4324 (SP1) , 8 Mins, 36 ºC
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 40mins
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT, Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 36 ºC

The Breast HER2 ICC Module Run 117

Amy Newman and Suzanne Parry

Antigen Assessed:	HER2
Sections Circulated:	4 Cell lines of varying Breast HER2 Expression Level (see table below)
Number of Registered Participants:	308
Number of Participants this Run	232 (75%)

Specific Guidelines Used in The Assessment of Slides:

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

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

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

Validation of Distributed UK NEQAS ICC & ISH Cell Lines

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

Assessment Scoring Procedure

UK NEQAS Specific Membrane Scoring Algorithm:

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

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

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

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

Numerical Scoring Criteria

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

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

Introduction

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

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

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

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

In-House Control Tissue Recommendations

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

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

Assessment Summary:

Pass rates for the assessment are summarised in the table below:

	Pass Rates Run 117:	
	NEQAS	**In-House**
Acceptable	72% (N=171)	64% (N=153)
Borderline	15% (N=36)	27% (N=63)
Unacceptable	13% (N=32)	9% (N=21)

As with previous runs the most popular antibody was the Ventana 4B5, used by 72% of participants and showed an overall acceptable pass rate of 82%. 12 laboratories are using the Dako HercepTest, with only 33% participant achieving an acceptable pass rate. 17 laboratories are using the Leica Oracle assay kit with an acceptable pass rate of 71%. 37 laboratories used laboratory devised tests (LTDs), using a variety of antibodies, pre-treatment methods and staining platforms. These laboratories showed an acceptable pass rate of 38%. All data is based on the methodology information submitted by participants, but unfortunately not all methodology was provided by all labs.

References

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

Acknowledgments

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

Selected Images showing Optimal and Sub-optimal Immunostaining

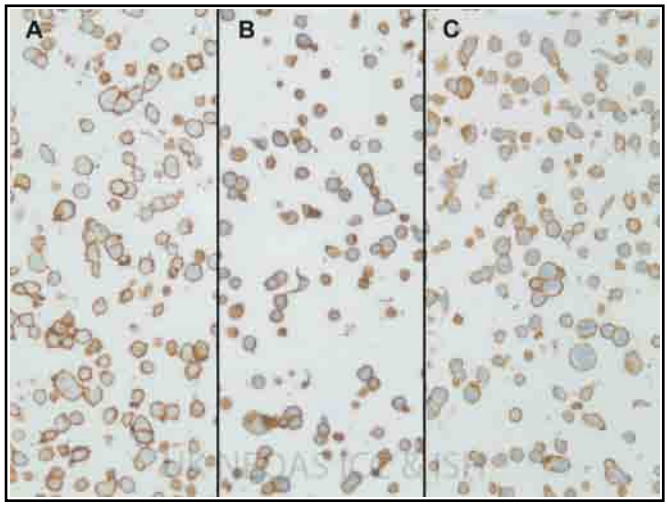


Figure 1: Excellent demonstration of the NEQAS 3+ cell line by each of the three FDA approved assays. All show clear, distinct strong membrane staining. A: Ventana 4B5; B: Dako HercepTest; C: Leica Oracle.

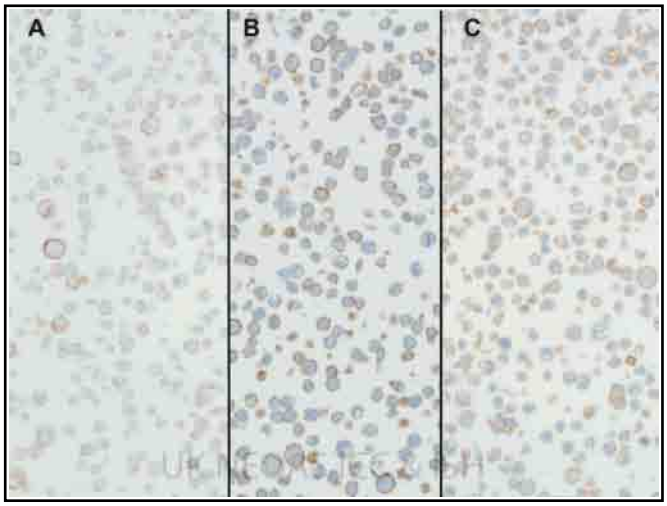


Figure 2: Excellent examples of the NEQAS 2+ cell line stained with each of the three FDA approved assays. All display moderate to strong circumferential staining. A: Ventana 4B5; Dako HercepTest; Leica Oracle.

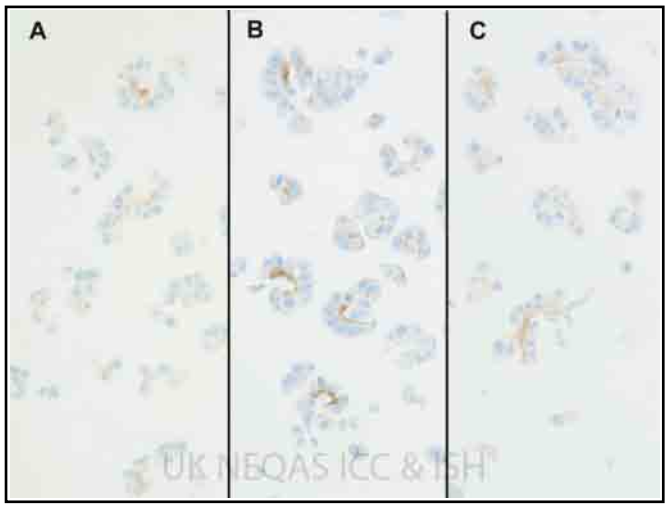


Figure 3: Optimal demonstration of the NEQAS 1+ staining using the three assays outlined in Figures 1 & 2. The staining pattern in incomplete/partial weak membrane staining. The 1+ HER-2 expression using the Ventana 4B5 (3A) is slightly weaker than the Dako HercepTest (3B) and the Leica Oracle (3C), however, this is a noted feature of this assay.

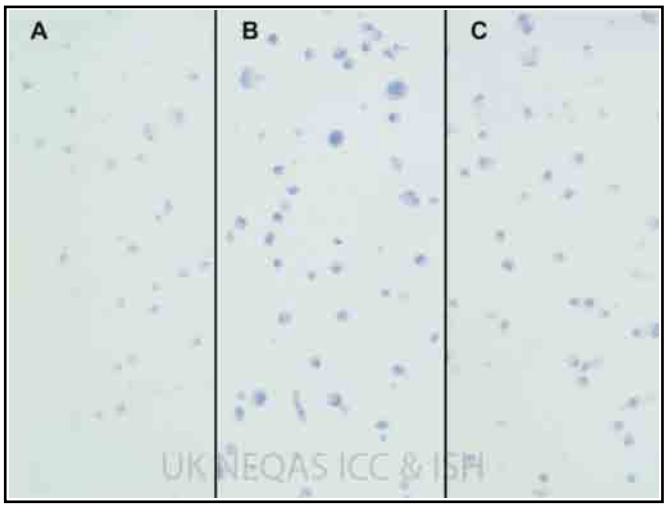


Figure 4: Negative demonstration of the NEQAS 0 HER-2 cell line which is free from any non-specific staining. Excellent demonstration from each of the approved assays. Figures 4A-C as described in Figures 1 & 2.

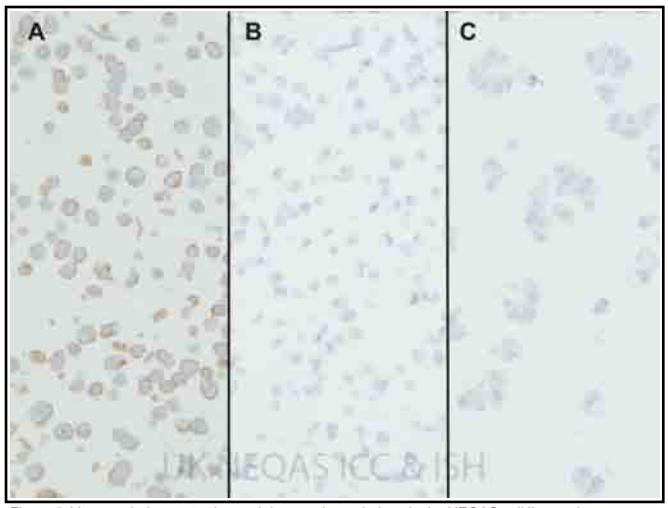


Figure 5: Very weak demonstration and damaged morphology in the NEQAS cell lines using an LTD. 5A: 3+ NEQAS cell line. Expression has dropped to a 2+. 5B: 2+ NEQAS cell line. There is noHER-2 expression. 5C: 1+ cell line with no staining. Method: Labvision (SP3) clone (1/50, 30'); Dako Autostainer (HMAR: PT Link pH8.4; Detection: NS).

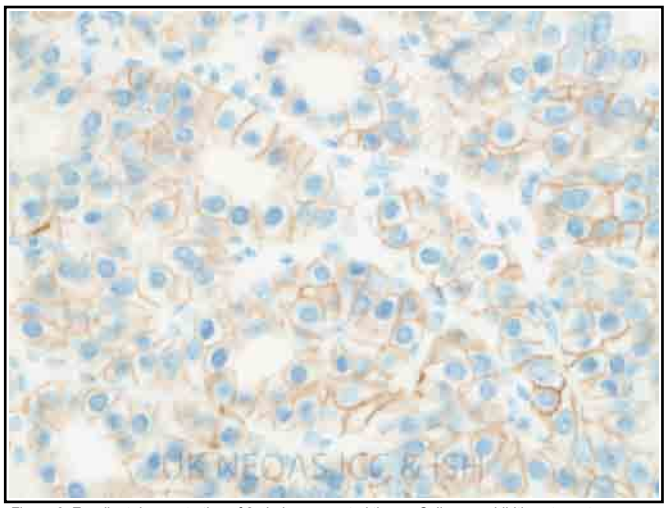
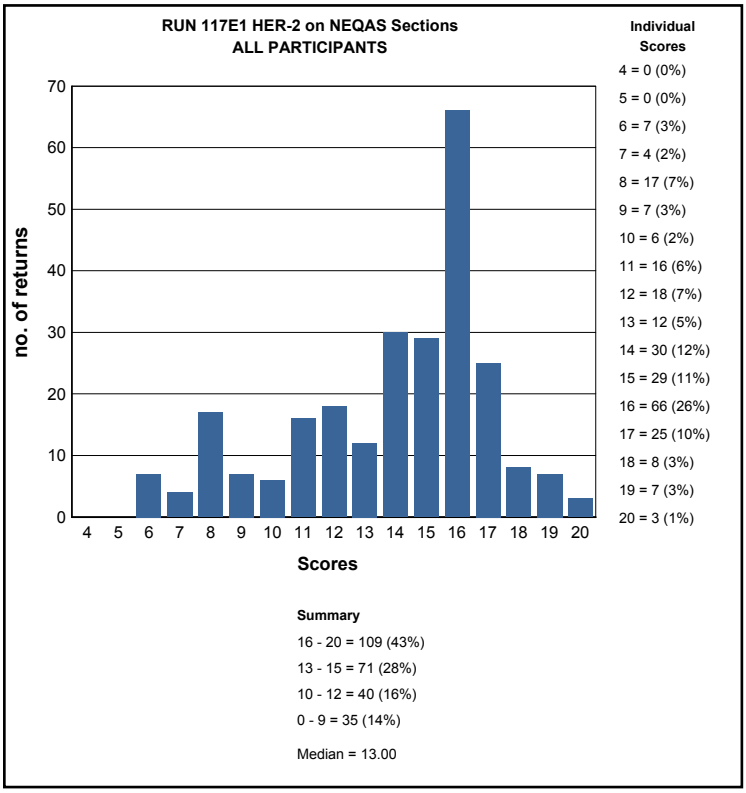
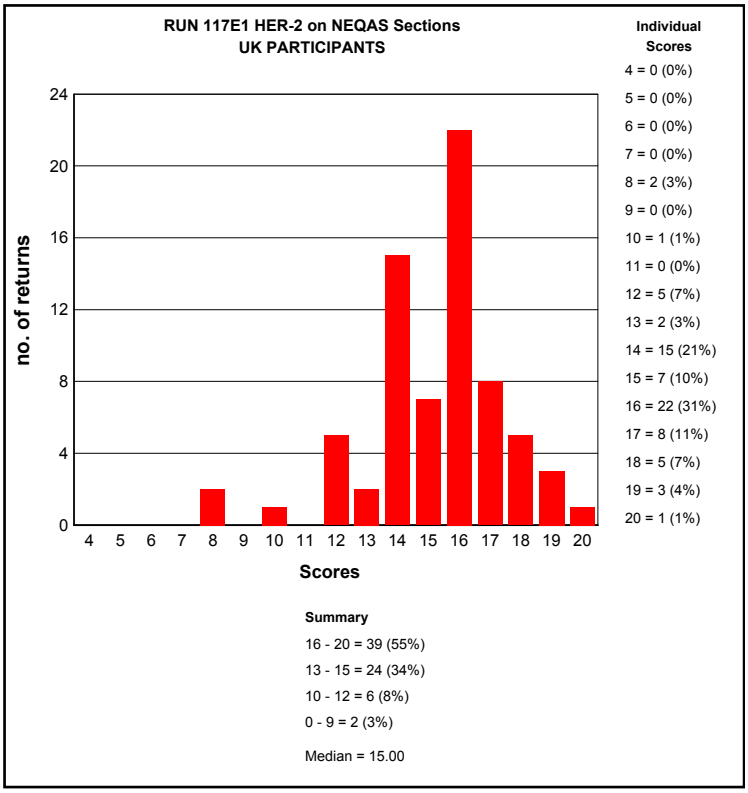
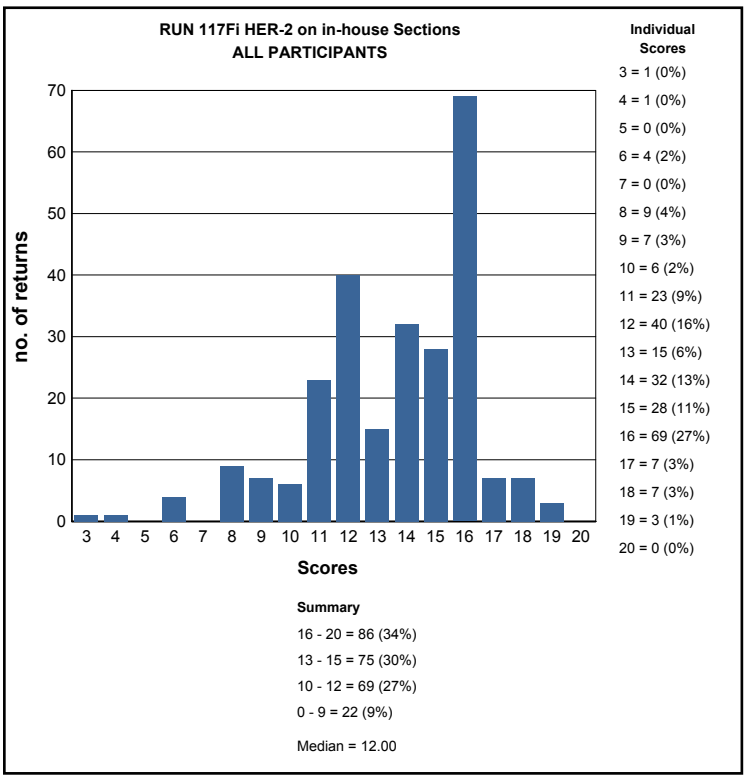


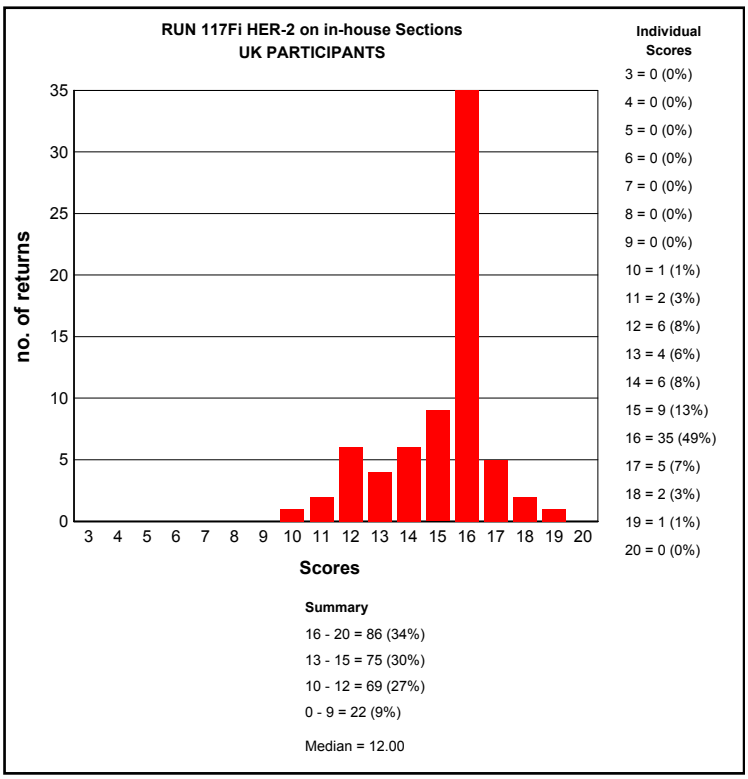
Figure 6: Excellent demonstration of 2+ in-house control tissue. Cells are exhibiting strong to moderate partial/incomplete membrane staining. Method: Dako A0485; Dako Omnis (HMAR: - Not specified; Detection: Dako EnVision FLEX).

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: 117

Primary Antibody	N	%
Dako HercepTest K5204 (poly)	2	0
Dako HercepTest K5205 (poly)	1	0
Dako HercepTest K5207 (poly)	1	0
Dako A0485 C-erB-2 (poly)	25	40
Cell Marque CMA 601 (CB11)	2	0
Ventana Pathway 790-100 (4B5)	9	78
Labvision / Neomarkers RM-9103 (SP3)	1	0
Leica Oracle HER2 Bond IHC (CB11)	17	71
Dako Link HercepTest SK001 (poly)	8	50
BioGenex (EP1045Y) rb mono	1	100
Ventana Confirm 790-4493 (4B5)	40	78
Ventana Pathway 790-2991 (4B5)	127	83
Novocastra NCL-L-CB11 (CB11)	4	0
Biocare CME 342 A,B (EP1045Y)	3	33
Other	7	43

Breast HER2 ICC Run: 117

Automation	N	%
Dako Autostainer	3	0
Dako Autostainer Link 48	14	50
Dako Autostainer plus	2	0
Dako Autostainer Plus Link	3	33
Dako Omnis	3	100
LabVision Autostainer	1	0
Leica Bond Max	11	55
Leica Bond-III	16	63
None (Manual)	11	9
Other	1	0
Shandon Sequenza	1	0
Ventana Benchmark GX	12	83
Ventana Benchmark ULTRA	101	85
Ventana Benchmark XT	69	72

Breast HER2 ICC Run: 117

Heat Mediated Retrieval	N	%
Biocare Decloaking Chamber	4	0
Dako Omnis	3	100
Dako PTLink	18	44
Lab vision PT Module	3	0
Leica ER1 10 mins	1	0
Leica ER1 20 mins	8	38
Leica ER1 25 mins	13	85
Leica ER1 30 mins	1	0
Leica ER1 40 mins	1	0
Leica ER2 10 mins	1	100
Leica ER2 30 mins	1	100
Microwave	3	33
None	3	67
Other	2	50
Ventana CC1 16mins	2	50
Ventana CC1 20mins	1	100
Ventana CC1 24mins	1	100
Ventana CC1 32mins	17	76
Ventana CC1 36mins	52	88
Ventana CC1 40mins	3	67
Ventana CC1 48mins	1	0
Ventana CC1 52mins	4	100
Ventana CC1 56mins	7	86
Ventana CC1 64mins	6	83
Ventana CC1 8mins	3	67
Ventana CC1 92mins	1	0
Ventana CC1 mild	62	79
Ventana CC1 standard	14	71
Ventana CC2 24mins	1	100
Ventana CC2 36mins	1	100
Water bath 95-98 OC	7	0

Breast HER2 ICC Run: 117

Detection	N	%
AS PER KIT	19	63
Biocare SLAB (STU HRP 700H,L10)	3	0
Dako HerCep Test (K5204)	1	0
Dako EnVision FLEX (K8000/10)	5	60
Dako EnVision FLEX+ (K8002/12)	8	50
Dako Envision HRP/DAB (K5007)	3	0
Dako HerCep Test Autor (SK001)	7	43
LabVision UltraVision ONE Polymer (TL-012/5-HDJ/T	1	0
Leica Bond Polymer Refine (DS9800)	17	47
Other	5	20
Ventana iView system (760-091)	5	80
Ventana OptiView Kit (760-700)	10	80
Ventana UltraView Kit (760-500)	157	80

Breast HER2 ICC Run: 117

Enzyme Retrieval	N	%
AS PER KIT	17	59
NOT APPLICABLE	136	75
Ventana Protease	2	50
Ventana Protease 1 (760-2018)	2	50

Breast HER2 ICC Run: 117

Chromogen	N	%
AS PER KIT	40	70
BioGenex liquid DBA (HK-124-7K)	1	100
DAKO DAB+	1	100
Dako DAB+ Liquid (K3468)	1	0
Dako EnVision Plus kits	1	0
Dako FLEX DAB	12	50
Dako REAL EnVision K5007 DAB	5	0
Leica Bond Polymer Refine kit (DS9800)	17	47
Other	10	40
Ventana DAB	7	71
Ventana iview	4	50
Ventana Ultraview DAB	149	80

BEST METHODS

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

HER-2 - Method 1

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

Primary Antibody:	Leica Oracle HER2 Bond IHC (CB11) , 30 Mins, amb ºC Prediluted
Automation:	Leica Bond Max
Method:	AS PER KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER1 25 mins
EAR:	
Chromogen:	AS PER KIT
Detection:	AS PER KIT Prediluted

HER-2 - Method 2

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

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

HER-2 - Method 3

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

Primary Antibody:	Ventana Pathway 790-2991 (4B5) , 24 Mins, amb ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.6
HMAR:	Ventana CC1 36mins
EAR:	
Chromogen:	Ventana Ultraview DAB, amb ºC., Time 1: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, amb ºC

HER-2 - Method 4

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

Primary Antibody:	Ventana Confirm 790-4493 (4B5) , 16 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark GX
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.4
HMAR:	Ventana CC1 mild
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500) Prediluted

The Gastric HER2 ICC Module Run 117

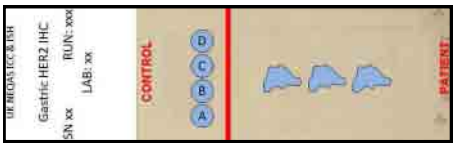
Suzanne Parry

Antigen Assessed:	HER2
Sections Circulated:	Composite slide consisting of 4 surgical intestinal gastric carcinomas with different levels of HER2 expression
Number of Registered Participants:	78
Number of Participants this Run	62 (79%)

Expected staining characteristics of the UK Neqas distributed tissue for Run 117:

Sample Position (from left to right on slide)	Expected HER2 IHC Expression Level
A B D	0 0 3+

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



Neqas Sections **In House Sections**

Please Note: Any variability and heterogeneity of HER2 expression throughout the block is taken into consideration when scoring participants slides. Core C was not included in the assessment due to stability of the tissue.

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

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

Validation of Distributed Samples

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).

Section Label From slide Label End	Staining Pattern with IHC	HER2 status by ISH
A	**0**	Non-Amplified
B	**0**	Non-Amplified
D	**3+**	Amplified

Table 2: Showing the HER2 IHC staining and ISH results

Assessment Procedure

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

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

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

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

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

Introduction

The American Society of Clinical Oncology (ASCO), the College of American Pathologists (CAP) and the American Society for Clinical Pathology (ASCP) have issued a **new** joint guideline on HER2 testing for patients with gastroesophageal cancers (GEA) on 14th of Nov 2016. Please see: www.jco.org. This guideline provides specific recommendations for assessment of *HER2* in patients with advanced GEA while addressing pertinent technical issues and clinical implications of the results. 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. 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) ISH techniques to confirm their IHC findings.

Assessment Results

Features Of Acceptable Staining: (Figs 1, 3, 5 & 6)

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

Features Of Suboptimal or Unacceptable Staining: (Figs 2& 4)

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

Additional Comment:

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

Pass Rates

The pass rates for the NEQAS distributed samples were (see table below):

NEQAS Pass Rates Run 117:	
Acceptable	96% (N=60)
Borderline	2% (N=1)
Unacceptable	2% (N=1)

There has been a remarkable significant improvement in pass rates over the past several runs. Improvement has increased from 73% in Run 113 to 96% in Run 117. Only 1 laboratory (2%) obtained an unacceptable result due to weak staining. In the clinical setting, false negative results may lead to suitable patients not being put forward for Herceptin therapy, and equally concerning is that many patients may be over-treated: For instance, a HER2 IHC result of 3+ will automatically mean that the patient is put forward for Herceptin therapy. Also, as IHC 2+ cases are also reflex tested, incorrect over-staining could mean that more samples than necessary are being put forward for ISH reflex testing.

Most labs are using the recommended standardised protocols for their particular automated systems, such as CC1 Mild antigen retrieval on the Ventana Benchmark platforms with the 4B5 pre-diluted antibody clone. However, a few labs are not using the recommended protocols, and this may have the potential to induce weaker or stronger membrane staining than expected.

Only one laboratory did not submit in-house control material for assessment. Participant results for those that did submit in -house results are summarised in the following table:

In-House Pass Rates Run 117:	
Acceptable	79% (N=48)
Borderline	20% (N=12)
Unacceptable	2% (N=1)

The overall pass rates on the in-house controls was lower than on the Neqas samples. More laboratories received a borderline pass on the in-house samples. Several of these borderline passes were given because the laboratories did not submit ideal composite control material, consisting of a 3+, 2+ and 1+/0 expressing gastric/breast control tumour sample. These labs were therefore given a maximum score of 12/20. Other labs were marked down for poor tissue quality or fixation. No labs failed on their in-house sections on this assessment. Recommendations for control tissue are described below.

Methodologies

The majority of labs are using the Ventana 4B5 standardised kit for their gastric HER2 testing: In this particular assessment run the Ventana assay was used by 89% of participants with an average acceptable pass rate of 98%. 3 laboratories used the Dako HercepTest with 67% acceptable pass rate.

Control Tissue and Recommendations

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

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

References:

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

Acknowledgments

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

Selected Images showing Optimal and Sub-optimal Immunostaining

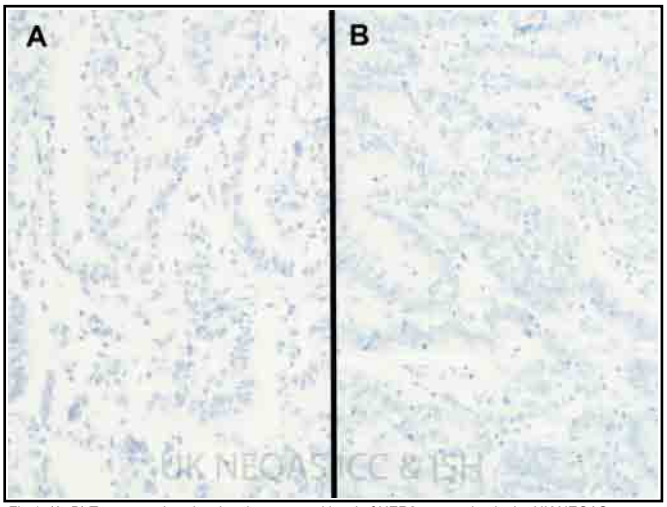


Fig 1. (A, B) Two examples showing the expected level of HER2 expression in the UK NEQAS negative gastric Her2 samples A and B. Both sections stained using the Ventana 4B5 assay with the recommended protocols.

Fig 2. Unacceptable staining in the UK NEQAS gastric HER2 negative sample A. The example shows inappropriate excessive membrane staining which is more representative of a 2+. It is unclear why the staining is not as expected as the recommended 4B5 assay protocol was used.

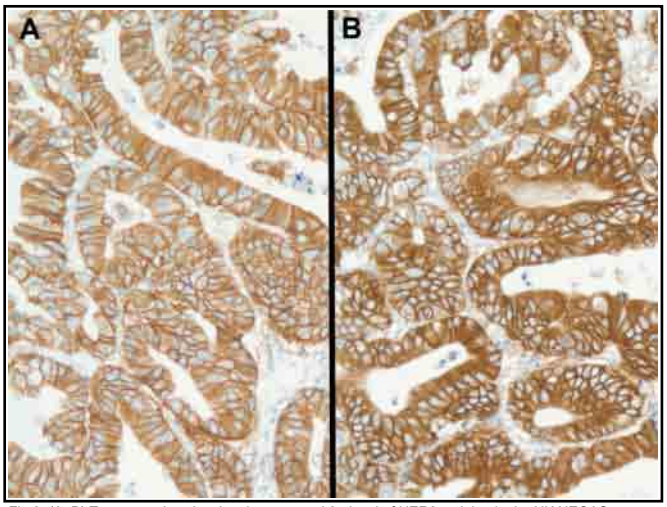


Fig 3. (A, B) Two examples showing the expected 3+ level of HER2 staining in the UK NEQAS gastric distributed sample D. (A) stained with the Ventana 4B5 pathway on the Benchmark XT, and (B) stained with the Dako HercepTest.

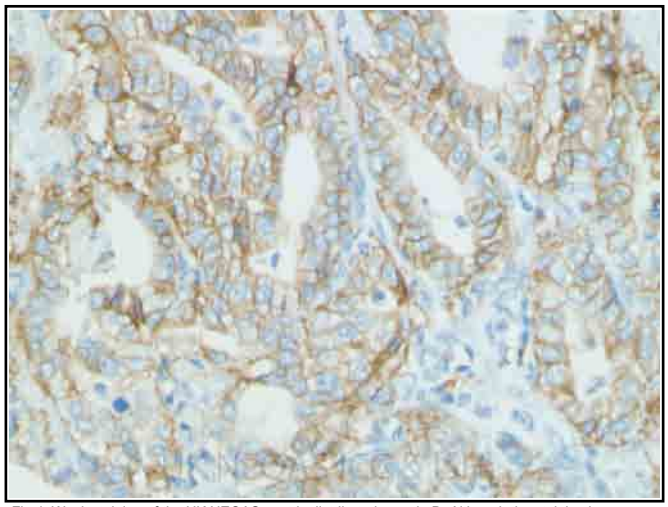


Fig 4. Weak staining of the UK NEQAS gastric distributed sample D. Although the staining is still a 3+, the staining is slightly weak. Stained using the Labvision Neomarkers SP3 clone on the Dako Autostainer with pre-treatment in the Labvision PT module.

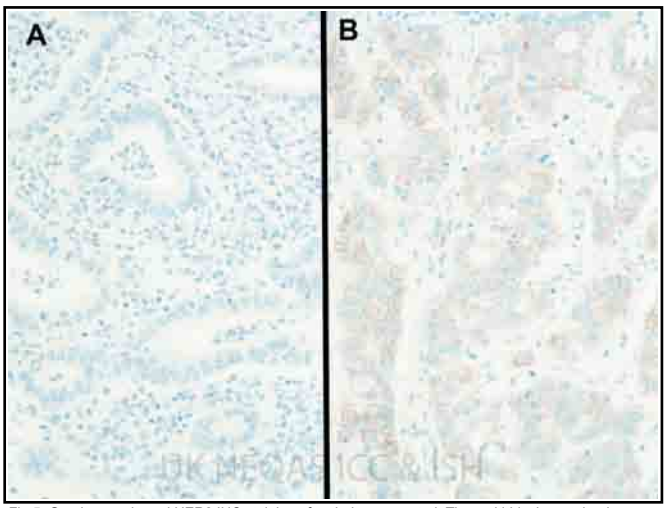


Fig 5. Good example and HER2 IHC staining of an in-house control. The multi-block contained (A) negative, (B) Her2 2+ and 3+ expressing gastric tumour (3+ tumour not shown). Stained with the Ventana 4B5 assay on the Benchmark ULTRA using the recommended protocol.

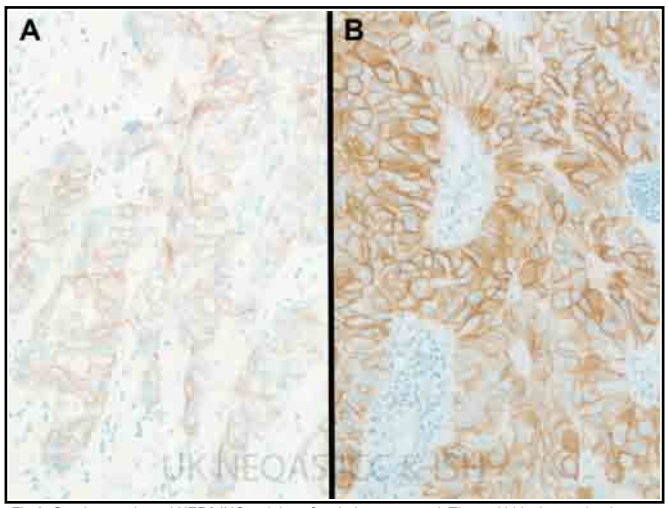
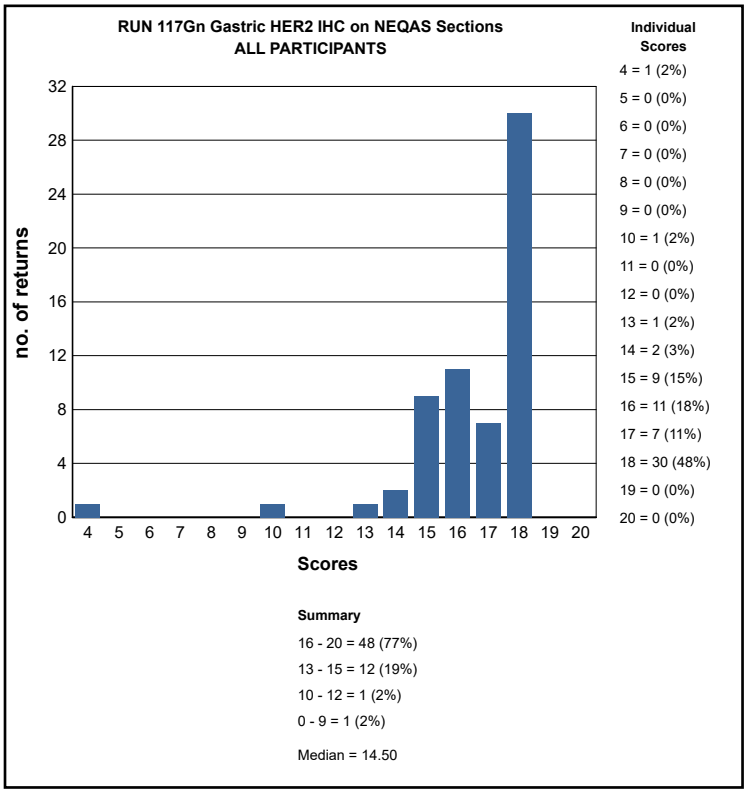
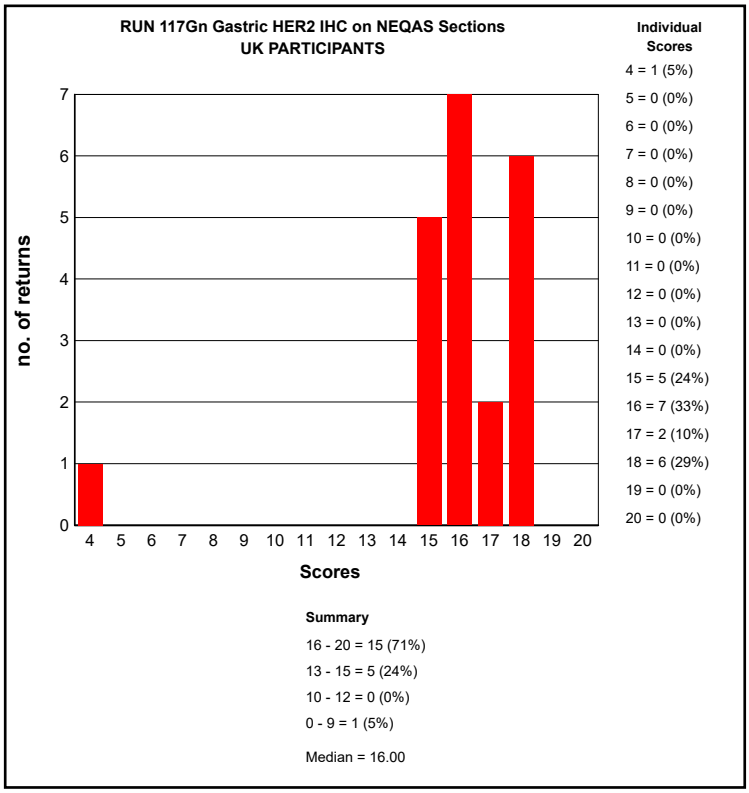
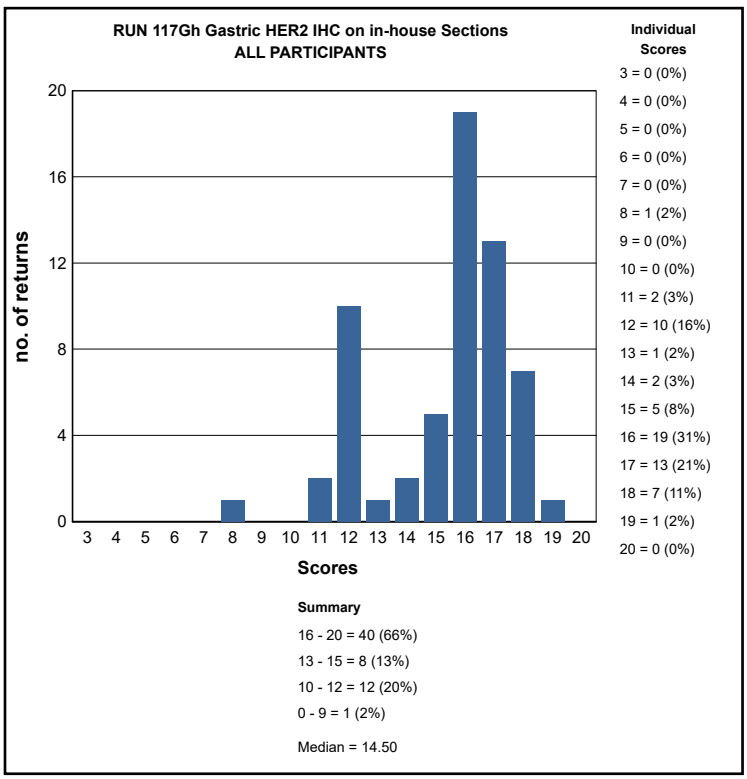


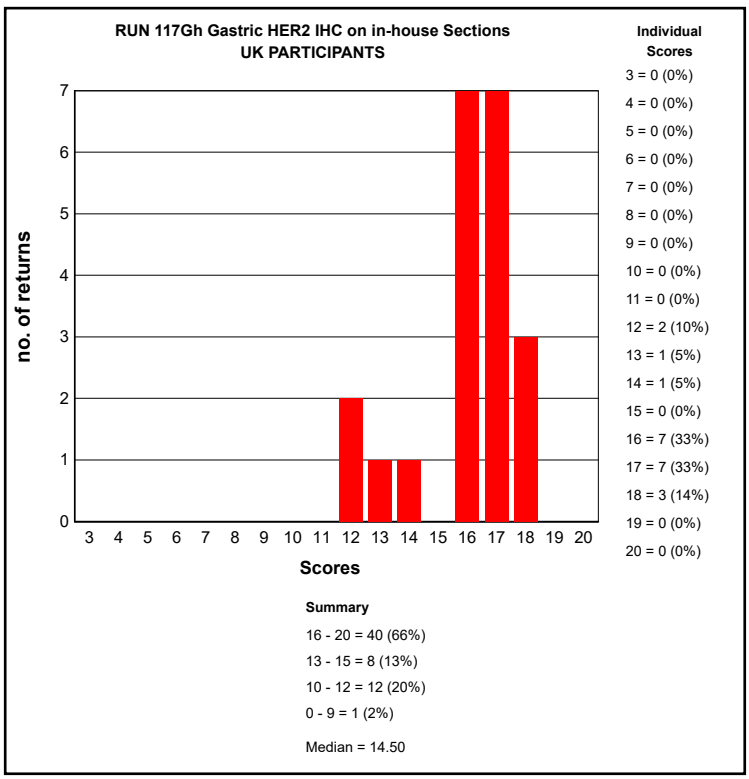
Fig 6. Good example and HER2 IHC staining of an in-house control. The multi-block contained (A) 2+, (B) 3+, and negative expressing gastric tumours (negative tumour not shown). Stained with the Ventana 4B5 assay on the Benchmark ULTRA, CC1 for 36 minutes.

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: 117

Primary Antibody	N	%
Dako A0485 C-erB-2 (poly)	1	100
Labvision / Neomarkers RM-9103 (SP3)	1	100
Dako Link HercepTest SK001 (poly)	3	67
Ventana Pathway 790-100 (4B5)	1	100
Ventana Pathway 790-2991 (4B5)	37	100
Other	1	100
Ventana Confirm 790-4493 (4B5)	17	94

Gastric HER2 ICC Run: 117

Automation	N	%
Dako Autostainer Link 48	1	100
Dako Autostainer plus	1	100
Dako Autostainer Plus Link	2	50
Dako Omnis	1	100
Leica Bond Max	1	100
None (Manual)	1	100
Ventana Benchmark GX	2	100
Ventana Benchmark ULTRA	32	100
Ventana Benchmark XT	20	95

Gastric HER2 ICC Run: 117

Heat Mediated Retrieval	N	%
Dako Omnis	1	100
Dako PTLink	3	67
Lab vision PT Module	2	100
Leica ER2 30 mins	1	100
Ventana CC1 16mins	1	100
Ventana CC1 24mins	1	100
Ventana CC1 32mins	4	100
Ventana CC1 36mins	17	100
Ventana CC1 56mins	2	100
Ventana CC1 64mins	5	100
Ventana CC1 mild	20	95
Ventana CC1 standard	4	100

Gastric HER2 ICC Run: 117

Detection	N	%
AS PER KIT	3	100
Dako EnVision FLEX+ (K8002/12)	1	100
Dako HerCep Test Autor (SK001)	2	50
Leica Bond Polymer Refine (DS9800)	1	100
Other	2	100
Ventana iView system (760-091)	2	100
Ventana OptiView Kit (760-700)	2	100
Ventana UltraView Kit (760-500)	46	98

Gastric HER2 ICC Run: 117

Enzyme Retrieval	N	%
AS PER KIT	5	100
NOT APPLICABLE	28	96
Ventana Protease 1 (760-2018)	2	100

Gastric HER2 ICC Run: 117

Chromogen	N	%
AS PER KIT	9	100
DAKO DAB+	1	100
Dako FLEX DAB	2	50
Leica Bond Polymer Refine kit (DS9800)	1	100
Other	2	100
Ventana DAB	2	100
Ventana iview	1	100
Ventana Ultraview DAB	43	98

BEST METHODS

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

Gastric HER2 IHC - Method 1

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

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

Gastric HER2 IHC - Method 2

Participant scored 18/20 (UK NEQAS Slide) and 18/20 (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 3

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

Primary Antibody:	Ventana Confirm 790-4493 (4B5) , 12 Mins, 36 ºC
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 36mins
EAR:	
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

David Blythe and Amy Newman

	Gold Standard	Second Antibody
Antigens Assessed:	BCL-2	CD30
Tissue Sections circulated:	Follicular lymphoma and reactive tonsil	Lymphoblastic Leukemia
Number of Registered Participants:	217	
Number of Participants this Run	187 (86%)	

Introduction

Gold Standard: BCL-2

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

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

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

Features of Sub-optimal Immunostaining (Fig 2, 4 & 6):

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

References:

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

Second Antigen: CD30

CD30 is a transmembrane cytokine receptor and plays a role in regulating the function, differentiation and/or proliferation of normal lymphoid cells[1]. Malignant cells in Hodgkin lymphoma are termed Hodgkin (mono-nuclear) or Reed-Sternberg (multi-nuclear) cells, although they can also be referred to as Hodgkin Reed-Sternberg (HRS) cells. The origin of HRS cells became clear when techniques were established to isolate single HRS cells from biopsy specimens and analyse them for rearranged immunoglobulin genes by single cell PCR. It is now known that these cells represent clonal populations of transformed germinal centre B-cells, classical Hodgkin lymphoma (CHL) but rarely in nodular lymphocyte predominant lymphomas (NLPHL). They are also positive in anaplastic large cell lymphomas (ALCL) arising from B, T and null cell lineage[2]. In summary, in CHL, the HRS cells are positive with CD30, often positive with CD15 (in approximately 80% of cases) and generally negative for B-cell antigens such as CD20 or CD79a (approximately 30% of CHL cases will show some staining with these markers). In NLPHL, the HRS cells (also known as L&H cells or popcorn cells) are negative for both CD30 and CD15 and are generally positive for B-cell antigens. CD30 staining is found in CHLs, ALCLs, germ cell tumours and in a varying proportion of activated T- and B-cells. The staining pattern of CD30 in CHL is very similar to that of CD15 but without the granulocyte positivity. Some plasma cells will also stain with this antibody.

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

- Membrane staining (granular) in most HRS.
- Golgi staining of some HRS.
- Clean background.

Features of Sub-optimal Immunostaining (Figs 8, 10 & 11):

- Weak, uneven or negative staining of the HRS.
- 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. de Bruin PC, et al. CD30 expression in normal and neoplastic lymphoid t issue: biological aspects and clinical implications (review). Leukaemia 1995;9: 1620-7.
2. Bishop P, Nodular lymphocyte predominant Hodgkin's lymphoma (NLPHL), www.e-immunohistochemistry.info; 2012

Assessment Summary:

BCL-2

187 laboratories submitted their slides for the BCL-2 assessment. Similarly to previous results, weak staining or poor localisation with diffuse uneven staining were the main reasons for receiving a borderline pass or in some cases, where there was very little staining at all, the labs received a failed score. This was not attributable to a particular antibody clone, but mostly due to an inappropriate dilution or antigen retrieval protocol.

All laboratories submitted an in-house control with a higher acceptable pass rate of 91% compared to the NEQAS scores for this run. No laboratories failed on their in-house material. Results are almost identical to the previous run (116).

Pass rates shown in the table below:

NEQAS Pass Rates Run 117: BCL-2	
Acceptable	81% (N=163)
Borderline	16% (N=33)
Unacceptable	3% (N=7)

A variety of antibodies and automated platforms were used in the assessment. However, overall the most popular antibody clone was the Dako M0887 (124), with 82 participants using this clone and showed a 77% acceptable pass rate.

David Blythe and Amy Newman

CD30

203 laboratories submitted both the NEQAS and in house sections for this assessment. The pass rates were 73% for the NEQAS sections and 87% for the in-house sections. The Borderline/Unacceptable pass rates on the in-house sections were much lower compared to the NEQAS sections. (summarised in table below):

NEQAS and In-house Pass Rates Run 117 CD30		
	NEQAS	In-house
Acceptable	73% (N=149)	87%(N= 124)
Borderline	19% (N=38)	11%(N=23)
Unacceptable	8% (N=16)	1 %(N=2)

It was noted that laboratories that received a borderline pass was due to sub-optimal staining of CD30 mainly due to weak or poorly localised antibody expression. The laboratories (8%) that failed had a combination of very weak, uneven staining with a lower percentage of cells staining than expected . One explanation for a higher failure rate on the NEQAS sections may be due to antigen instability. If slides are not stained as soon as received in the laboratory and are left at room temperature for a few weeks, dependent on the robustness of the antigen to be demonstrated can affect the antigenicity of the slide. The most commonly used primary antibody for this CD30 was the Dako M0751 (Ber-H2) antibody with 69 participants using this clone and this showed an average acceptable pass rate of 77%. Other product versions from Dako, Ventana and Cell Marque of the Ber-H2 clone were commonly used by 51 participants.

Selected Images showing Optimal and Sub-optimal Immunostaining

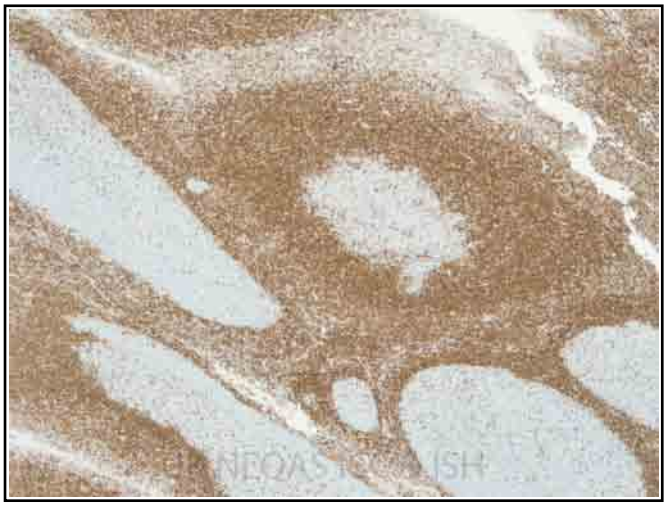


Figure 1: Excellent demonstration of BCL-2 on NEQAS reactive tonsil. Strong to moderate cytoplasmic staining in the lymphocytes in the inter-folllicular areas, scattered T cells in the germinal centres and mantle zone B cells. Germinal centre B cells are negative. Method: Dako M0887 (124) (1/80; 30'); Dako Link 48 (HMAR: PT Link, pH9.0; Detection: Dako Flex).

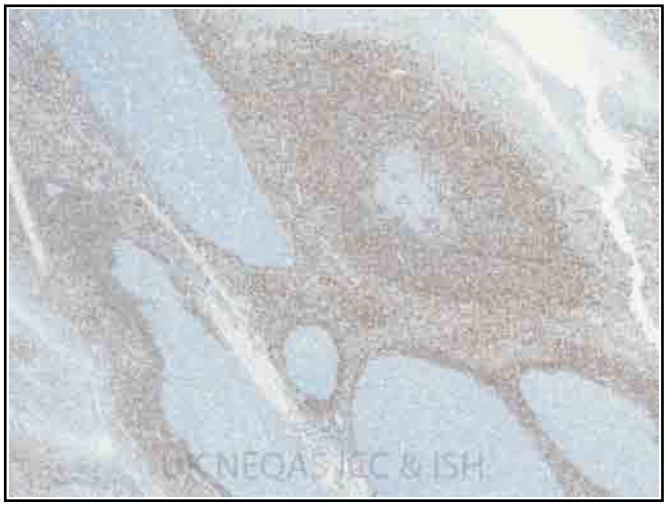


Figure 2: Weak demonstration of BCL-2 on NEQAS reactive tonsil. Staining is weak and diffuse. Borderline score of 12/20. Method: Dako FLEX IR614 (124) clone (RTU; 20'); Dako Omnis (HMAR: Omnis High pH TRS; Detection: Dako FLEX).

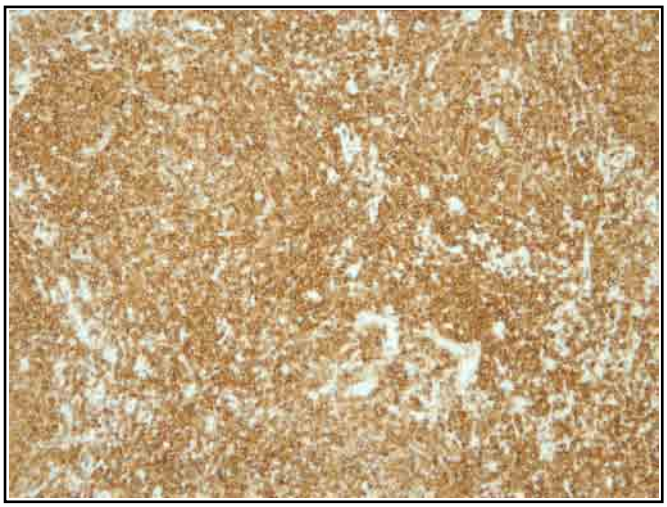


Figure 3: Optimal staining of BCL-2 on NEQAS follicular lymphoma. The majority of neoplastic cells show well localised cytoplasmic staining. Protocol identical to Figure 1.

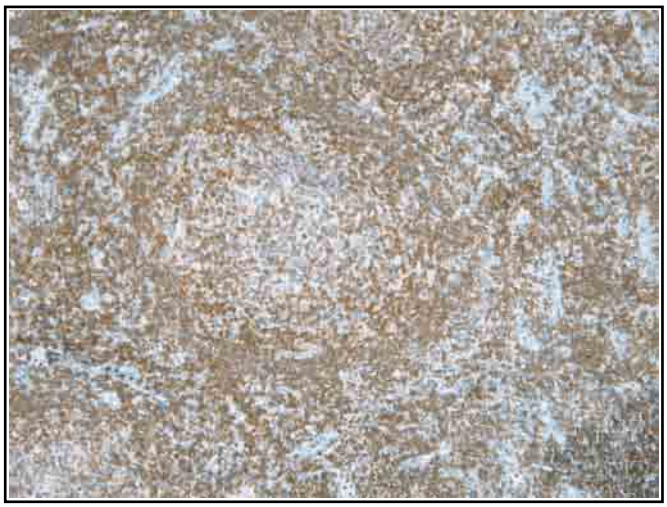


Figure 4: Sub-optimal demonstration of BCL-2 on the NEQAS follicular lymphoma. The staining pattern in diffuse and the antigen is poorly localised. However, this was not considered to interfere with the diagnosis. Overall score 12/20. Method: Dako M0887 (124) clone; Menarini-Intellipath FLX (HMAR: Dako pH6.0, water bath; Detection: Dako REAL HRP/DAB).

Figure 5: Low magnification of Figure 3, demonstrating crisp localisation of BCL-2 antigen in neoplastic cells. Method described in Figure 1.

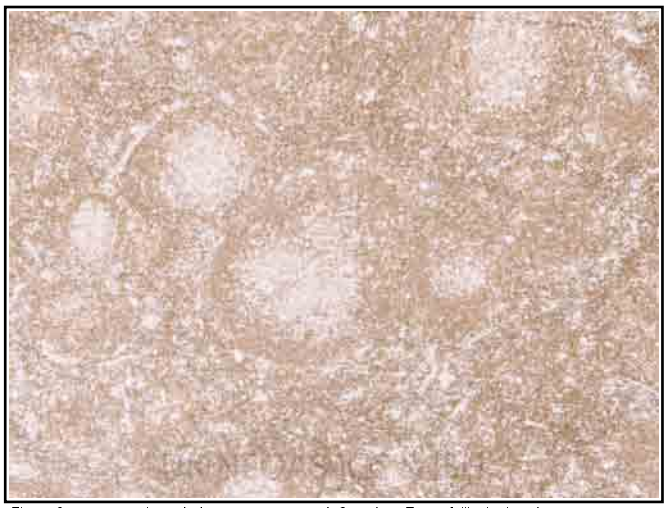


Figure 6: Sub-optimal, weak demonstration of BCL-2 on the NEQAS follicular lymphoma. Staining is weaker and more diffuse than Figure 5. With some negative neoplastic cells within the follicular centres, which may lead to an incorrect diagnosis. Over all score: 10/20. Method: Ventana (124) (RTU, 20'); Ventana ULTRA (HMAR: CC1 32'; Detection: Optiview).

Selected Images showing Optimal and Sub-optimal Immunostaining

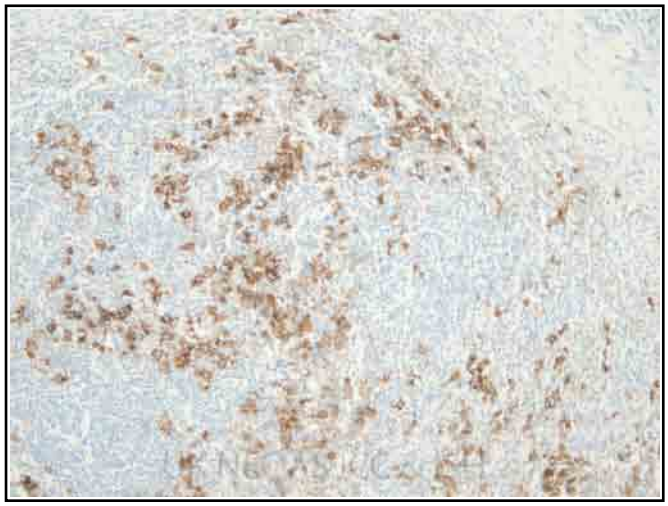


Figure 7: Excellent demonstration of CD30 on the NEQAS Hodgkin's lymphoma. Clear distinct membrane staining of Reed-Sternberg cells. Staining within the Golgi apparatus is also clear on low power. Method: Leica CD30 (RTU; 15'), Leica Bond III (HMAR: ER1, 20'; Detection: Leica Bond Polymer Refine).

Figure 8: Weak demonstration of CD30 on NEQAS Hodgkin's lymphoma. Staining is absent in many of the Reed-Sternberg cells when compared to Figure 7. Method: Dako M0751 (Ber-H2) clone (1/10, 30'); Dako Autostainer Link 48 (HMAR: Envision pH9.0, Dako PT Link; Detection: Dako EnVision FLEX+).

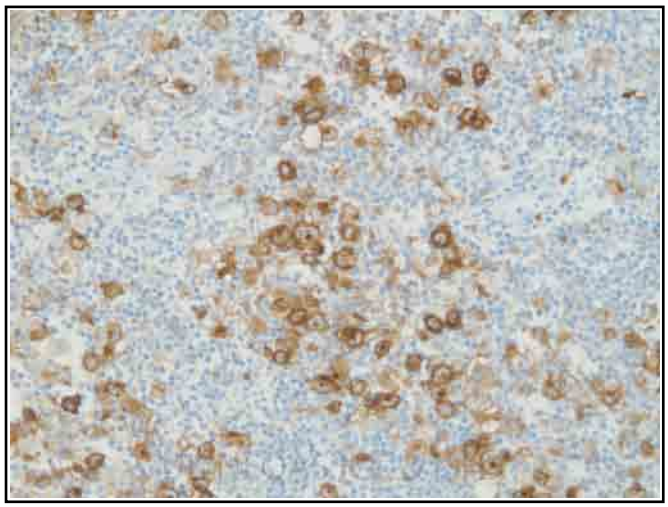


Figure 9: Good demonstration of NEQAS Hodgkin's lymphoma at high magnification. The paranuclear focus of the Golgi apparatus is very distinct. Method described in Figure 7.

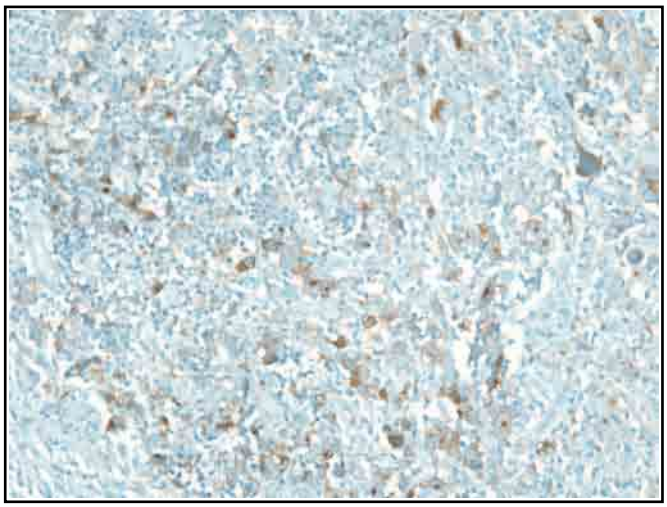


Figure 10: Very poor demonstration of NEQAS Hodgkin's lymphoma. The morphology appears damaged and over-retrieved. Method: Dako M0751 (Ber-H2) clone; Ventana Benchmark XT (HMAR: CC1 mild; Detection; Ventana Ultraview).

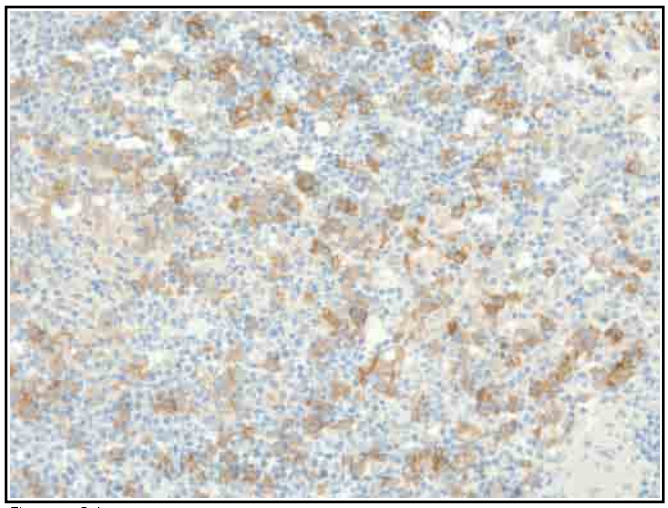


Figure 11: Sub-optimal demonstration of NEQAS Hodgkin's lymphoma. There is poor localisation of the CD30 antigen and the Golgi apparatus are failing to show satisfactory staining. Method: Leica NCL-L-CD30 (1G12) Clone (1/40, 30'); Leica Bond III (HMAR: ER2 20'; Detection: Leica Bond Polymer Refine).

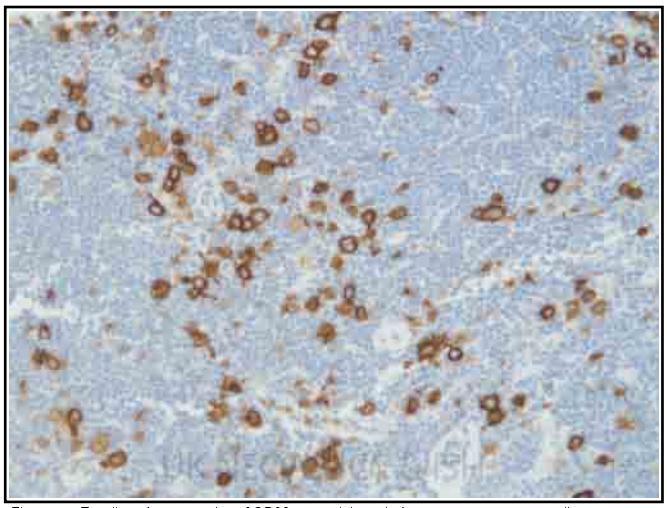
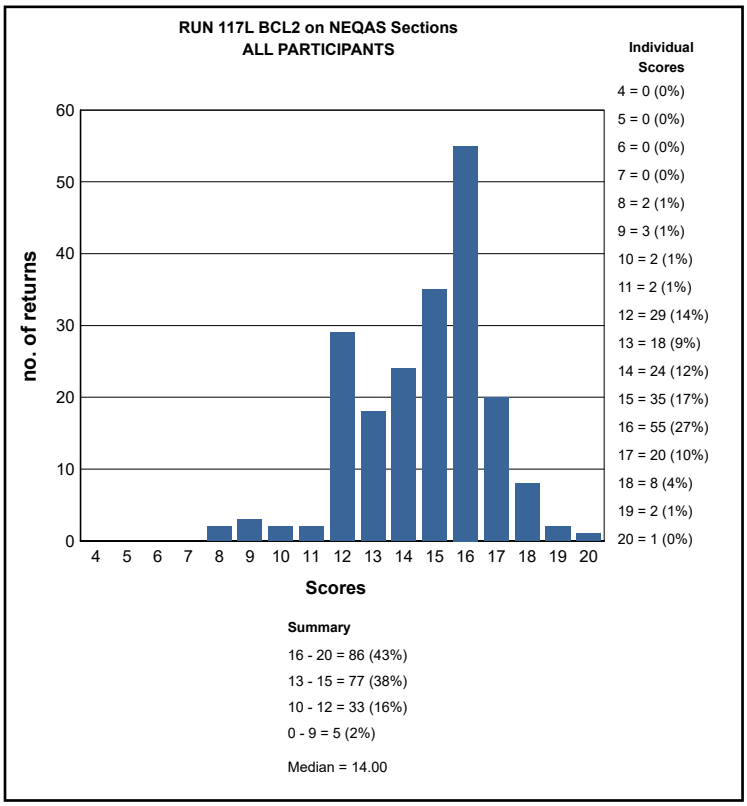
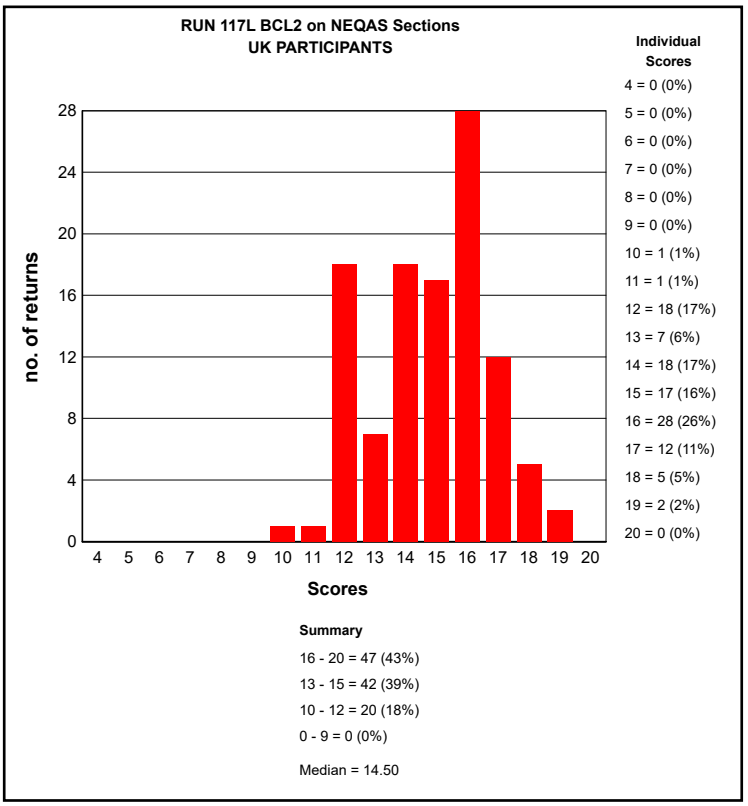
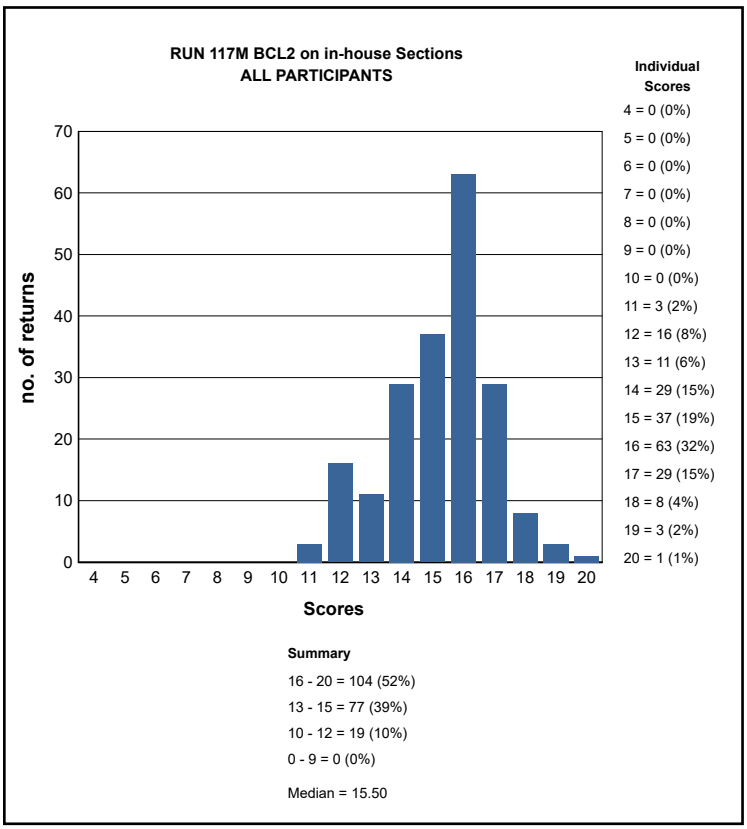


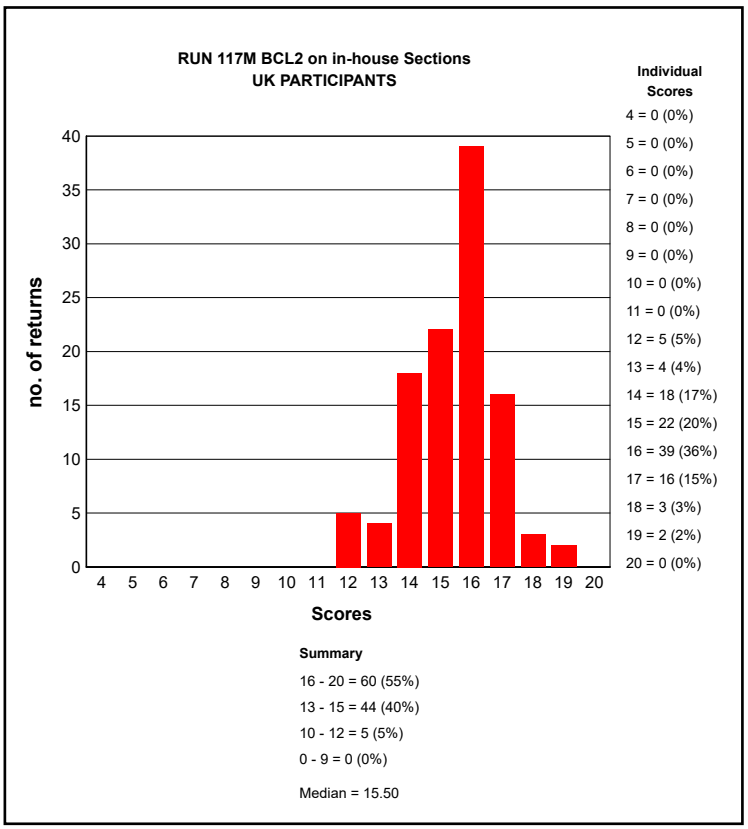
Figure 12: Excellent demonstration of CD30 on participant in-house control. Strong, well localised membrane staining in Hodgkin's tumour cells. Method as per Figure 7.

GRAPHICAL REPRESENTATION OF PASS RATES

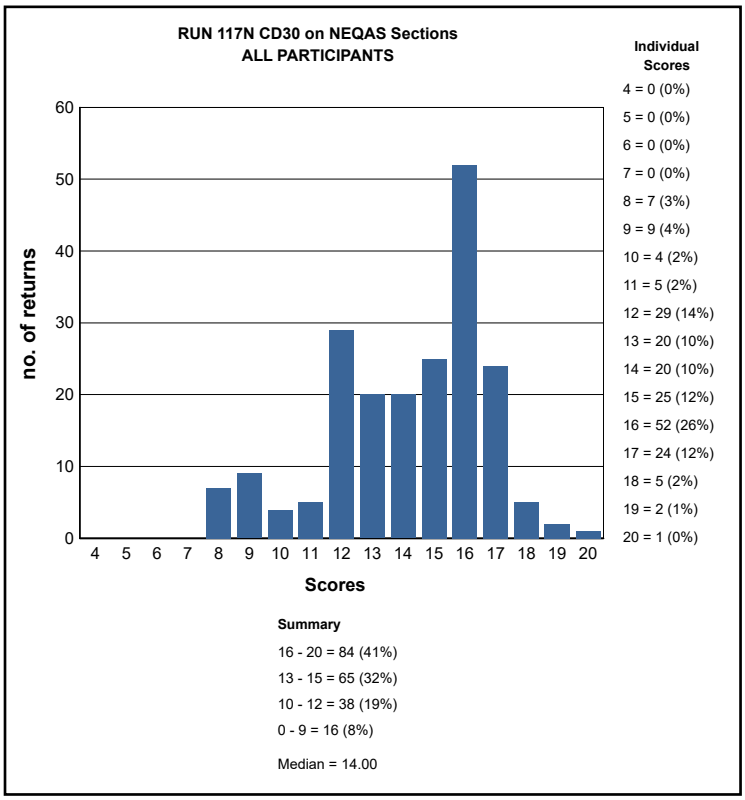


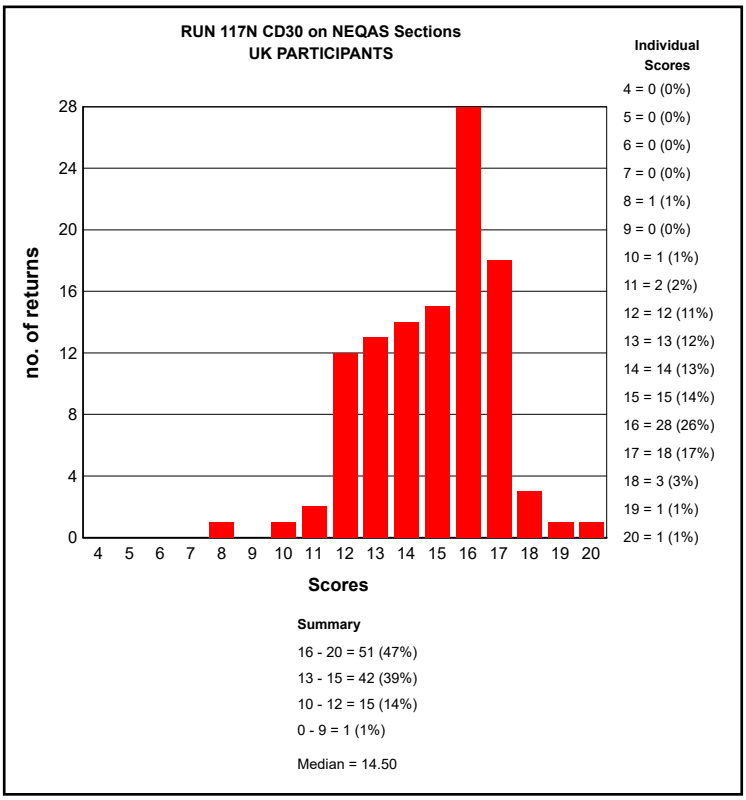


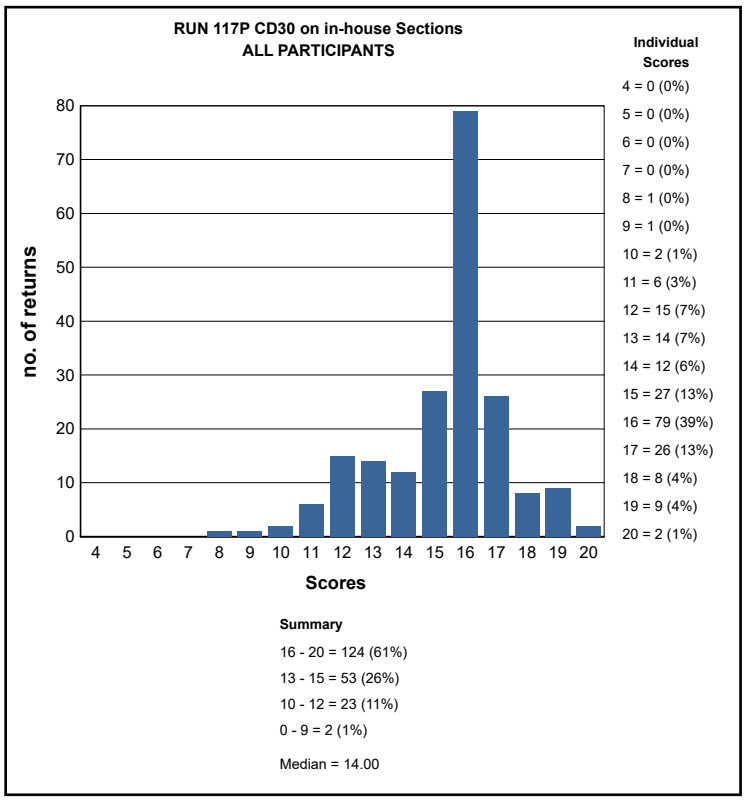


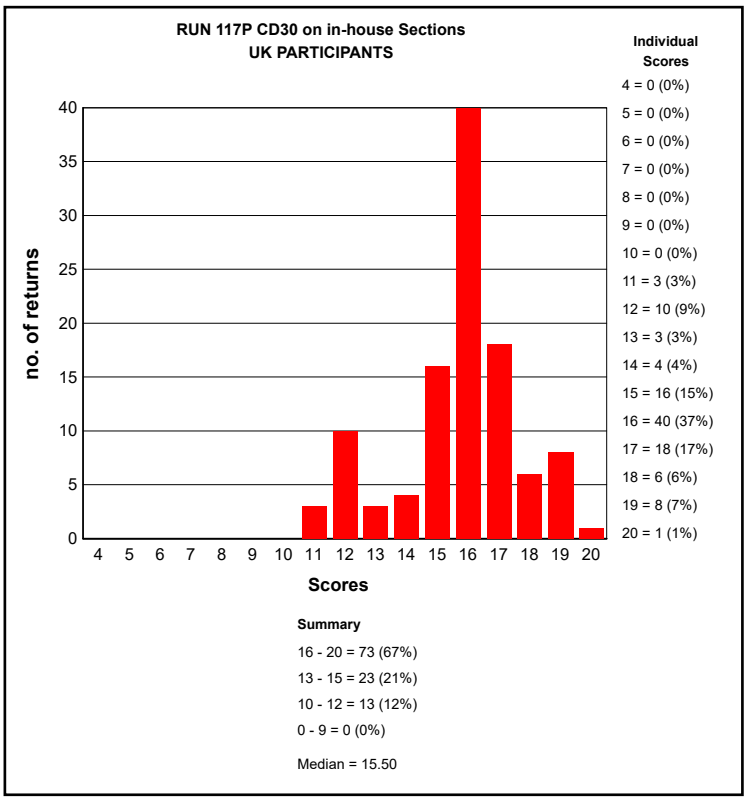


GRAPHICAL REPRESENTATION OF PASS RATES









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: 117

Primary Antibody : BCL2

Antibody Details	N	%
Dako M0887 (124)	75	77
Labvision/Neomarkers MS-123-P (100/D5)	1	0
Leica/Novocastra NCL-BCl-2 (BCl2/100/D5)	15	73
Ventana (SP66) 790-4604	29	93
Ventana (P2D11F11) 760-2693	1	100
Ventana 760-4240 (124)	2	100
Other	7	57
Cell marque CMC329 (124)	1	100
Dako FLEX IR614 (124)	15	80
Leica/Novocastra NCL-BCl-2-486 (3.1)	3	100
Leica RTU (BCl2/100/D5) PA0117	21	90
Ventana (124) 790-4464	24	83
Cell Marque 226R-26 (SP66)	4	50
Abcam ab32124 (E17)	1	100

Lymphoma Run: 117

Primary Antibody : CD30

Antibody Details	N	%
Dako IR/IS602 (Ber-H2)	17	53
Dako M0751 (Ber-H2)	69	77
Dako N1558 (Ber-H2)	1	100
labvn/Thermo MS-1857-P (HRS4)	1	100
Leica/Novocastra NCL-CD30 (1G12)	3	33
Leica/Novocastra NCL-CD30-365 (15B3)	3	100
Leica/Novocastra NCL-L-CD30 (1G12)	3	33
Leica/Novocastra NCL-L-CD30-591 (JCM182)	11	100
Leica/Novocastra Bond RTU PA0153 (1G12)	3	33
Leica/Novocastra RTU-CD30 (1G12)	5	100
Other	23	65
Ventana 790 2926 (Ber-H2)	28	71
Cell Marque 130M (Ber-H2)	9	67
Leica/Novocastra Bond RTU PA0790 (JCM182)	20	100

Lymphoma Run: 117

Heat Mediated Retrieval	BCL2 N	BCL2 %	CD30 N	CD30 %
_Leica BondMax ER1	0	0	1	100
Dako Omnis	9	67	8	88
Dako PTLink	21	95	21	71
Leica ER1 10 mins	1	100	1	0
Leica ER1 20 mins	2	50	18	89
Leica ER1 30 mins	5	40	6	100
Leica ER2 10 mins	0	0	2	100
Leica ER2 20 mins	46	80	16	69
Leica ER2 30 mins	8	100	14	86
Leica ER2 40 mins	1	100	2	50
Microwave	1	100	2	50
Other	1	100	3	33
Pressure Cooker	1	100	1	0
Pressure Cooker in Microwave Oven	1	0	0	0
Ventana CC1 16mins	0	0	2	0
Ventana CC1 24mins	1	100	1	0
Ventana CC1 32mins	11	73	10	90
Ventana CC1 36mins	3	33	1	100
Ventana CC1 40mins	3	100	2	100
Ventana CC1 44mins	0	0	2	100
Ventana CC1 48mins	15	80	3	67
Ventana CC1 52mins	3	100	0	0
Ventana CC1 56mins	3	100	7	86
Ventana CC1 64mins	32	81	39	74
Ventana CC1 72mins	5	80	2	50
Ventana CC1 76mins	5	60	3	67
Ventana CC1 80mins	0	0	3	100
Ventana CC1 88mins	1	100	2	50
Ventana CC1 92mins	1	100	2	50
Ventana CC1 extended	6	67	3	67
Ventana CC1 mild	1	0	3	33
Ventana CC1 standard	10	100	12	75
Ventana CC2 32mins	0	0	1	100
Ventana CC2 56mins	1	100	0	0
Water bath 95-98 OC	2	50	2	50

Lymphoma Run: 117

Enzyme Mediated Retrieval	BCL2 N	BCL2 %	CD30 N	CD30 %
AS PER KIT	3	100	6	67
Dako Protease (S2019)	0	0	1	100
NOT APPLICABLE	124	81	105	76

Lymphoma Run: 117	BCL2		CD30	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	12	83	12	83
Dako EnVision FLEX (K8000/10)	6	67	5	80
Dako EnVision FLEX+ (K8002/12)	17	94	18	61
Dako Envision HRP/DAB (K5007)	1	100	1	0
Dako Envision+ HRP mouse K4004/5/6/7	2	100	1	100
Dako REAL HRP/DAB (K5001)	1	0	1	100
Leica Bond Polymer Define (DS9713)	2	100	0	0
Leica Bond Polymer Refine (DS9800)	56	80	58	79
Other	4	50	5	80
Ventana iView system (760-091)	1	0	2	50
Ventana OptiView (760-700) + Amp. (7/860-099)	7	86	13	85
Ventana OptiView Kit (760-700)	50	82	41	80
Ventana UltraView Kit (760-500)	40	80	38	61

Lymphoma Run: 117	BCL2		CD30	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	1	100	0	0
Dako Autostainer Link 48	20	100	21	71
Dako Autostainer Plus Link	2	50	2	50
Dako Omnis	9	67	8	88
LabVision Autostainer	1	0	0	0
Leica Bond Max	24	63	28	79
Leica Bond-III	39	90	37	81
Menarini - Intellipath FLX	1	0	1	100
None (Manual)	2	100	2	0
Ventana Benchmark GX	2	100	1	0
Ventana Benchmark ULTRA	74	81	73	75
Ventana Benchmark XT	25	76	24	67

Lymphoma Run: 117	BCL2		CD30	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	28	82	30	80
BioGenex liquid DBA (HK-124-7K)	1	100	1	0
DAKO DAB+	1	100	2	50
Dako EnVision Plus kits	3	67	1	100
Dako FLEX DAB	21	86	21	76
Dako REAL EnVision K5007 DAB	2	100	2	0
Dako REAL K5001 DAB	1	0	1	100
Leica Bond Polymer Refine kit (DS9800)	55	78	57	81
Other	14	64	11	100
Ventana DAB	29	93	23	70
Ventana iview	1	0	1	0
Ventana Ultraview DAB	43	79	47	66
Vision BioSystems Bond X DAB	1	100	0	0

BEST METHODS - Gold Standard Antibody

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

BCL2 - Method 1

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

Primary Antibody: Dako M0887 (124) , 15 Mins Dilution 1: 50
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)

BCL2 - Method 2

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

Primary Antibody: Dako M0887 (124) , 30 Mins, Room ºC Dilution 1: 50
Automation: Dako Autostainer Link 48
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer, PH: 7.6
HMAR: Dako PTLink, Buffer: EnVision FLEX High pH, PH: 9
EAR: NOT APPLICABLE
Chromogen: Dako EnVision Plus kits, Room ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection: Dako EnVision FLEX+ (K8002/12) , 15 Mins, Room ºC Prediluted

BCL2 - Method 3

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

Primary Antibody:	Ventana (124) 790-4464 , 24 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 32mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700) Prediluted

BCL2 - Method 4

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

Primary Antibody:	Leica/Novocastra NCL-BCl-2 (BCl2/100/D5) , 32 Mins, 21 ºC Dilution 1: 25
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 32mins, Buffer: CC1
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB, Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 21 ºC Prediluted

BEST METHODS - Secondary Antibody

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

CD30 - Method 1

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

Primary Antibody:	Leica/Novocastra NCL-CD30-365 (15B3) , 15 Mins Dilution 1: 50
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)

CD30 - Method 2

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

Primary Antibody:	Dako M0751 (Ber-H2) , 25 Mins, 22 ºC Dilution 1: 25
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: FLEX TRS High pH
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 22 ºC., Time 1: 10 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 20 Mins, 22 ºC Prediluted

CD30 - Method 3

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

Primary Antibody:	Dako M0751 (Ber-H2) Dilution 1: 20
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 30 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

CD30 - Method 4

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

Primary Antibody:	Ventana 790 2926 (Ber-H2) , 36 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB, RT ºC., Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, RT ºC Prediluted

The Neuropathology Module Run 117

Neil Bilbe

	Gold Standard	Second Antibody
Antigens Assessed:	GFAP	Beta Amyloid
Tissue Sections circulated:	GBM	Alzheimer's brain.
Number of Registered Participants:	62	
Number of Participants this Run	62 (100%)	

Introduction

Gold Standard: GFAP

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

Features of Optimal Immunostaining:

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

Features of Sub-optimal Immunostaining:

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

References:

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

Second Antigen: β Amyloid

Beta amyloid is an extracellular filamentous protein deposit found in the brain. It is the major protein component of amyloid cores, neuritic plaques and is also found as a deposit in neurofibrillary tangles. Alzheimer's diseased brain tissue is characterised by an abundance of neurofibrillary tangles, neuropil threads and abnormal neuritis in senile plaques. Neurofibrillary tangles represent dense accumulations of ultrastructurally distinct paired helical filaments whose major component is a microtubule-associated tau protein. Positive staining can be observed in senile plaque cores, plaque periphery and diffuse plaques. In some cases staining can be observed in vessel walls and in extracellular neurofibrillary tangles.

Features of Optimal Immunostaining:

- Staining of amyloid in senile plaque cores and periphery
- Occasional staining in cerebral vasculature
- Clean background with minimal non-specific staining.

Features of Sub-optimal Immunostaining:

- Weak or uneven staining of Amyloid protein
- Diffuse, poorly-localised staining
- Excessive background or non-specific specific staining of glia

References:

1. Akiyama H, Hiroshi M, Saido T, Kondo H, Ikeda K, McGeer PL. Occurrence of the diffuse amyloid β-protein (A4) deposits with numerous A4-containing glial cells in the cerebral cortex of patients with Alzheimer's disease. Glia 1999;25:324-31.
2. Kang J, Lemaire H-G, Unterbeck A, Salbaum JM, Colin L, Masters CL, et al. The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. Nature 1987;325:733-6.
3. Bayer TA, Wirths O, Majtényi K, Gartmann T, Multhaup G, Beyreuther K, et al. Key factors in Alzheimer's disease: β-amyloid precursor protein processing, metabolism and intraneuronal transport [review]. Brain Pathol 2001;11:1-11.
4. Morishima-Kawashima M, Ihara Y. Alzheimer's disease: β-amyloid protein and tau [review]. J Neurosci Res 2002;70:392-401.
5. Akiyama H, Schwab C, Kondo H, Mori H, Kametani F, Ikeda K, et al. Granules in glial cells of patients with Alzheimer's disease are immunopositive for C-terminal sequences of β-amyloid protein. Neurosci let 1996;206:169-72.

Assessment Summary:

All 62 participants submitted GFAP (**G & H**) slides. 17 (27%) labs submitted Synaptophysin as an alternative to β amyloid, 8 (13%) of participants did not submit any 2nd antigen. A total of 232 slides/sections were assessed: **G=62, H=62, J=54 K=54**

The overall pass rate was 93% (216 slides), borderline 4% (10), and the failed slides rate was 3% (6). The average score for all slides was 16/20.

(1) Summary Table - All Slides:

Slide	Antigen	Pass	Borderline	Fail
G (NEQAS)	GFAP (62)	94% (58)	5% (3)	2% (1)
H (In-House)	GFAP (62)	97% (60)	3% (2)	0% (0)
J (NEQAS)	β Amy/Syn(54)	93% (50)	2% (1)	6% (3)
K (In-House)	β Amy/Syn(54)	89% (48)	7% (4)	4% (2)
Total (Average)	**232**	**93%**	**4%**	**3%**

GFAP (**G & H**)

The NEQAS (**G**) GFAP results are better than for Run 116. Pass rate is up to 94% (90% Run 116), and there are fewer borderline slides. Five (8%) down to three (5%). All had weak staining $^{+}/_{-}$ some background.

Just a single NEQAS (**G**) slide failed the assessment. (see Fig 4-Left). This lab employed the Dako polyclonal at 1:2500, no retrieval, on the Leica Bond platform. The tumour is insufficiently demonstrated, and some of the vessels have stained. Interestingly, their in-house (Fig 4-Right) was nicely stained.

Only one slide was assessed as 20/20, this participant used the RTU Ventana 760-4345 (EP672Y) antibody, CC1 20 mins, on the Ventana Benchmark ULTRA. Several other of the NEQAS **(G)** slides received scores of >16/20.

β Amyloid (**J & K**)

β Amyloid was last requested in 2013 (Run 100). The Run 117 results were better than for Run 100 on the NEQAS (**J**) material, with three slides failing (seven previously), and with a pass rate of 89% improved from 75% (Run 100). Those submitting Synaptophysin had a 100% pass rate for their NEQAS **(J)** slides.

(2) Summary Table - β Amyloid Protein slides only (N = 37):

Slide	Antigen	Pass	Borderline	Fail
J (NEQAS)	β Amy(37)	89% (33)	3% (1)	8% (3)
K (In-House)	β Amy(37)	86% (32)	8% (3)	5% (2)
Total (Average)	**74**	**88%**	**6%**	**7%**

Three slides failed the assessment. One using the Chemicon/ Millipore MAB1561 (4G8), was far too weak (Fig 10), The assessors questioned if the 'appropriate' antibody had been employed. The second, the Novocastra NCL-B-AMY monoclonal on Labvision Autostainer (no other details), again staining was too weak. The third had used the Amyloid A marker IR605 and *not* one directed against β Amyloid (Fig 11). A single lab attaining a borderline score also used the Novocastra, but with CC2 76 mins.

Selected Images showing Optimal and Sub-optimal Immunostaining

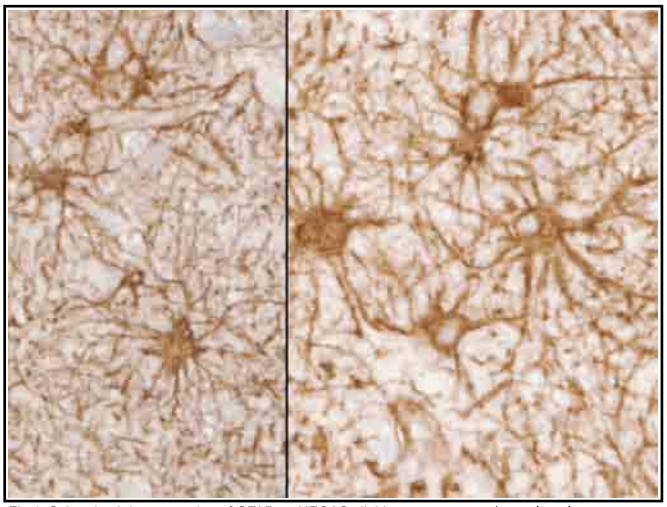


Fig 1. Suboptimal demonstration of GFAP on NEQAS glioblastoma; staining is weak and uneven, further compromised by a weak counterstain. Slide assessed as a low pass. Novocastra PA0026 RTU (GA5), 20 mins, with Leica ER2 30 mins, Leica Bond-III, and a pre-diluted Bond Polymer Refine (DS9800) for 8 mins.

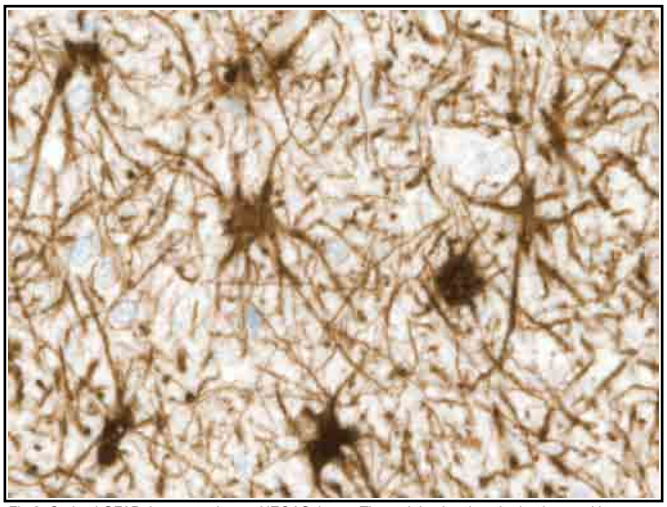


Fig 2. Optimal GFAP demonstration on NEQAS tissue. The staining is crisp, the background is clean, and more selective, even though the same marker as above is used: Novocastra PA0026 RTU (GA5), 20 mins, though with a reduced Leica ER2 to 10 mins, and again the Bond III and Bond Refine kit.

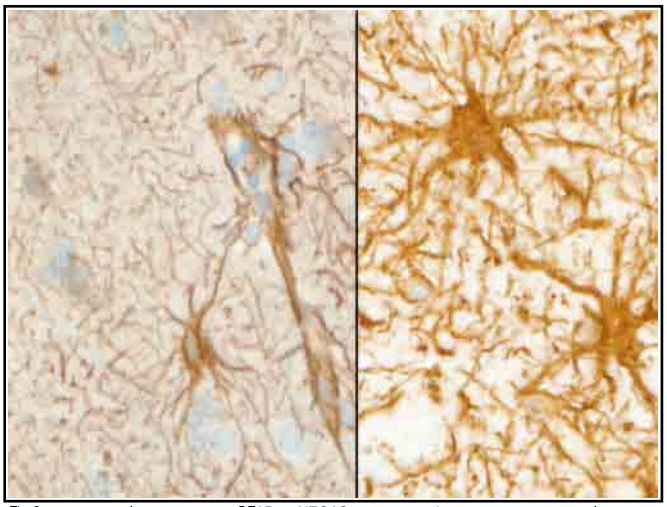


Fig 3. Suboptimal demonstration of GFAP on NEQAS tumour sample. Staining is patchy and uneven (low and high power views), but just suitable for diagnostic purposes. Dako Z0334 polyclonal, 1:10000, 20 mins, Dako Omnis HMAR, 30 mins, on the Dako Omnis platform, using a RTU Dako EnVision FLEX (K8000/10), 20 mins.

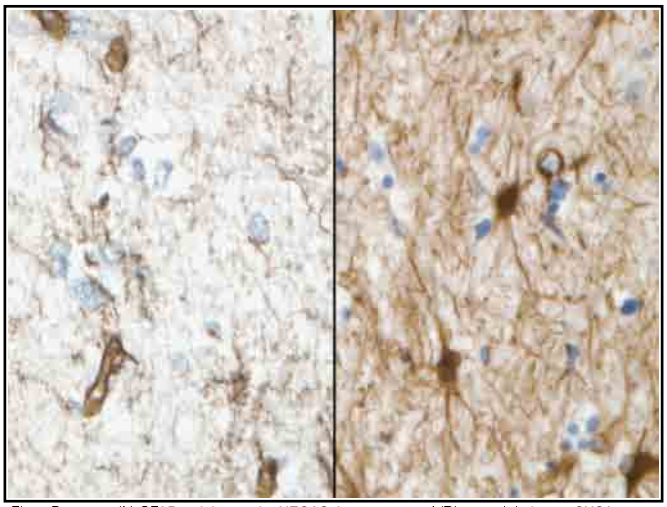


Fig 4. Both poor (L) GFAP staining on the NEQAS tissue, but good (R) on their in-house CUSA material from a glioma, for the same participant. Dako polyclonal at 1:2500, no time given; without use of retrieval, on a Leica Bond Max, and using a Leica Bond Polymer Refine (DS9800), again no additional details given.

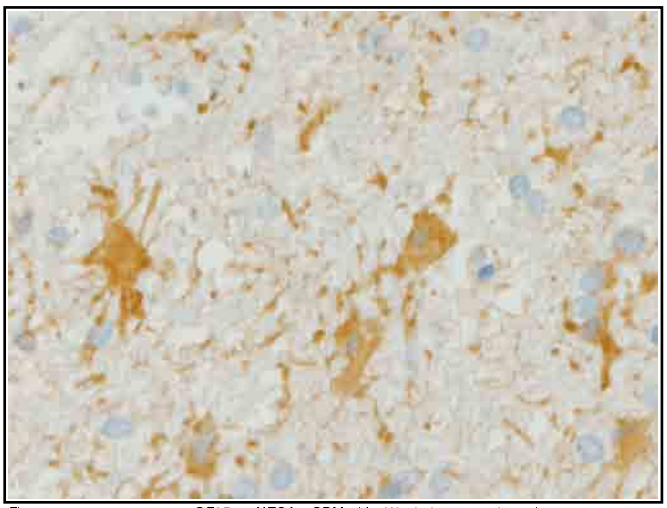


Fig 5. Suboptimal staining of GFAP on NEQAS GBM slide. Weak demonstration, with many processes not demonstrated at all. Borderline assessment outcome. Dako polyclonal, 1:10000, 30 mins, retrieved using a water bath at 95-98 °C, no time given, on a Dako Autostainer Link 48, and RTU Flex kit.

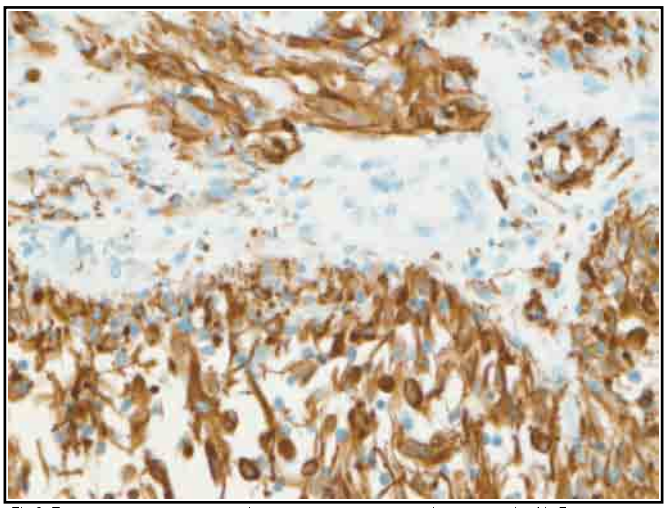


Fig 6. Excellent demonstration on an in-house control section of glioblastoma, fixed in 7.5% formalin for 24 hours, resulting in precise and clean expression of GFAP. Dako poly again, 1:800, 24 mins, no retrieval, on a Ventana Benchmark ULTRA, and a RTU Ventana UltraView Kit (760-500) for 8 mins.

Selected Images showing Optimal and Sub-optimal Immunostaining

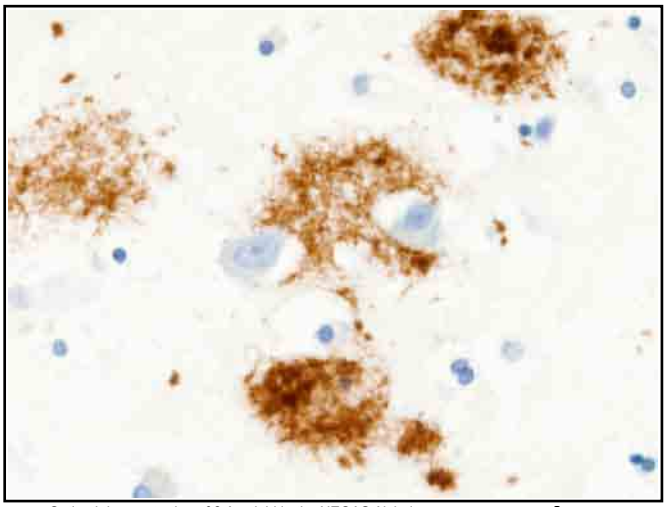


Fig 7. Optimal demonstration of ß Amyloid in the NEQAS Alzheimer's tissue plaques. Staining is very selective and clean (compare Fig 8). Dako M0872 primary, 1:400, 40 mins, no HMAR, on a Dako Autostainer Plus Link, with a prediluted Dako EnVision FLEX+ (K8002/12), for 30 mins.

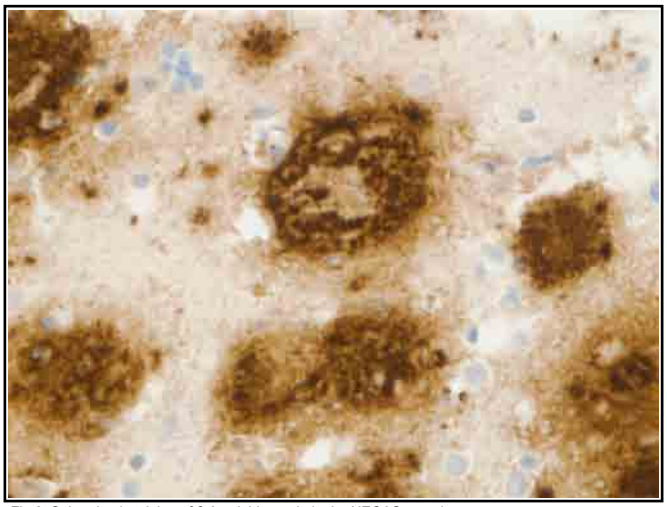


Fig 8. Suboptimal staining of ß Amyloid protein in the NEQAS sample. Plaque staining is seen, but there is also a non-specific background wash. Slide is acceptable in the diagnostic setting. Dako monoclonal, 1:100, 15 mins, but with Pressure Cooker pretreatment, Leica Bond Max, and RTU Refine kit for 8 mins.

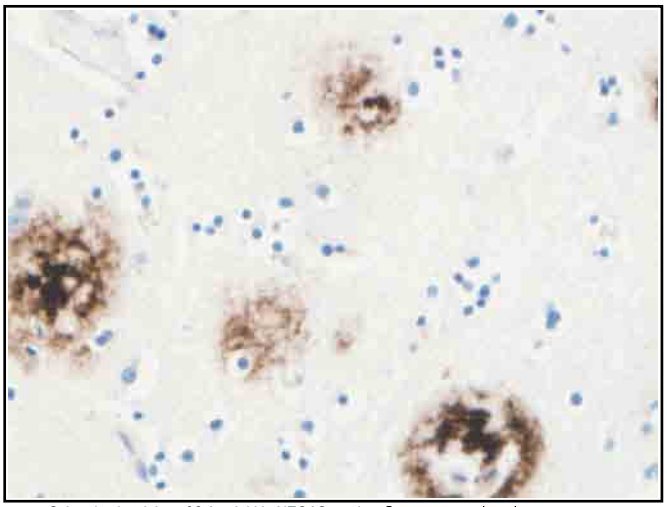


Fig 9. Suboptimal staining of ß Amyloid in NEQAS section. Staining is weak and some plaques were missing or incomplete. Dako monoclonal, 1:200, 20 mins, no pretreatment, on a Shandon Sequenza (rare these days), and the RTU Dako Envision HRP/DAB (K5007) kit, 30 mins.

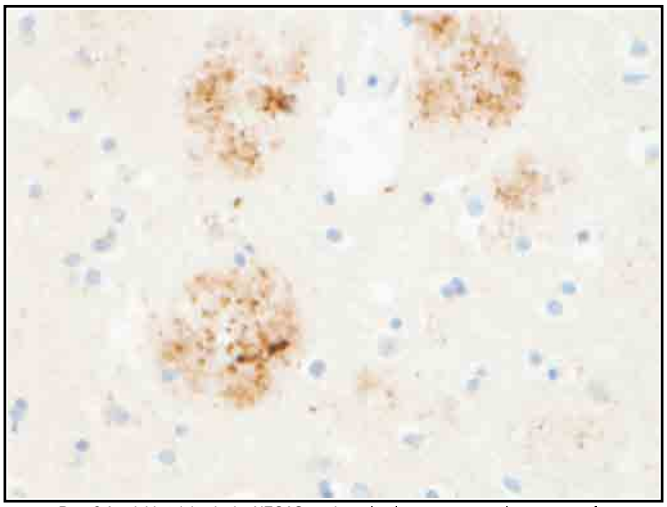


Fig 10. Poor ß Amyloid staining in the NEQAS section. The demonstration in the plaques is far too weak, and there is a slight background hue. The slide failed the assessment. Chemicon/Millipore MAB1561 (4G8), no further details, with Dako PTLink, Dako Autostainer Link 48, and the Dako EnVision FLEX+ (K8002/12) kit.

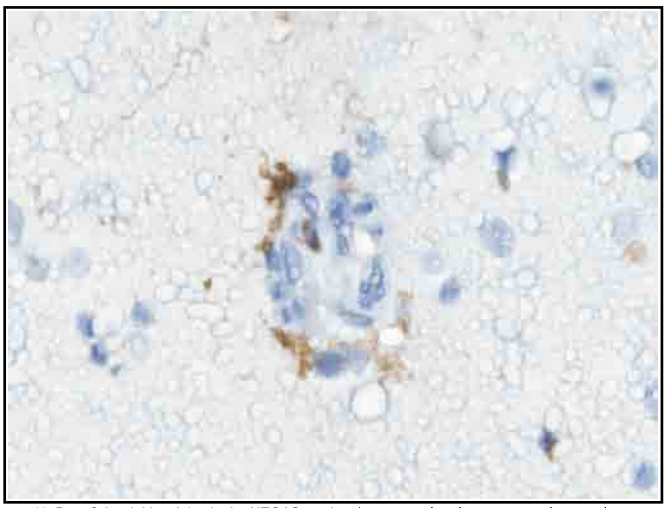


Fig 11. Poor ß Amyloid staining in the NEQAS section. It appears that the participant has used a marker for Amyloid A (Dako IR605). There is also some morphological damage and vacuolation; they had also used a Dako PTLink for 45 mins. Obviously the slide failed the assessment.

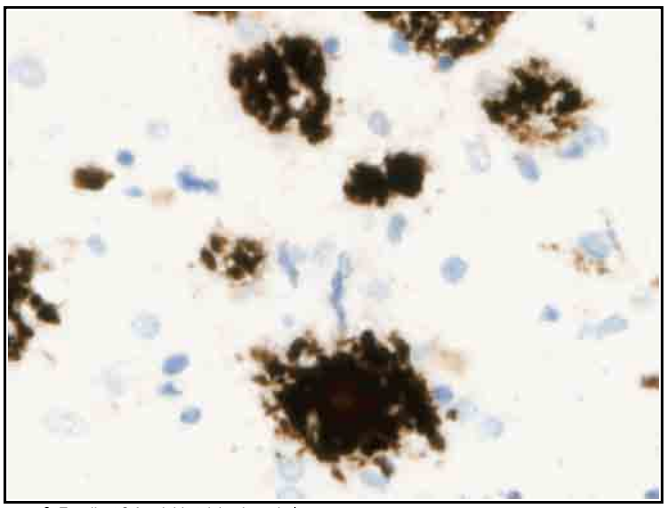
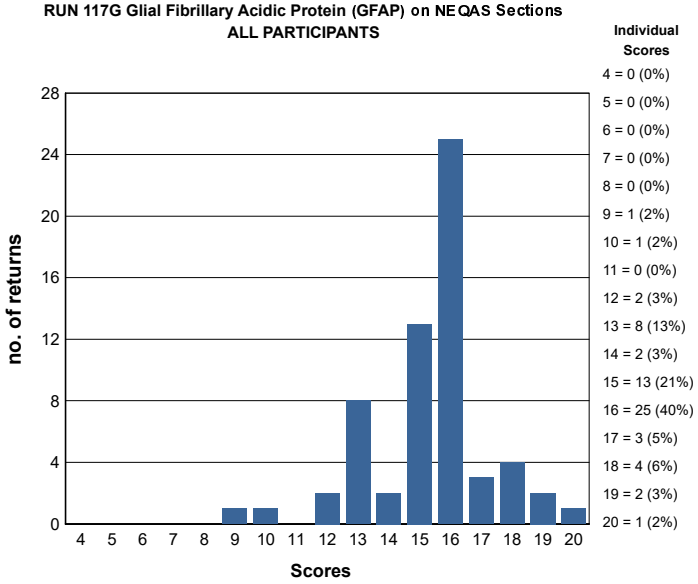


Fig 12. Excellent ß Amyloid staining in an in-house section. Plaques are clearly seen against a clean background. Novocastra NCL-B AMYLOID, 1:50, 15 mins, Leica ER2 40 mins, Leica Bond-III, and the prediluted Leica Bond Polymer Refine (DS9800), 8 mins.

GRAPHICAL REPRESENTATION OF PASS RATES

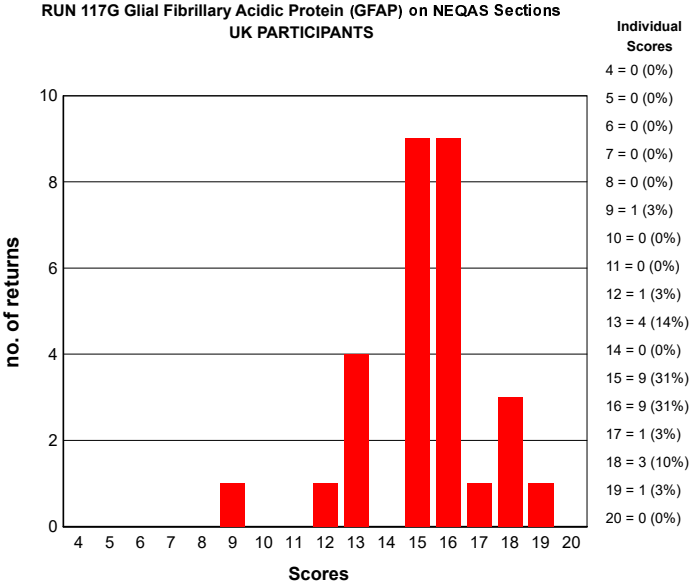
RUN 117G Glial Fibrillary Acidic Protein (GFAP) on NEQAS Sections
ALL PARTICIPANTS



Individual Scores
4 = 0 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 0 (0%)
8 = 0 (0%)
9 = 1 (2%)
10 = 1 (2%)
11 = 0 (0%)
12 = 2 (3%)
13 = 8 (13%)
14 = 2 (3%)
15 = 13 (21%)
16 = 25 (40%)
17 = 3 (5%)
18 = 4 (6%)
19 = 2 (3%)
20 = 1 (2%)

Summary
16 - 20 = 35 (56%)
13 - 15 = 23 (37%)
10 - 12 = 3 (5%)
0 - 9 = 1 (2%)
Median = 15.00

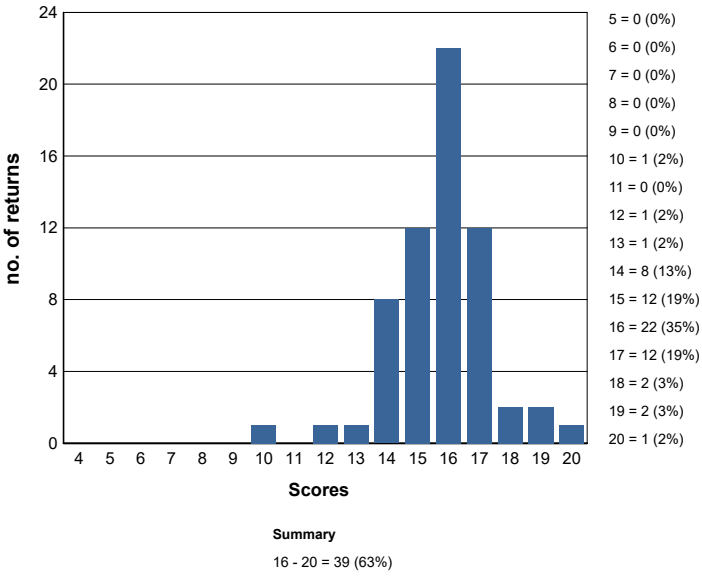
RUN 117G Glial Fibrillary Acidic Protein (GFAP) on NEQAS Sections
UK PARTICIPANTS



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

Summary
16 - 20 = 14 (48%)
13 - 15 = 13 (45%)
10 - 12 = 1 (3%)
0 - 9 = 1 (3%)
Median = 15.50

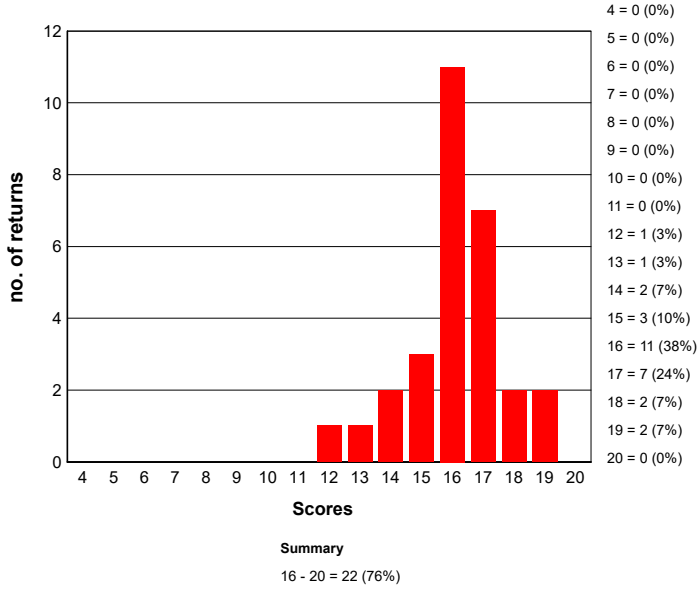
RUN 117H Glial Fibrillary Acidic Protein (GFAP) on in-house Sections
ALL PARTICIPANTS



Individual Scores
4 = 0 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 0 (0%)
8 = 0 (0%)
9 = 0 (0%)
10 = 1 (2%)
11 = 0 (0%)
12 = 1 (2%)
13 = 1 (2%)
14 = 8 (13%)
15 = 12 (19%)
16 = 22 (35%)
17 = 12 (19%)
18 = 2 (3%)
19 = 2 (3%)
20 = 1 (2%)

Summary
16 - 20 = 39 (63%)
13 - 15 = 21 (34%)
10 - 12 = 2 (3%)
0 - 9 = 0 (0%)
Median = 15.50

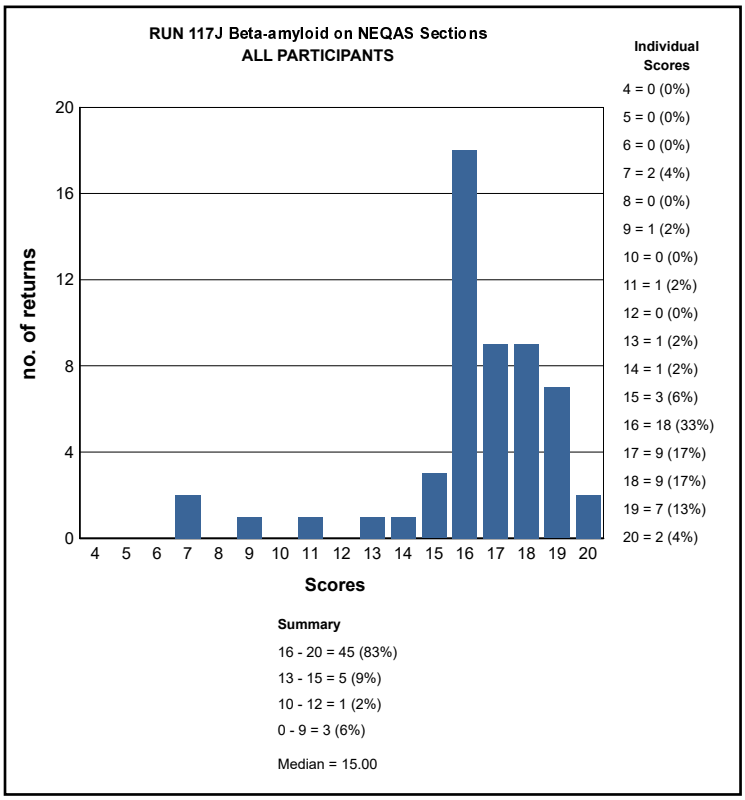
RUN 117H Glial Fibrillary Acidic Protein (GFAP) on in-house Sections
UK PARTICIPANTS

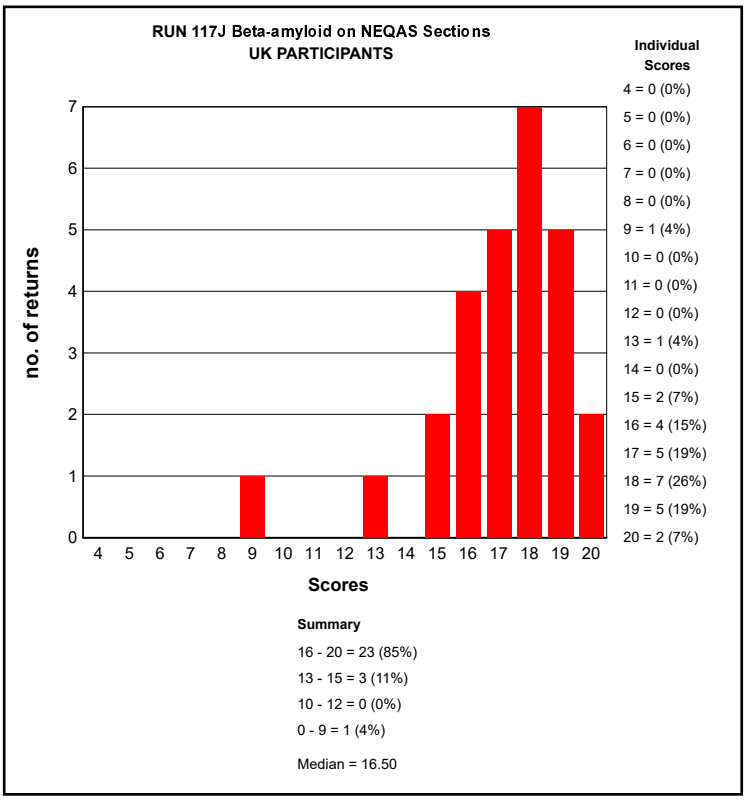


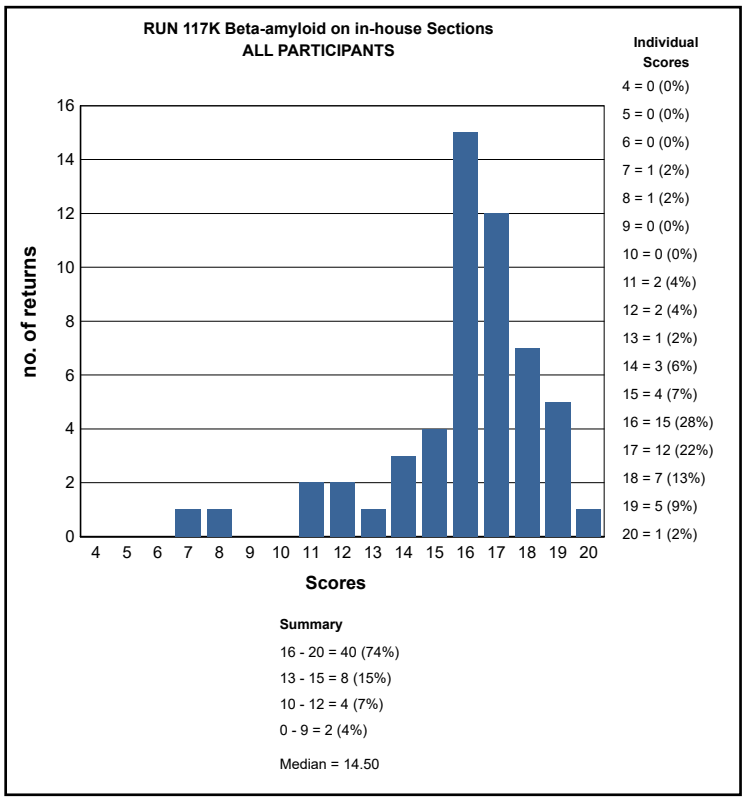
Individual Scores
4 = 0 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 0 (0%)
8 = 0 (0%)
9 = 0 (0%)
10 = 0 (0%)
11 = 0 (0%)
12 = 1 (3%)
13 = 1 (3%)
14 = 2 (7%)
15 = 3 (10%)
16 = 11 (38%)
17 = 7 (24%)
18 = 2 (7%)
19 = 2 (7%)
20 = 0 (0%)

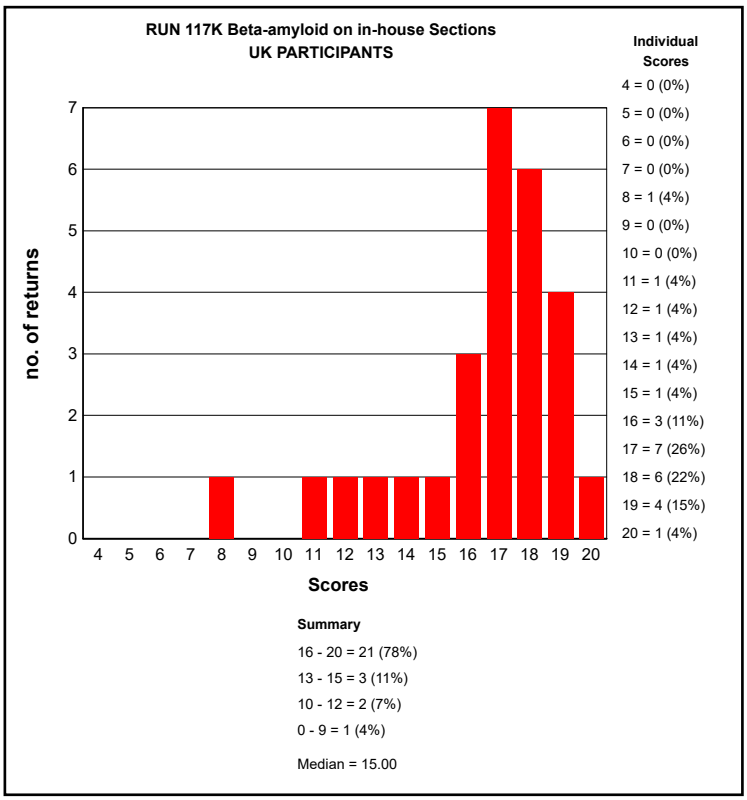
Summary
16 - 20 = 22 (76%)
13 - 15 = 6 (21%)
10 - 12 = 1 (3%)
0 - 9 = 0 (0%)
Median = 15.50

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: 117

Primary Antibody : Glial Fibrillary Acidic Protein (GFAP)

Antibody Details	N	%
Dako M0761 (6F2)	12	100
Dako Z0334 (R Poly)	26	85
Immunon 490740RB	1	100
Novocastra NCL-GFAP-GA5 (GA5)	2	100
Sigma G3895 (GA5)	1	100
Zymed/Invitrogen 08-1021 (ZCG29)	1	100
Dako IR524 (R Poly)	2	100
Novocastra PA0026 RTU (GA5)	4	100
Ventana 760-4345 (EP672Y)	7	100
Cell Marque (EP672Y) 258R	2	100
Dako Omnis GA524 (R Poly)	3	100

Neuropathology Run: 117

Primary Antibody : Beta-amyloid

Antibody Details	N	%
Dako M0872	23	100
Novocastra NCL-B AMYLOID	9	78
Other	15	93
Chemicon/Millipore MAB1561 (4G8)	3	67

Neuropathology Run: 117

Heat Mediated Retrieval	Beta-amyloid N	Beta-amyloid %	Glial Fibrillary Acidic Protein (GFAP) N	Glial Fibrillary Acidic Protein (GFAP) %
Dako Omnis	1	100	7	100
Dako PTLink	3	0	5	80
Leica ER1 10 mins	0	0	1	100
Leica ER1 20 mins	0	0	3	100
Leica ER1 30 mins	0	0	1	100
Leica ER1 40 mins	1	100	0	0
Leica ER2 10 mins	0	0	1	100
Leica ER2 20 mins	2	100	4	100
Leica ER2 30 mins	3	100	1	100
Leica ER2 40 mins	2	100	0	0
Microwave	1	100	2	100
None	12	92	10	90
Other	4	100	1	100
Pressure Cooker	1	100	0	0
Ventana CC1 20mins	0	0	1	100
Ventana CC1 32mins	2	100	2	100
Ventana CC1 36mins	0	0	3	100
Ventana CC1 40mins	0	0	2	100
Ventana CC1 52mins	1	100	0	0
Ventana CC1 56mins	1	100	0	0
Ventana CC1 64mins	7	100	3	100
Ventana CC1 76mins	0	0	1	100
Ventana CC1 8mins	0	0	4	100
Ventana CC1 mild	3	100	4	100
Ventana CC1 standard	1	100	2	100
Ventana CC2 76mins	1	0	0	0
Water bath 95-98 OC	0	0	1	0

Neuropathology Run: 117

Enzyme Mediated Retrieval	Beta-amyloid N	Beta-amyloid %	Glial Fibrillary Acidic Protein (GFAP) N	Glial Fibrillary Acidic Protein (GFAP) %
AS PER KIT	1	100	0	0
Enzyme digestion + HIER	1	100	0	0
NOT APPLICABLE	17	94	32	97
Other	10	100	1	100
VBS Bond Enzyme 1	0	0	3	33
Ventana Protease 1 (760-2018)	1	100	6	100

Neuropathology Run: 117 Detection	Beta-amyloid N	Beta-amyloid %	Glial Fibrillary Acidic Protein (GFAP) N	Glial Fibrillary Acidic Protein (GFAP) %
AS PER KIT	5	100	6	83
Dako EnVision FLEX (K8000/10)	4	50	5	80
Dako EnVision FLEX+ (K8002/12)	3	33	4	100
Dako Envision HRP/DAB (K5007)	3	100	2	100
Leica Bond Polymer Define (DS9713)	1	100	0	0
Leica Bond Polymer Refine (DS9800)	13	100	16	88
Other	2	50	2	100
Vector Elite ABC (PK-6101)	1	100	0	0
Vector Elite Universal ABC (PK-6200)	1	100	0	0
Vector ImmPRESS Universal (MP-7500)	1	100	0	0
Ventana iView system (760-091)	1	100	0	0
Ventana OptiView (760-700) + Amp. (7/860-099)	2	100	0	0
Ventana OptiView Kit (760-700)	4	100	10	100
Ventana UltraView Kit (760-500)	10	90	16	100

Neuropathology Run: 117 Automation	Beta-amyloid N	Beta-amyloid %	Glial Fibrillary Acidic Protein (GFAP) N	Glial Fibrillary Acidic Protein (GFAP) %
Dako Autostainer Link 48	5	20	6	67
Dako Autostainer Plus Link	1	100	1	100
Dako Omnis	2	100	7	100
LabVision Autostainer	1	0	0	0
Leica Bond Max	5	100	6	67
Leica Bond-III	10	100	11	100
None (Manual)	6	100	2	100
Other	1	100	0	0
Shandon Sequenza	1	100	0	0
Ventana Benchmark ULTRA	13	92	21	100
Ventana Benchmark XT	6	100	7	100

Neuropathology Run: 117 Chromogen	Beta-amyloid N	Beta-amyloid %	Glial Fibrillary Acidic Protein (GFAP) N	Glial Fibrillary Acidic Protein (GFAP) %
AS PER KIT	6	83	9	89
DAKO DAB+	0	0	1	100
Dako DAB+ REAL Detection (K5001)	1	100	1	100
Dako EnVision Plus kits	2	0	3	100
Dako FLEX DAB	4	75	7	86
Dako REAL EnVision K5007 DAB	2	100	1	100
Leica Bond Polymer Refine kit (DS9800)	14	100	16	88
Other	5	80	3	100
Sigma DAB (D5637)	1	100	0	0
Vector DAB (SK-4100)	1	100	0	0
Ventana DAB	2	100	3	100
Ventana Enhanced Alk. Phos. Red Detection Kit	1	100	0	0
Ventana iview	1	100	0	0
Ventana Ultraview DAB	11	91	17	100

BEST METHODS - Gold Standard Antibody

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

Glial Fibrillary Acidic Protein (GFAP) - Method 1

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

Primary Antibody: Novocastra NCL-GFAP-GA5 (GA5) , 20 Mins Dilution 1: 200
Automation: Dako Omnis
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako Omnis, Buffer: Dako Low pH TRS
EAR: NOT APPLICABLE
Chromogen: Dako FLEX DAB, Time 1: 5 Mins, Time 2: 5 Mins
Detection: Other , 20 Mins Prediluted

Glial Fibrillary Acidic Protein (GFAP) - Method 2

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

Primary Antibody: Novocastra PA0026 RTU (GA5) , 8 Mins, 25 ºC Prediluted
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 10 mins
EAR: NOT APPLICABLE
Chromogen: Leica Bond Polymer Refine kit (DS9800), 25 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins, 25 ºC Prediluted

Glial Fibrillary Acidic Protein (GFAP) - Method 3

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

Primary Antibody:	Ventana 760-4345 (EP672Y)
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 20mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

Glial Fibrillary Acidic Protein (GFAP) - Method 4

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

Primary Antibody:	Dako Z0334 (R Poly)
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	AS PER KIT
HMAR:	
EAR:	Ventana Protease 1 (760-2018)
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

Beta-amyloid - Method 1

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

Primary Antibody:	Dako M0872 , 40 Mins Dilution 1: 400
Automation:	Dako Autostainer Plus Link
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	None
EAR:	Other Digestion Time NEQAS: 5 Mins. In-House: 5 Mins
Chromogen:	Dako FLEX DAB, Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 30 Mins Prediluted

Beta-amyloid - Method 2

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

Primary Antibody:	Novocastra NCL-B AMYLOID , 15 Mins, 25 ºC Dilution 1: 50
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	None
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, 25 ºC

Beta-amyloid - Method 3

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

Primary Antibody:	Dako M0872 , 15 Mins, r/t ºC Dilution 1: 40
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	None
EAR:	Other, r/t ºC. Digestion Time NEQAS: 8 Mins. In-House: 8 Mins
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

Beta-amyloid - Method 4

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

Primary Antibody:	Chemicon/Millipore MAB1561 (4G8) Dilution 1: 6000
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Other
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	AS PER KIT

Neil Bilbe and Irena Srebotnik Kirbis

	Gold Standard	Second Antibody
Antigens Assessed:	CK	Calretinin
Sample circulated; cytospins and cell block sections:	Three different effusion samples with carcinoma cells, mesothelial cells, lymphocytes, macrophages and erythrocytes and human pharynx squamous cell carcinoma.	
Number of Registered Participants:	84 - Cell block 62 (74%), Cytospin 22 (26%)	
Number of Participants this Run	82 (98%)	

Introduction

Gold Standard: Cytokeratin

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

Features of Optimal Immunostaining:
- Intense cytoplasmic staining of tumour cells.
- Clean background.
- No non-specific staining of other cell type
- 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. MJ Goddard et al. Comparison of commercially available cytokeratin antibodies in normal and neoplastic adult epithelial and non-epithelial tissues. J Clin Pathol 1991; 44:660-6632.
2. LJ Fowler & WA Lachar Application of immunocytochemistry to cytology. Archives of Pathology & Laboratory Medicine. 2008; 132(3): 373-38.
3. PA Fetsch & A Abati Immunocytochemistry in effusion cytology. Cancer Cytopathology. 2001; 93(5): 293-308.

Second Antigen: Calretinin

Calretinin is a 29 kDa calcium-binding protein involved in calcium signalling. It is found abundantly in neurons and the thymus, and is associated with the kinetocore during the cell cycle. Outside the nervous system, calretinin is found in a number of cells with varying expression, including mesothelial cells, steroid producing cells, testicular cells, ovarian surface epithelium, some neuroendocrine cells, breast glands, and hair follicle cells. Diagnostically, calretinin is used as a positive marker for both benign mesothelium and in malignant mesothelioma (Saydan *et al*). It's use in the identification of mesothelioma in cytological preparations (Doglioni *et al*), and in the differential diagnosis between malignant mesothelioma and adenocarcinoma in FFPE cell blocks of cytological fluids, washings and aspirates (Wiezorekd and Krane) have been described. Calretinin can also be used to help differentiate lung tumours (Marchevsky), and also to distinguish between different types of brain tumour, i.e. neuronal rather than glial differentiation (Leong *et al*).

Features of Optimal Immunostaining
- Strong cytoplasmic staining of the mesothelial cells.
- Clean background with no non-specific staining of other cell types not expected to stain.
- Adequate nuclear counterstain.

Features of Sub-optimal Immunostaining
- Weak or absent staining in the mesothelial cells.
- Uneven staining.
- Excessive background staining.
- Excessive non-specific staining of cell types or components not expected to satin.
- Inadequate nuclear counterstain.

References

1. Saydan N, et al. Expression of calretinin in human mesothelioma cell lines and cell cycle analysis by flow cytometry. Anticancer Res 21 (1A): 181-8.
2. Doglioni C, et al. Calretinin: a novel immunocytochemical marker for mesothelioma. Am J Surg pathol 1996; 20: 1037-1046.
3. Wieczorek TJ, et a.. Diagnostic utility of calretinin immunohistochemical marker for mesothelioma. Am J Surg pathol 1996; 20: 1037-1046.
4. Marchevsky AM. Application of immunohistochemistry to the diagnosis of malignant mesothelioma. Arch Pathol Lab Med. 132 (3): 397-401.
5. Leong A, et al. Manual of Diagnostic Cytology (2nd ed.) Greenwich Medical Media Ltd. pp. 45-46.

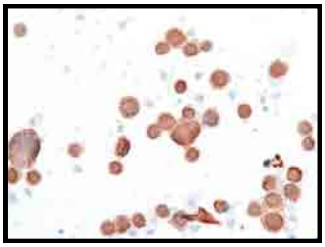
References (cell blocks in cytology)

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

Assessment Summary:

84 labs were registered on the Cytopathology module for Run 117, the last round of the 2016/2017 EQA year. Two participants did not submit any slides. Two participants failed to send in in-house **(S & U)** samples, and another did not submit a Calretinin control. Other slides were incorrectly or unlabelled. Four of the labs using cell blocks placed their in-house controls on the NEQAS slide, resulting in a total of 323 slides being scored: **R = 82, S = 80, T = 82, U = 79.**

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



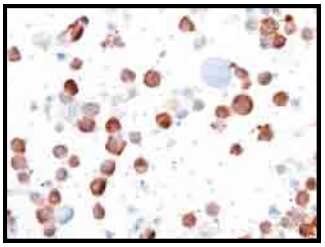


Fig 1 (CK) & Fig 2 (Calretinin): on Cytospins Run 117

Approximately 80% of the cell population was positive for CK, and unusually, a similar level of cells also stained to varying degrees with Calretinin, due to the contents, components, and morphological integrity of this particular sample's cocktail.

Assessment Outcomes:

The overall pass rate was 89% (289 slides), 8% borderline (25), and 3% failure rate (9).

The average score for all slides was 16/20.

(1) Summary Table - All Slides:

Slide	Antigen	Pass	Borderline	Fail
R (NEQAS)	CK (82)	87% (71)	9% (7)	5% (4)
S (In-House)	CK (80)	99% (79)	1% (1)	0% (0)
T (NEQAS)	Calret (82)	76% (62)	18% (15)	6% (5)
U (In-House)	Calret (79)	97% (77)	3% (2)	0% (0)
Average (Total)	323	89% (289)	8% (25)	3% (9)

No in-house slides (**S & U)** failed the assessment, and only three were scored as borderline. For the sake of this review therefore, these were not included in subsequent discussions.

CK (R)

Four participants scored <10/20 for their NEQAS panCK assessment. Three of these participants submitted cell blocks , and one a cytospin; but all had similar comments, there was either none, or very weak demonstration of the antigen. The three participants who stained the cell block samples all used different primary antibodies (Dako MNF116, Ventana AE1/AE3, and a Dako AE1/AE3).
The participant who failed on the cytospin sample, used a Novocastra CK7 OV-TL (12/30 clone).

45 out of 82 participants used the AE1/AE3 clone from a variety of manufacturers, obtaining an 89% pass rate. The Dako M3515 (AE1/AE3) being the most commonly employed. The second most common panCK was the Dako M0821 (MNF116) used by 27% of participants, with a 100% pass rate.

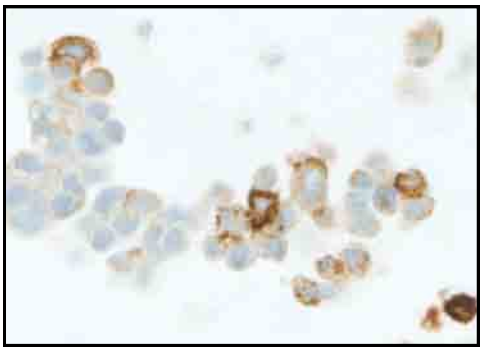


Fig 3: Example of a failed CB section stained with CK. Only a very low percentage of epithelial cells are suitably demonstrated.

The number of slides scored as borderline on the NEQAS samples was the most striking outcome of the assessment. For Cytokeratin (**R**) seven slides (9%) were scored as borderline; four cytospins (18% of users) and three cell block slides (5% of users). There was more evidence of non-specific reactions, and additionally, the use of pre-treatment on some of the cytospin samples (see Image Reports Fig 5), giving rise to sub-optimal reactions, rather than because of weak or poor demonstration of cytokeratin as seen in Fig 3 above.

Calretinin (T)

There were five failed slides for the Calretinin submissions. These failures were all by laboratories who submitted the NEQAS cell block material. The issue for all of these failures was very weak or no demonstration of the Calretinin antigen. This is most probably due to insufficient antigen retrieval or inappropriate antibody dilution. A failure indicates the sample is inadequate for diagnostic purposes. Borderline 10-12/20 were also predominantly attributed to weak staining of Calretinin (see Images report Fig 8), rather than some of the non-specific reactions seen with the Cytokeratin slides.

The most frequently used antibody was the Ventana 790-4467 (SP65), used by 25 participants, who achieved a 100% pass rate. The second most common antibody was the Dako M7245 (DAK-Calret 1), however, only 59% of these achieved a pass rate of 13/20 or above, and three of these slides using the DAK-Calret 1 clone failed the assessment (Example Images Report Fig 9), including one who used the RTU FLEX marker.

There were 15 (18%) NEQAS Calretinin (**T**) slides scored as borderline: six cytospins (27% of users) and nine cell blocks (15% of users). Again a range of different suppliers and clones were employed.

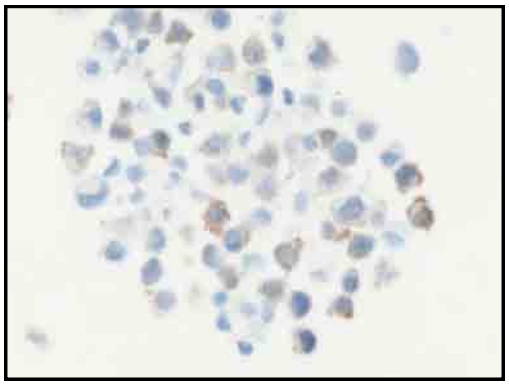


Fig 4: Example of a borderline CB section stained with Calretinin. Staining is weak, albeit adequate for diagnostic use

Important note

Although 74% of laboratories submitted cell blocks, for both Cytokeratin and Calretinin, a disproportionate number of participants stated that they *did not* perform any type of antigen retrieval.

It is important to note, that **cell blocks should be treated identically to FFPE blocks** and should therefore receive the same antigen retrieval as a standard FFPE protocol. Participants who received individual comments regarding weak staining and did not perform antigen retrieval should perhaps review their protocols, or conversely, ensure that they accurately state when returning their protocol data that the field: Antigen Retrieval on NEQAS samples Yes or No is accurately indicated, in order that the assessors can give meaningful feedback using the data provided when this relates to the use, lack of, or over use of any antigen retrieval or pre-treatment method used.

Selected Images showing Optimal and Sub-optimal Immunostaining

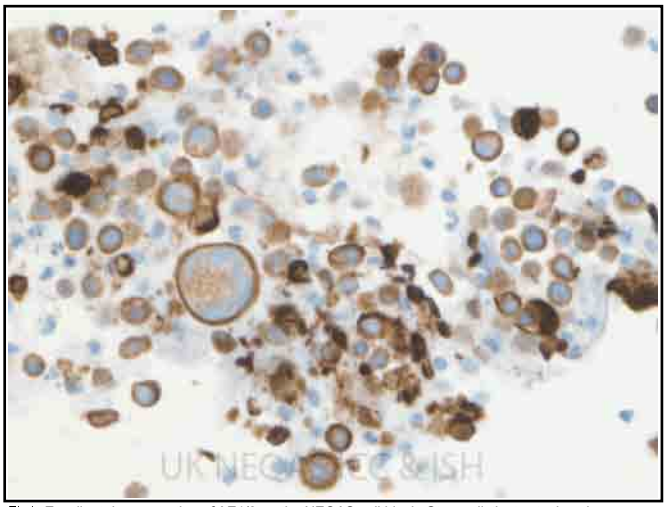


Fig1: Excellent demonstration of AE1/3 on the NEQAS cell block. Strong distinct cytoplasmic staining of the tumour cells with clear background and adequate counter stain. Method: Dako M3515 (AE1/3) clone; Leica Bond III (HMAR: ER1 30'; Detection: Leica Bond Polymer Refine).

Fig2: Poor demonstration of AE1/3 on the NEQAS cell block. The tumour cells are negative and there is non-specific granular staining in some cells. Methods: Ventana 760 2135 (AE1/AE3/PCK26) clone; Ventana Benchmark ULTRA (HMAR: None; Detection: Ventana Optiview Kit).

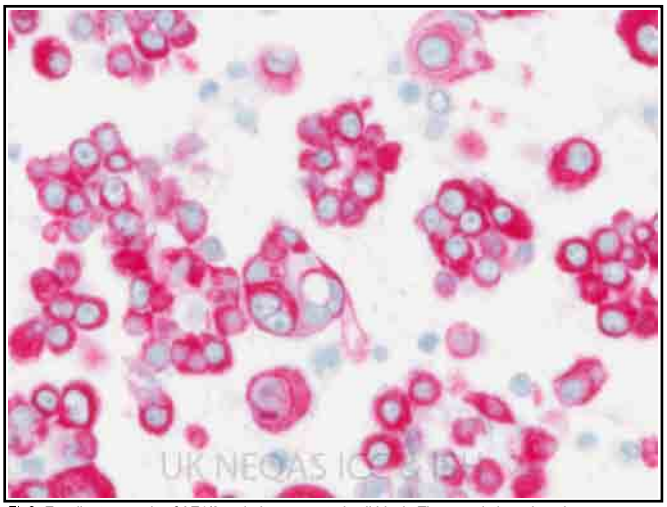


Fig3: Excellent example of AE1/3 on in-house control cell block. The sample is a pleural effusion containing reactive mesothelial cells. The reactive cells show clear distinct cytoplasmic staining and a clean background. Method: Dako M3515 (AE1/3) clone; Ventana Benchmark XT (HMAR: CC1; Detection: Not specified).

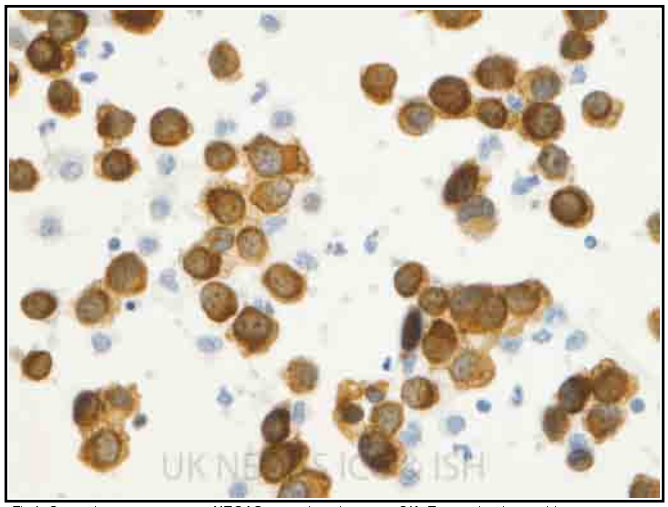


Fig4: Optimal demonstration of NEQAS cytospin using a panCK. Expression is specific to the tumour cells and the background is clear. Method: ImmunoBS MM-1012 (CK cocktail); Leica Bond Max (HMAR: None; Detection: Leica Bond Polymer Refine).

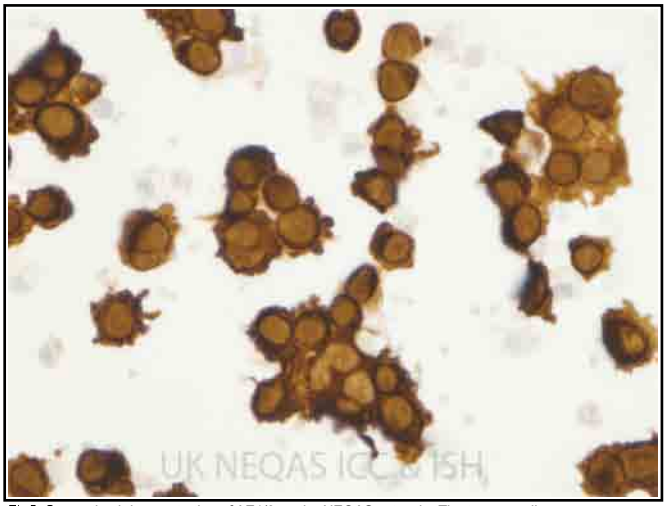


Fig5: Sub-optimal demonstration of AE1/3 on the NEQAS cytospin. The tumour cells are excessively stained and there is evidence of morphology damage. Most likely caused from excessive heat during antigen retrieval. Method: Ventana 760 2595 (AE1/AE3/PCK26) clone; Ventana Benchmark Ultra (HMAR: CC1 20'; Detection: Ventana Ultraview). Score 12/20

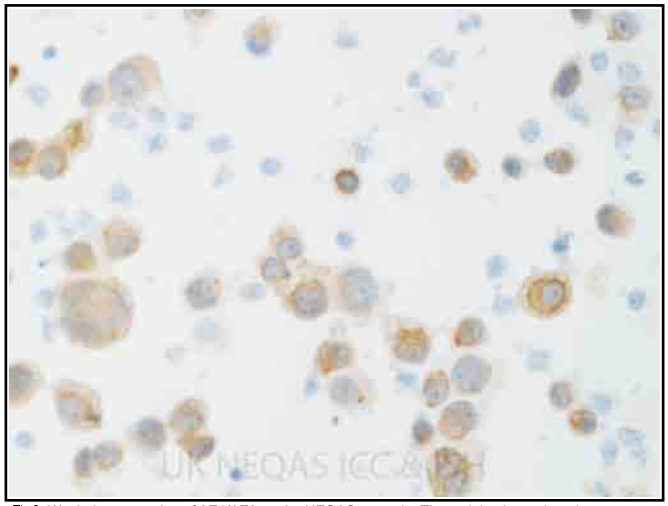


Fig6: Weak demonstration of AE1/AE3 on the NEQAS cytospin. The staining is weak and poorly localised. Method: Leica NCL-AE1/AE3 clone; Leica Bond Max (HMAR: None; Detection: Leica Bond Polymer Refine). Score 13/20

Selected Images showing Optimal and Sub-optimal Immunostaining

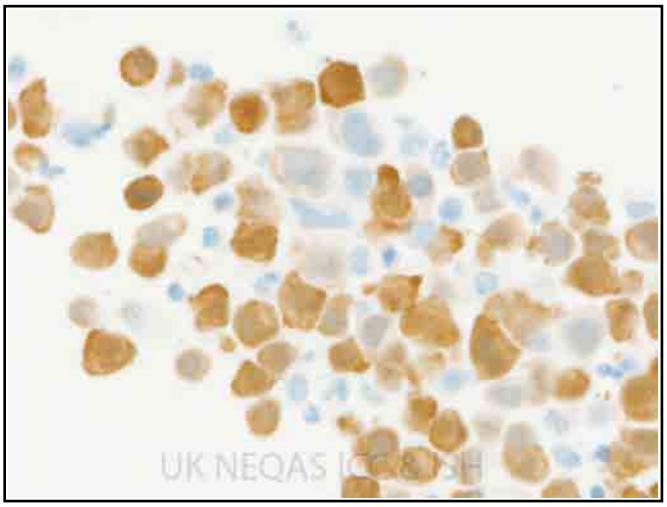


Fig7: Optimal demonstration of Calretinin on the NEQAS cell block. The staining is specific to the mesothelial cells. Method: Ventana (SP65) clone (RTU 12'); Ventana Benchmark Ultra (HMAR: CC1 mild; Detection: Ventana UltraView).

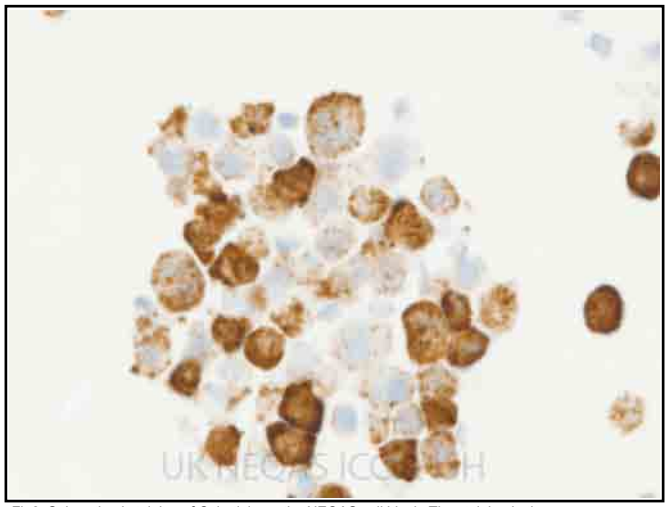


Fig8: Sub-optimal staining of Calretinin on the NEQAS cell block. The staining in the mesothelial cells is granular and there is some non-specific staining in other cells which should be negative. Method: Dako (DAK-Calret 1) clone (1/25 32'): Ventana Benchmark XT (HMAR: CC1 standard; Detection: Ventana Optiview). Score 14/20

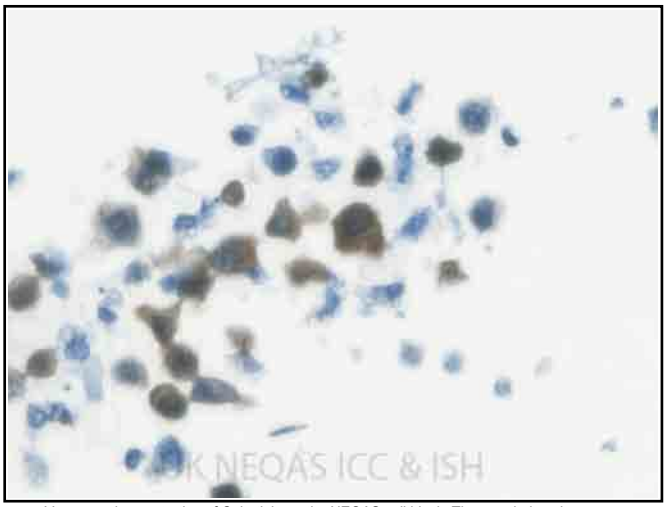


Fig9: Very poor demonstration of Calretinin on the NEQAS cell block. The morphology is severely damaged and the counterstain is excessive. Method: Dako (DAK-Calret 1) clone (1/50 30'); Dako Autostainer Link 48 (HMAR: PT Link pH9.0; Detection: Dako EnVision FLEX+). Score 7/20

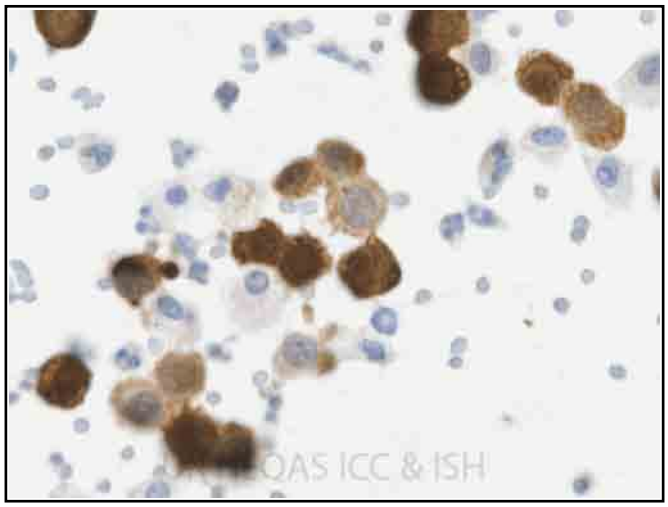


Fig10: Acceptable demonstration of Calretinin on the NEQAS cytospin. The mesothelial cells show slightly strong cytoplasmic staining. However, there is a slight background present. Method: Zymed/Invitrogen 18 0211 (DC8) clone (1/50 60'): Manually stained (HMAR: None; Detection: Dako EnVision + HRP).

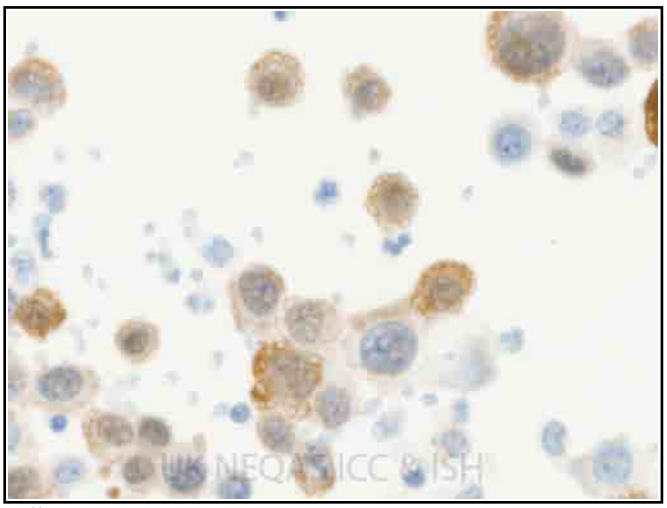


Fig11: Slightly weak demonstration of Calretinin on NEQAS cytospin. The staining is slightly weak and there is some background on negative cells. Method: Leica NCL-L-CALRET 556 (CAL6); Leica Bond III (HMAR: None; Detection: Leica Bond Polymer Refine).

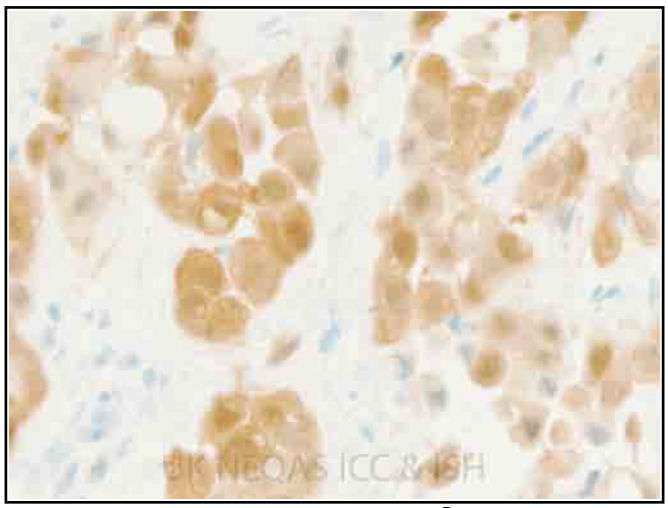
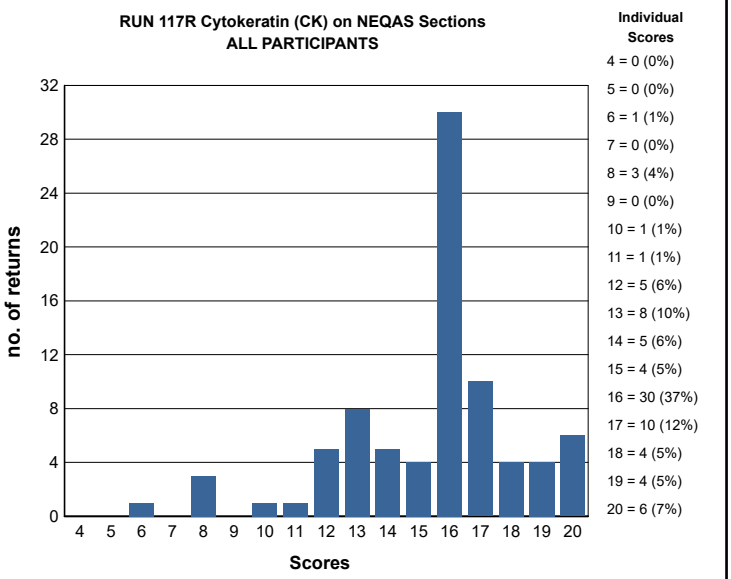


Fig12: Optimal demonstration of in-house Calretinin control on a FFPE mesothelioma. The cytoplasmic staining is specific to the tumour cells. Method: Invitrogen 18-0211 (DC8) clone (1/250 44'); Ventana Benchmark Ultra (HMAR: CC1 36';

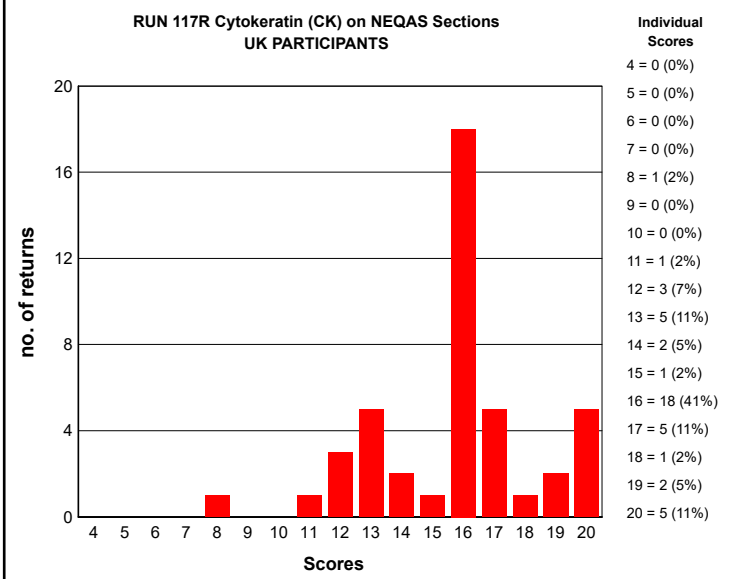
GRAPHICAL REPRESENTATION OF PASS RATES



Summary

16 - 20 = 54 (66%)
13 - 15 = 17 (21%)
10 - 12 = 7 (9%)
0 - 9 = 4 (5%)

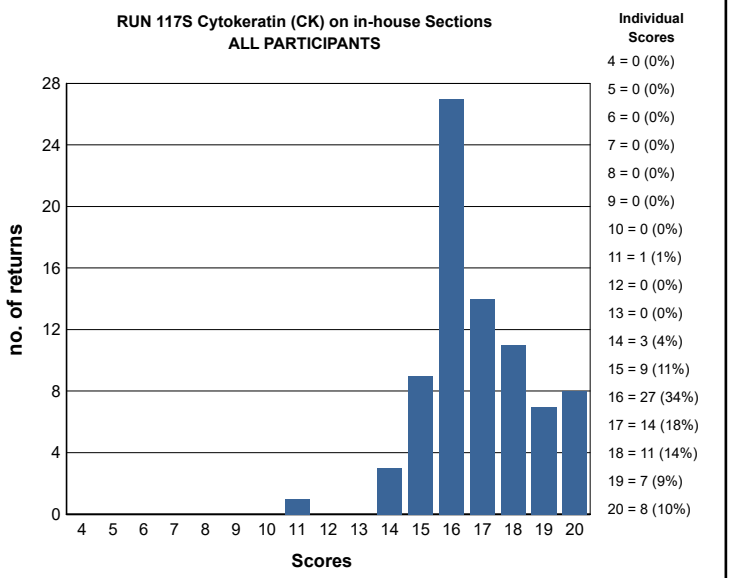
Median = 14.00



Summary

16 - 20 = 31 (70%)
13 - 15 = 8 (18%)
10 - 12 = 4 (9%)
0 - 9 = 1 (2%)

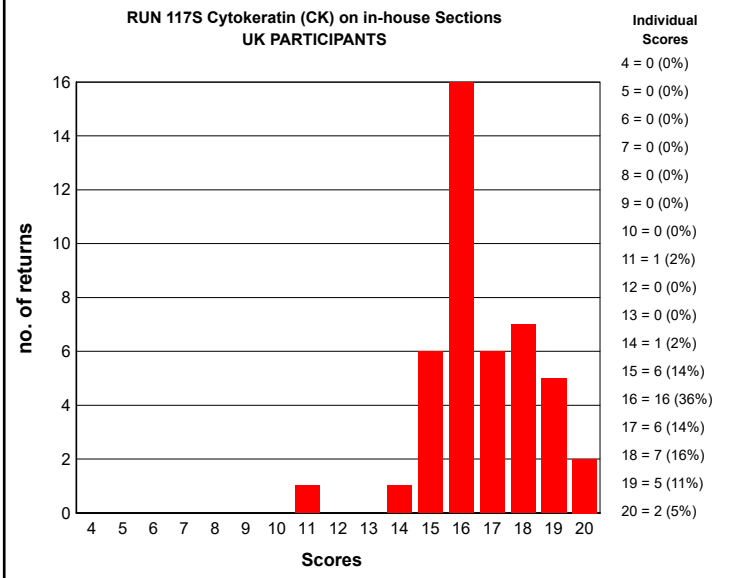
Median = 15.00



Summary

16 - 20 = 67 (84%)
13 - 15 = 12 (15%)
10 - 12 = 1 (1%)
0 - 9 = 0 (0%)

Median = 16.50

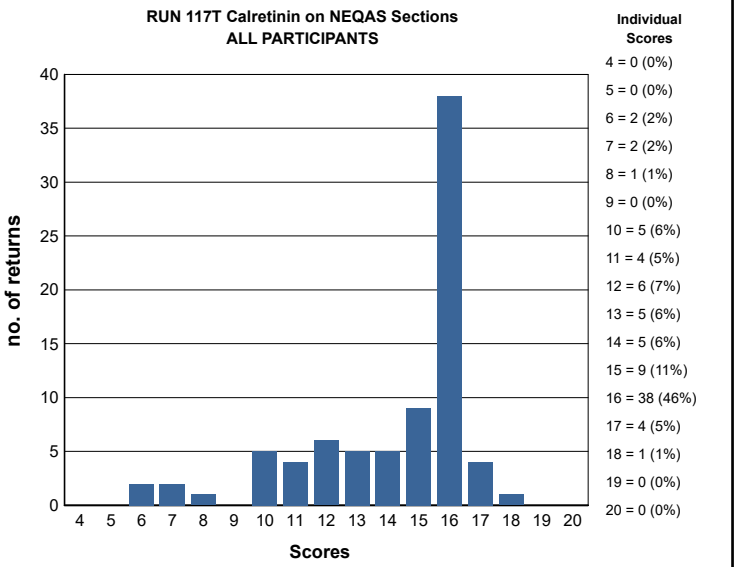


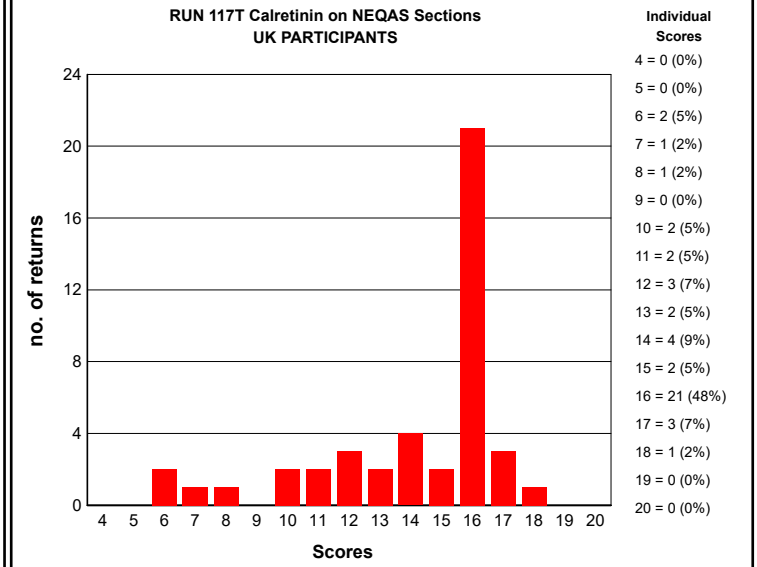
Summary

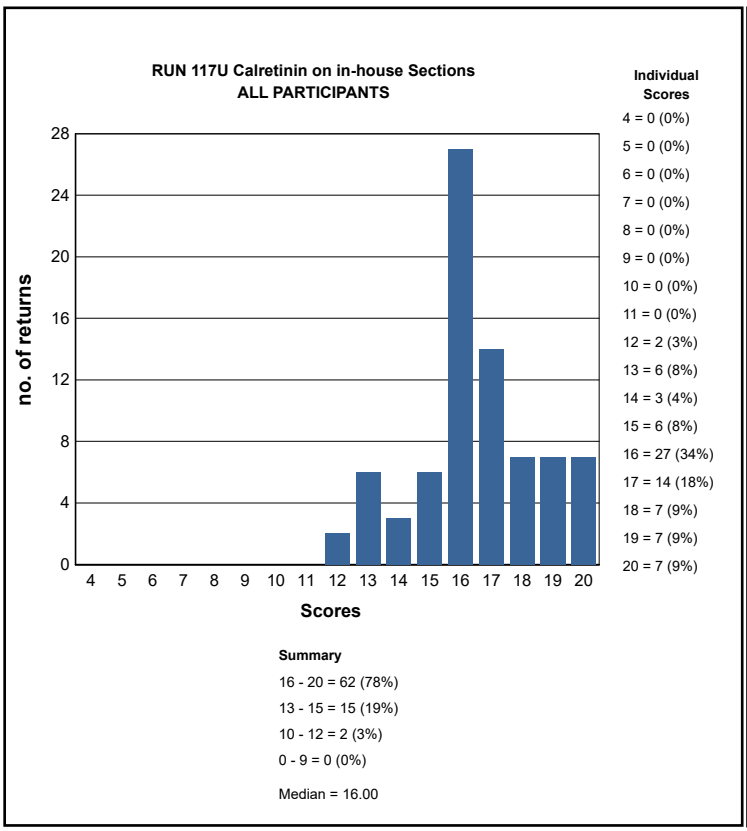
16 - 20 = 36 (82%)
13 - 15 = 7 (16%)
10 - 12 = 1 (2%)
0 - 9 = 0 (0%)

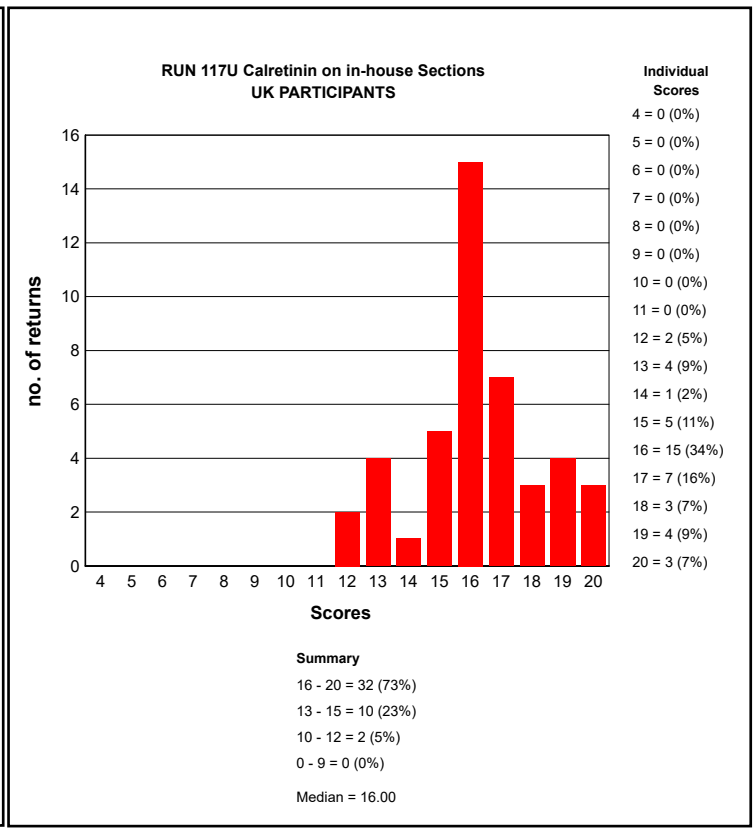
Median = 16.50

GRAPHICAL REPRESENTATION OF PASS RATES









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

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

Cytology Run: 117

Primary Antibody : Cytokeratin (CK)

Antibody Details	N	%
Becton Dickinson 349205 (CAM5.2)	3	33
BioGenex MU071-UC (clones AE1/AE3)	1	100
Dako M3515 (AE1/AE3)	19	84
Dako M0630 (34BE12)	1	100
Dako M0821(MNF116)	22	95
Leica/Novocastra RTU PA0909 (AE1/AE3)	2	100
Leica/Novocastra NCL-L-AE1/AE3	3	67
Ventana 760 2135 (AE1/AE3/PCK26)	3	67
Ventana 760 2595 AE1/AE3/PCK26	7	86
Other	5	60
Cell Marque 313M- (AE1/AE3)	1	100
Leica/Novocastra NCL-AE1/AE3	2	100
Dako FLEX RTU IR053 (AE1/AE3)	4	100
ImmunoBS MM-1012 (CK cocktail)	1	100
Ventana 790-4555 (CAM 5.2)	3	100
Leica/Novacastra NCL- L-CK5/6/8/18 (Multi 5D3/LP34	1	100
Dako Omnis FLEX GA053 (AE1/AE3)	3	100

Cytology Run: 117

Primary Antibody : Calretinin

Antibody Details	N	%
Dako M7245 (DAK-Calret 1)	17	59
Novocastra NCL-CALRET (5A5)	4	50
Zymed/Invitrogen 18 0211(DC8)	5	80
Other	6	100
Cell Marque - CMC757/758/759	1	0
Novocastra/Leica RTU-CALRET (5A5)	2	50
Cell Marque 232A-74/75/76/77/78 (R Poly)	1	0
Dako IS627 RTU FLEX (DAK-Calret 1)	6	83
Invitrogen18-0211 (DC8)	5	60
Novocastra/Leica NCL-L-CALRET-566 (CAL6)	9	56
Ventana 790-4467 (SP65)	25	100

Cytology Run: 117

Primary Antibody : Cytokeratin (CK)

Antigen Retrieval	N	%
YES	26	32
NO	56	68
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	26	
Not Specified	0	

Cytology Run: 117

Primary Antibody : Calretinin

Antigen Retrieval	N	%
YES	22	27
NO	60	73
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	22	
Not Specified	0	

Cytology Run: 117

Heat Mediated Retrieval

Cytology Run: 117

Heat Mediated Retrieval

Cytology Run: 117

Enzyme Mediated Retrieval

Cytology Run: 117

Enzyme Mediated Retrieval

Cytology Run: 117

Detection	Calretinin		Cytokeratin (CK)	
	N	%	N	%
AS PER KIT	9	100	5	100
Dako EnVision FLEX (K8000/10)	1	0	2	100
Dako EnVision FLEX+ (K8002/12)	7	86	7	86
Dako Envision+ HRP mouse K4004/5/6/7	1	100	1	100
Leica Bond Polymer AP Red Detection (DS9305)	0	0	1	100
Leica Bond Polymer Refine (DS9800)	19	53	20	85
Other	3	100	3	33
Ventana iView system (760-091)	2	100	2	100
Ventana OptiView (760-700) + Amp. (7/860-099)	0	0	1	100
Ventana OptiView Kit (760-700)	18	78	15	87
Ventana UltraView Kit (760-500)	18	78	21	90

Cytology Run: 117

Automation	Calretinin		Cytokeratin (CK)	
	N	%	N	%
Dako Autostainer Link 48	9	89	9	89
Dako Autostainer Plus Link	0	0	1	100
Dako Omnis	3	67	3	100
Leica Bond Max	8	63	8	88
Leica Bond-III	14	57	15	80
None (Manual)	3	67	1	0
Ventana Benchmark GX	2	100	2	50
Ventana Benchmark ULTRA	31	81	30	90
Ventana Benchmark XT	11	82	12	92

Cytology Run: 117

Chromogen	Calretinin		Cytokeratin (CK)	
	N	%	N	%
AS PER KIT	15	93	10	90
DAKO DAB+	2	100	2	50
Dako EnVision Plus kits	1	100	0	0
Dako FLEX DAB	7	71	10	90
Dako REAL EnVision K5007 DAB	1	100	0	0
Leica Bond Polymer Refine kit (DS9800)	19	47	22	86
Other	6	83	5	80
Ventana DAB	8	100	9	78
Ventana iview	2	50	2	100
Ventana Ultraview DAB	20	75	21	90

BEST METHODS - Gold Standard Antibody

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

Cytokeratin (CK) - Method 1

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

Primary Antibody:	Dako M3515 (AE1/AE3)
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER1 30 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

Cytokeratin (CK) - Method 2

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

Primary Antibody:	Dako Omnis FLEX GA053 (AE1/AE3) , 12.5 Mins, 32 ºC Prediluted
Automation:	Dako Omnis
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis, Buffer: Dako TRS pH high
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 32 ºC.
Detection:	Dako EnVision FLEX (K8000/10) , 20 Mins, 32 ºC Prediluted

Cytokeratin (CK) - Method 3

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

Primary Antibody:	Dako FLEX RTU IR053 (AE1/AE3)
Automation:	Dako Autostainer Plus Link
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB
Detection:	Dako EnVision FLEX+ (K8002/12)

Cytokeratin (CK) - Method 4

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

Primary Antibody:	Ventana 760 2595 AE1/AE3/PCK26 , 4 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.6
HMAR:	Ventana CC1 16mins, Buffer: Ultra CC1 (100c) Ventana, PH: 8.4
EAR:	Other, 36 ºC. Digestion Time NEQAS: 4 Mins. In-House: 4 Mins
Chromogen:	Other, PH: 7.3, 36 ºC., Time 1: 8 Mins, Time 2: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 36 ºC Prediluted

BEST METHODS - Secondary Antibody

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

Calretinin - Method 1

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

Primary Antibody:	Dako M7245 (DAK-Calret 1) , 20 Mins, 22 ºC Dilution 1: 200
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: FLEX TRS High pH
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 22 ºC., Time 1: 10 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 20 Mins, 22 ºC Prediluted

Calretinin - Method 2

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

Primary Antibody:	Ventana 790-4467 (SP65) , 20 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 mild
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC Prediluted

Calretinin - Method 3

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

Primary Antibody:	Novocastra/Leica NCL-L-CALRET-566 (CAL6) , 15 Mins, 20 ºC Dilution 1: 150
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	None
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800), 20 ºC., Time 1: 8 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins Prediluted

Calretinin - Method 4

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

Primary Antibody:	Invitrogen18-0211 (DC8)
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	
EAR:	
Chromogen:	Ventana DAB
Detection:	Ventana UltraView Kit (760-500)

The Alimentary Tract Module: GIST Run 117

Amy Newman

	First Antibody	Second Antibody
Antigens Assessed:	CD117	CD34
Tissue Sections circulated:	Normal appendix, GIST and Desmoid	
Number of Registered Participants:	115	
Number of Participants this Run	113 (98%)	

Introduction

Gold Standard: CD117

CD117 (c-Kit) is a transmembrane tyrosine kinase growth factor receptor involved in cell differentiation. It is expressed by melanocytes, mast cells and interstitial cells of Cajal[1]. Gastrointestinal stromal tumours (GISTs) are thought to arise from the interstitial cells of Cajal and occur within the bowel wall and encompass a group of heterogeneous neoplasms with differing morphology and biologic characteristics[2]. CD117 is used for a differential diagnosis of GIST from other spindle like neoplasms such as leiomyomas and leiomyosarcomas which are negative for CD117[3]. Approximately 95% of GISTs are positive with CD117. Expression can vary from strong and diffuse (Spindle subtype) to focal and weakly positive in a dot-like pattern (epithelioid subtype)[3]. Glivec (Imatinib), originally developed for chronic myeloid leukaemia (CML), has proved effective in the targeted treatment of GISTs by directly inhibiting the action of CD117[4]. Although surgery remains the standard of care for patients for patients with localised GIST, imatinib can delay recurrence and is used in the advanced and metastatic setting as the standard of care[5].

Features of Optimal Immunostaining: **(See Figs 1, 3a, 4 & 6)**

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

Features of Sub-optimal Immunostaining: **(See Fig 2, 3b & 5)**

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

Second Antibody: SMA

Smooth muscle actin (SMA) is part of the microfilament system of cytoskeletal proteins. In normal cells, it is expressed in the smooth muscle cells of vascular walls, intestinal muscularis mucosae, the muscularis propria, myofibroblasts and the myoepithelial cells lining the epithelial surface[6, 7].
SMA is traditionally used within an IHC panel for the differential diagnosis of GISTs to confirm a tumour of myogenic origin as opposed to neurogenic origin. SMA expression in GISTs varies according to the site of GIST origin. It is most commonly positive (~50%) in those arising from the duodenum and less so in those of rectal and oesophageal origin (~10-13%)[8].

Features of Optimal Immunostaining (See Figs 7, 9a&b, 10a)

- Strong cytoplasmic staining in the vessel walls and muscle
- layers and lamina mucosa in the appendix and colon
- Good localisation in muscle cells lining the epithelial surface
- Minimal background staining

Features of Suboptimal Immunostaining (See Figs 8, 9c, 10b)

- Weak or negative staining of the vessels or muscularis layers
- Staining in epithelial cells
- Excessive background / non specific staining
- Staining of the tumour cells in the desmoid tumour

Tissue Distribution and Assessment Procedure

Composite slides comprising of a GIST, appendix and desmoid tumour were distributed to all participants to be stained with the requested antibodies CD117 and SMA 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:

CD117

Results from the **CD117** assessment showed an overall acceptable pass rate of 75%. This is considerably lower than the previous 2 runs on the Neqas distributed material. The main reason for sub-optimal marks was due to very weak staining of the GIST. Non-specific and inappropriate false-positive staining was often observed in the desmoid tumour which should be negative for CD117 expression. Inappropriate antibody dilution factor and antigen retrieval were the main causes of these issues. The staining in the appendix TMA control showed to be the least variable in terms of expression. The most popular CD117 antibody choice still remains the Dako polyclonal, used by 91 participants and showed an acceptable pass rate of 86% in this assessment run.

NEQAS Pass Rates Run 116 v 117 CD117		
Run no	116	117
Acceptable	83% (N=93)	75% (N=85)
Borderline	13% (N=15)	18% (N=20)
Unacceptable	4% (N=4)	7% (N=8)

SMA

Results from the SMA assessment showed an acceptable pass rate of 95%. This is predominantly due to assessors only being able to reliably assess the Appendix and desmoid NEQAS tissue. The NEQAS GIST tumour stained using SMA presented a broad spectrum of expression ranging from negative and weakly positive to strongly positive as demonstrated in Figure 9. The differences in expression appeared to reflect the combination of primary antibody and platform employed. As SMA is not a specific marker for GIST, rather part of a panel used for differential diagnosis of other stromal or mesenchymal tumours, (as described previously), the assessors felt that some lenience should be allowed to participants with regard to demonstration of SMA in this core. The expected expression was also not known to participants

The Gold standard and expected expression for the SMA in this particular GIST shows moderate to strong staining. The method employed was the Dako (1A4) SMA clone at 1/50 on the Bond III, ER1 20'.

Analysis of the participants' methods data, shows the best method to capture the SMA antigen within the GIST was the Dako (1A4) clone either as an RTU or concentrate with a dilution of 1/200-1/300 using ER2 20' on the Leica Bond system. The Dako (1A4) RTU and concentrate on the Dako platform tended to show strong expression (Figure 9A) using a high pH antigen retrieval, however, if the antibody dilution was too high (1/50), the staining was too strong. Again, 1/200-1/300 gave optimal results The Dako and Ventana (1A4) clone used on the Ventana system had a tendency to produce very weak to negative results, despite optimal results on the appendix and desmoid tumour. Participants employing these methods may benefit from re-calibrating their SMA protocol using tissue with low level SMA antigen expression to optimise their sensitivity levels.

References

1. Cordless et al., Biology of Gastrointestinal Stromal Tumours. J Clin Oncol 2004, 22(18): 3813-3825.
2. Ostrowski J et al, Functional features of gene expression profiles differentiating gastrointestinal stromal tumours according to KIT mutations and expression, BMC Cancer, 2009; 9:413.
3. Lamba G et al, Current Management and Prognostic Features for Gastrointestinal Stromal Tumour (GIST), Exp Hematol Oncol 2012; 1:14.
4. 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.
5. Pisters PW and Patel SR., Gastrointestinal stromal tumours: current management, J Surg Oncol., 2010 Oct 1: 102(5):530-8.
6. DAKO, FLEX Ready-to-Use, Atlas of Stains—3rd Edition, 2008
7. Leica Biosystems, http://www.leicabiosystems.com/ihc-ish-fish/immunohistochemistry-ihc-antibodies-novocastra-reagents/primary-antibodies/products/sma-alpha-smooth-muscle-actin.
8. Miettinen et al, Immunohistochemical spectrum of GISTs at different sites and their differential diagnosis with a reference to CD117 (KIT). Mod Pathol. 2000 Oct;13(10):1134-42.

Selected Images showing Optimal and Sub-optimal Immunostaining

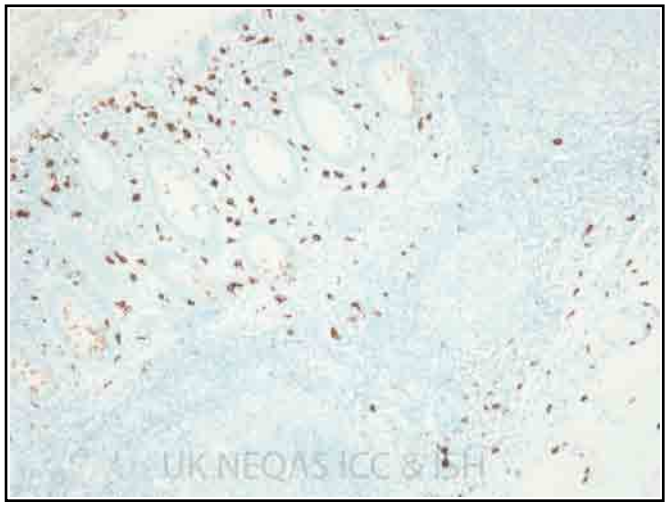


Figure 1: Excellent demonstration of CD117 on the NEQAS appendix. Well localised membrane and cytoplasmic staining in the mast cells can be seen. Method: Dako A4502 (1/200 32'); Ventana Benchmark ULTRA (HMAR: CC1 Standard; Detection: Ventana OptiView).

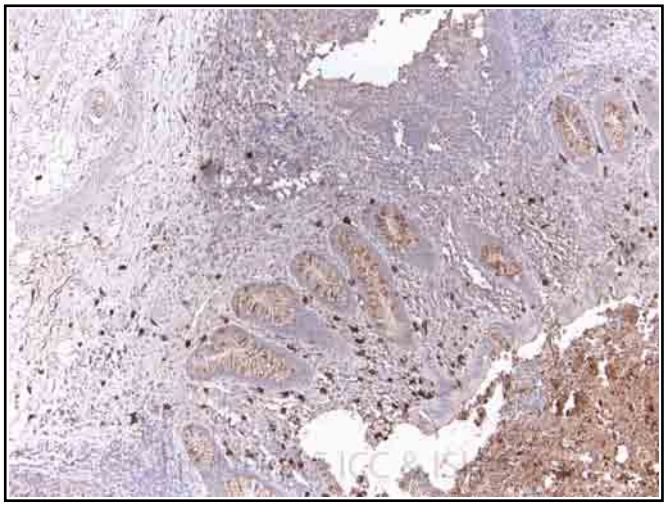


Figure 2: Poor demonstration of CD117 on the NEQAS appendix. There is demonstration of the mast cells, however, there is excessive non-specific and background staining. The morphology also appears damaged, possible due to excessive HMAR. Method: Dako A4502 clone (1/100 20'); Leica Bond III (HMAR: ER2 20'; Detection; Leica Bond Polymer Refine). Score 9/20.

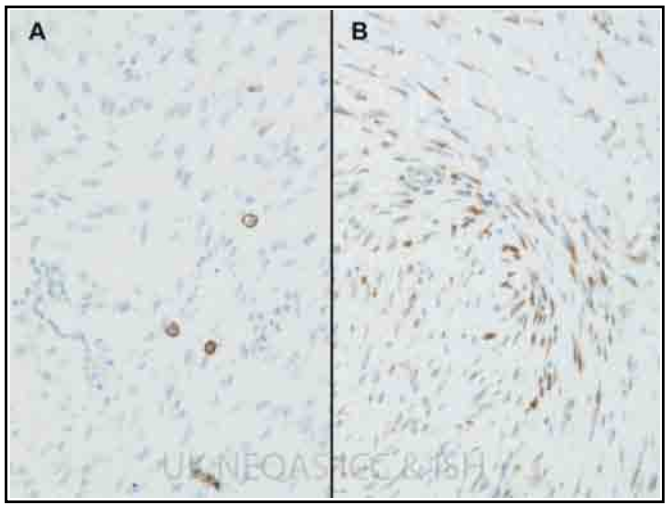


Figure 3: NEQAS negative desmoid tumour with CD117. 3A: Good demonstration with occasional mast cell positivity. Method identical to Figure 2. Figure 3B: Poor demonstration displaying false-positive staining of the desmoid cells. Method: Dako A4502 (1/50 30'); Dako Autostainer Link 48 (HMAR: Dako PT Link high pH; Detection: Dako EnVision FLEX+).

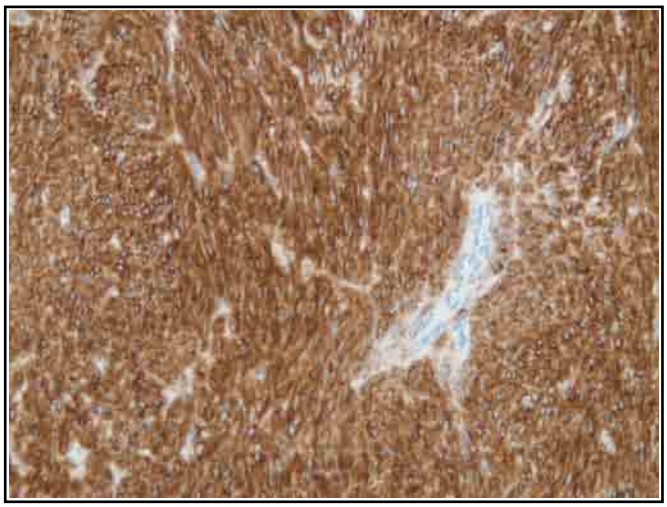


Figure 4: Excellent demonstration of CD117 on the NEQAS GIST. The antibody is well localised to the tumour cells. Method: Dako A4502 (1/100); Dako Omnis (HMAR: Not specified; Detection: Dako EnVision FLEX).

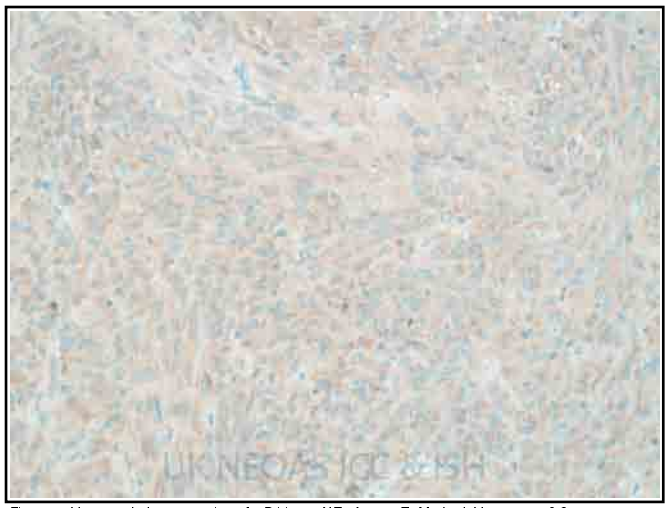


Figure 5: Very weak demonstration of CD117 on NEQAS GIST. Method: Ventana 790-2939; Ventana Benchmark ULTRA (HMAR: CC1 36'; Detection: Ventana UltraView). Score 9/20.

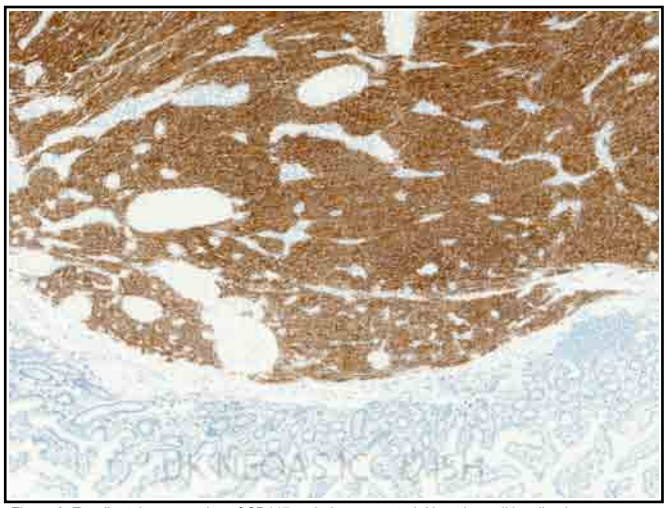


Figure 6: Excellent demonstration of CD117 on in-house control. Note the well localised staining to the GIST and the contrast with the normal gastric epithelium showing positive staining in the cells of Cajal. Method: Leica NCL-CD117 (T595) clone (RTU); Leica Bond III (HMAR: ER1 30'; Detection: Leica Bond Polymer Refine). Score 20/20.

Selected Images showing Optimal and Sub-optimal Immunostaining

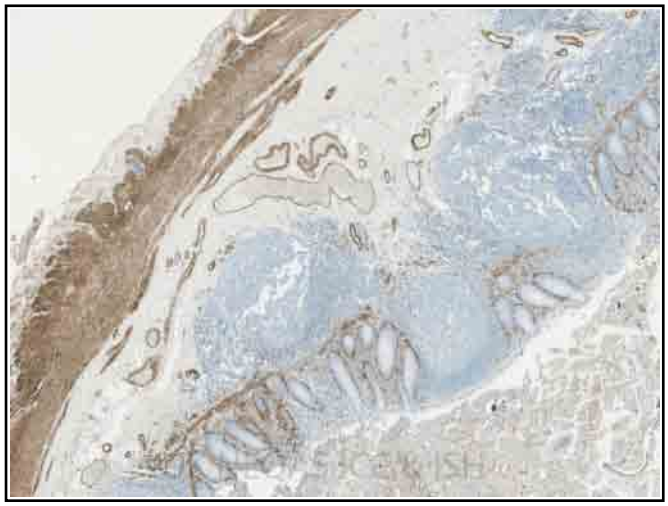


Figure 7: Acceptable staining on the submitted NEQAS tonsil for SMA. There is appropriate localisation to the lamina propria, vessel walls and smooth muscle lining the epithelial surface. Stained using the Leica PA0943 RTU clone; Leica Bond III (HMAR: None; Detection: Leica Bond Polymer Refine kit).

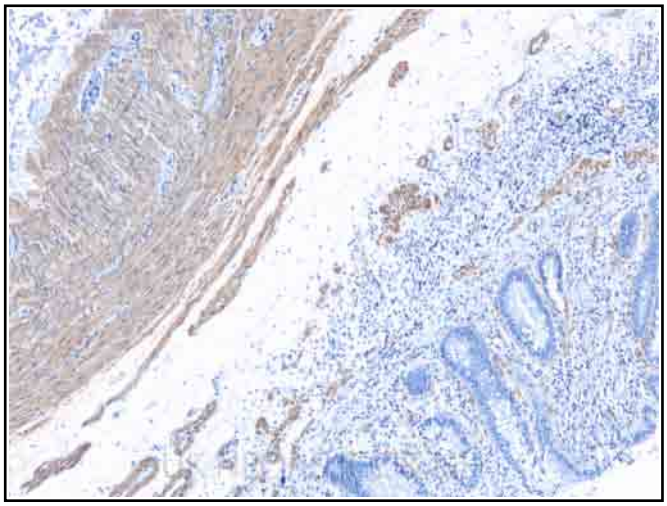


Figure 8: Weak SMA staining on the NEQAS tonsil. Particularly, the muscles lining the epithelial cells could be stronger. Method: Thermo Scientific/Neomarkers MS (1A4) clone (1/40; 36'); Ventana Benchmark XT (HMAR: None; Detection: Ventana UltraView Kit).

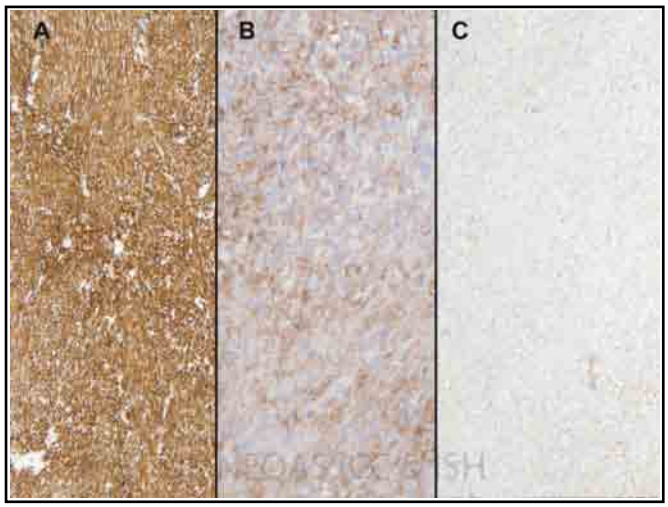


Figure 9: Variations observed in SMA expression in the NEQAS distributed GIST. A) Strong expression using the Dako RTU (1A4); Dako Link 48 (HMAR: Dako PT Link pH9.0; Detection: EnVision FLEX+). B) Leica RTU (ASM-1); Leica Bond III (HMAR: None; Detection: Polymer Refine). C) Ventana RTU (1A4); Benchmark XT (HMAR: None; Detection: UltraView).

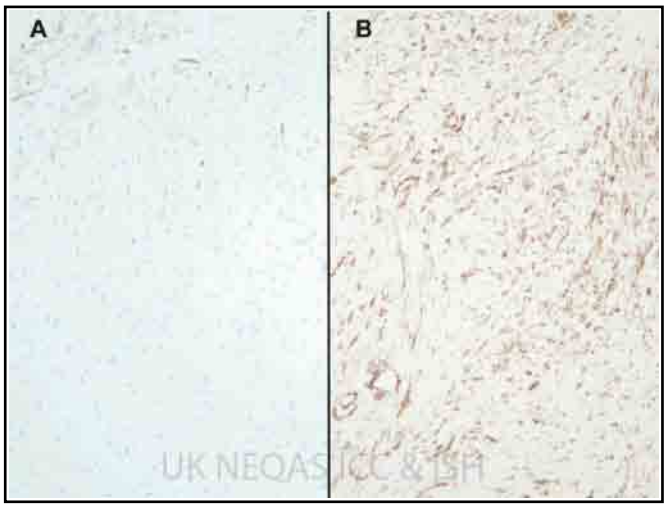
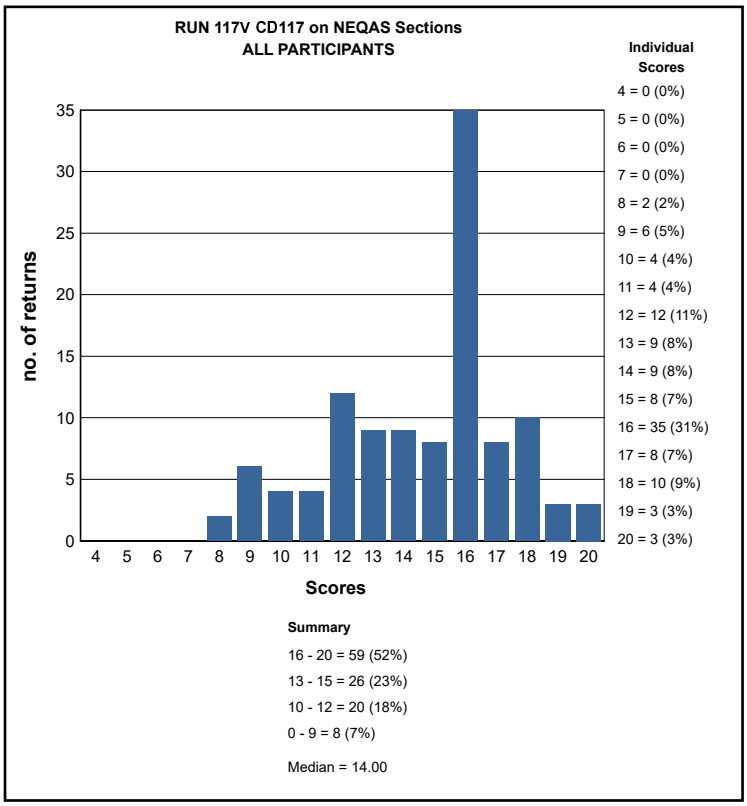
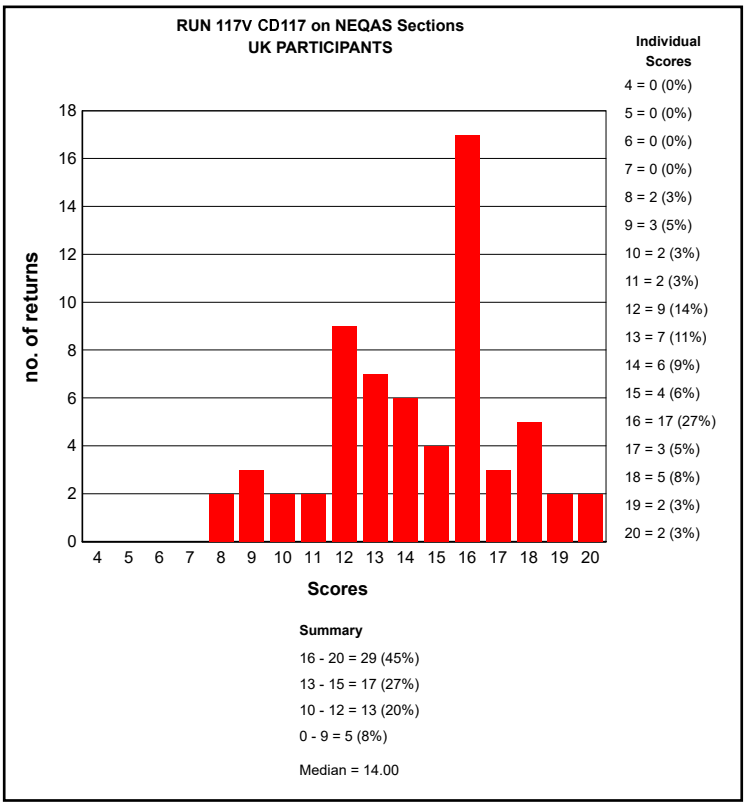
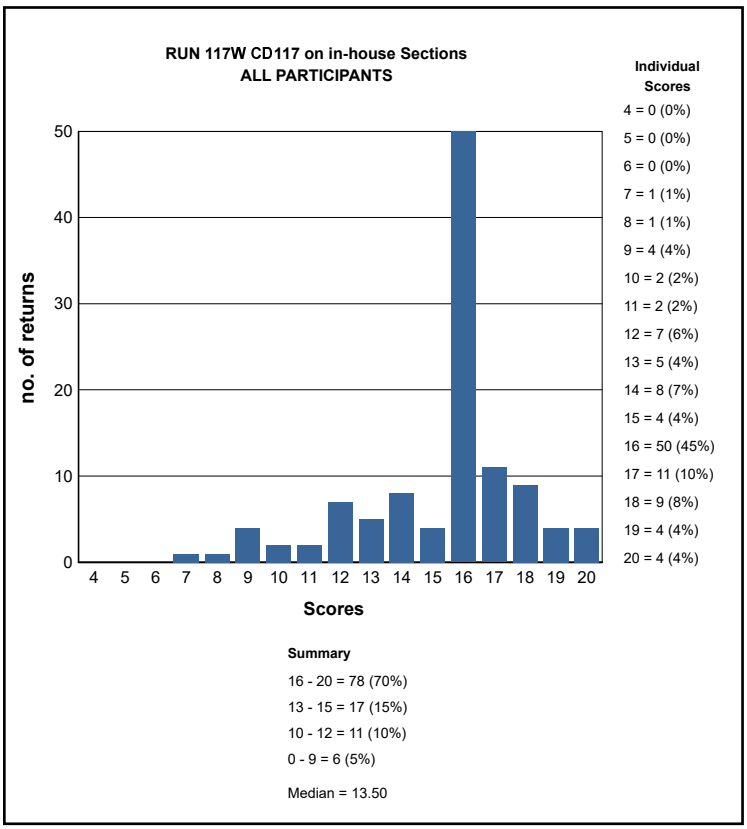


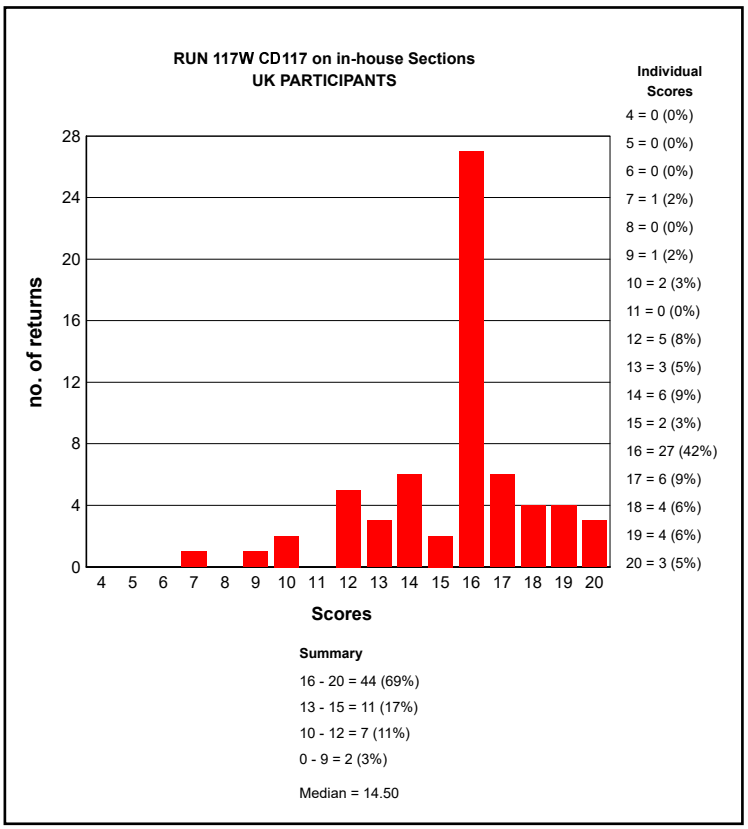
Figure 10: Optimal staining (A) and unacceptable staining (B) of the UK NEQAS Desmoid sample. A typical desmoid should be negative for SMA with positivity in the vessel walls. In (B), there is staining in the desmoid stromal cells which could potentially lead to a false diagnosis. This overstaining was noted on the same sample as the very weak GIST (Figure 9C).

GRAPHICAL REPRESENTATION OF PASS RATES

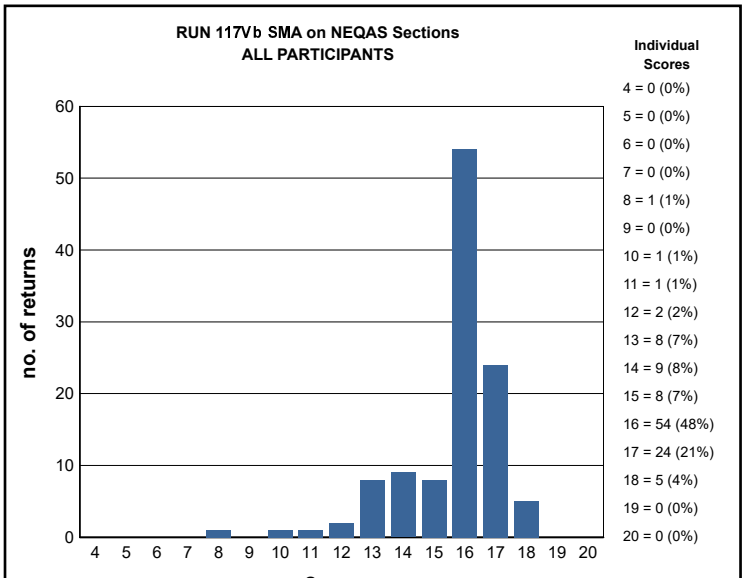


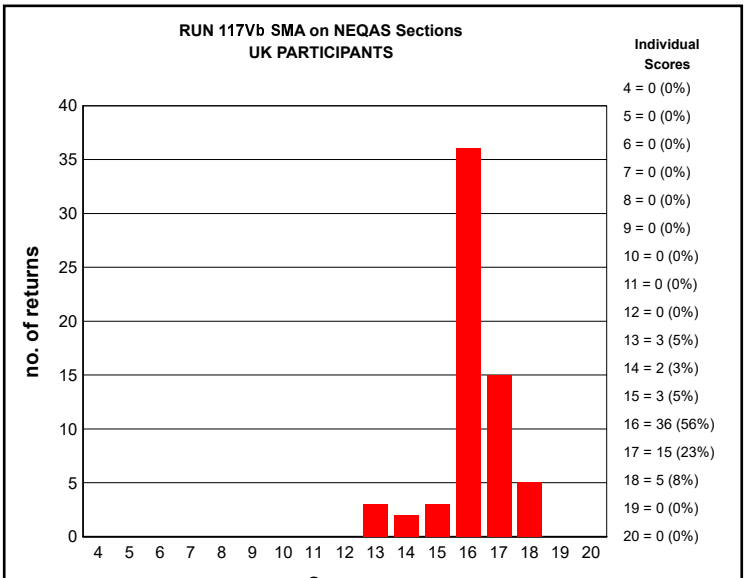


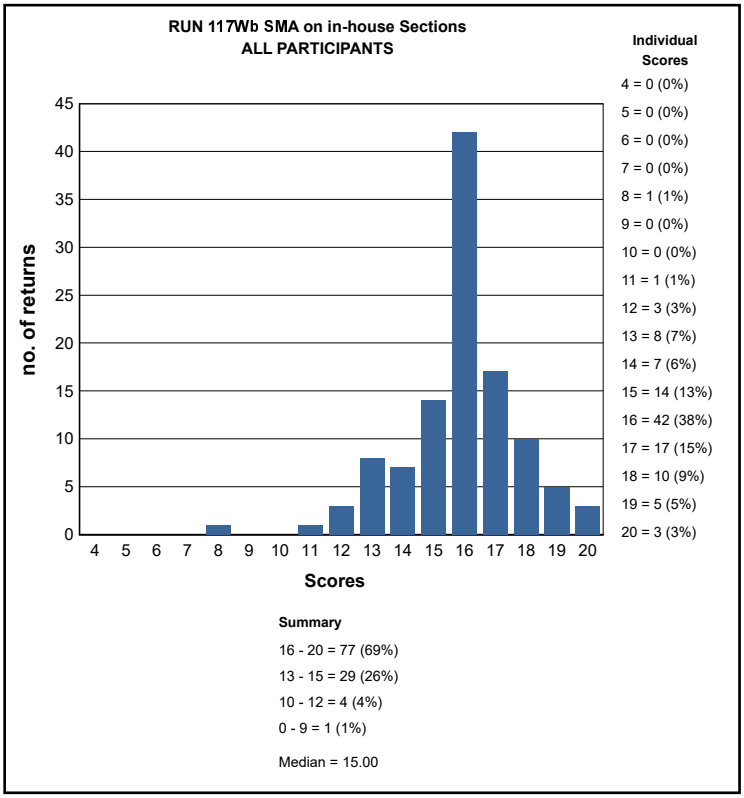


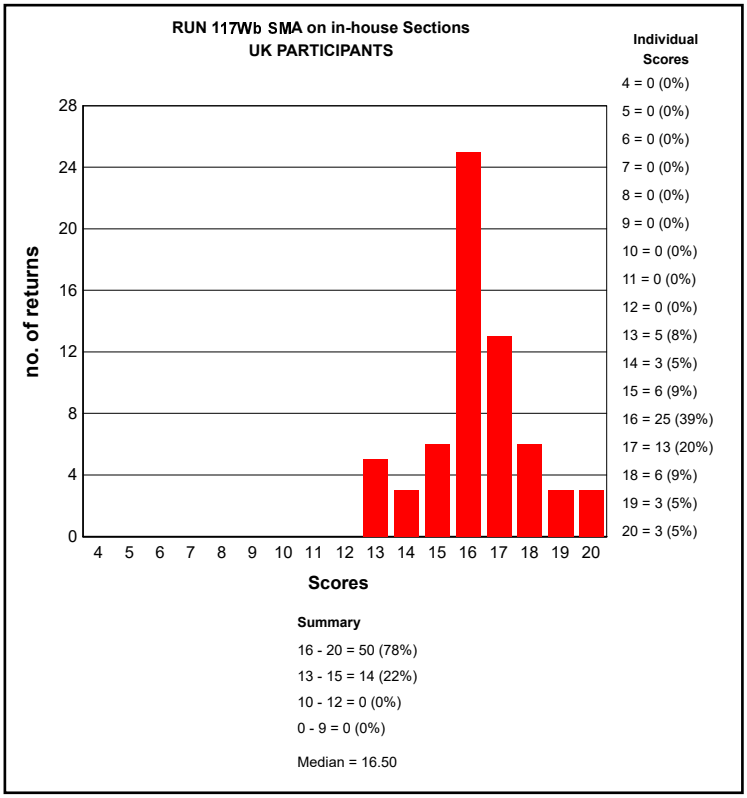


GRAPHICAL REPRESENTATION OF PASS RATES









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: 117

Primary Antibody : CD117

Antibody Details	N	%
Dako A4502 (rb poly)	86	76
Leica/Novocastra NCL-CD117 (T595)	2	50
Ventana 790-2939 (rb poly)	1	0
Cell Marque 117R/S-xx (YR145)	9	78
Leica/Novocastra RTU-CD117 (T595)	1	100
Ventana 790-2951 (9.7)	7	86
Leica RTU (EP10) PA0007	6	83

Alimentary Tract Pathology Run: 117

Primary Antibody : SMA

Antibody Details	N	%
Cell Marque 201M (HHF35)	1	100
Cell Marque 202M (1A4)	6	100
Dako M0851 SMA (1A4)	33	94
Dako 1R611 RTU Flex Link (1A4)	17	100
Dako M0635 SMA (HHF35)	10	90
Invitrogen 18-0106 (1A4)	1	100
Novocastra RTU-SMA (asm-1)	1	100
Leica PA0943 RTU (ASM-1)	11	100
Sigma (1A4) A2547	4	100
Thermo Scientific/Neomarkers MS 113-R (1A4)	1	0
Thermo Scientific/Neomarkers MS 113-P (1A4)	1	100
Novocastra NCL-SMA (asm-1)	4	100
Ventana 760 2833 (1A4)	20	95
Ventana 760-2601 (HHF35)	1	100

Alimentary Tract Pathology Run: 117

Heat Mediated Retrieval	CD117 N	CD117 %	SMA N	SMA %
Dako Omnis	4	100	4	100
Dako PTLink	13	85	13	100
Lab vision PT Module	0	0	1	100
Leica ER1 10 mins	1	100	2	100
Leica ER1 20 mins	4	75	6	100
Leica ER1 30 mins	4	75	1	100
Leica ER2 10 mins	2	100	1	100
Leica ER2 20 mins	21	67	7	100
Leica ER2 30 mins	6	83	1	100
None	3	33	37	97
Other	0	0	3	100
Pressure Cooker	1	0	1	100
Pressure Cooker in Microwave Oven	0	0	1	100
Ventana CC1 16mins	0	0	3	67
Ventana CC1 24mins	0	0	1	100
Ventana CC1 32mins	8	75	3	33
Ventana CC1 36mins	6	50	0	0
Ventana CC1 40mins	1	100	0	0
Ventana CC1 48mins	2	50	0	0
Ventana CC1 52mins	1	0	0	0
Ventana CC1 56mins	4	100	0	0
Ventana CC1 64mins	13	85	2	100
Ventana CC1 72mins	2	100	0	0
Ventana CC1 88mins	1	100	0	0
Ventana CC1 8mins	1	0	15	93
Ventana CC1 92mins	1	100	0	0
Ventana CC1 extended	0	0	1	100
Ventana CC1 mild	4	75	2	100
Ventana CC1 standard	8	88	3	100
Ventana CC2 mild	1	100	0	0

Alimentary Tract Pathology Run: 117

Enzyme Mediated Retrieval	CD117 N	CD117 %	SMA N	SMA %
AS PER KIT	0	0	6	100
NOT APPLICABLE	84	75	57	95
Ventana Protease 1 (760-2018)	0	0	1	100

Alimentary Tract Pathology Run: 117	CD117		SMA	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	8	63	13	92
BioGenex SS Polymer (QD 430-XAKE)	1	0	0	0
Dako EnVision FLEX (K8000/10)	3	100	6	100
Dako EnVision FLEX+ (K8002/12)	8	88	7	100
Dako Envision+ HRP rabbit K4008/9/10/11	1	100	0	0
Leica Bond Polymer Refine (DS9800)	37	76	33	100
Other	2	50	2	100
Ventana iView system (760-091)	1	100	1	100
Ventana OptiView (760-700) + Amp. (7/860-099)	1	100	2	100
Ventana OptiView Kit (760-700)	24	83	21	86
Ventana UltraView Kit (760-500)	25	68	24	96

Alimentary Tract Pathology Run: 117	CD117		SMA	
Chromogen	**N**	**%**	**N**	**%**
A. Menarini Liquid Stable DAB kit	1	0	0	0
AS PER KIT	17	82	21	86
DAKO DAB+	1	100	1	100
Dako EnVision Plus kits	0	0	2	100
Dako FLEX DAB	11	82	10	100
Leica Bond Polymer Refine kit (DS9800)	37	76	33	100
Other	5	60	4	100
Ventana DAB	12	92	12	92
Ventana iview	1	100	1	100
Ventana Ultraview DAB	27	67	26	96

Alimentary Tract Pathology Run: 117	CD117		SMA	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	1	0	0	0
BioGenex Optimax	0	0	1	100
Dako Autostainer Link 48	11	82	13	100
Dako Autostainer Plus Link	3	67	0	0
Dako Omnis	4	100	4	100
LabVision Autostainer	0	0	2	100
Leica Bond Max	14	71	13	100
Leica Bond-III	25	76	23	100
Ventana Benchmark GX	1	100	2	100
Ventana Benchmark ULTRA	38	74	34	88
Ventana Benchmark XT	15	80	17	94

BEST METHODS - Gold Standard Antibody

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

CD117 - Method 1

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

Primary Antibody: Dako A4502 (rb poly) , 20 Mins, 20 ºC Dilution 1: 100
Automation: Dako Omnis
Method: Dako FLEX kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako Omnis
EAR: NOT APPLICABLE
Chromogen: AS PER KIT
Detection: AS PER KIT

CD117 - Method 2

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

Primary Antibody: Dako A4502 (rb poly) , 32 Mins, 36 ºC Dilution 1: 200
Automation: Ventana Benchmark ULTRA
Method: Ventana Optiview
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 standard, Buffer: Ventana Reaction Buffer
EAR: NOT APPLICABLE
Chromogen: Ventana DAB, RT ºC., Time 1: 8 Mins, Time 2: 8 Mins
Detection: Ventana OptiView Kit (760-700) , 8 Mins, RT ºC Prediluted

CD117 - Method 3

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

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

CD117 - Method 4

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

Primary Antibody:	Leica RTU (EP10) PA0007 , 15 Mins, 21 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), 21 ºC., Time 2: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, 21 ºC Prediluted

BEST METHODS - Secondary Antibody

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

SMA - Method 1

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

Primary Antibody:	Dako M0851 SMA (1A4) , 32 Mins Dilution 1: 1:200
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 8mins
EAR:	
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

SMA - Method 2

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

Primary Antibody:	Leica PA0943 RTU (ASM-1) , 15 Mins, 21 ºC
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	None
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), 21 ºC., Time 1: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, 21 ºC

SMA - Method 3

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

Primary Antibody:	Dako 1R611 RTU Flex Link (1A4) , 20 Mins, 32 ºC Prediluted
Automation:	Dako Omnis
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis, Buffer: Dako TRS High PH
EAR:	
Chromogen:	Dako FLEX DAB, 32 ºC., Time 1: 5 Mins
Detection:	Dako EnVision FLEX (K8000/10) , 20 Mins, 32 ºC Prediluted

SMA - Method 4

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

Primary Antibody:	Cell Marque 202M (1A4) , 40 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark GX
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 8mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700) , 12 Mins, 37 ºC Prediluted

Lynch Syndrome/HNPCC Run 117

Keith Miller and Amy Newman

	Gold Standard	Second Antibody
Antigens Assessed:	MSH2	MSH6
Tissue Sections circulated:	Positive and negative colonic tumours plus normal appendix	
Number of Registered Participants:	99	
Number of Participants This Run:	MSH2: 82 (82%), MSH6: 85 (85%)	

General Introduction

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

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

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

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

Mismatch Repair Markers

Where possible, IHC should be carried out using an automated staining machine. Manual IHC is prone to significant variation, both within and between batches. Whereas automated immunostaining generates more consistent and reproducible staining patterns. There are 4 MMR antibodies available to aid in the differential identification of colorectal cancers. These are MLH1, MSH2, MSH6 & PMS2, and the use of all 4 antibodies is recommended rather than just using the 2 markers (MLH1 & MSH2). There is a heterodimeric association of proteins, such that a loss of MLH1 expression is almost always accompanied by the loss of PMS2 expression, and a loss of MSH2 is almost always accompanied with the loss of MSH6. Therefore, the expression changes to the heterodimeric binding partners of these proteins acts as a very useful confirmatory finding. The antigenic epitopes for the four anti-MMR antibodies are particularly fixation-sensitive, so immunostaining patterns should only be assessed in well-fixed regions of the tissue section, for example where the adjacent normal epithelium or the intra- or peri-tumoural lymphoid cells or stromal cells, such as fibroblasts, show clear, strong nuclear immune-positivity 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 immune-positivity in tumour

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

Assessment Procedure:

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

Features of Optimal Immunostaining: (Figs 1-3, 7-9)
Appendix: (Figs 1, 7)
Strong nuclear staining of the epithelium at the base and lower third-half of the crypts with fading of nuclear staining intensity in the middle and upper thirds of the crypts, to negative or weak staining of epithelial nuclei at the luminal surface.

- Strong staining of lymphoid follicles.

Tumour without loss of MMR protein: (Figs 3, 9)

. Strong nuclear staining in the tumour cells.

- Strong nuclear staining in the lymphocytes and stromal cells.

Tumour with loss of MMR protein: (Figs 2, 8)

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

Features of Suboptimal Immunostaining: (Figs 4-6, 10-12)
Appendix: (Figs 4, 10)

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

Tumour without loss of MMR protein: (Figs 6, 12)

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

Tumour with loss of MMR protein: (Figs 5, 11)

- 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 NEQAS section pass rates for the **MSH2** assessment were similar to the previous time (Run 114) this antibody was assessed, with 76% of labs achieving an acceptable pass, and a further 15% of participants receiving a borderline score (10-12/20), and therefore an overall pass of 91%. There was a fail rate of 9%. However, similarly to previous runs, the main reason for a failed assessment was due to either weak staining or inappropriate non-specific staining. The in-house MSH2 had an excellent pass rate, with only one laboratory having a score below 10/20. The Ventana (G219-1129) clone was the most popular choice of antibody, and showed a pass rate of 82%. The Dako (FE11) clone was also popular , used by 31 labs and showed a pass rate of 77%.

The **MSH6** assessment showed a higher pass rate to that of the previous run (Run 114). 84% of participants received an acceptable pass, and a further 12% received a borderline pass. Similarly to the **MSH2** assessment, the fail rate was quite low with 4 laboratories (5%) receiving a score of under 10. The scores overall were higher than the last time **MSH6** was assessed. Again, weak staining was the main reason for failure or borderline scores (depending on the severity). The Ventana 44 clone was the most popular choice of MSH2 antibody used in this assessment by 29 labs, and showed a pass rate of 86%. The DakoEP49 clone was also commonly used, and showed an overall pass rate of 90%.

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

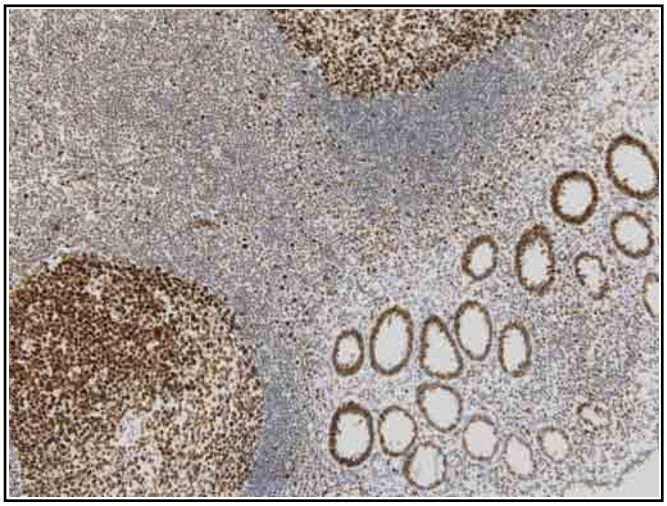


Figure 1: Optimal demonstration of MSH2 on the NEQAS appendix. There is strong nuclear staining in the follicles and within the lower crypts.

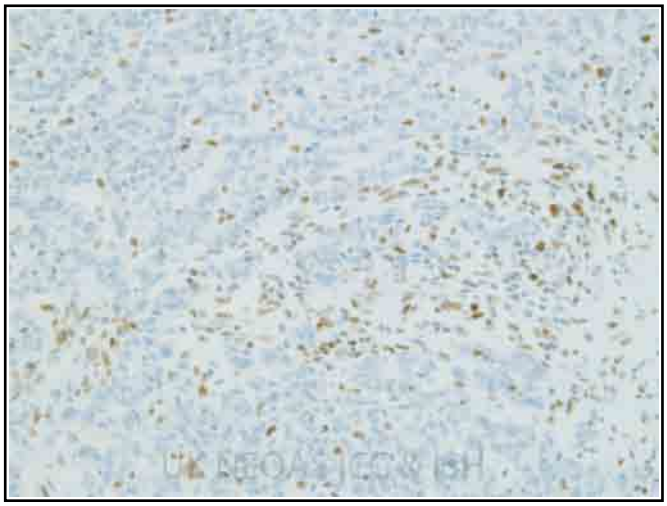


Figure 2: Good demonstration of NEQAS tumour with loss of MSH2 expression. There is strong staining in the internal positive controls of lymphocytes and stromal cells with negative tumour nuclei.

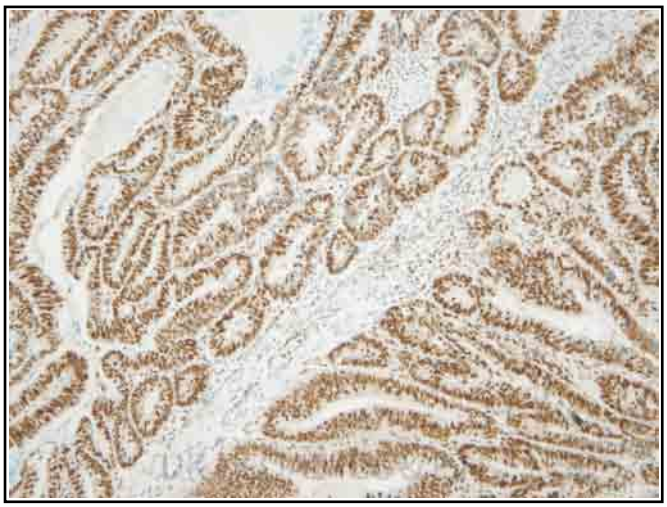


Figure 3: Excellent demonstration of the NEQAS MSH2 positive tumour. Good localisation of the MSH2 antigen to the tumour nuclei, lymphocytes and tumour cells.

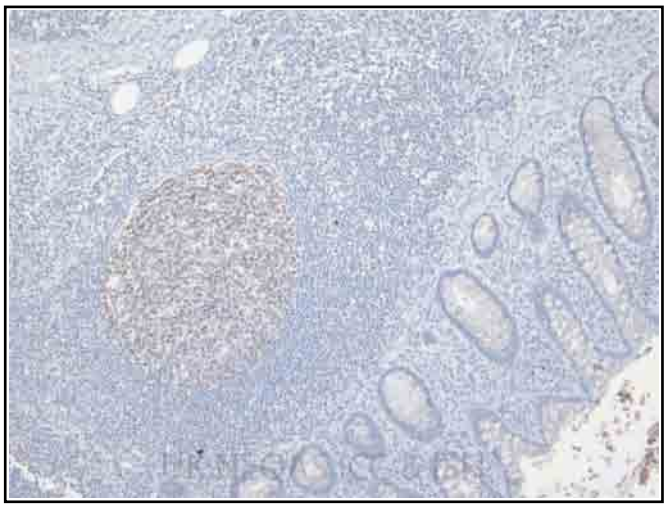


Figure 4: Weak demonstration of MSH2 on the NEQAS appendix. The staining is weak in the follicle centres and the staining is absent in the crypt epithelium. Method: NCL-MSH2 (25D12) clone (1/50); Leica Bond III (HMAR: ER2, 30'; Detection: Bond Polymer Refine Kit).

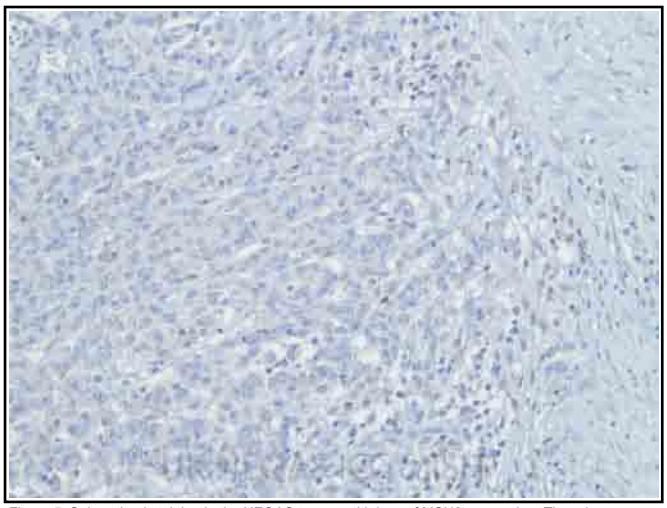


Figure 5: Sub-optimal staining in the NEQAS tumour with loss of MSH2 expression. There is occasional weak expression in some stromal cells and lymphocytes. Method as in Figure 4.

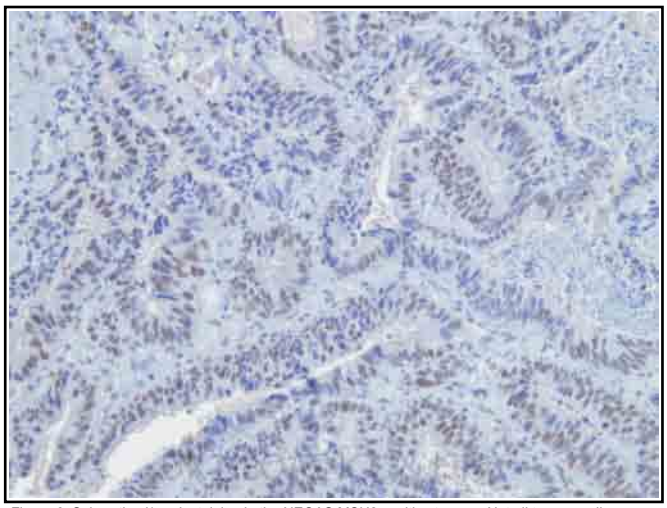


Figure 6: Sub-optimal/weak staining in the NEQAS MSH2 positive tumour. Not all tumour cells are positive as expected. The counterstain is also excessive, which can mask the protein positivity. Method as in Figure 4.

Selected Images showing Optimal and Sub-optimal Immunostaining

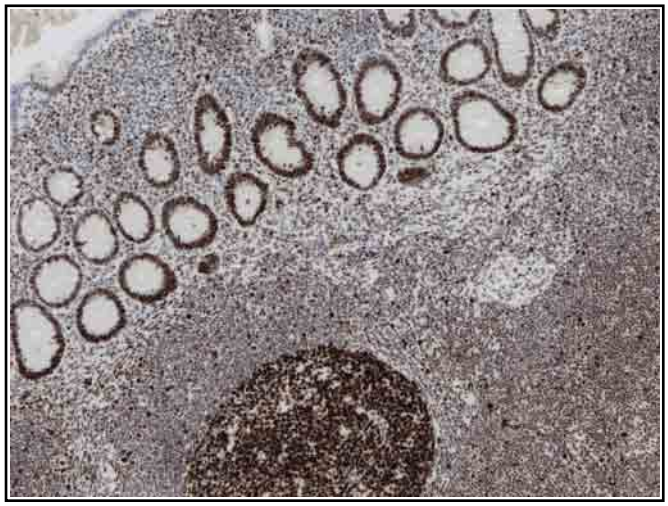


Figure 7: Good demonstration of MSH6 on the NEQAS tonsil. Similar to MSH2, there is strong nuclear staining in the follicles and lower crypts. Method: Dako M3646 (EP49) clone (1/30; 30'); Leica Bond III (HMAR: ER2 30'; Detection: Leica Bond Polymer Refine kit).

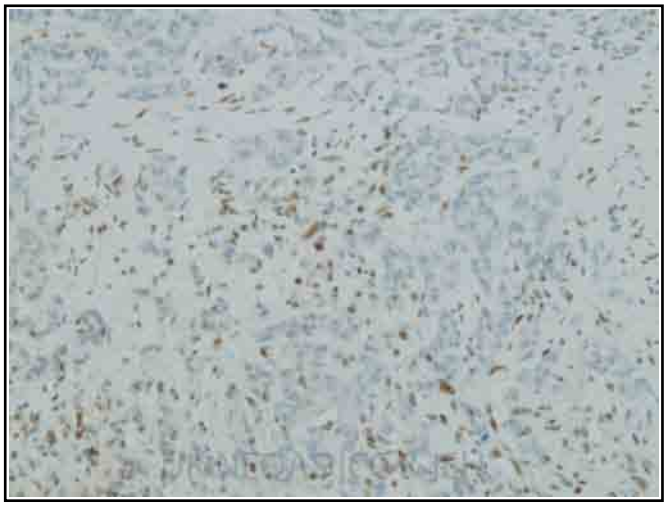


Figure 8: Excellent demonstration of the NEQAS tumour with loss of MSH6 expression. There is strong staining in the internal positive controls of lymphocytes and stromal cells with negative tumour nuclei. Method as described in Figure 7.

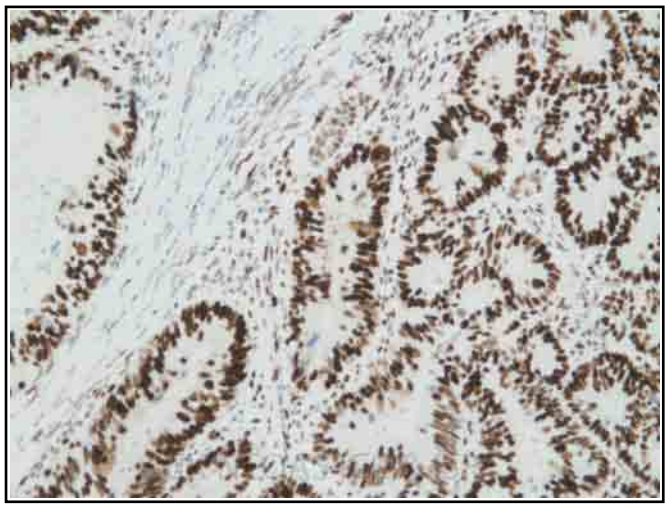


Figure 9: Excellent demonstration of the MSH6 antibody on the NEQAS positive tumour. Good localisation of the MSH2 antigen to the tumour nuclei, lymphocytes and tumour cells. No background staining is present. Method as described in Figure 7.

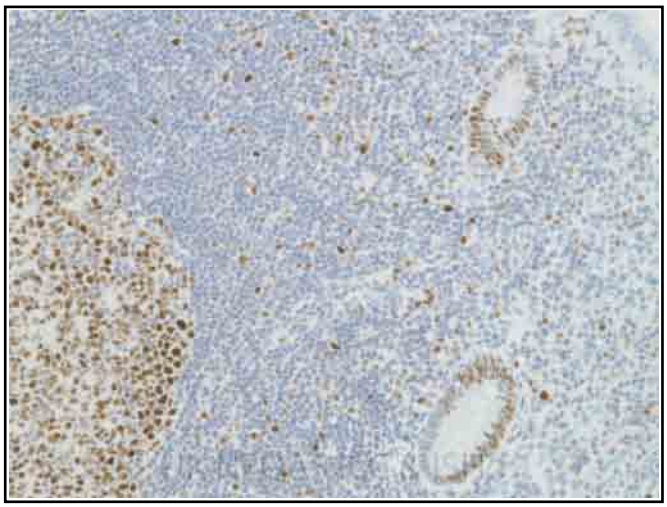


Figure 10: Slightly weak (borderline) staining with MSH6 on the NEQAS appendix. There is good localisation, however, the epithelial crypts are weaker as expected and more lymphocytes could be staining in the inter-follicular areas.

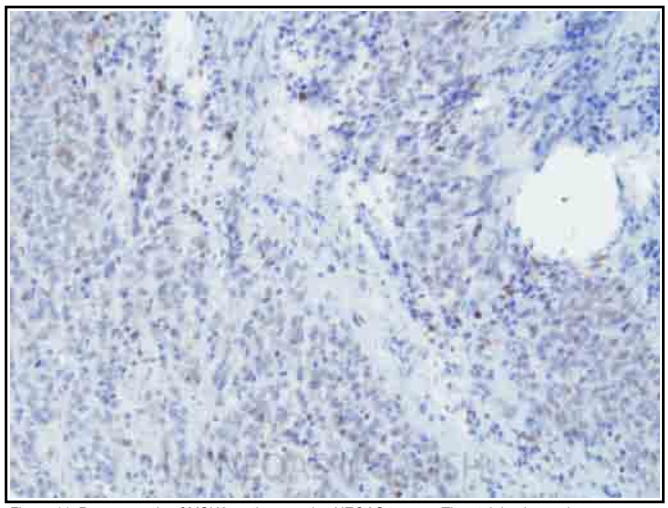


Figure 11: Poor example of MSH6 on the negative NEQAS tumour. The staining is poorly localised and non-specific. Method: Ventana CONFIRM (44) clone (RTU; 20'); Ventana Benchmark Ultra (HMAR: CC1 80'; Detection: Ventana Optiview).

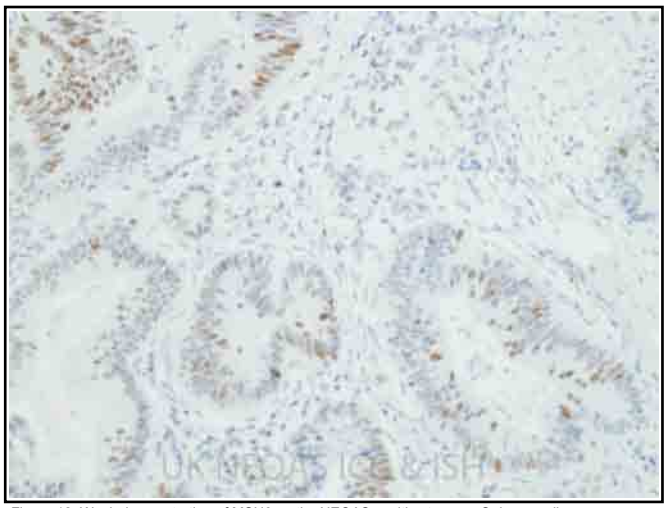
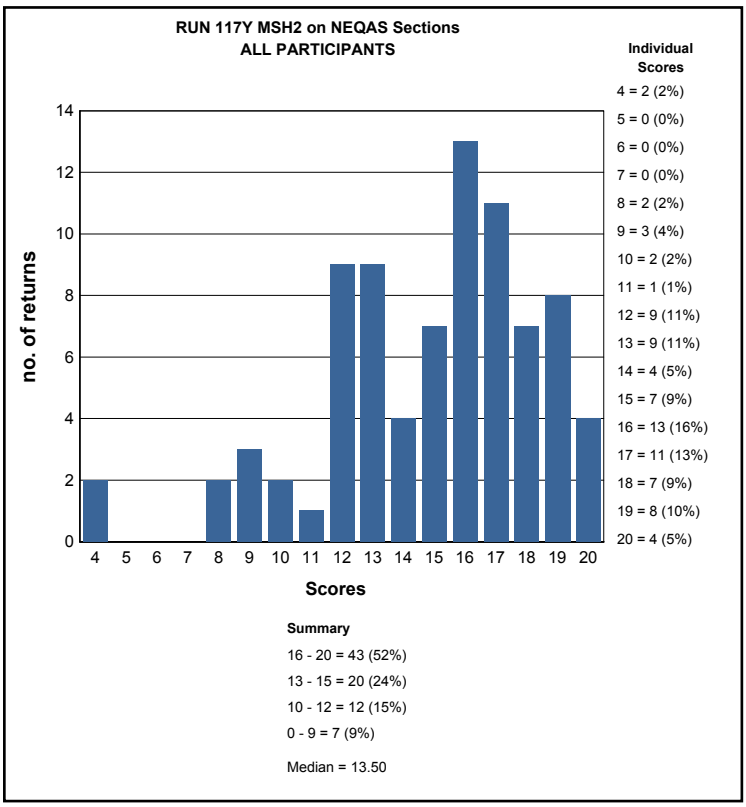
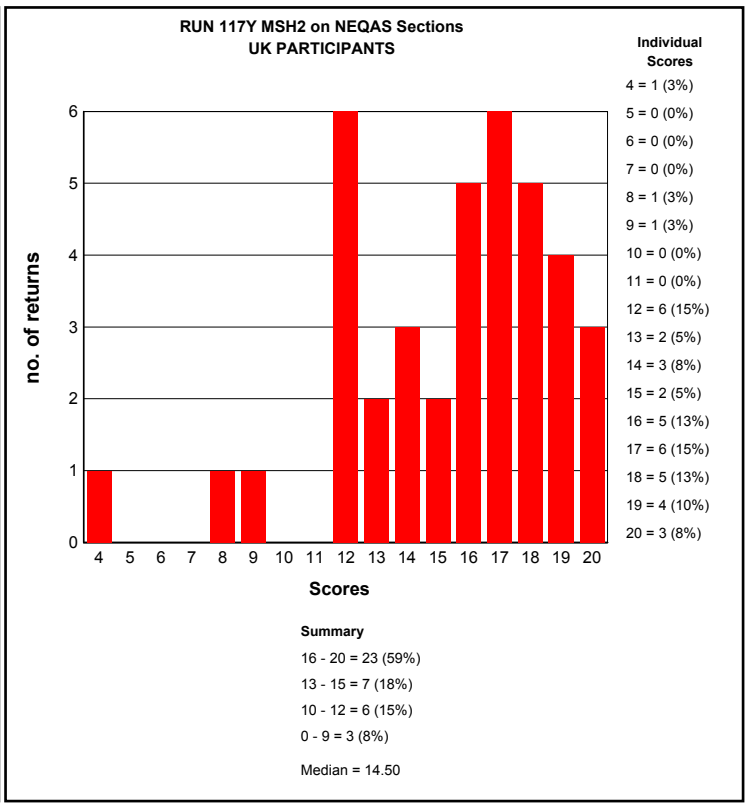
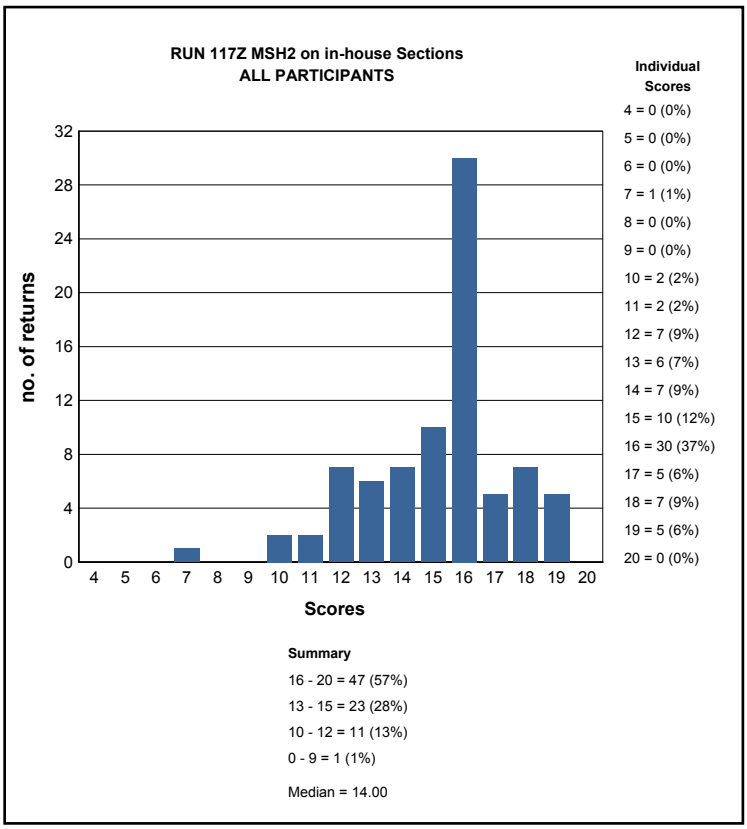


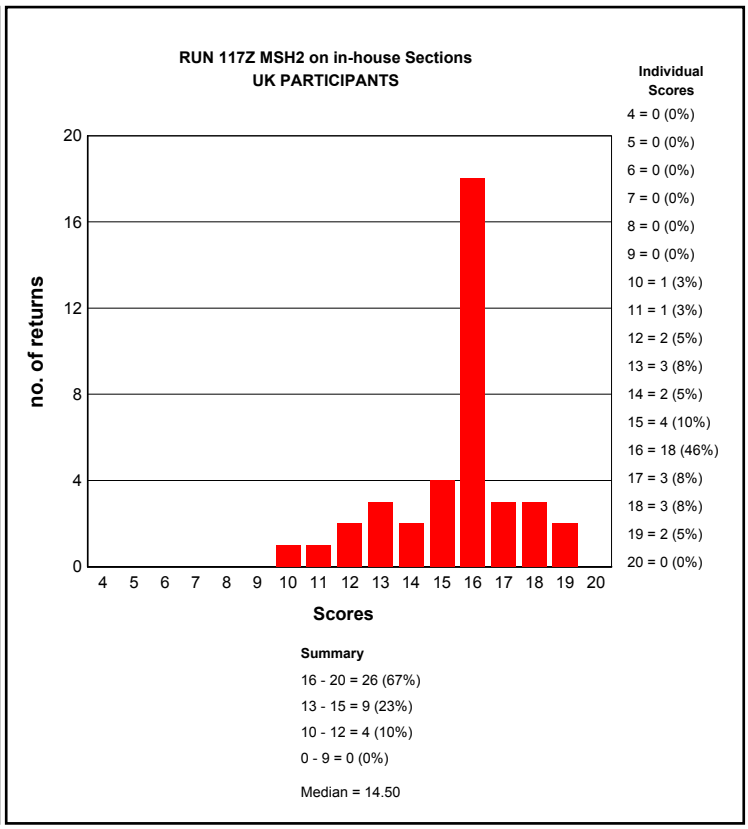
Figure 12: Weak demonstration of MSH6 on the NEQAS positive tumour. Only a small percentage of tumour cells show staining which is weak. The internal positive controls, stromal cells and lymphocytes are negative.

GRAPHICAL REPRESENTATION OF PASS RATES

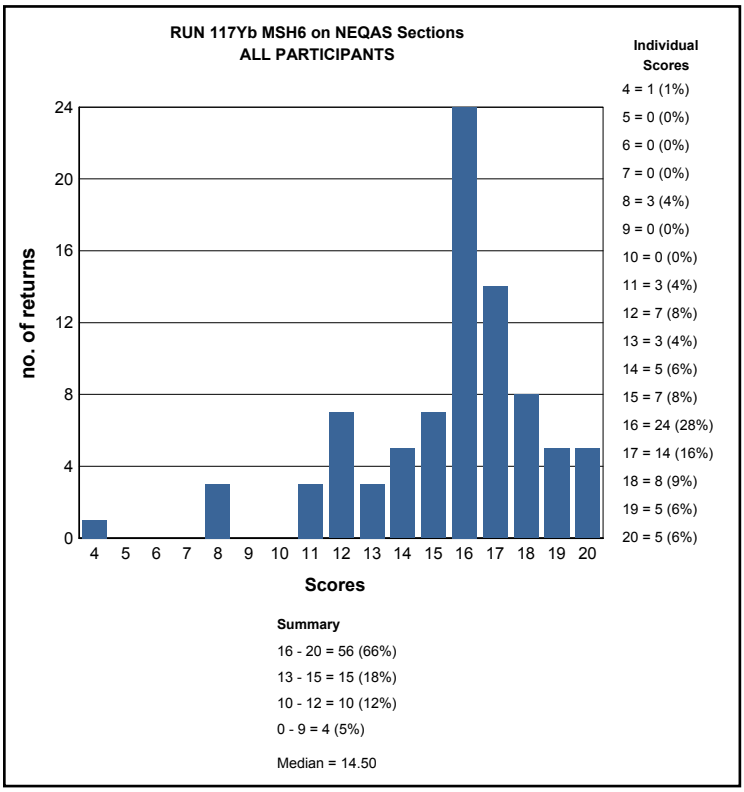


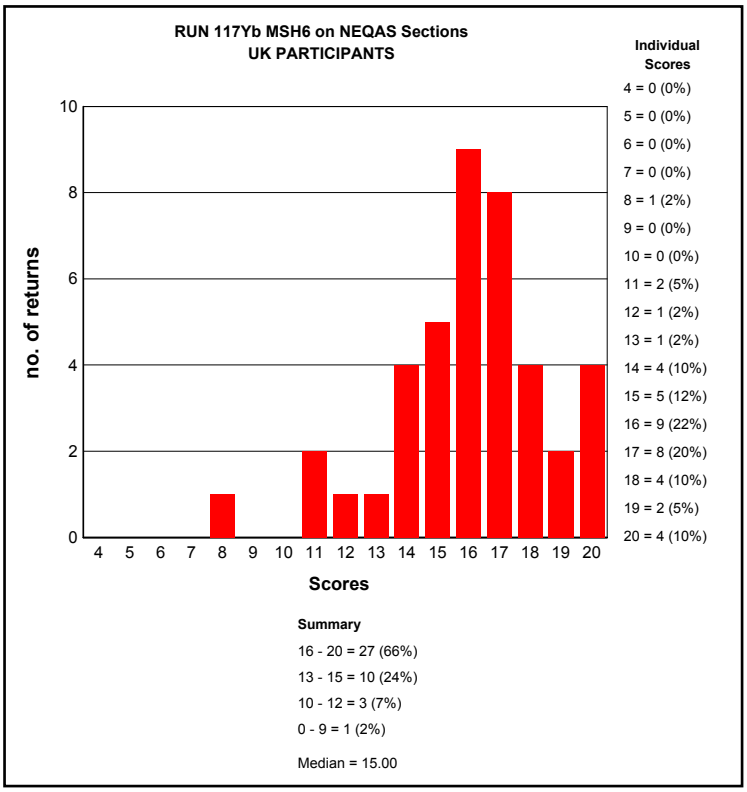


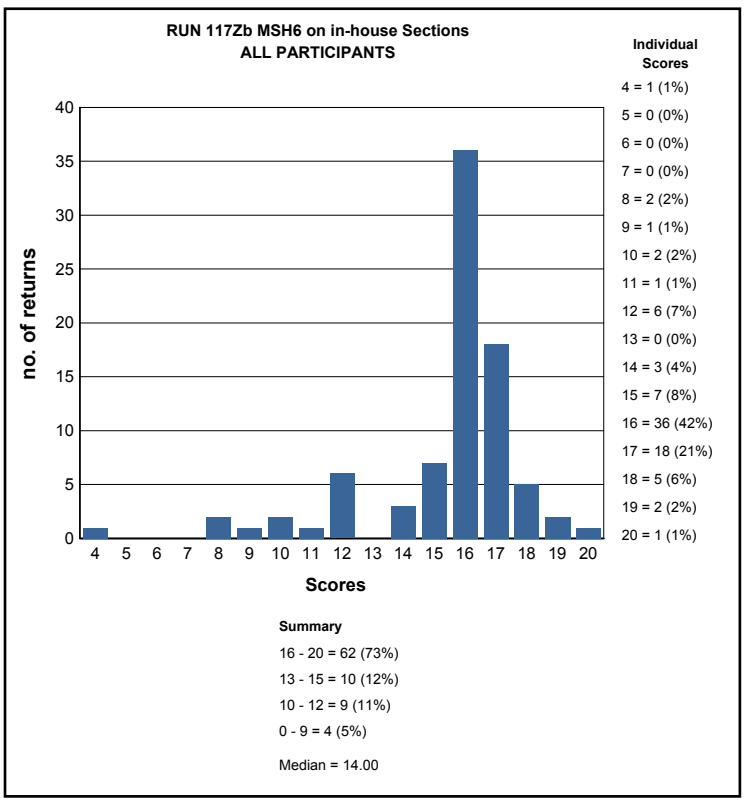


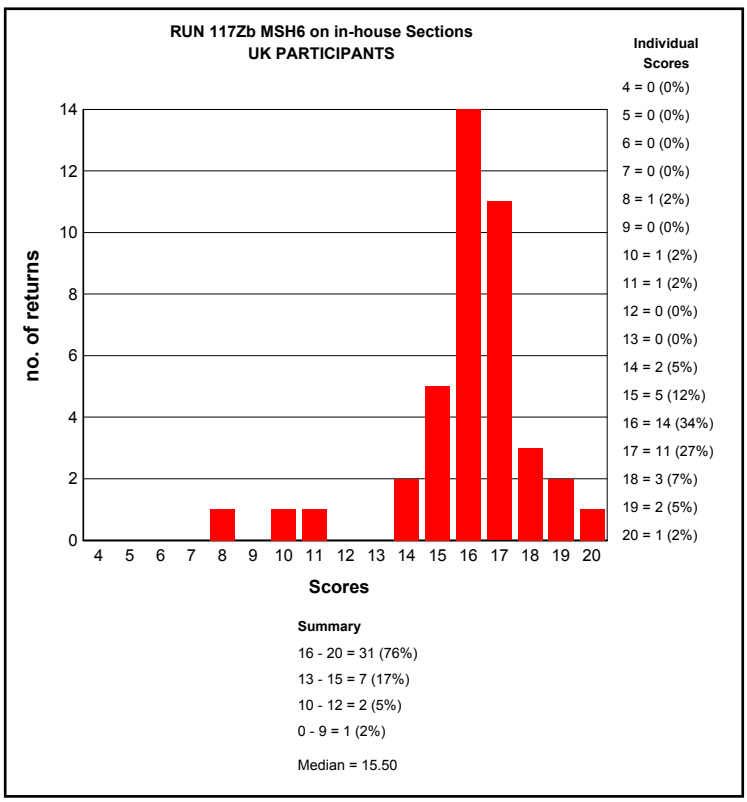


GRAPHICAL REPRESENTATION OF PASS RATES









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: 117

Primary Antibody : MSH2

Antibody Details	N	%
Other	2	100
BD Bio/Pharmingen 556349 (G219-1129)	4	100
Leica/Novocastra NCL-MSH2 (25D12)	4	50
Invitrogen 33-7900 (FE11)	1	100
Ventana 760-4265 (G219-1129)	33	82
Cell Marque CMAx/Cx (G219-1129)	5	60
Biocare medical CM/PM 219x (FE11)	1	0
Dako FLEX RTU IR085 (FE11)	17	82
Dako M3639 (FE11)	14	71

Primary Antibody : MSH6

Antibody Details	N	%
Other	3	100
Abcam AB92471 (EPR3945)	2	100
Leica/Novocastra NCL-L-MSH6 (PU29)	1	0
Ventana CONFIRM 790-4455 (44)	29	86
BD T. Labs/BioSci/Pharmingen 610918 (44)	3	0
Cell Marque 287R-24/25/26 (SP93)	2	100
Cell Marque 287R-27/28 RTU (SP93)	1	0
Epitomics AC-0047 (EP49)	3	100
Dako FLEX RTU IR086 (EP49)	17	100
Dako M3646 (EP49)	23	83

HNPCC Run: 117

Heat Mediated Retrieval	MSH2 N	MSH2 %	MSH6 N	MSH6 %
Biocare Decloaking Chamber	1	100	1	100
Dako Omnis	2	100	3	100
Dako PTLink	8	100	8	100
Leica ER1 20 mins	1	100	0	0
Leica ER1 30 mins	0	0	3	67
Leica ER2 20 mins	12	67	8	63
Leica ER2 30 mins	8	75	11	64
Leica ER2 40 mins	5	60	3	100
Pressure Cooker	1	100	1	100
Ventana CC1 24mins	2	0	0	0
Ventana CC1 32mins	11	73	3	100
Ventana CC1 40mins	5	60	3	67
Ventana CC1 48mins	2	100	5	100
Ventana CC1 52mins	1	0	0	0
Ventana CC1 56mins	2	100	4	100
Ventana CC1 64mins	16	75	13	85
Ventana CC1 72mins	1	100	3	100
Ventana CC1 80mins	0	0	2	50
Ventana CC1 88mins	1	100	2	100
Ventana CC1 92mins	1	100	4	100
Ventana CC1 standard	2	100	3	100
Ventana CC2 64mins	0	0	1	100
Ventana CC2 92mins	0	0	2	50

HNPCC Run: 117

Enzyme Mediated Retrieval	MSH2 N	MSH2 %	MSH6 N	MSH6 %
AS PER KIT	4	75	4	50
NOT APPLICABLE	44	80	46	83
Ventana Protease 1 (760-2018)	0	0	1	100

HNPCC Run: 117	MSH2		MSH6	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	8	100	7	100
Dako EnVision FLEX (K8000/10)	1	100	2	100
Dako EnVision FLEX+ (K8002/12)	5	100	6	100
Dako Envision HRP/DAB (K5007)	1	100	1	100
Leica Bond Polymer Refine (DS9800)	24	67	24	71
Other	3	100	3	67
Ventana OptiView (760-700) + Amp. (7/860-099)	7	71	9	100
Ventana OptiView Kit (760-700)	28	75	24	88
Ventana UltraView Kit (760-500)	4	50	5	80

HNPCC Run: 117	MSH2		MSH6	
Automation	**N**	**%**	**N**	**%**
Dako Autostainer Link 48	6	100	6	100
Dako Autostainer plus	1	100	1	100
Dako Autostainer Plus Link	1	100	1	100
Dako Omnis	2	100	3	100
Leica Bond Max	8	75	7	57
Leica Bond-III	18	67	18	72
None (Manual)	2	100	2	100
Ventana Benchmark GX	2	50	2	100
Ventana Benchmark ULTRA	36	78	37	89
Ventana Benchmark XT	7	57	7	86

HNPCC Run: 117	MSH2		MSH6	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	19	89	20	90
Dako EnVision Plus kits	1	100	1	100
Dako FLEX DAB	6	100	6	100
Dako REAL EnVision K5007 DAB	2	100	3	100
Leica Bond Polymer Refine kit (DS9800)	25	68	24	71
Other	7	86	7	86
Ventana DAB	18	61	16	94
Ventana Ultraview DAB	5	60	7	71

BEST METHODS - Gold Standard Antibody

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

MSH2 - Method 1

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

Primary Antibody: Dako FLEX RTU IR085 (FE11) , 20 Mins, 20 ºC Prediluted
Automation: Dako Autostainer Link 48
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako PTLink
EAR: NOT APPLICABLE
Chromogen: Dako EnVision Plus kits
Detection: Dako EnVision FLEX+ (K8002/12)

MSH2 - Method 2

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

Primary Antibody: Cell Marque CMAx/Cx (G219-1129) , 32 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:
Chromogen: AS PER KIT, 37 ºC., Time 1: 8 Mins
Detection: Ventana OptiView Kit (760-700) , 8 Mins, 37 ºC Prediluted

MSH2 - Method 3

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

Primary Antibody:	Dako FLEX RTU IR085 (FE11) , 15 Mins, RT ºC Prediluted Dilution 1: RTU
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 30 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

MSH2 - Method 4

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

Primary Antibody:	Ventana 760-4265 (G219-1129) , 32 Mins, 36 ºC Prediluted Dilution 1: 1
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700)

BEST METHODS - Secondary Antibody

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

MSH6 - Method 1

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

Primary Antibody:	Dako M3646 (EP49) , 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 30 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), Time 1: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, rt ºC

MSH6 - Method 2

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

Primary Antibody:	Ventana CONFIRM 790-4455 (44) , 20 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 80mins
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	Ventana OptiView Kit (760-700)

MSH6 - Method 3

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

Primary Antibody:	Epitomics AC-0047 (EP49) , 60 Mins, amb ºC Dilution 1: 500
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), amb ºC., Time 2: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 15 Mins Prediluted

MSH6 - Method 4

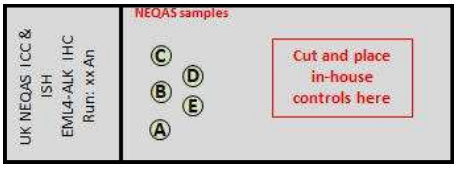
Participant scored 20/20 (UK NEQAS Slide) and 17/20 (In House slide) using this method.

Primary Antibody:	Dako FLEX RTU IR086 (EP49)
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	AS PER KIT
HMAR:	Dako PTLink
EAR:	
Chromogen:	AS PER KIT
Detection:	AS PER KIT

Suzanne Parry

Antigen Assessed:	ALK
Sections Circulated:	Composite slide consisting of cell lines and Non-small cell lung carcinoma (NSCLC) tissue samples with different levels of ALK expression, and normal appendix tissue
Number of Registered Participants:	66
Number of Participants this Run:	56 (85%)

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



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

Introduction

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

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

Assessment Criteria

Same slide NEQAS & In-house controls

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

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

Interpretation criteria incorporating staining intensity

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

Assessment scoring guidelines

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

Table 2: Assessment interpretation

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

Results & Discussion

Distributed NEQAS Sample Results

There was a continued increase in number of participants from the previous run, with an 88% (N=50) acceptable pass rate for the NEQAS section. 5 laboratories (9%) received a borderline pass and 2 laboratories (4%) failed the assessment. This was mainly due to weak or very weak staining compared to the expected level of staining this was mainly due to laboratories not following the recommended staining protocols or inadequate antigen retrieval. The most popular antibody of choice was the Ventana/Roche (D5F3) with 47 participants using this antibody with a 94% pass rate. The Novocastra 5A4 antibody was used by 6 participants and achieved a 67% pass rate.

Features of Expected Staining (Figs 1,3,5A,6a,7,9&10)

- Moderate to strong cytoplasmic staining of the ganglion cells in the appendix
- Moderate to strong granular staining of the positive tumour sample
- Moderate to strong granular staining of the positive tumour cell line samples . The ALK negative cells within the mixed cell line should remain unstained.
- No staining in the negative tumour sample
- No background or inappropriately localised staining

.

Features of Sub-optimal Staining (Figs 2,4,5b,6b&8b)

- False negative or absence of ALK staining where tumour cells should be staining positive
- Non-specific / Excessive Tyramide staining
- Absence of staining in appendix

In-house Control Results

Of the 56 participants taking part in this assessment, 41(72%) achieved an acceptable result. 15 (26%) participants obtained a borderline pass result and 1 participant failed on their in-house sections.

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 to gauge both the sensitivity & specificity, but should not be used 'alone' as a single control.

- Commercially available control material (e.g. cell lines or xenografts) with varying ALK IHC expression may also be used as an acceptable alternative alongside a representative piece of your own material.
- In all cases the control material should also be validated using FISH.
- It is also recommended that control material should be cut and placed alongside the clinical sample being tested.

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

Note: Lymphoma is not recommended as a control in the lung setting. The use of a lymphoma control can lead to potential false-negative result when used in the lung setting.

References:

1. Ibrahim M, et a. ALK Immunohistochemistry in NSCLC: Discordant Staining Can Impact Patient Treatment Regimen. J Thorac Oncol. 2016 Dec;11 (12):2241-2247. doi: 10.1016/j.jtho.2016.07.012. Epub 2016 Jul 25.
2. Jemal A, Bray F, Center MM, et al. Global cancer statistics. CA Cancer J Clin 2011;61:69–90
3. American Cancer Society. Cancer Facts & Figures 2011. Atlanta: American Cancer Society; 2011
4. Camidge DR, Christensen J, Bang YJ. Addressing right drug-right target-right patient in phase I studies to accelerate bench to clinical benefit time: ALK gene rearrangements and the development of PF-02341066 in NSCLC. J Thorac Oncol 2010;5:S233.
5. Bang Y, Kwak EL, Shaw AT, et al. Clinical activity of the oral ALK inhibitor PF-02341066 in ALK-positive patients with non-small cell lung cancer (NSCLC). J Clin Oncol 2010;28:18s.
6. Soda M, Choi YL, Enomoto M, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 2007; 448:561–566.
7. Palmer RH, Vernersson E, Grabbe C, et al. Anaplastic lymphoma kinase: signalling in development and disease. Biochem J 2009;420: 345–361.
8. Wong DW, Leung EL, So KK, et al. The EML4-ALK fusion gene is involved in various histologic types of lung cancers from nonsmokers with wild-type EGFR and KRAS. Cancer 2009;115:1723–1733.
9. Inamura K, Takeuchi K, Togashi Y, et al. EML4-ALK lung cancers are characterized by rare other mutations, a TTF-1 cell lineage, an acinar histology, and young onset. Mod Pathol 2009;22:508 –515.
10. Boland JM, Erdogan S, Vasmatzis G, et al. Anaplastic lymphoma kinase immunoreactivity correlates with ALK gene rearrangement and transcriptional up-regulation in non-small cell lung carcinomas. Hum Pathol 2009;40:1152–1158.
11. Takeuchi K, Choi YL, Soda M, et al. Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. Clin Cancer Res 2008;14:6618–6624.
12. Paik JH, Choe G, Kim H, et al. Screening of anaplastic lymphoma kinase rearrangement by immunohistochemistry in nonsmall cell lung cancer: correlation with fluorescence in situ hybridization. J ThoracOncol 2011;6:466-472.
13. Savic S, Diebold J, Zimmermann AK, Jochum W, Baschiera B, Grieshaber S, Tornillo L, Bisig B, Kerr K, Bubendorf L. Screening for ALK in non-small cell lung carcinomas: 5A4 and D5F3 antibodies perform equally well, but combined use with FISH is recommended.. Lung Cancer. 2015 May (ahead of print).
14. Fiona H. Blackhall, Solange Peters, Lukas Bubendorf et al., (2014) Prevalence and Clinical Outcomes for Patients With ALK-Positive Resected Stage I to III Adenocarcinoma: Results From the European Thoracic Oncology Platform Lungscape Project. J.Clin. Oncol 32 (25): 2780 - 2788.
15. Georg Hutarew, Cornelia Hauser-Kronberger, Felix Strasser et al., (2014) Immunohistochemistry as a screening tool for ALK rearrangement in NSCLC: evaluation of five different ALK antibody clones and ALK FISH. histopahology 65:398-407.

Acknowledgements

We are grateful to Novartis Pharmaceuticals UK Limited for providing an educational grant which was used to help setup the NSCLC ALK IHC EQA module.

Selected Images showing Optimal and Sub-optimal Immunostaining

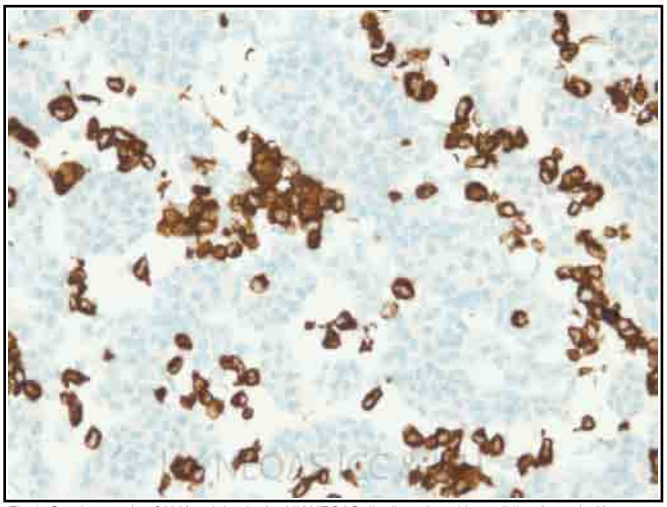


Fig 1. Good example of ALK staining in the UK NEQAS distributed positive cell line (sample A). The section shows strong membranous and cytoplasmic staining of the expected proportion of neoplastic cells. Stained with the Ventana D5F3 assay on the Benchmark ULTRA using the recommended protocol with OptiView detection and an amplification step.

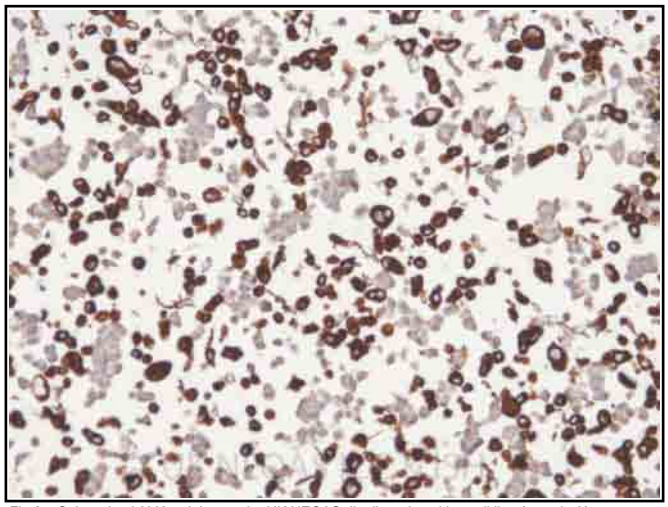


Fig 2. . Sub-optimal ALK staining on the UK NEQAS distributed positive cell line (sample A) (compare to Fig 1). Although the tumour cells are staining positive as expected, the negative cells show background staining, and the morphology of the cells is damaged. The section appears to be over-heated.

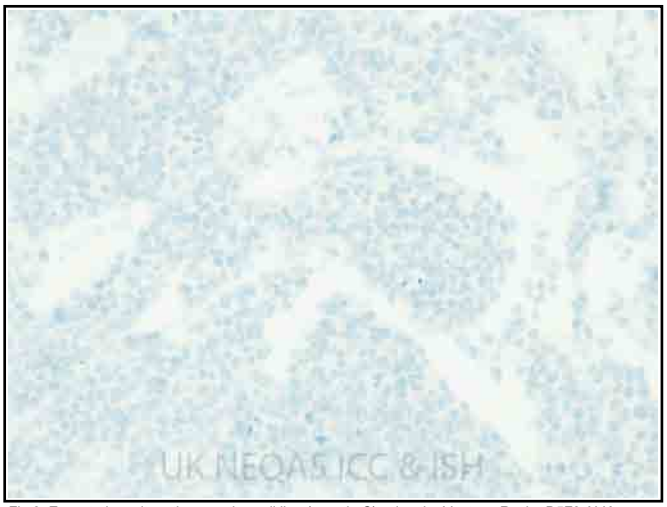


Fig 3. Expected result on the negative cell line (sample C) using the Ventana Roche D5F3 ALK assay. All cells are negative and the sample is clean. (Same protocol as Fig 1).

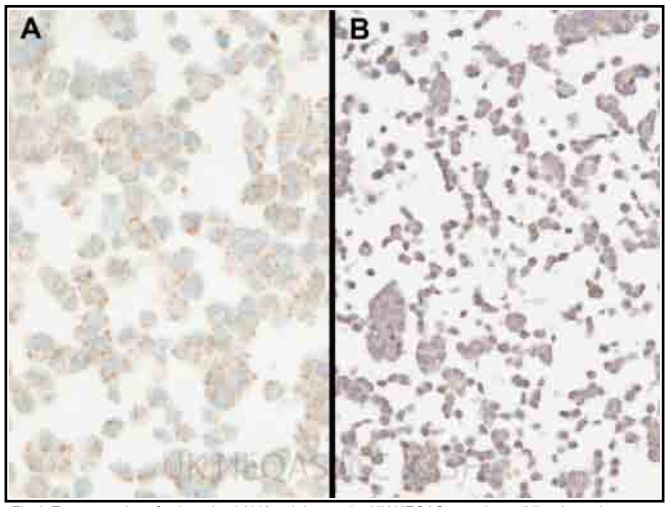


Fig 4. Two examples of sub-optimal ALK staining on the UK NEQAS negative cell line (sample C): Example A shows excessive teramide deposit, while the section in example B shows morphology damage. Both examples were carried out using the Ventana Roche D5F3 ALK assay.

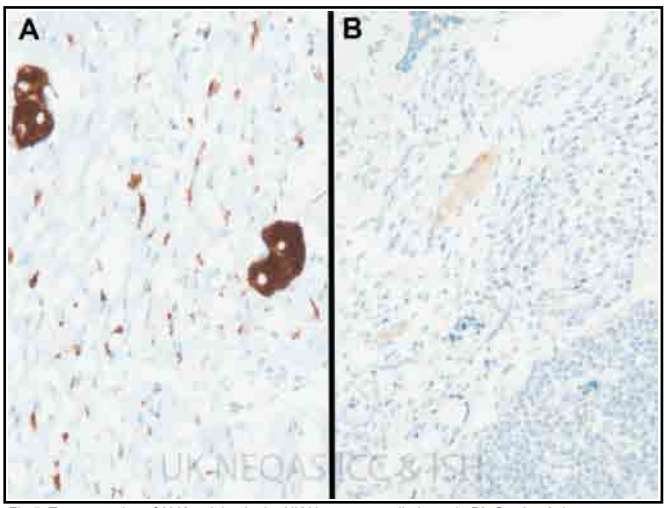


Fig 5. Two examples of ALK staining in the UK Neqas appendix (sample D): Section A the staining is optimal and shows the expected strong positive staining of ganglion cells and axons. While the staining in section B is very weak, with many of the expected elements not staining.

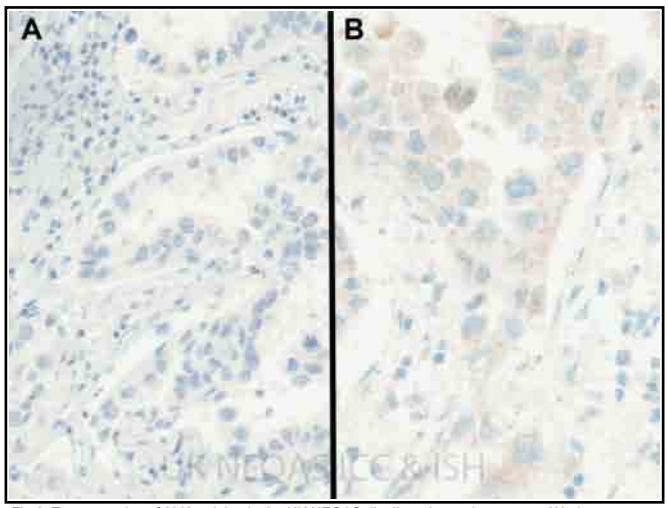


Fig 6. Two examples of ALK staining in the UK NEQAS distributed negative tumour. (A) shows the expected result and is therefore negative for ALK. Whereas the image in (B) shows excessive teramide deposit, and therefore received a borderline pass. Both samples were stained using the Ventana D5F3 assay.

Selected Images showing Optimal and Sub-optimal Immunostaining

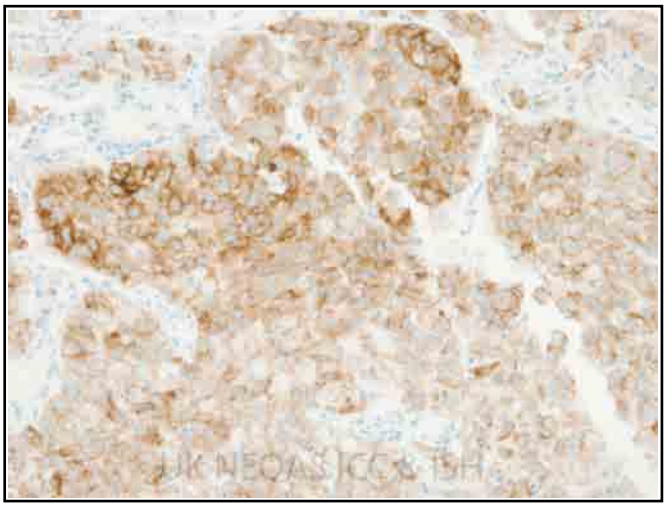


Fig 7. Expected level of staining in the UK NEQAS distributed positive tumour sample. The example shows moderate with some areas of strong membranous and cytoplasmic staining of the neoplastic cells. The section was stained using the Ventana D5F3 assay on the Benchmark with OptiView detection and amplification.

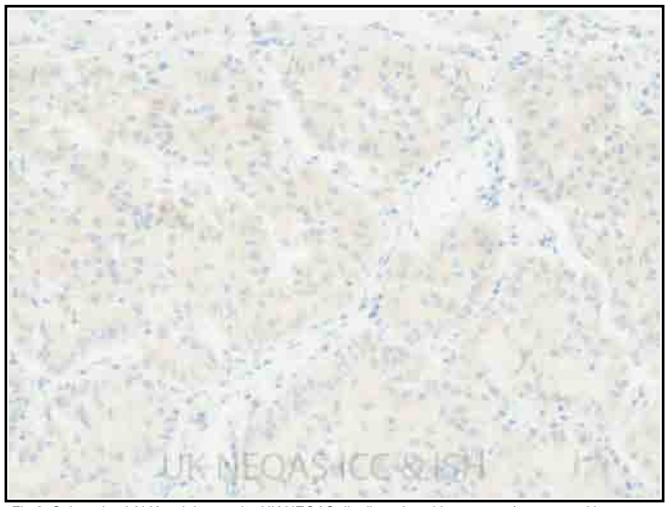


Fig 8. Sub-optimal ALK staining on the UK NEQAS distributed positive tumour (compare with Fig 7). Although, still positive, the staining is much weaker than expected. This section was stained using a lab-developed assay; Leica 5A4 RTU antibody on the Leica Bond III, ER2 40 mins.

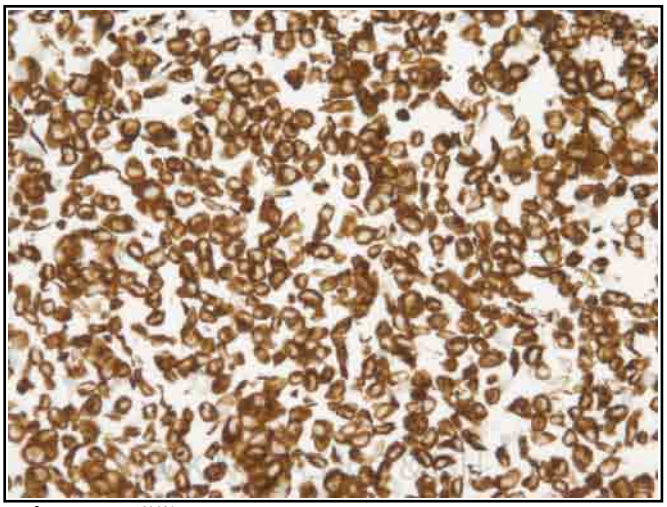


Fig 9. Good NSCLC ALK in-house positive control (see also Fig 10 for the accompanying in-house negative tumour and appendix controls). Stained using the Ventana D5F3 assay on a Benchmark ULTRA with the recommended protocol.

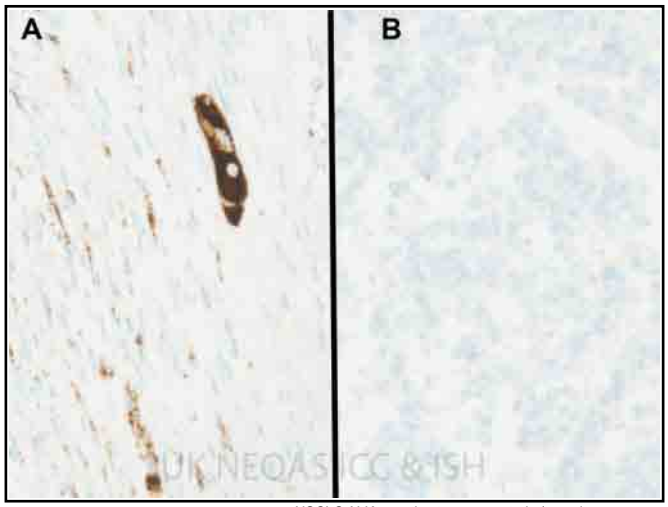
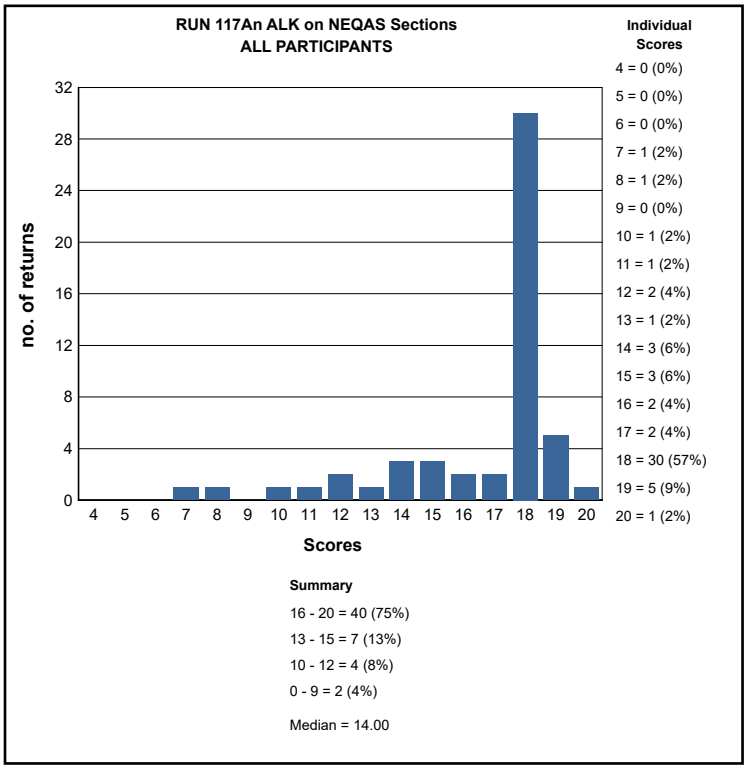
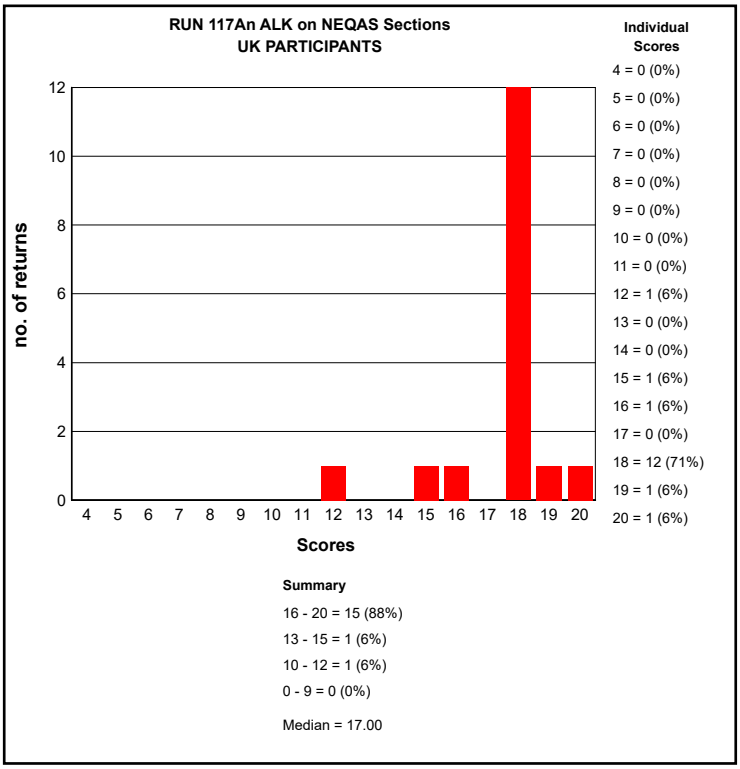
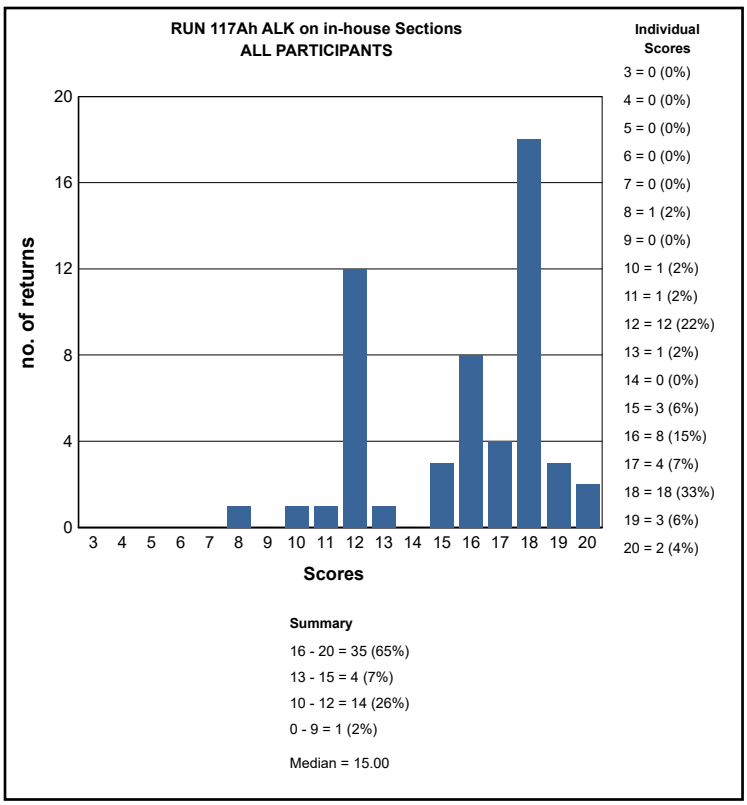


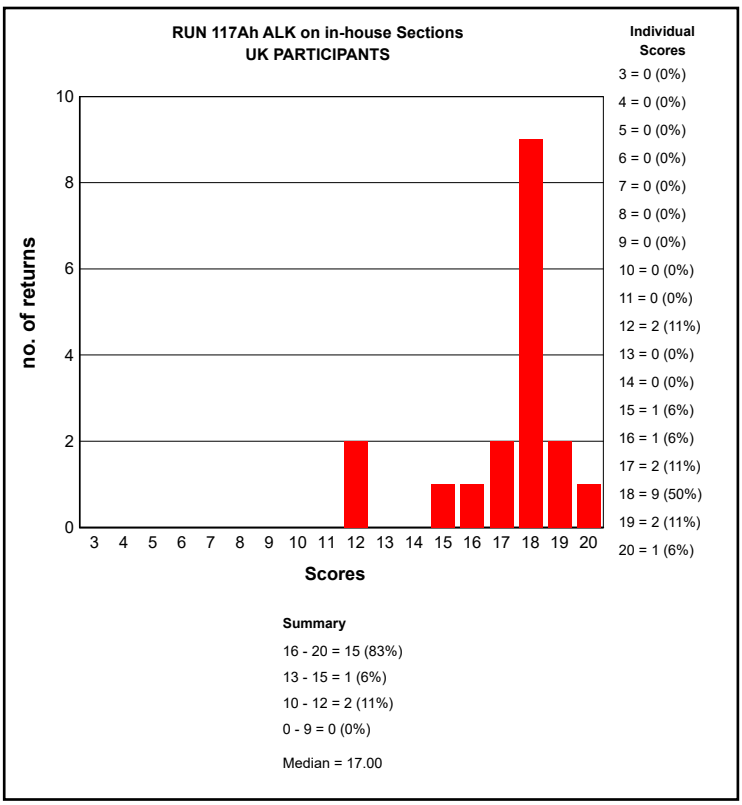
Fig 10. Good in-house (A) appendix and (B) NSCLC ALK negative tumour controls (see also Fig 9 for the accompanying ALK positive control). Fig 10. Good in-house (A) appendix and (B) NSCLC ALK negative tumour controls (see also Fig 9 for the accompanying ALK positive control). Fig 10. Good in-house (A)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

GRAPHICAL REPRESENTATION OF PASS RATES









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

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

ALK NSCLC Run: 117

Primary Antibody	N	%
Cell Sign. Tech. (D5F3)	1	100
Novocastra NCL-ALK (5A4)	6	67
Origene (1A4)	1	0
Other	1	0
Ventana/Roche (D5F3)	43	95

ALK NSCLC Run: 117

Automation	N	%
Dako Autostainer Link 48	2	50
Leica Bond-III	1	0
Ventana Benchmark GX	4	75
Ventana Benchmark ULTRA	20	95
Ventana Benchmark XT	25	92

ALK NSCLC Run: 117

Heat Mediated Retrieval	N	%
Dako PTLink	2	50
Leica ER2 40 mins	1	0
Other	1	100
Ventana CC1 32mins	1	0
Ventana CC1 64mins	1	0
Ventana CC1 88mins	1	100
Ventana CC1 92mins	37	95
Ventana CC1 extended	6	100
Ventana CC1 standard	2	100

ALK NSCLC Run: 117

Detection	N	%
AS PER KIT	2	50
Dako EnVision FLEX (K8000/10)	1	100
Leica Bond Polymer Refine (DS9800)	1	0
Ventana OptiView (760-700) + Amp. (7/860-099)	28	93
Ventana OptiView Kit (760-700)	18	94
Ventana UltraView Kit (760-500)	1	0

ALK NSCLC Run: 117

Enzyme Retrieval	N	%
AS PER KIT	2	50
NOT APPLICABLE	30	87
Ventana Protease	1	100

ALK NSCLC Run: 117

Chromogen	N	%
AS PER KIT	18	83
Dako EnVision Plus kits	1	100
Leica Bond Polymer Refine kit (DS9800)	1	0
Ventana DAB	28	96
Ventana Ultraview DAB	4	75

BEST METHODS

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

ALK - Method 1

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

Primary Antibody:	Ventana/Roche (D5F3) , 16 Mins Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	
Main Buffer:	
HMAR:	Ventana CC1 92mins
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	Ventana OptiView Kit (760-700)

ALK - Method 2

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

Primary Antibody:	Ventana/Roche (D5F3) , 16 Mins Prediluted
Automation:	Ventana Benchmark XT
Method:	
Main Buffer:	
HMAR:	Ventana CC1 92mins
EAR:	
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700)

The HER2 ISH Module - Interpretive Run 46

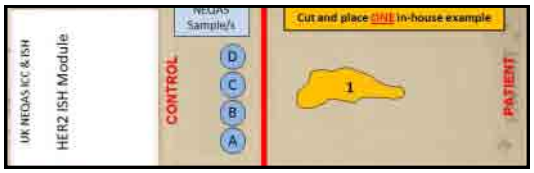
Suzanne Parry and Amy Newman

Gene Assessed:	HER2
Sections Circulated:	Composite slide consisting of 4 breast carcinoma sections (see table below)
Number of Registered Participants:	175
Number of Participants This Run:	143 (81%)

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.

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

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



Introduction

Breast cancer HER2 status should be determined in all newly diagnosed and recurrent and metastatic breast cancers[1] . In-situ hybridization (ISH), using either fluorescent (FISH)[4] or brightfield chromogenic methods (CISH)[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 ASCO/CAP (USA) and UK Recommendations and Guidelines[6,7]. It is advisable that these guidelines are followed and the processes of introducing and maintaining a clinically validated HER2 ISH assay or laboratory developed test (LDT) are properly validated within the prior to their introduction into the laboratory as a diagnostic test.

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

All laboratories employed a dual probe and a ratio scoring algorithm. 66% of participants achieved excellent or acceptable results. 26% received a borderline pass and eleven laboratories (8%) had an unacceptable interpretation result. There were no unacceptable results from any UK laboratory. The most common brightfield method was the Ventana DDISH with 49 (34%) laboratories using this technique. The most popular FISH method was the Pathvysion Vysis Kit with 43 (30%) laboratories using this technique. The acceptable pass rates for these two assays were 59% and 70% respectively.

There was a slight increase in the unacceptable rate for the current run 46, compared to that of Run 45 where only 4 labs (3%) failed on the interpretation. The current run showed 11 labs (8%) providing unacceptable counts.

Frequency Histograms

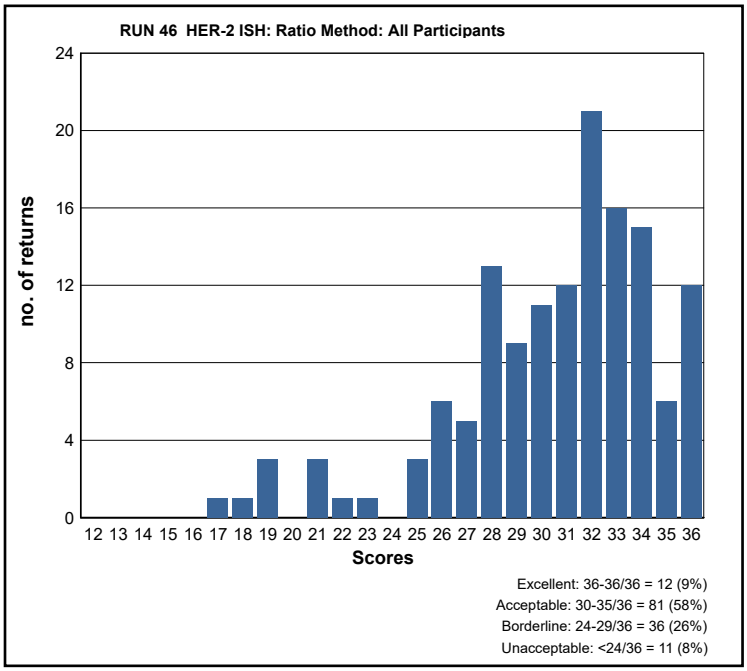
Frequency histograms of the assessment results are also provided showing the distribution of HER2 copy and Cen17 counts and ratio calculations. Participants are encouraged to use the provided frequency histograms to gauge their own performance and variability in counts for each of the distributed samples.

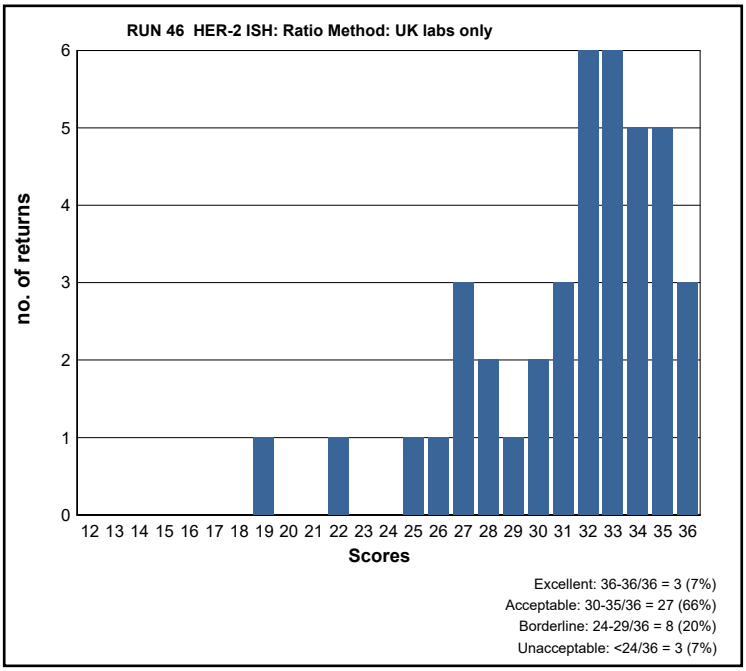
HER2 ISH Method and Probe Enumeration

Table 1 shows the various ISH methods employed along with mean HER2, Cen17 counts and ratio calculations.

References
1. Walker RA, Bartlett JM, Dowsett M, Ellis IO, Hanby AM, Jasani B, Miller K. Pinder SE. (2015) HER2 testing in the UK: further update recommendations. J Clin Pathol;61818-824.
2. Bartlett JM, Starczynski J, Atkey N, Kay E, O'Grady A, Gandy M, Ibrahim M, Jasani B, Ellis IO, Pinder SE, Walker RA. (2011) HER2 testing in the UK: recommendations for breast and gastric in-situ hybridisation methods. J Clin Pathol; 64: 649-653.
3. Moore, D. S. and McCabe, G. P. Introduction to the Practice of Statistics, 4th ed. New York: W. H. Freeman, 2002.

GRAPHICAL REPRESENTATION OF PASS RATES (ratio methods only)





METHODS USED and PASS RATES

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

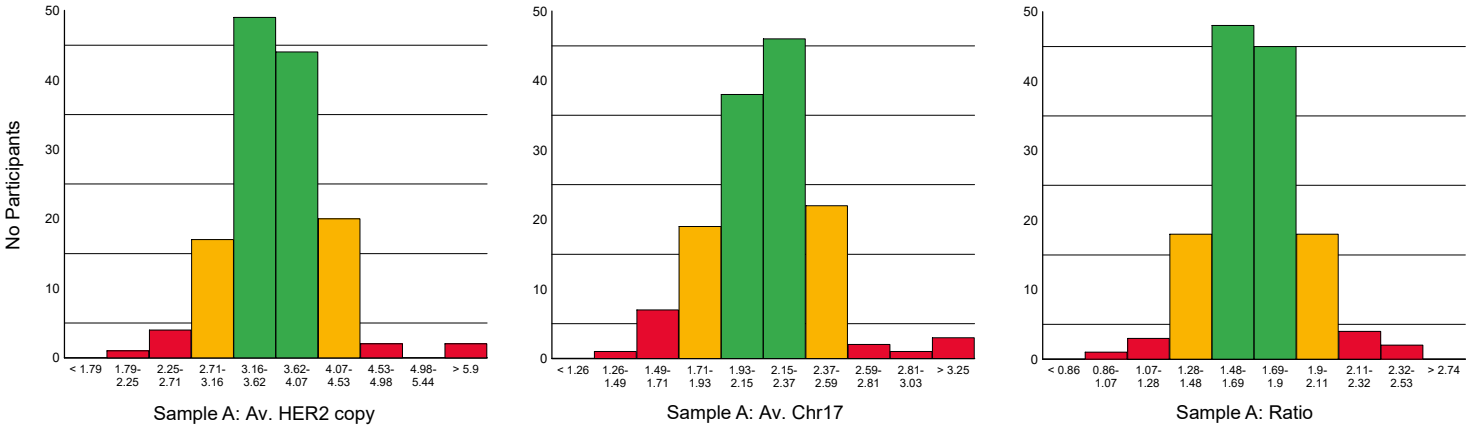
Ratio Method	N	% Pass (score ≥ 30/36)
Ratio: Dako IQFISH pharmDX	11	64%
Ratio: Dako Pharm Dx	3	67%
Ratio: In house FISH	1	100%
Ratio: Kreatech Probes	5	100%
Ratio: Leica HER2 FISH TA9217	7	71%
Ratio: Other - FISH	3	67%
Ratio: Pathvysion Vysis Kit	43	72%
Ratio: Ventana BDISH 800-098/505	3	67%
Ratio: Ventana DDISH (780/800-4422)	48	56%
Ratio: Ventana Inform Silver ISH	6	67%
Ratio: Zytovision ZytoDot 2C	1	0%
Ratio: Zytovision ZytoLight	9	78%

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

FREQUENCY HISTOGRAMS

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

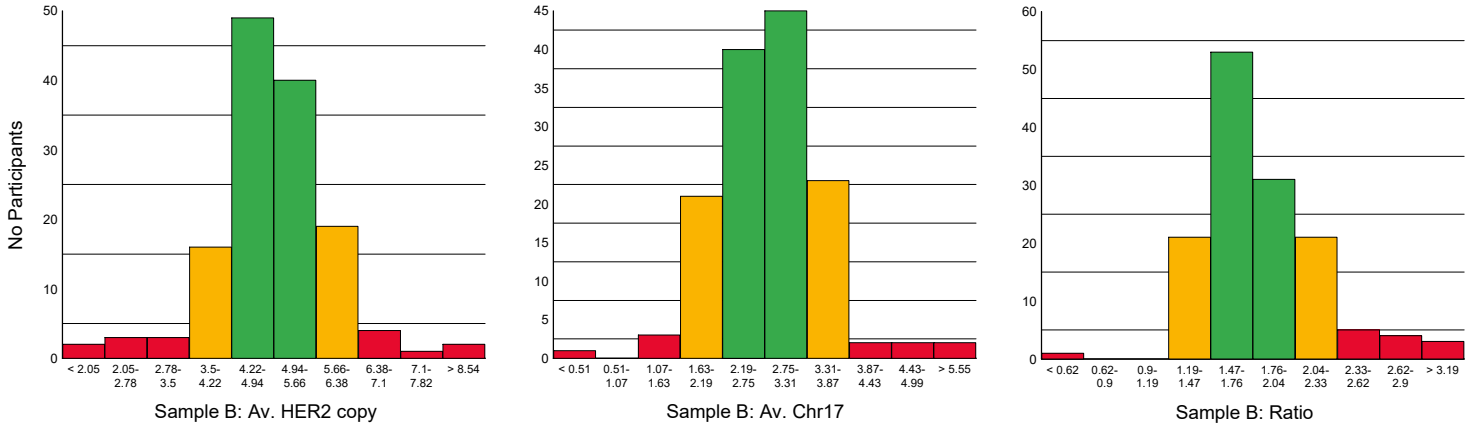
Green = mean +/- 1 Stdev Amber = mean +/- 2 Stdev Red = mean ≥ +/- 3 stdev



Sample A: Av. HER2 copy

Sample A: Av. Chr17

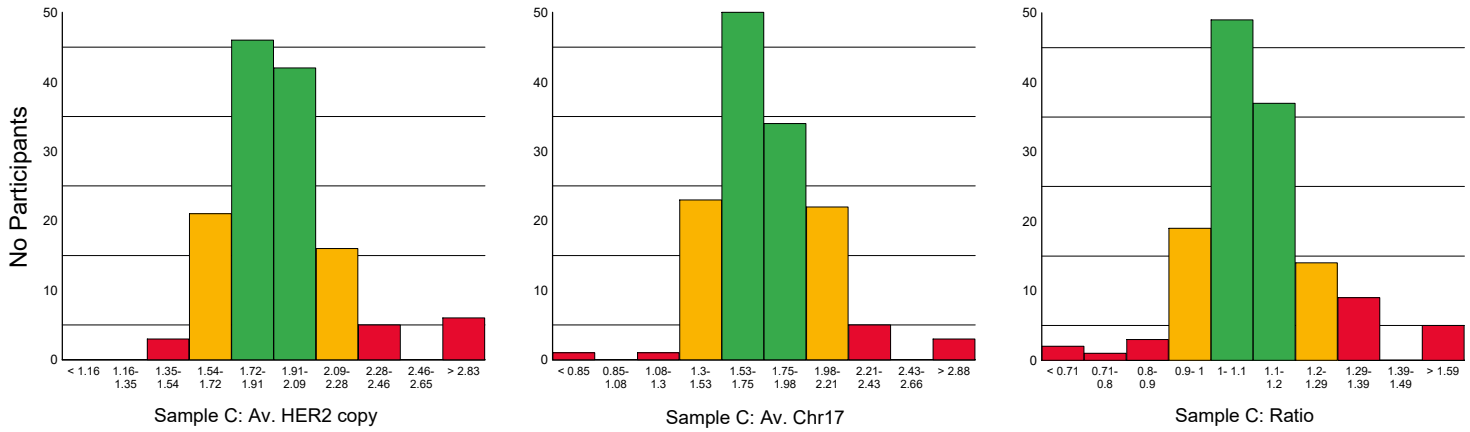
Sample A: Ratio



Sample B: Av. HER2 copy

Sample B: Av. Chr17

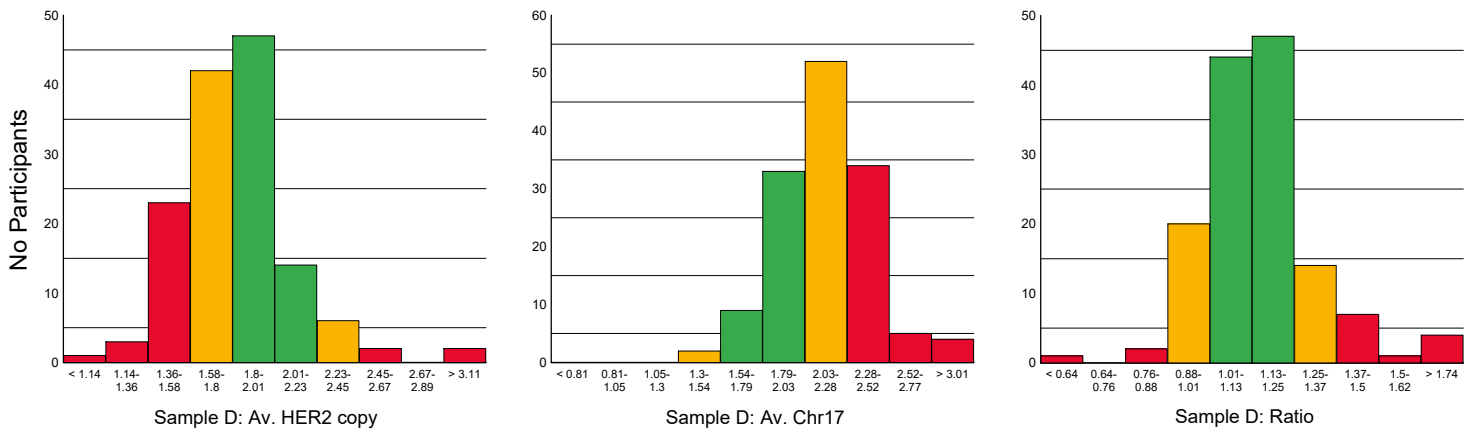
Sample B: Ratio



Sample C: Av. HER2 copy

Sample C: Av. Chr17

Sample C: Ratio



Sample D: Av. HER2 copy

Sample D: Av. Chr17

Sample D: Ratio

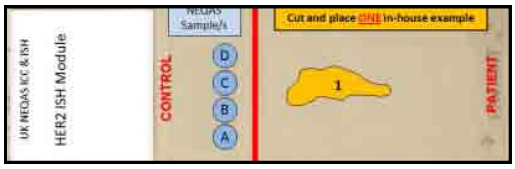
The HER2 ISH Module - Technical Run 46

Suzanne Parry and Amy Newman

Gene Assessed:	HER2
Sections circulated:	Composite slide consisting of 4 breast carcinoma sections (see table below)
Number of Registered Participants:	175
Number of Participants Taking Part this Run	141 (80%) (55% Fluorescent and 45% 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+**	Non-amplified
C	**2+**	Amplified
D	**2+**	Non-amplified



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

Assessment Procedure

Brightfield ISH (CISH / SISH / BDISH / DDISH etc.) was assessed around a multi-header microscope. Each slide was reviewed by 4 independent assessors, each providing scores from 1-5 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 score was given out of 20.

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 showed an improvement in pass rates compared to the previous run (Run 45). 38 (59%) of laboratories achieved an acceptable pass rate on this Run (46) compared to 50% on previous run on the UK NEQAS distributed material. The failures were predominantly due to weak or no Cen17 signals. Several labs that received borderline scores showed silver deposit outside of the nuclei, or weak, but readable Cen17 signals. These observational results refer to the Ventana DDISH or BDISH inform methods, which 54 (84%) of laboratories have adopted who submitted brightfield ISH slides for this technical assessment.

The Dako DuoCISH method was used by a small number of laboratories, but only two of these laboratories correctly submitted their methodology details. Other slides using this assay were easily recognised as being stained with the Dako DuoCISH kit due to the distinct HER2 signals being red and the Cen17 signals being blue. The cytoplasm also has a very red blush which is inherent of this method. The Zytovision ZytoDot 2C method was used by one laboratory, and this achieved an unacceptable pass due to uninterpretable HER2 signals in several of the cores, and very weak or no Cen 17 signals. This CISH methodology is easily recognised by the assessment team, as the HER2 signals are green and the Cen17 signals are red.

Important: Whichever Brightfield ISH methodology is being used, any laboratory experiencing staining problems should contact the relevant company for further support.
Important: By ensuring that the relevant and correct methodology is entered onto the UK NEQAS database, a more precise analysis of the data can be procured.

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

Scoring Interpretation	
Acceptable Individual Assessor 4-5/5 or Overall score ≥13/20	The NEQAS distributed samples and/or in-house tissue show a good/very good standard of staining and are suitable for interpretive analyses.
Borderline Individual Assessor 3/5 or Overall Score = 10-12/20	The NEQAS distributed samples and/or in-house tissue is borderline interpretable with 2 out of the 4 assessors indicating that improvements can be made with respect for example: Excessive / Weak Nuclear staining Level of probe hybridisation Excessive background staining **Also see assessor comments on your report**
Unacceptable Individual Assessor 1-2/5 or Overall score ≤9/20 =	The NEQAS distributed samples and/or in-house tissue is unacceptable for interpretation with at least 3 out of the 4 assessors indicating that there are hybridisation issues, which could be due: Excessive / Weak Nuclear staining Poor probe hybridisation Missing HER2 copy no. / CEN 17 Excessive background staining **Also see assessor comments on your report**
Score = 0	Slide Not submitted for assessment

FISH Results

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

The current Run 46 assessment showed a lower acceptable pass rate of 62% (N=45), compared to that of the previous Run (45), where 73% achieved acceptable passes. There was still a higher than expected fail rate of 27% (N=21). As with the previous Run (45), several slides showed weak or no HER2 and/or Cen17 signals, which contributed to a higher number of laboratories receiving an unacceptable mark. This problem is presumed to be a stability or transport issue, as this does not appear to be assay specific. The Pathvysion Vysis Kit still remains the most commonly used FISH assay by laboratories. Pass rates for this assay are lower than for the previous run, with 50% of labs receiving an acceptable pass, compared to 62% in the previous assessment. The Dako IQFISH and Leica FISH assays were used by 16% and 9% of FISH users respectively. They both achieved a very good acceptable pass rates of 92% and 100% respectively. Other FISH assays used include the Dako Pharm Dx, Kreatech Probes and the Zytovision ZytoLight.

Validating ISH

It is crucial to emphasise the importance of undertaking a robust validation and verification protocol when introducing ISH into a diagnostic laboratory for the first time or a new assay/method is being introduced. For example, changing from a FISH assay to a brightfield ISH assay, the new technique must be fully validated and staff must undergo extra training to gain expertise with the new method employed.
The UK NEQAS data indicates that there is a definite move towards the use of the Ventana DDISH method and laboratories introducing either this method or another ISH method should make sure that:

- Staff perform a minimum of 100 ISH evaluations in parallel with an experienced member of staff to ensure scoring consistency before working independently.
- A minimum concordance of 95% is recommended for both diagnostic results (amplified and non-amplified status) and
- numerical results (for both HER2 and Cen17).
- Clinical and UK NEQAS samples should be assessed for quality of hybridisation prior to interpretation. You should make sure that the probe signals are easily discernible, normal cells show the expected HER2 and/or Cen17 signal, and there is minimal background and leaching of the signal.
- Further validation guidelines can be found in: Bartlett et al., (2011) HER2 testing in the UK: Recommendations for Breast and Gastric In-situ Hybridisation Methods. J Clin Pathol.64(8):649-653.

Recommendations for Returning FISH Slides for NEQAS Assessments

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

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

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

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

Selected Images showing Optimal and Sub-optimal Immunostaining

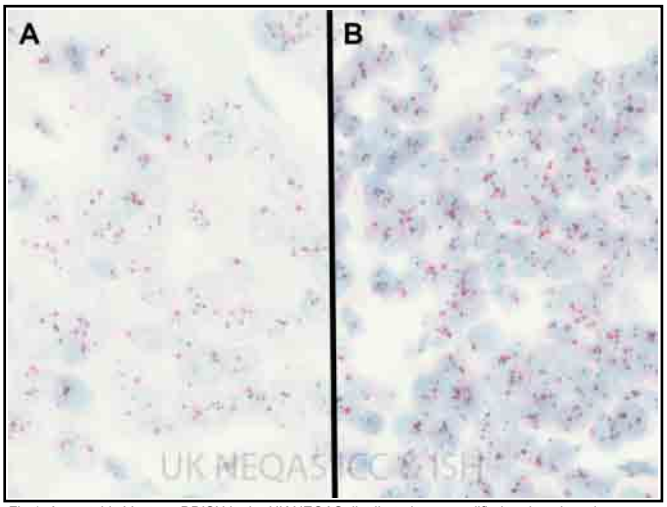


Fig 1. Acceptable Ventana DDISH in the UK NEQAS distributed non-amplified and equivocal samples A and B. Both examples show distinct HER2 signals (black) and Chr17 signals (red) at the expected signal level.

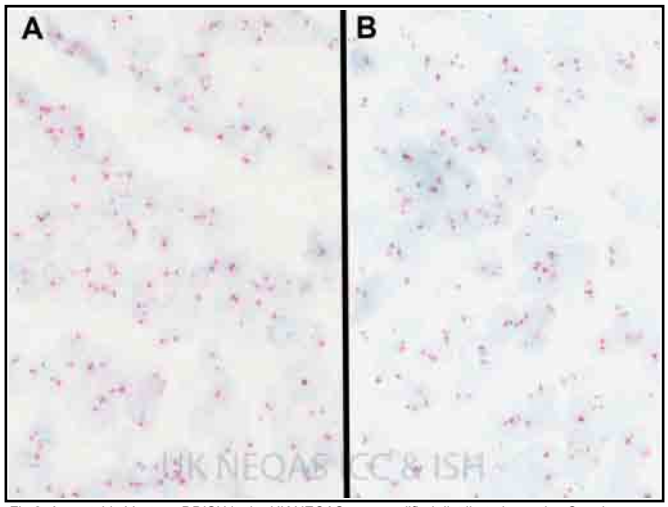


Fig 2. Acceptable Ventana DDISH in the UK NEQAS non-amplified distributed samples C and D. Both samples show strong HER2 signals (black) and Chr17 signals (red) with the expected average copy numbers per cell.

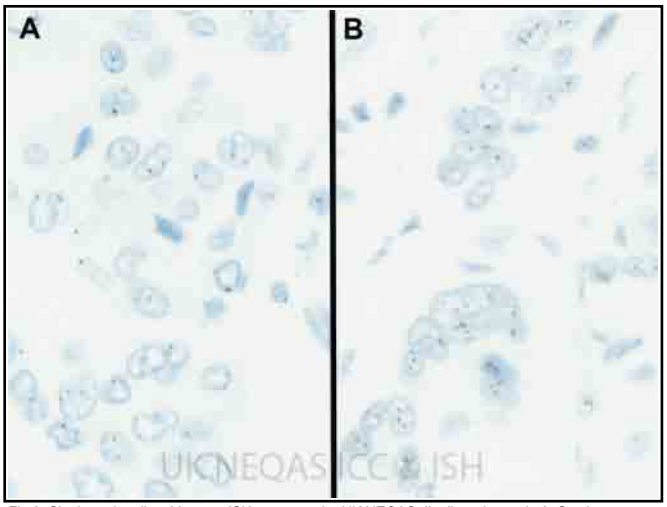


Fig 3. Single probe silver Ventana ISH assay on the UK NEQAS distributed sample A. Section A shows the Chr17 probe, while section B is the HER2 copy number. The section is non-amplified.

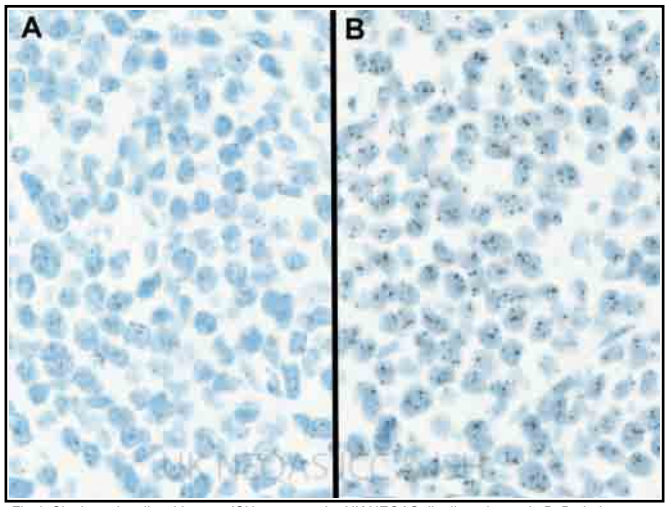


Fig 4. Single probe silver Ventana ISH assay on the UK NEQAS distributed sample B. Both the Chr17 (A) and the HER2 (B) probes show the good strong black signals at the expected level.

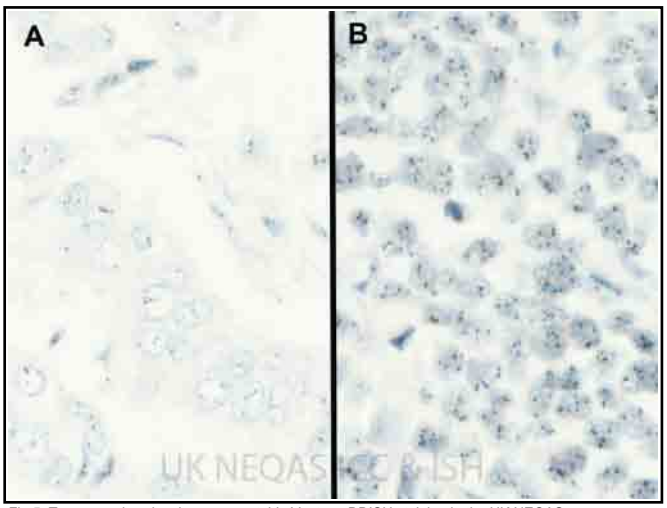


Fig 5. Two examples showing unacceptable Ventana DDISH staining in the UK NEQAS non-amplified samples (A and B): There are no Chr17 signals and therefore the samples are uninterpretable.

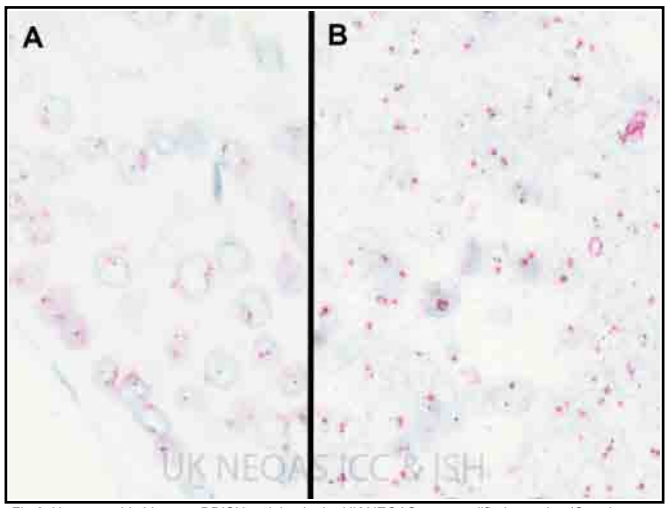


Fig 6. Unacceptable Ventana DDISH staining in the UK NEQAS non-amplified samples (C and D): Example A shows leaching of the Chr17 probe, and in example B there is excessive precipitate and background.

Selected Images showing Optimal and Sub-optimal Immunostaining

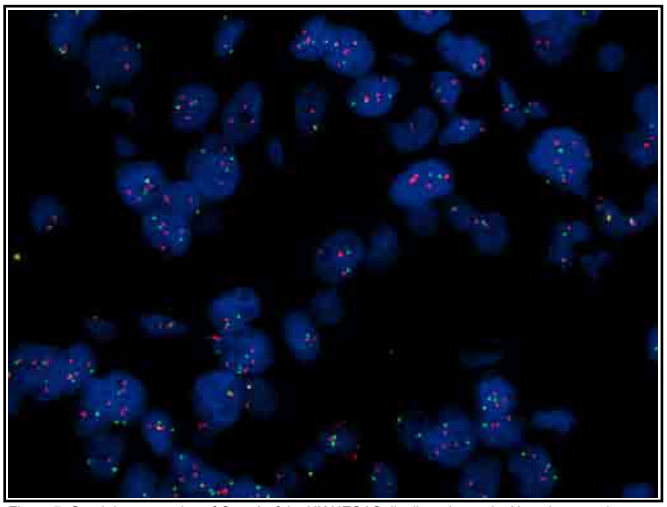


Figure 7: Good demonstration of Core A of the UK NEQAS distributed sample. Negative sample with an expected ratio of 1.48-1.9 (Avg. Her-2 copy 3.16-4.07; Avg. Cen17 copy 1.93-2.37). Method: PathVysion Vysis kit; Pre-treatment: HCl, 20' RT; NaSCN, 30' 830C; Enzyme: Pepsin: 50' 370C. Images captured using the BioView Encore FISH Image Analysis.

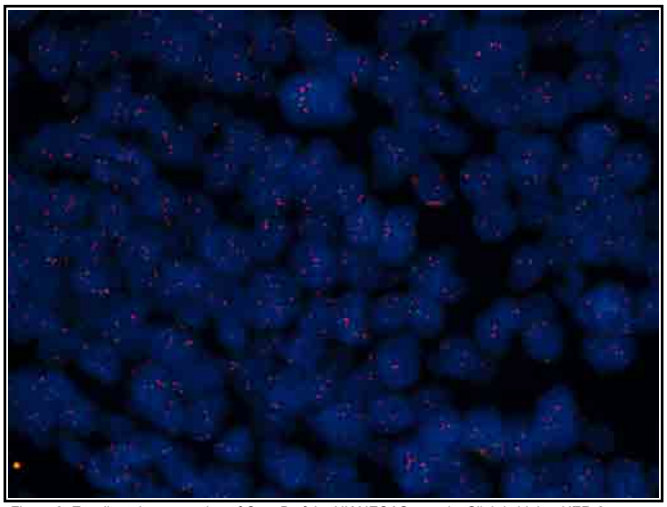


Figure 8: Excellent demonstration of Core B of the UK NEQAS sample. Slightly higher HER-2 and CEP17 count than Core A (Figure 1). Average Her-2 and Cen17, 4.22-5.66 and 2.19-3.31 respectively. Method identical to Figure 1.

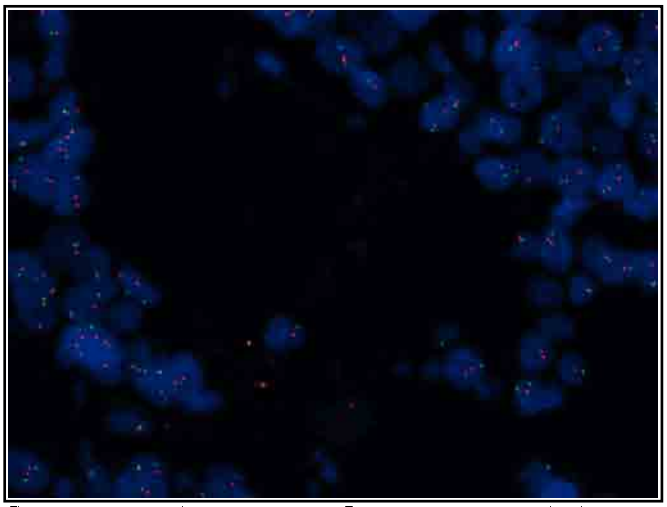


Figure 9: Good demonstration of Core C of the UK NEQAS sample. Method as described in Figure 1.

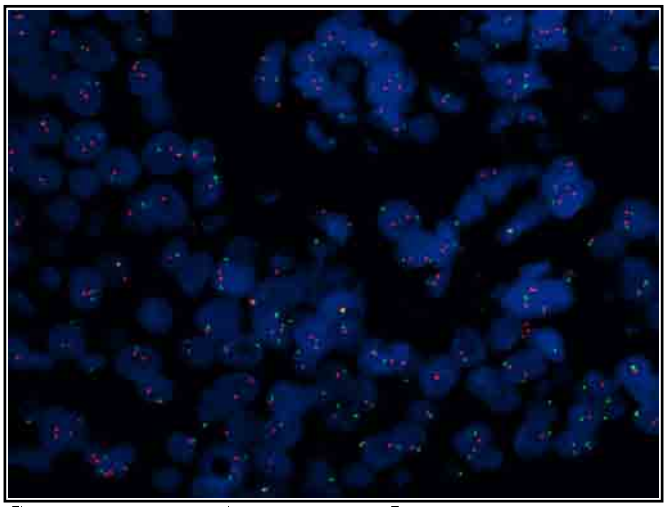


Figure 10: Acceptable demonstration of Core D of the UK NEQAS sample. Method as described in Figure 1.

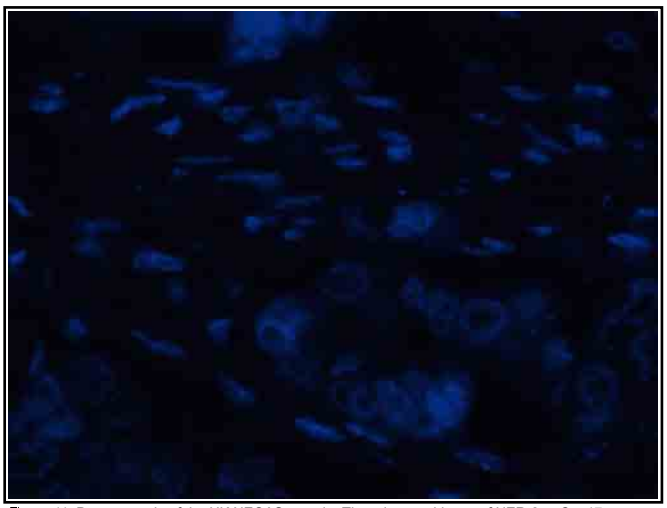


Figure 11: Poor example of the UK NEQAS sample. There is no evidence of HER-2 or Cen17 signal and the tumour nuclei appear pale and indistinct. Method: Zytovision ZytoLight; Pretreatment: HCl 2' 370C; Enzyme Pre-treatment: Pepsin 1' 370C.

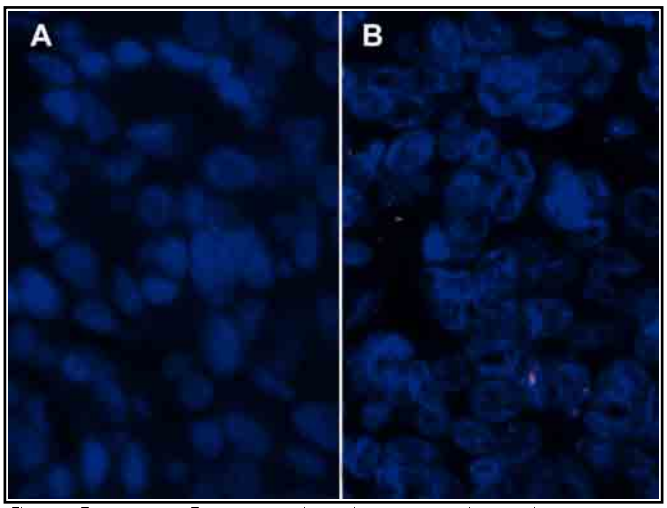
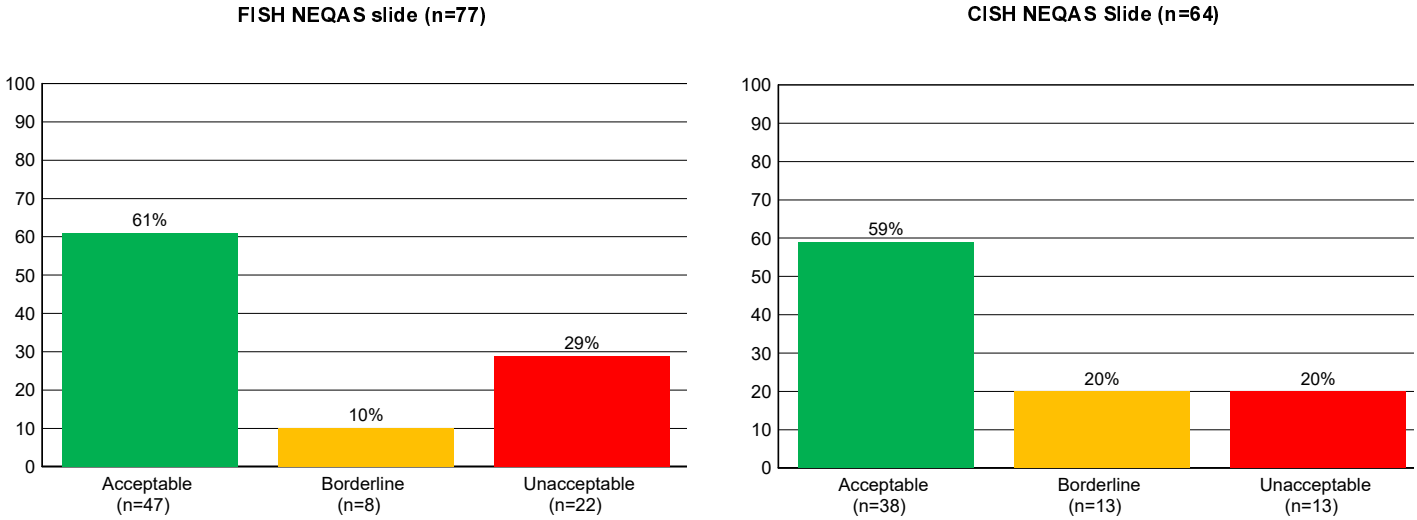
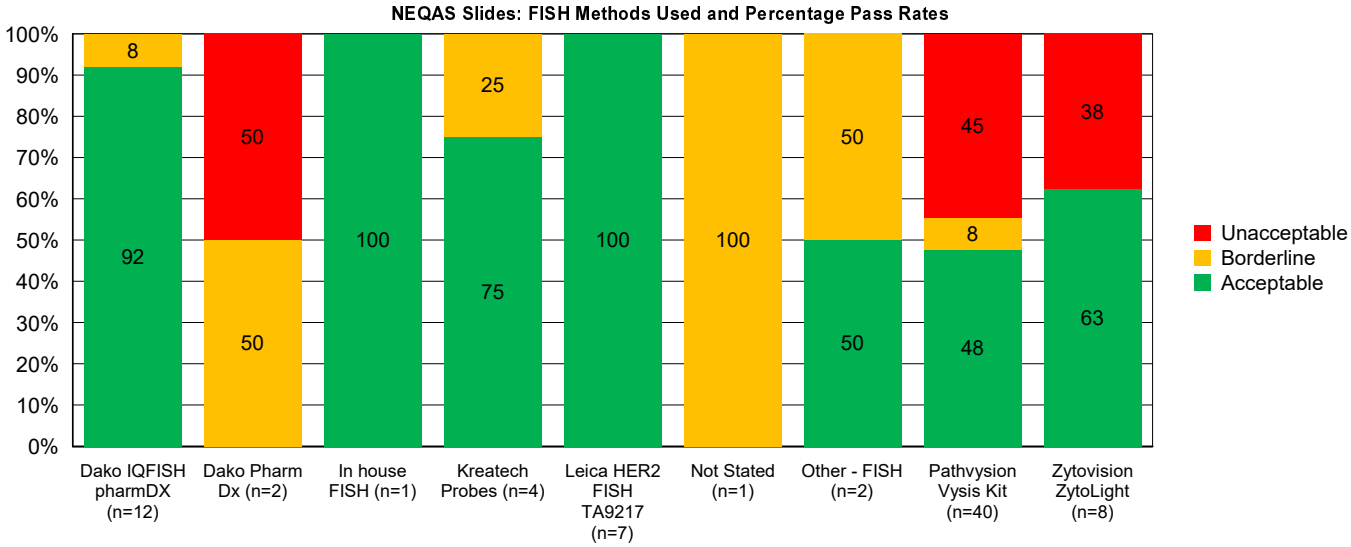


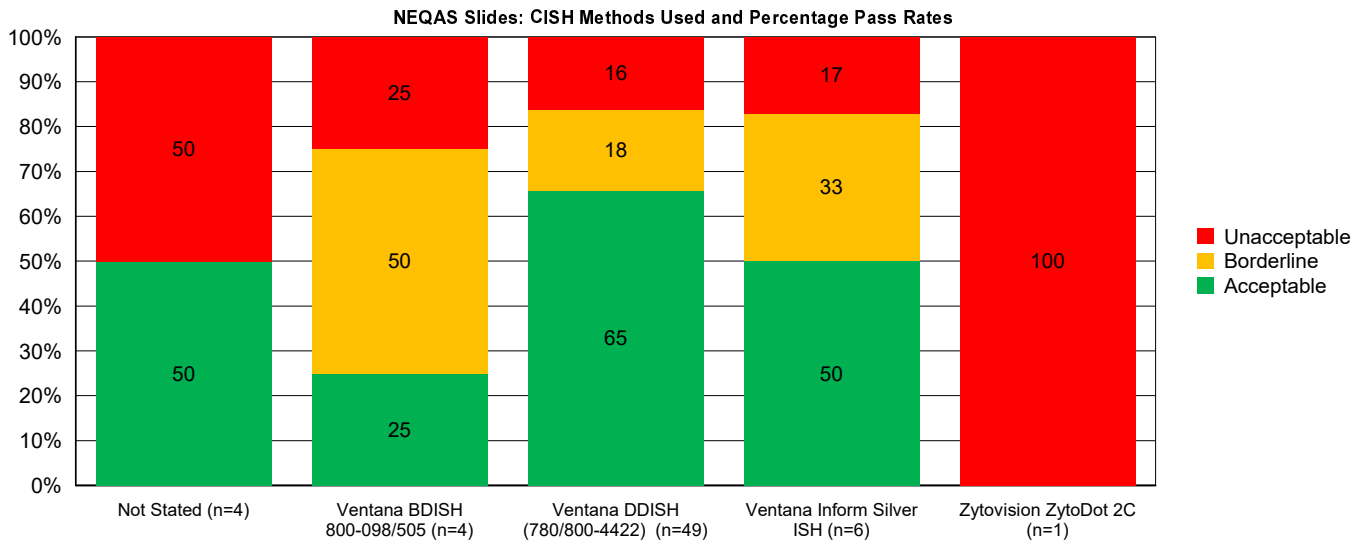
Figure 12: Example of UK NEQAS sample which received 34/36 on the interpretation module, however signal had faded by the date of technical assessment. Method: PathVysion Vysis kit: Enzyme Pre-treatment: 10', 370C.

Technical ISH: Pass Rates and Methods

Overall Pass Rates





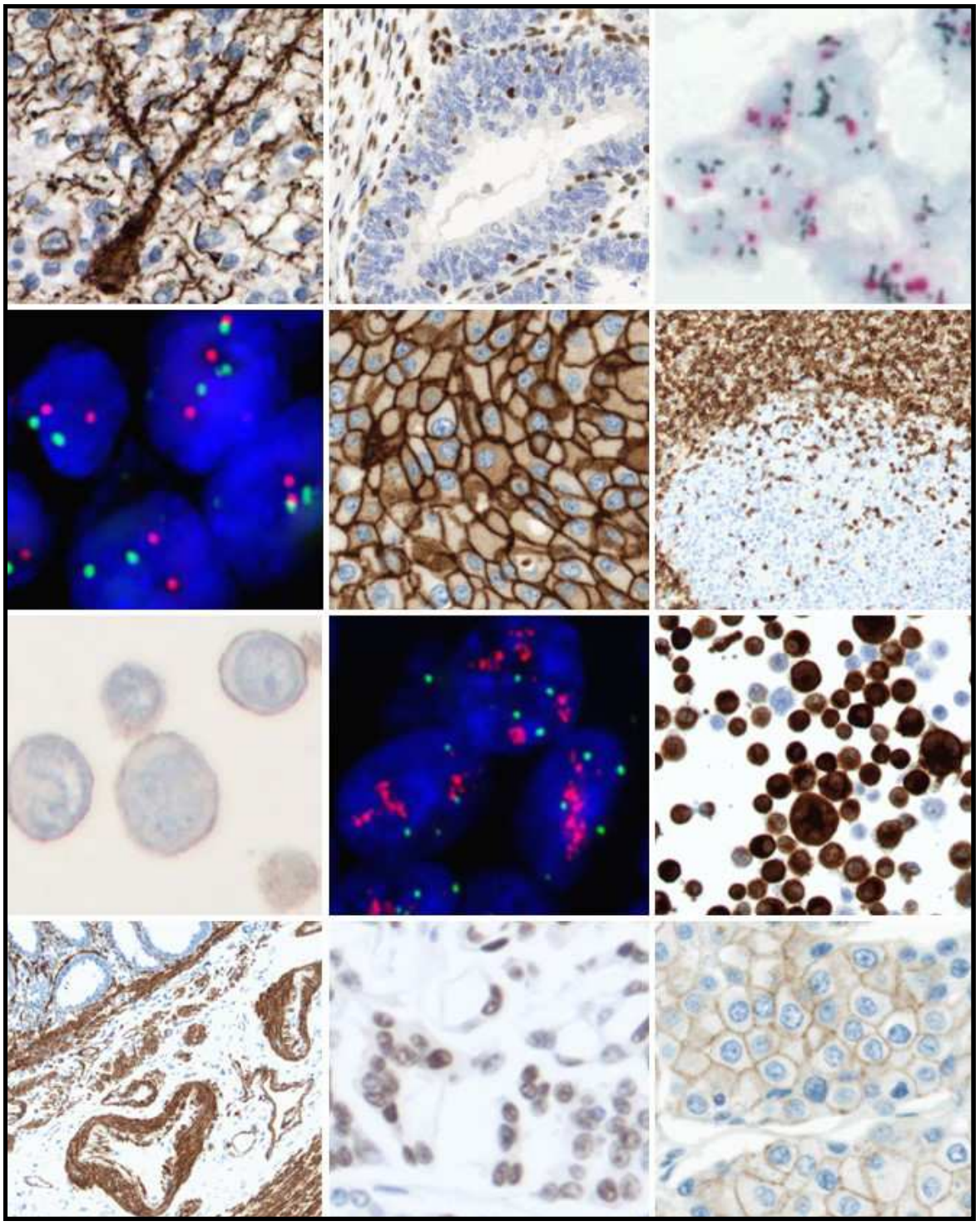


UK NEQAS
International Quality Expertise

7833

UK NEQAS ICC & ISH

Improving Immunocytochemistry for over 25 Years



For advertising opportunities contact:
Neil Bilbe: n.bilbe@ucl.ac.uk or nbilbe@ukneqasiccish.org