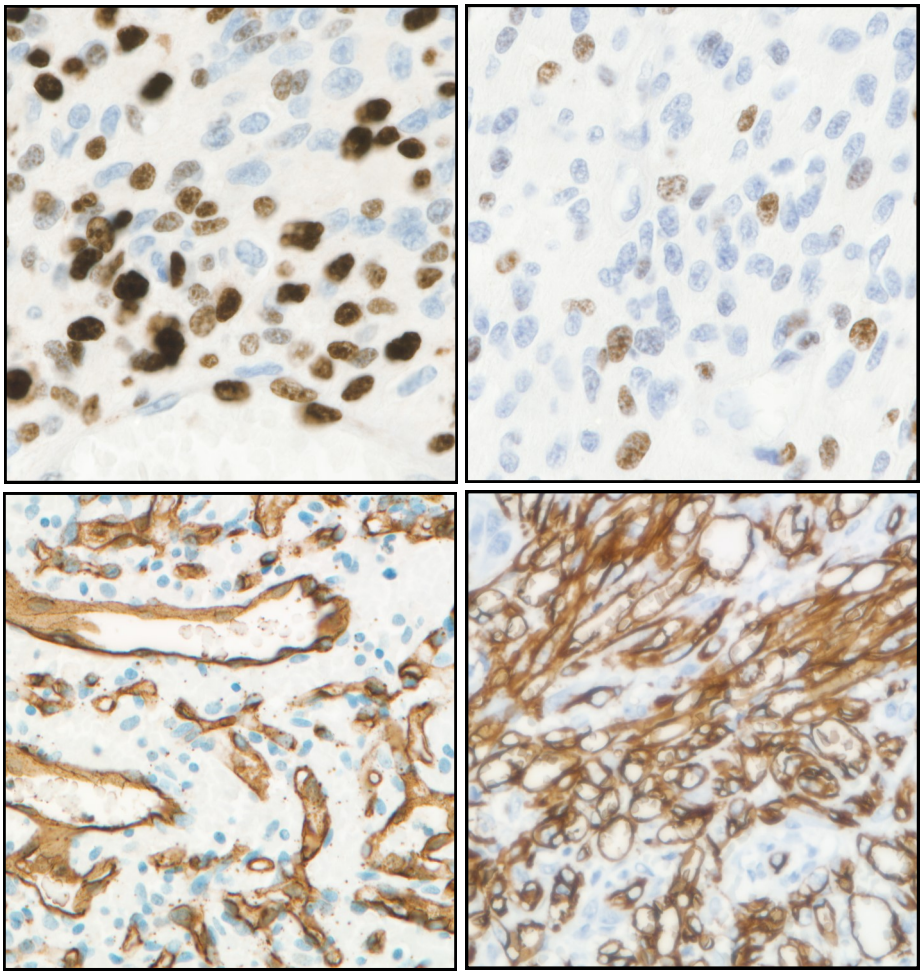
UK NEQAS
International Quality Expertise

Immunocytochemistry

Improving Immunocytochemistry for Over 25 Years

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

Assessment Dates: 2nd — 18th January 2018



Cover Photo: Taken from the : Neuropathology Module

Top Left: Optimal Ki 67 staining on the NEQAS GBM sample (G)
Top Right: Sub-optimal Ki 67 staining on the NEQAS GBM sample (G)
Bottom Left: Optimal CD34 demonstration on the NEQAS haemangioblastoma (J)
Bottom Right: Excellent CD34 staining on an in-house control from a KS case (K)

General Information

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

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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
Dr B M Araujo, Cambridge
Mr N Bilbe, London
Mr D Blythe, Leeds
Dr C Cardozo, Preston
Ms A Clayton, Preston
Mr A Dodson, London
Mr R Fincham, Cambridge
Mr D Fish, Reading
Ms S Forrest, Liverpool
Dr I Frayling, Edinburgh
Dr C Gillett, London
Mr J Hughes, London
Prof. R Hunt, Liverpool
Dr J Joseph, Preston
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Ms H McBride, Belfast
Ms J McMillan, Glasgow
Mr C Marsh, Newcastle
Mr K Miller, London
Ms A Newman, London
Dr G Orchard, London
Ms S Parry, London
Ms A Patterson, Belfast
Dr M Pitt, Cambridge
Ms J Pritchard, Birmingham
Ms A Riley, Glasgow
Mr G Rock, Birmingham
Ms L Skaetes, Cambridge
Dr J Starczynski, Birmingham
Ms G Valentine, London
Dr P Wencyk, Nottingham
Ms H White, Maidstone
Ms M Whitney, Nottingham
Ms J Williams, Southampton

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

Germany
Dr I Nagelmeier, Kassel

Netherlands
Dr Erik Thunnissen, Amsterdam

Portugal
Mr J Matos, Lisbon
Mr R Roque, Lisbon

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A TOWERING PERFORMANCE!

UK NEQAS ICC & ISH: Autumn Conference 2018

21st Century Slide Based Pathology & Molecular Diagnostics:
From the Laboratory to the Patient

UK NEQAS ICC & ISH Autumn Conference 2018: Tower Hotel, London - November 9th-11th

UK NEQAS ICC & ISH held its first major conference since the Windsor meeting back in 2012, at the Tower Hotel, London, in early November 2018. Over 140 delegates (excluding trade), from around the globe, consisting of a mixture of UK NEQAS ICC & ISH participants, pathologists, and other scientific staff, enjoyed a successful weekend of highly informative talks, presentations and workshops; accompanied by an excellent social programme.

The conference was further enhanced by the presence of 13 trade stands, and around 40 representatives, from a variety of companies, whose support and sponsorship added greatly to the weekend.

The event was focused on the many changes occurring in the field and these included current and new companion diagnostics and their related therapies. Also advances in digital pathology, the need to improve pre-analytics, improvements in handling cytological samples and aspects of EQA.

Friday afternoon

The meeting was formally opened by **Keith Miller,** UK NEQAS ICC & ISH Scheme Director; which was followed by an address from **Liam Whitby,** UK NEQAS President-Elect. Liam outlined the structure and purpose of UK NEQAS, and its importance to the profession.

The first session continued with a series of talks under the banner **Lung Cancer: Pathology, personalised therapy & related biomarkers** given by **Dr Phillipe Taniere**, **Dr Shobhit Baijal**, and **Suzanne Parry.**

The rest of the Friday programme was split into two separate sessions: (1) **Presentations by commercial companies** given by **Nikos Lioutas,** MSD UK, and **Dr Ian Milton,** Histocyte Laboratories; and (2) **PD-L1 Biomarkers in Bladder Cancer and Personalised Healthcare** presented by **Dr Mark Kockx, Dr Philippe Taniere**, **Dr Corrado D'Arrigo**, and **Professor Syed Hussain**

The day finished with an informal buffet dinner in the Tower Suite, overlooking the Thames.

Saturday

Visits to the Trade continued before proceedings, and was interspersed throughout the day, during coffee breaks, and an excellent buffet lunch.

The programme for Saturday again consisted of two parallel sessions: In Conference Room 1: **Targos & Roche Joint Symposium: Predictive Cancer Pathology of Lung & Urothelial Cancer** consisting primarily of talks, and incorporating workshops on PD-L1. Speakers were: **Dr Eslie Dennis, Professor Bharat Jasani** (who also gave the Gerry Reynolds Memorial Lecture), **Dr Gudrun Baenfer, Dr Erik Thunnissen, Dr Kathrina Alexander, Dr Rolf Diezko,** and **Dr Bryce Portier**.

Conference Room 2 held a session titled: **Targos & UK NEQAS ICC & ISH Joint Symposium: Predictive Cancer Pathology of Breast & GI Tract Cancer** presentations were given by: **Professor Josef Rueschoff**, **Dr Ian Frayling**, **Professor Mark Arends**, **Andrew Dodson**, **Jamie Hughes**, and **Hilary McBride**.

Professor Bharat Jasani of Targos, and Dr Eslie Dennis of Roche, Arizona, introducing the joint Targos & Roche Symposium.

The final part of this session had a series of **Selected Topics** with talks given by **Dr Mark Kockx, Julia Pagliuso**, and **Dr Sarah Wedden.**

These concluded the educational proceedings for day 2.

Professor Josef Rueschoff of Targos giving an update on HER2

On the Saturday evening a Gala Dinner with Entertainment was held in the main Conference Room. A pleasant three course meal with wine, was followed by some lively entertainment, with a comedian starting proceedings, followed by a 6 piece band which kept everyone happy until the wee small hours.

Sunday Morning

Sunday morning consisted of a single session of talks, held in Conference Room 1, and divided into two themes: (1) **Digital Pathology,** with talks given by **Dr Bethany Williams,** and **Martin Kristensson;** and (2) **Selected Topics**, with presentations by **Dr Jenni Fairley, Dr Guy Orchard,** and **Dr Patricia Carrigan.**

Workshops

Throughout the weekend a number of workshops were held outside of the main conference lecture halls. These were housed in smaller meeting rooms on the 12th floor of the hotel, and designed for a an audience of about 20 participants.

The first of these was held during Saturday lunchtime: **Visiopharm Workshop: Digital Image Analysis for Clinical and Quality Control Measurements** which was run by **Andreas Schønau**. This proved a popular lunchtime session.

Sunday morning saw two more workshops on the 12th floor:
(1) **Roche Digital Slide Viewing and Education** (in the Discovery Room) consisting of five short sessions, each of 35 minutes, and overseen by **Dr Richard Huang** and **Dr Bryce Portier**; and
(2) **Non-Gynae Workshop: Is Brown/Red Good Enough** (Britannia Room) run jointly by **Dr Irena Srebotnik Kirbis** and **Neil Bilbe.**

Both these well received sessions continued until 1 pm, after which everyone went downstairs to lunch before departing.

To access the talks go to: www.uknegasiccish.org/downloads/

N.B. Not all presentations are available, due to various restrictions.

UK NEQAS ICC & ISH would like to thank all the speakers, the Trade, MeetingVoice for organising much of the event, along with the excellent AV support, all of which helped to make this a successful and highly productive weekend conference.

Suzanne Parry

	Gold Standard	Second Antibody
Antigens Assessed:	CD45	CD56
Tissue Sections circulated:	Tonsil and known negative tumour	Appendix and neuroendocrine tumour
Number of Registered Participants:	310	
Number of Participants this Run	300 (97%)	

Introduction

Gold Standard: CD45

CD45 is a transmembrane glycoprotein expressed on most nucleated cells of haematopoietic origin, and is an essential regulator of T- and B-cell antigen receptor signalling, The CD45 cluster of antibodies recognise a group of proteins known as the leucocyte common antigens (LCA), which are found exclusively around the cytoplasmic membrane of haematopoetic/lymphoid cells and their progenitors, except maturing erythrocytes and megakaryocytes. Antibodies against CD45 stain precursor cells, mature T- and B-lymphocytes, granulocytes, monocytes and macrophages, and the main diagnostic use of CD45 is to differentiate lymphomas from other malignant tumours, such as carcinomas. CD45 is therefore positively expressed in lymphomas, B-cell chronic lymphocytic leukaemia, hairy cell leukaemia, and acute non-lymphocytic leukaemia.

Features of Optimal Immunostaining: (Figures 1, 2, 3 & 5)

- Strong, crisp, uniform membrane staining of virtually all lymphocytes in the UK NEQAS tonsil.
- Strong, crisp membrane staining of scattered lymphocytes around carcinoma tissue in the colorectal tumour.
- Clean background.
- No non-specific staining of other cell types not expected to stain, including the colorectal carcinoma.
- Adequate nuclear counter-stain.

Features of Sub-optimal Immunostaining: (Figures 4 & 6)

- Weak, diffuse or partial membrane staining of lymphocytes.
- Uneven staining.
- Excessive background staining.
- Non-specific staining of cell types or components not expected to stain.
- Inadequate nuclear counter-stain.

References

1. Kurtin PJ, Pinkus GS. Leukocyte common antigen - a diagnostic discriminant between hematopoietic and nonhematopoietic neoplasms in paraffin sections using monoclonal antibodies: Correlation with immunologic studies and ultrastructural localization. Hum Pathol 1985;16:353.
2. Michie SA, Spagnolo DV, Dunn KA, Warnke RA, Rouse RV. A panel approach to the evaluation of the sensitivity and specificity of antibodies for the diagnosis of routinely processed histologically undifferentiated human neoplasms. Am J Clin Pathol 1987;88:457-62.

Second Antigen: CD56

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

Features of Optimal Immunostaining:

Appendix (Figures 7 & 11A)

- Strong staining in virtually all of the peripheral nerves
- No background staining

Neuroendocrine tumour: (Figures 10 & 12)

- Moderate to strong nuclear staining in virtually all of the tumour cells
- Minimal background staining

Sub-optimal Immunostaining: (Figures 8 & 9)

- Weak, uneven or no staining
- Diffuse staining
- Non-specific staining of cell types not expected to stain
- Excessive background staining

References:

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

Assessment Summary

CD45

300 laboratories submitted slides for the CD45 assessment, and all but 2 laboratories submitted their in-house control sections for this run. The results show an increase of 2% in acceptable results from Run 118, and a similar unacceptable rate, as shown in the table below:

CD45 Pass Rates : NEQAS section		
	Run 118	Run 120
Acceptable	86%(N=255)	88%(N=272)
Borderline	13%(N=38)	7%(N=21)
Unacceptable	2%(N=6)	2%(N=7)

The in-house samples also showed similar pass rates to the NEQAS distributed material for run 120; 88% acceptable pass rate, 11% borderline pass, and only 3 laboratories (1%) failed the assessment.

Once again, the most popular clone was the Dako 2B11+PD7/26, used by 60% of laboratories, and showed an acceptable pass rate of 94%. A variety of automated

Suzanne Parry

platforms and antigen retrieval methods were used with the various clones.

CD56

297 laboratories submitted slides for the assessment, and all but 2 laboratories also submitted their in-house material. The results showed disappointing results. Only 41% of laboratories (N=121) achieved an acceptable pass on the NEQAS distributed material. A further 163 laboratories (55%) achieved a borderline pass, and 13 laboratories (4%) failed the assessment. The pass rates were much lower than when CD56 was last assessed in the general module (Run 111), 79% of laboratories received an acceptable pass. The result for both assessment runs are summarised in the table below:

CD56 Pass Rates: NEQAS section		
	Run 111	Run 120
Acceptable	79% (N=244)	41%(N=121)
Borderline	12% (N=37)	55%(N=163)
Unacceptable	9% (N=27)	4%(N=13)

The reason for the higher level of unacceptable or borderline scores was due to the inability to stain the neuroendocrine tumour section appropriately. Some laboratories demonstrated no staining at all in this tumour, and therefore failed the assessment, even though they may have achieved acceptable staining in the NEQAS distributed appendix section on the same slide. Many more laboratories showed weak or patchy staining of the neuroendocrine tumour. These laboratories tended to achieve a borderline score. Overall, these findings highlight the importance of using appropriate controls. As appendix contains a lot more of the protein, it may show staining, even if the assay is not sensitive enough. Therefore, using appendix alone may not highlight when the assay is not optimal. Most laboratories would have passed the assessment with acceptable results if NEQAS had only distributed the appendix sample alone. The neuroendocrine tumour was the key sample to highlight issues with the sensitivity of laboratories' methods. UK NEQAS therefore recommends using a combination of controls, such as tonsiland tumour. The tonsil is a good positive control, and should show good strong membranous staining of the Natural Killer cells, and the CD4 and CD8 T cells. No other lymphocytes should stain positive.

In this particular assessment, the Leica CD564 clone was the most popular antibody choice. This was used by 80 laboratories, and showed an overall acceptable pass rate of 34%. The Ventana MRQ-42 antibody, was also a popular clone. This was used by 45 laboratories, and showed a pass rate of 89%. The MRQ-42 clone by Cell Marque, showed a similar pass rate of 81%, and was used by 16 participants. Many laboratories also used the Leica/Novocastra antibody, 1B6 clone (N=42), but this only showed an acceptable pass rate of 21%. Also popular was the Dako 123C3 clone, used by 47 participants, but only showed a pass rate of 20%.

Weak or no demonstration of the antigen was the over-riding reason for unacceptable marks or failure of the assessment. This was predominantly caused by insufficient antigen retrieval methods used. Use of the Ventana MRQ-42 antibody, in combination with an extended antigen retrieval protocol, appeared to achieve the expected results much better than any other systems. However, other platforms, such and the Dako autostainer with the PT Link and the Leica Bond machines also achieved optimal results with the correct optimised methods.

Most laboratories performed better on their in-house material, and therefore received higher scores. However, some laboratories also received sub-standard results on their in-house controls, and this was either due to weak staining, background staining or poor quality of tissue. Many laboratories are only using appendix as their in-house controls, and showed the expected strong staining of the peripheral nerves. Those laboratories showing weaker staining in their in-house appendix also failed to demonstrate the fine delicate fibres expected to stain.

It is important to highlight that using an appendix alone as an in-house control can lead to a false sense of security that the assay is working optimally. It may not highlight when an assay is not sensitive enough due to the higher level of protein within appendix tissue. Using a combination of tissues, such as tonsil, and a known positive tumour, would be a better combination of controls to gauge the sensitivity of the assay.

Selected Images showing Optimal and Sub-optimal immunostaining

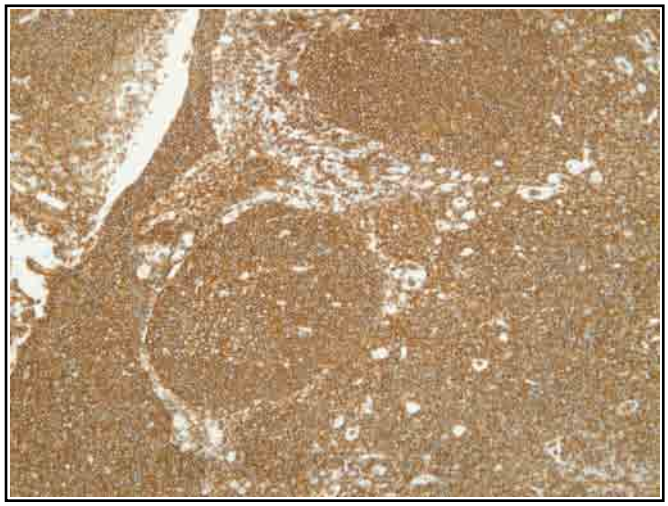


Figure 1: Optimal demonstration of CD45 in the UK NEQAS distributed tonsil. Even at low power it is clear to see the strong and well-localised staining in both the B- and T-cells. Stained with the Ventana RP2/18 RTU clone on the ULTRA, CC1 24 mins, UltraView detection.

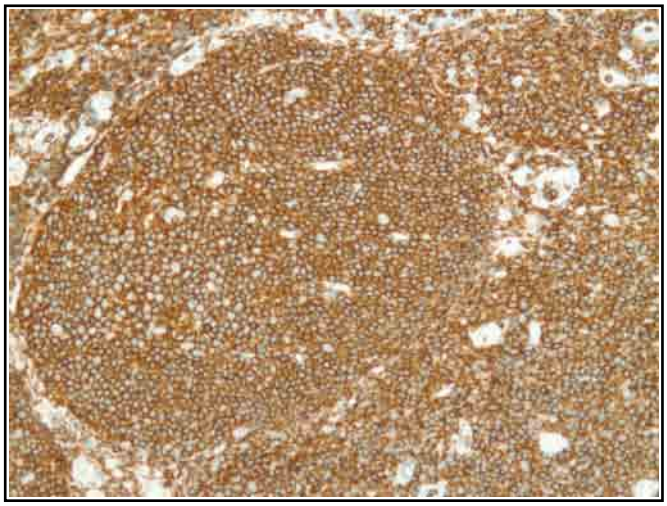


Figure 2: Higher power image of the UK NEQAS tonsil stained with CD45. The example highlights the strong crisp membranous staining of the B- and T-cells. (Same protocol as Fig 1).

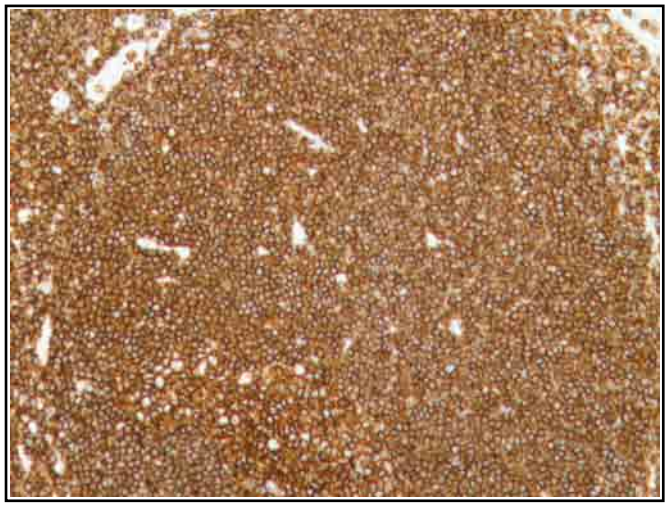


Figure 3: Good CD45 staining in the UK NEQAS distributed tonsil. The example shows strong and distinct membranous staining of both B- and T-cells. Stained with the Dako antibody (2B11+PD7/26 clone), 1:180, on the autostainer Link48, pre-treatment in the PT link with high pH buffer.

Figure 4: Sub-optimal demonstration of CD45 in the UK NEQAS distributed tonsil (compare to Figs 1-3).The staining is weak and diffuse with many of the lymphocytes expected to stain not demonstrated. The antibody dilution factor is far to weak (Dako 2B11+PD7/26 clone, used at 1:950).

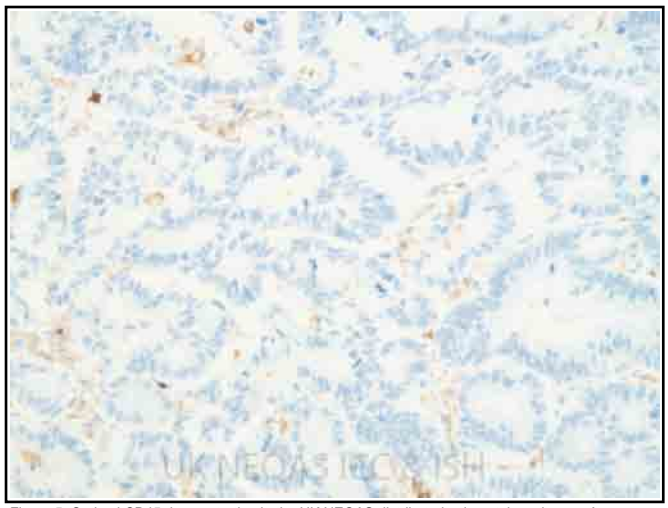


Figure 5: Optimal CD45 demonstration in the UK NEQAS distributed colorectal carcinoma. As expected the tumour is negative, and only some of the surrounding scattered lymphocytes show strong to moderate distinct staining. (Same protocol as Fig 3).

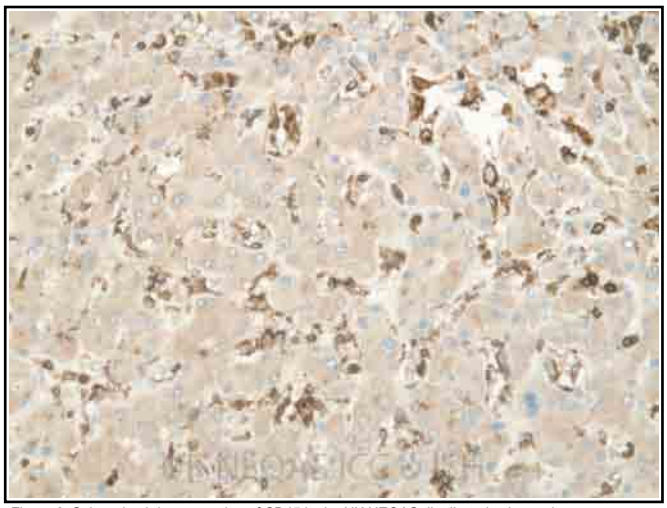


Figure 6: Sub-optimal demonstration of CD45 in the UK NEQAS distributed colorectal carcinoma. While the tumour is negative as expected, the sections shows excessive background staining.

Selected Images showing Optimal and Sub-optimal {@stainingtext}

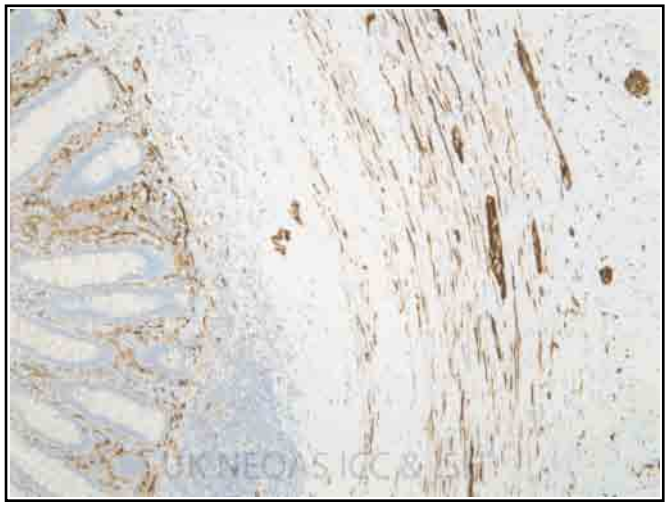


Figure 7: Optimal demonstration CD56 in the UK NEQAS appendix sample. The example shows strong staining in virtually all of the peripheral nerves while the background remains clean. Stained using the Dako RTU 123C3 antibody, on the autostainer Plus Link.

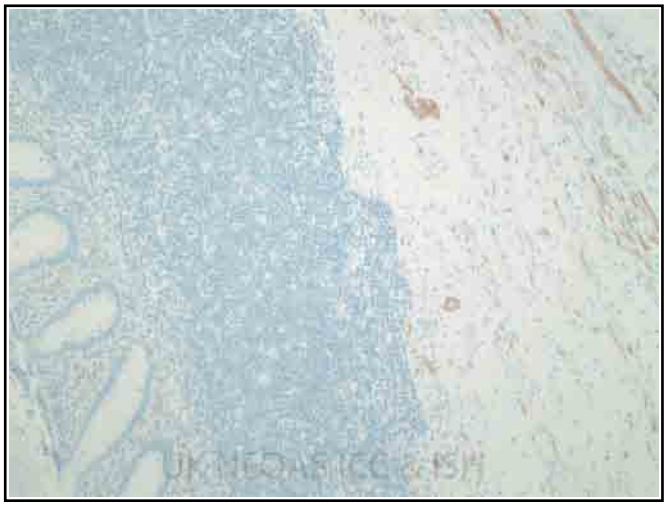


Figure 8: Unacceptable staining with CD56 in the UK NEQAS appendix sample (compare to Fig 7). The staining is weak or moderate in some nerve fibres, and then many of the finer peripheral nerves are not staining at all. Stained with the Leica RTU CD564 antibody on the Ventana Ultra.

Figure 9: Sub-standard staining with CD56 in the UK NEQAS appendix sample. Even though the nerve fibres are demonstrated, the section shows excessive background staining. Stained with the Leica (clone 1B6), on the Bond with ER2 pre-treatment for 30 minutes.

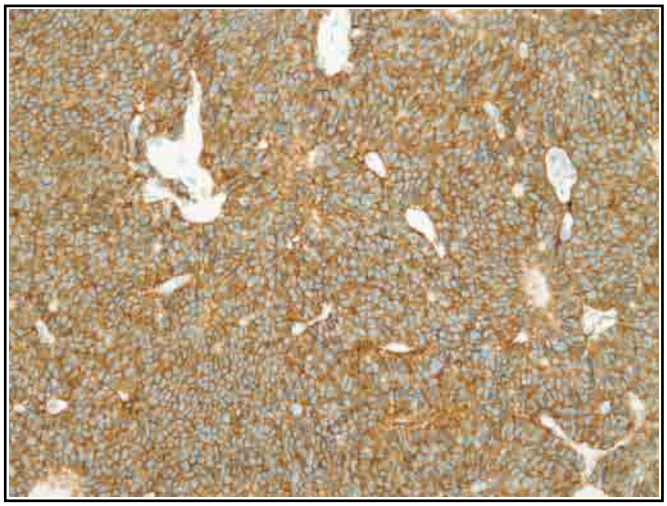


Figure 10: Optimal demonstration of CD56 in the UK NEQAS neuroendocrine tumour sample. All neoplastic cells show a strong and distinct membranous staining pattern. Stained using the Ventana MRQ-42 RTU antibody, CC1 for 72 minutes on the Ultra.

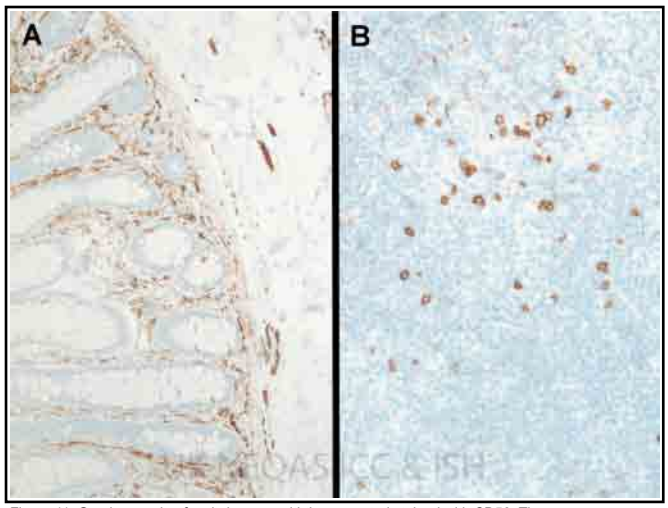


Figure 11: Good example of an in house multi-tissue control stained with CD56. The nerve fibres in the colon (A) and the NK cells of the tonsil (B) show a strong and distinct staining reaction, while the background remains clean. Stained with the Ventana MRQ-42 RTU antibody, CC1 for 32 minutes on the Benchmark Ultra.

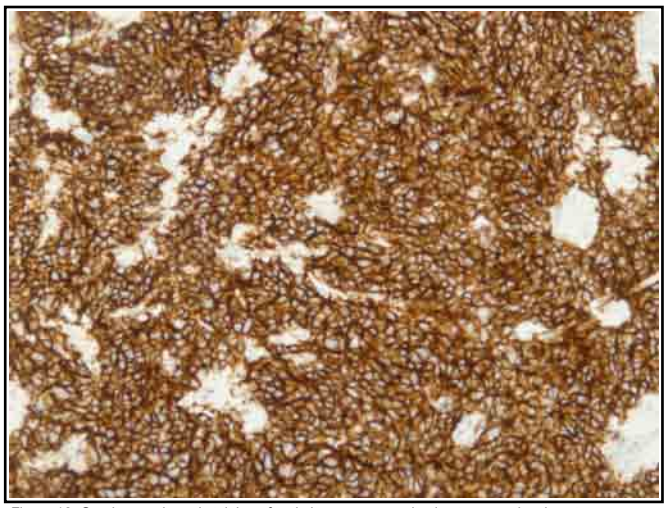
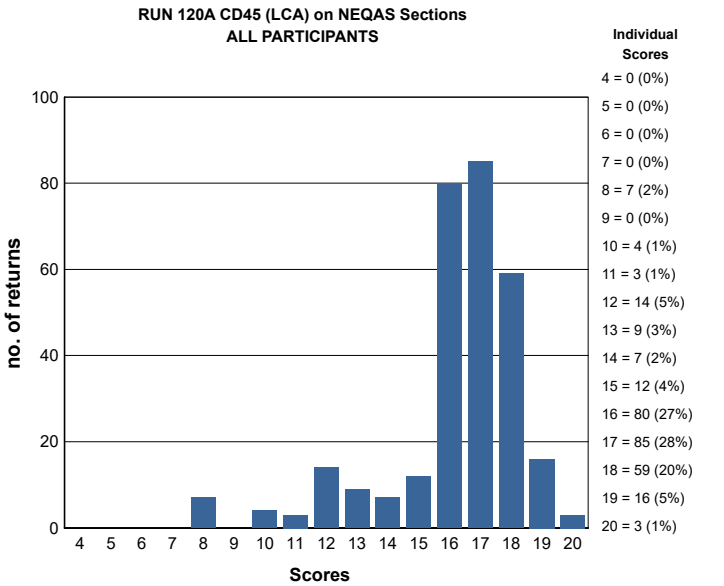
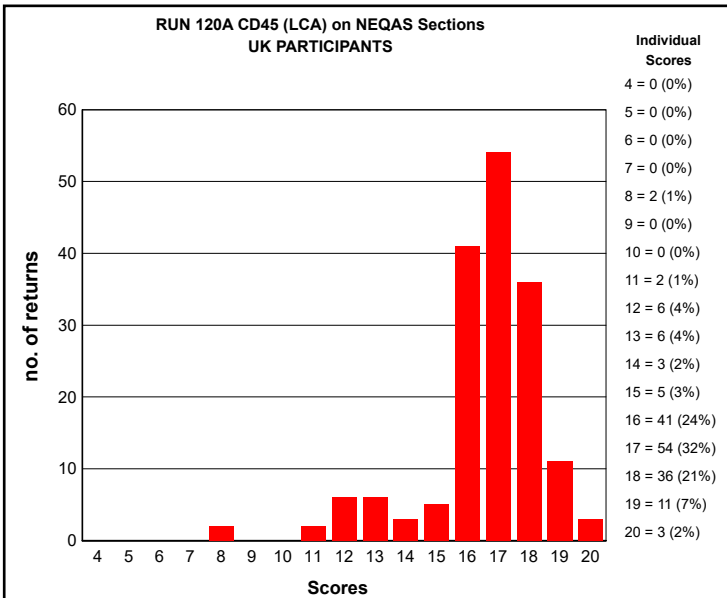
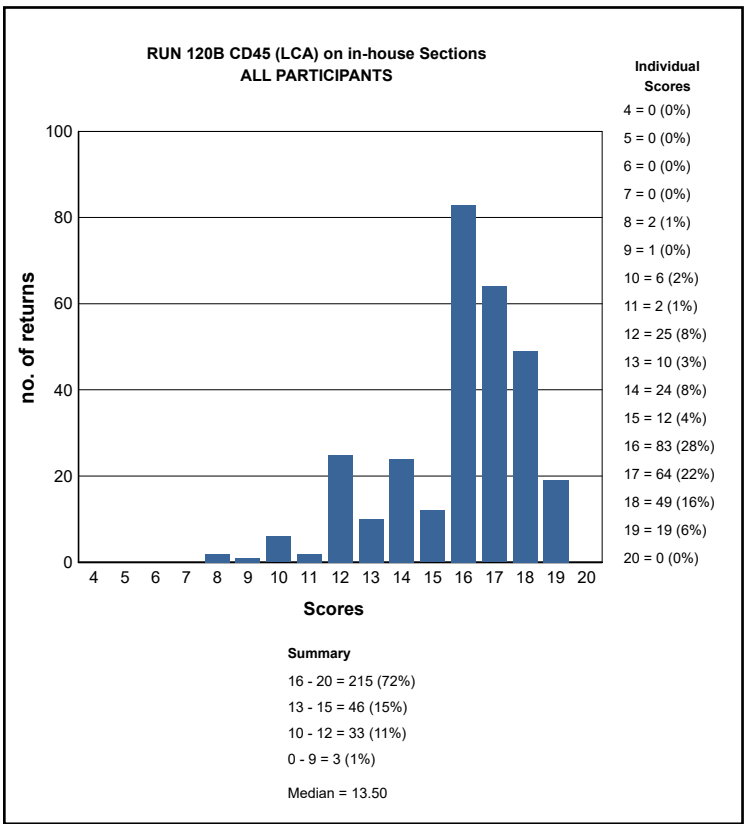


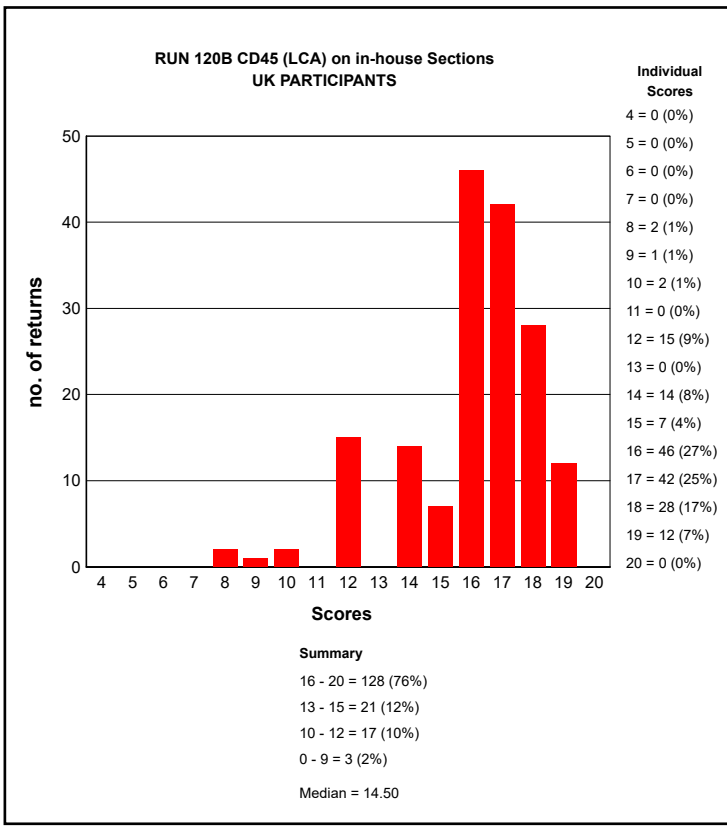
Figure 12: Good example and staining of an in house neuroendocrine tumour, showing strong and crisp membranous staining. (Same protocol as Fig 7).

GRAPHICAL REPRESENTATION OF PASS RATES

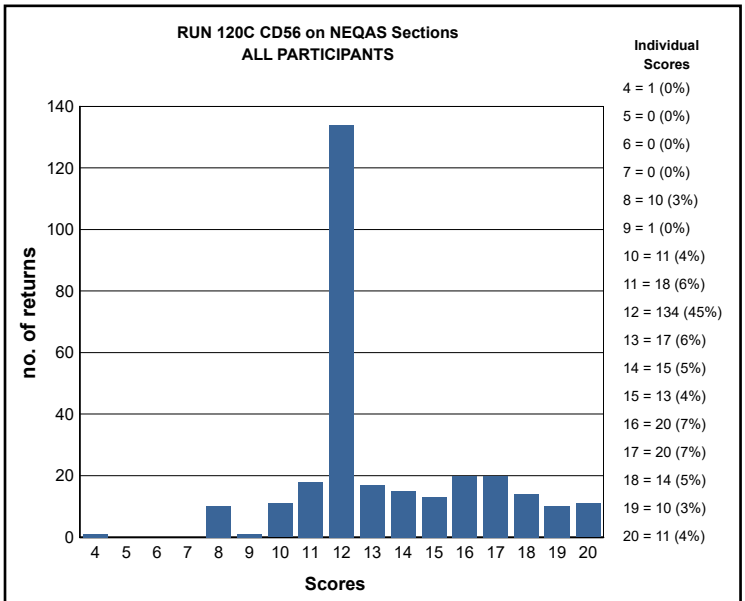








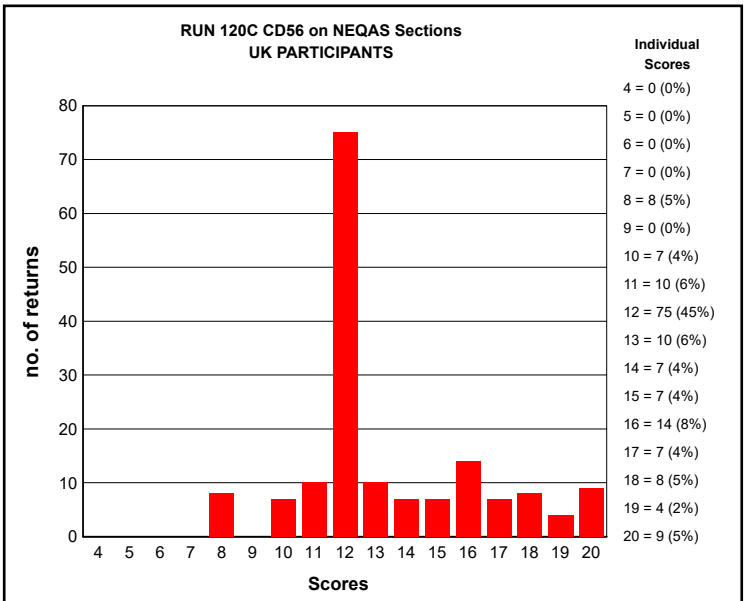
GRAPHICAL REPRESENTATION OF PASS RATES



Summary

16 - 20 = 75 (25%)
13 - 15 = 45 (15%)
10 - 12 = 163 (55%)
0 - 9 = 12 (4%)

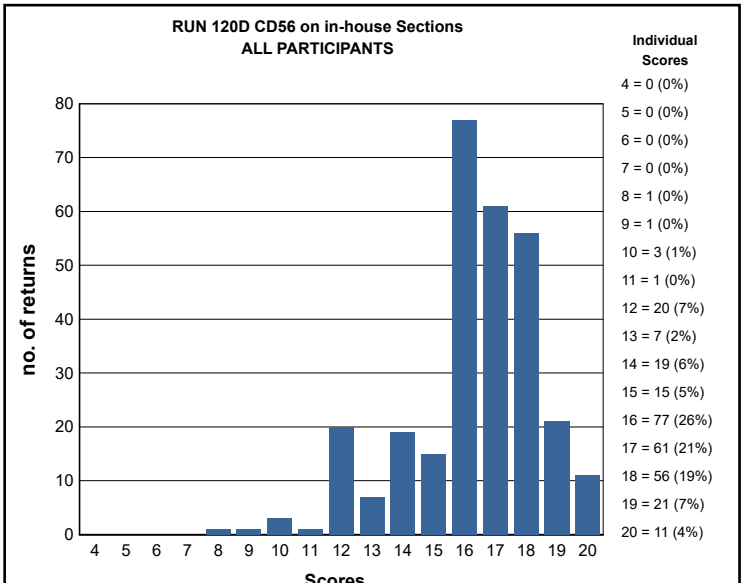
Median = 13.50



Summary

16 - 20 = 42 (25%)
13 - 15 = 24 (14%)
10 - 12 = 92 (55%)
0 - 9 = 8 (5%)

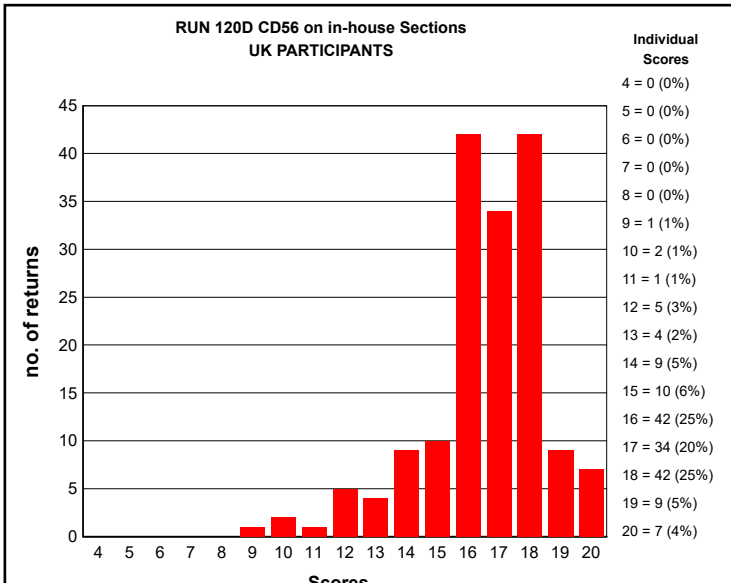
Median = 14.50



Summary

16 - 20 = 226 (77%)
13 - 15 = 41 (14%)
10 - 12 = 24 (8%)
0 - 9 = 2 (1%)

Median = 14.00



Summary

16 - 20 = 134 (81%)
13 - 15 = 23 (14%)
10 - 12 = 8 (5%)
0 - 9 = 1 (1%)

Median = 14.50

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: 120

Primary Antibody : CD45 (LCA)

Antibody Details	N	%
Dako M0701 (clones 2B11+PD7/26)	159	91
Dako M0754 (clone 4KB5) CD45RA	3	100
Novocastra NCL-LCA	4	100
Ventana 760 2505	42	90
BioGenex AM111-5M	1	100
BioGenex MU371-UC	1	100
Cell Marque clone (PD7/26&2B11)760-4279	4	100
Dako IS751 (2B11 + PD/726) Plus	6	83
Leica Bond PA0042 CD45 (X16/99)	26	100
Ventana Confirm LCA RP2/18	15	93
Ventana 760 4279	14	71
Cell Marque CMA 304	1	0
Dako RTU Omnis GA751 (2B11+PD7/26)	10	100
Dako IR751 (2B11 + PD/726) Link48	6	100
Biocare CM016C (CD45RO [PD7/26])	1	100

General Pathology Run: 120

Primary Antibody : CD56

Antibody Details	N	%
Other	10	50
Cell Marque CMA 361 (clone 123C3 D5)	1	0
Medite MOB 261 (clone 123C3 D5)	1	0
Monson MOH9006-1 (clone 123C3 D5)	1	0
Neomarkers MS 1149 (clone BC45C04)	2	0
Neomarkers MS 204 (clone 123C3 D5)	1	0
Novocastra/Leica NCL-CD56 (clone 1B6)	41	20
Ventana 760 (clone IB6)	1	0
Ventana 790 4465 (NCAM) (clone 123C3)	37	8
Zymed/Invitrogen 18-0152	1	0
Dako M7304 (123C)	26	27
Dako RTU FLEX Link IR628 (123C3)	19	32
Dako RTU FLEX Plus IS628 (123C3)	2	0
Cell Marque 156R-94,95,96,97,98 (MRQ-42)	16	81
Novocastra/Leica NCL-CD56-504 (CD564)	48	50
Novocastra/Leica RTU PA0191(CD564)	31	19
Ventana 760-4596 (MRQ-42)	45	89

General Pathology Run: 120

Heat Mediated Retrieval	CD45 (LCA) N	CD45 (LCA) %	CD56 N	CD56 %
_Leica BondMax ER2	1	100	0	0
Biocare Decloaking Chamber	1	100	1	0
Dako Omnis	13	100	10	0
Dako PTLink	26	96	29	45
Lab vision PT Module	2	50	2	50
Leica ER1 10 mins	5	100	1	0
Leica ER1 20 mins	37	95	40	18
Leica ER1 25 mins	1	100	0	0
Leica ER1 30 mins	16	100	16	50
Leica ER1 40 mins	1	100	0	0
Leica ER2 10 mins	3	67	2	50
Leica ER2 20 mins	18	83	21	52
Leica ER2 30 mins	4	100	7	43
Microwave	5	60	3	33
None	4	75	0	0
Other	1	0	1	100
Pressure Cooker	1	100	0	0
Pressure Cooker in Microwave Oven	1	100	1	100
Steamer	1	100	1	0
Ventana CC1 16mins	6	100	3	67
Ventana CC1 20mins	2	50	1	0
Ventana CC1 24mins	11	82	6	33
Ventana CC1 32mins	34	97	32	47
Ventana CC1 36mins	10	80	11	64
Ventana CC1 40mins	4	100	5	40
Ventana CC1 44mins	1	100	0	0
Ventana CC1 48mins	3	67	8	63
Ventana CC1 52mins	3	100	3	33
Ventana CC1 56mins	3	100	5	60
Ventana CC1 64mins	24	96	28	25
Ventana CC1 72mins	1	100	1	100
Ventana CC1 76mins	2	100	1	100
Ventana CC1 8mins	6	100	4	25
Ventana CC1 92mins	0	0	1	100
Ventana CC1 extended	1	0	2	50
Ventana CC1 mild	15	93	17	41
Ventana CC1 standard	15	100	14	43
Ventana CC2 16mins	1	0	0	0
Ventana CC2 24mins	1	0	0	0
Ventana CC2 32mins	1	100	0	0
Ventana CC2 56mins	0	0	1	100
Ventana CC2 64mins	1	0	2	50
Ventana CC2 standard	0	0	1	100
Water bath 68 OC	1	100	1	0

General Pathology Run: 120

Enzyme Mediated Retrieval	CD45 (LCA) N	CD45 (LCA) %	CD56 N	CD56 %
AS PER KIT	4	100	5	60
Dako Protease (S2019)	1	100	0	0
NOT APPLICABLE	156	93	131	39
Ventana Protease 1 (760-2018)	1	100	0	0

General Pathology Run: 120	CD45 (LCA)		CD56	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	18	89	23	35
BioGenex HRP (HK 519-06K)	1	100	0	0
Dako EnVision FLEX (K8000/10)	7	100	7	29
Dako EnVision FLEX+ (K8002/12)	23	96	21	29
Dako Envision HRP/DAB (K5007)	5	60	2	0
Dako Envision+ HRP mouse K4004/5/6/7	2	100	4	75
LabVision UltraVision LP HRP (TL 125 HLJ)	1	0	1	0
Leica Bond Polymer Refine (DS9800)	78	95	74	38
None	1	100	1	0
NOT APPLICABLE	1	100	0	0
Novocastra Novolink PDS (RE7-140/150/280/290-K)	2	100	2	0
Other	6	83	6	33
Ventana iView system (760-091)	2	100	2	0
Ventana OptiView (760-700) + Amp. (7/860-099)	3	100	2	50
Ventana OptiView Kit (760-700)	67	91	63	49
Ventana UltraView Kit (760-500)	70	87	67	40

General Pathology Run: 120	CD45 (LCA)		CD56	
Automation	**N**	**%**	**N**	**%**
Dako Autostainer	2	100	2	50
Dako Autostainer Link 48	23	96	28	46
Dako Autostainer plus	3	100	0	0
Dako Autostainer Plus Link	1	100	2	50
Dako Omnis	13	100	10	0
LabVision Autostainer	3	67	2	50
Leica Bond Max	36	86	32	22
Leica Bond-III	51	98	55	42
None (Manual)	8	75	5	20
Other	1	0	1	100
Shandon Sequenza	1	100	1	0
Ventana Benchmark GX	6	83	6	83
Ventana Benchmark ULTRA	105	90	101	42
Ventana Benchmark XT	41	93	39	44
Ventana NexES	1	100	0	0

General Pathology Run: 120	CD45 (LCA)		CD56	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	37	89	38	55
BioGenex liquid DBA (HK-124-7K)	1	100	0	0
Dako DAB K3468	1	0	0	0
DAKO DAB+	1	100	2	50
Dako DAB+ Liquid (K3468)	3	67	3	0
Dako EnVision Plus kits	2	100	6	50
Dako FLEX DAB	29	97	26	27
Dako REAL EnVision K5007 DAB	4	75	3	33
LabVision (TA-125-HD)	1	0	1	100
Leica Bond Polymer Refine kit (DS9800)	80	95	76	36
Other	10	90	8	13
Sigma DAB (D5637)	1	100	1	0
Ventana DAB	46	96	41	56
Ventana iview	3	100	1	0
Ventana Ultraview DAB	75	87	78	35

BEST METHODS - Gold Standard Antibody

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

CD45 (LCA) - Method 1

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

Primary Antibody: Leica Bond PA0042 CD45 (X16/99) , 15 Mins, ROOM ºC Prediluted
Automation: Leica Bond-III
Method: AS PER KIT
Main Buffer: AS PER KIT
HMAR: Leica ER1 20 mins
EAR: NOT APPLICABLE
Chromogen: Leica Bond Polymer Refine kit (DS9800), ROOM ºC., Time 1: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins, ROOM ºC Prediluted

CD45 (LCA) - Method 2

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

Primary Antibody: Dako M0701 (clones 2B11+PD7/26) , 30 Mins, 23 ºC Dilution 1: 180
Automation: Dako Autostainer Link 48
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer, PH: 7.6
HMAR: Dako PTLink, Buffer: ENVISION TR SOLUTION, PH: 9
EAR:
Chromogen: Dako FLEX DAB, 23 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection: Dako EnVision FLEX+ (K8002/12) , 35 Mins, 23 ºC Prediluted

CD45 (LCA) - Method 3

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

Primary Antibody:	Dako RTU Omnis GA751 (2B11+PD7/26)
Automation:	Dako Omnis
Method:	Dako FLEX+ kit
Main Buffer:	AS PER KIT
HMAR:	Dako Omnis
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB
Detection:	Dako EnVision FLEX (K8000/10)

CD45 (LCA) - Method 4

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

Primary Antibody:	Ventana 760 2505 , 16 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins, Buffer: 0
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB, 36 ºC., Time 1: 8 Mins, Time 2: 4 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

CD56 - Method 1

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

Primary Antibody:	Dako M7304 (123C) , 20 Mins, 22 ºC Dilution 1: 100
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

CD56 - Method 2

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

Primary Antibody:	Ventana 760-4596 (MRQ-42) , 28 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins, Buffer: 0
EAR:	
Chromogen:	Ventana DAB, 36 ºC., Time 1: 8 Mins, Time 2: 4 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 36 ºC Prediluted

CD56 - Method 3

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

Primary Antibody:	Novocastra/Leica NCL-CD56-504 (CD564) , 30 Mins, 20 ºC Dilution 1: 100
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 30 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), 20 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 20 Mins, 20 ºC Prediluted

CD56 - Method 4

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

Primary Antibody:	Cell Marque 156R-94,95,96,97,98 (MRQ-42)
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 32mins
EAR:	
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700)

The Breast Hormonal Receptor Module Run 120

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:	255
Number of Participants This Run	266 (96%)

Circulated Tissue
The table below shows the staining characteristics of the tissue sections circulated during Run 120. This composed of three infiltrating ductal carcinomas (IDCs) with differing levels of receptor expression along with a section of tonsil. The staining of the breast tumours were characterised using the Leica 6F11, Ventana SP1 and Dako EP1 clones.

Sections	% Positivity	Expected Staining Intensity	Allred / Quick Score
A. IDC	>95%	High	8
B. IDC	11-33%	Mid	4 (the expression level varied depending on the serial section received)
C. IDC	0%	Negative	0
D. Tonsil	≤ 3%	Weak to Medium	0 (Negative)

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

General Guideline Used in The Assessment of slides:

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

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

In-House Tissue Recommendations:

Participants in-house control tissue MUST consist of composite breast tissue placed onto a single slide. Cell line controls are an acceptable alternative when used alongside a piece of your own in-house tissue. Commercial kit/assay controls are not accepted as the 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 4-6)
3. Negative tumour, ideally with normal positively stained glands (Allred/Quick score '0')

Participants NOT using a composite control are scored a maximum 'borderline' pass (score of 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 Optimal Immunostaining (Figures 1, 2, 5 & 6)

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

Features of Suboptimal Immunostaining (Figures 3, 4, 7, 8 & 10)

- 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, such as lymphocytes, fibroblasts

NEQAS Section Assessment Results

255 laboratories submitted their slides for the ER assessment. There was a slight increase in the acceptable pass rate since the previous ER run (Run 118), and higher than the assessment before that also (Run 117). Please see table below:

ER NEQAS Pass Rates :			
	Run 117	**Run 118**	**Run 120**
Acceptable	81%(N=217)	87%(N=222)	90%(N=230)
Borderline	13%(N=35)	10%(N=26)	8%(N=20)
Unacceptable	6%(N=15)	4%(N=10)	2%(N=5)

The borderline and failed marks for this assessment were mostly due to weak staining, particularly in the mid-expressing tumour. 2 laboratories failed due to inappropriate staining seen in all the tumours, with one showing false positive staining in the known negative tumour.

In-House Tissue Assessment Results

All 255 participants also submitted their in-house controls for assessment. Overall the staining quality and results were similar to that of the NEQAS distributed material. However, the pass rates for acceptable staining were lower, and the borderline rate higher, due to several laboratories not submitting the required and recommended range of in-house controls, and therefore received a maximum borderline score of 12/20. In particular, many laboratories are still unable to provide a mid-expressing tumour.

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

Another factor to take into consideration is that the sensitivity of a participant's assay would have been validated on their own in-house tissue. However, this does not always mean that the assay is optimal. A protocol should be in place that will stain effectively on both the NEQAS samples and the laboratories' own samples.

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

Comparing NEQAS Sample Scores and In-house Control Scores.

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

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

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

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

Selected Images showing Optimal and Sub-optimal immunostaining

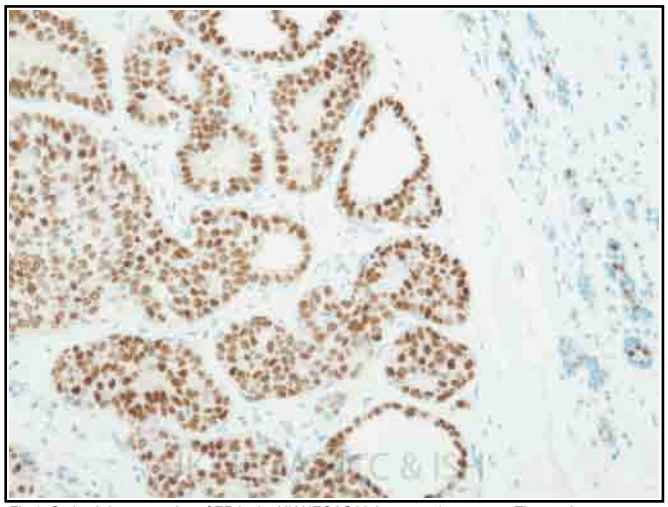


Fig 1. Optimal demonstration of ER in the UK NEQAS high-expressing tumour. The section shows intense staining in over 95% of neoplastic cells and the expected level of staining in the normal glands. Stained using the Ventana SP1 antibody on the Benchmark ULTRA, CC1 retrieval for 64 minutes.

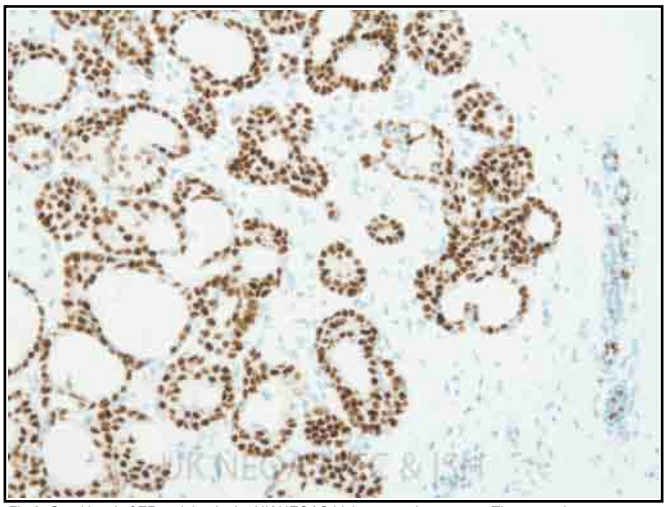


Fig 2. Good level of ER staining in the UK NEQAS high-expressing tumour. The example shows strong nuclear staining in over 95% of the neoplastic cells and the expected level of staining in the normal ducts. Stained with the Dako EP1 clone, 1:50, with antigen retrieval in the PT link using high pH buffer solution.

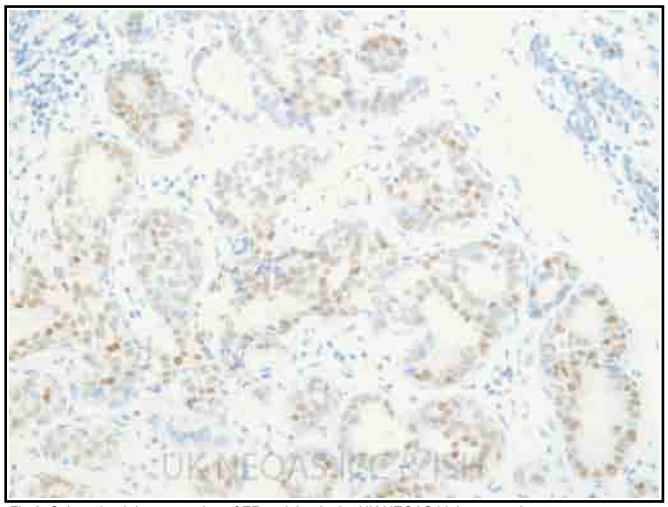


Fig 3. Sub-optimal demonstration of ER staining in the UK NEQAS high-expressing tumour (compare to Figs 1&2). The staining is very weak with less tumour nuclei staining than expected. The normal glandular cells also show a low percentage of cells staining. The Dako EP1 clone was used but unfortunately no further protocol details were provided.

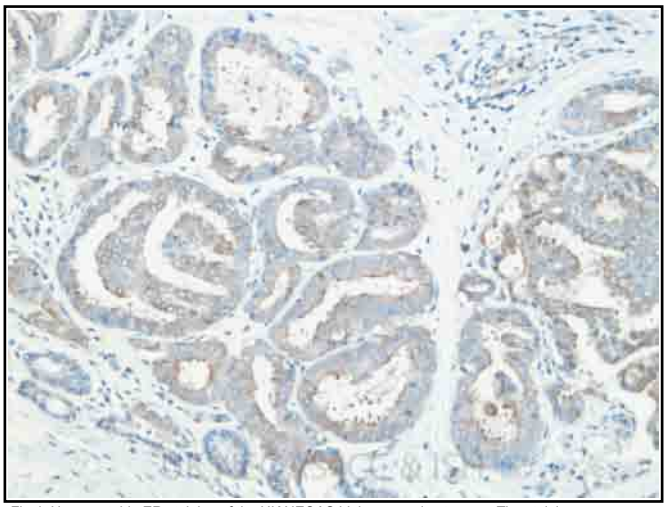


Fig 4. Unacceptable ER staining of the UK NEQAS high expressing tumour. The staining pattern is mostly cytoplasmic rather than the expected nuclear localisation. The assessment team questioned antibody contamination to be the cause of this inappropriate staining.

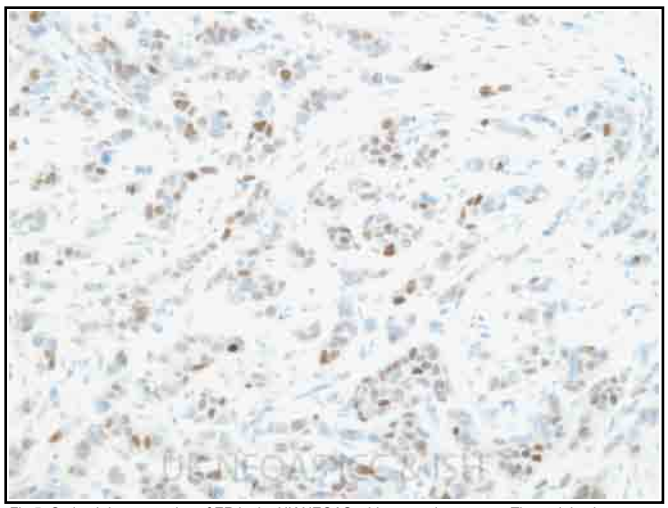


Fig 5. Optimal demonstration of ER in the UK NEQAS mid-expressing tumour. The staining is of varying intensity in over 60% of neoplastic cells. Staining was carried out using the Leica 6F11 antibody, 1:20, on the Bond III with antigen retrieval in ER1 for 30 minutes.

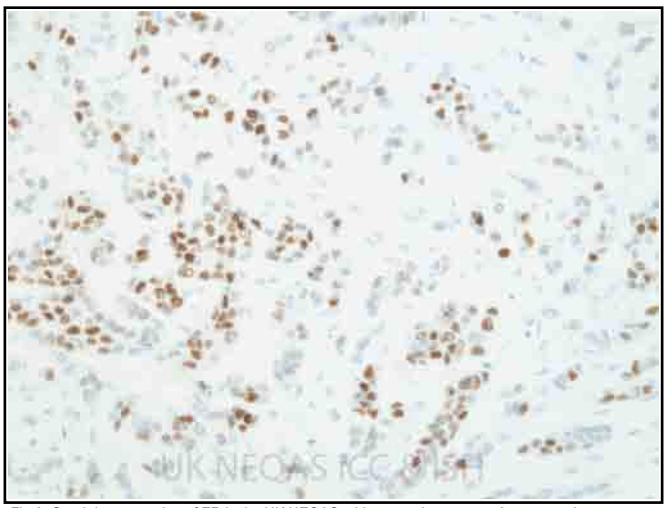


Fig 6. Good demonstration of ER in the UK NEQAS mid-expressing tumour. As expected over 60% of the neoplastic cells show weak to moderate, with a few strong nuclear staining, while the background remains clean. (Same protocol as Fig 2).

Selected Images showing Optimal and Sub-optimal {@stainingtext}

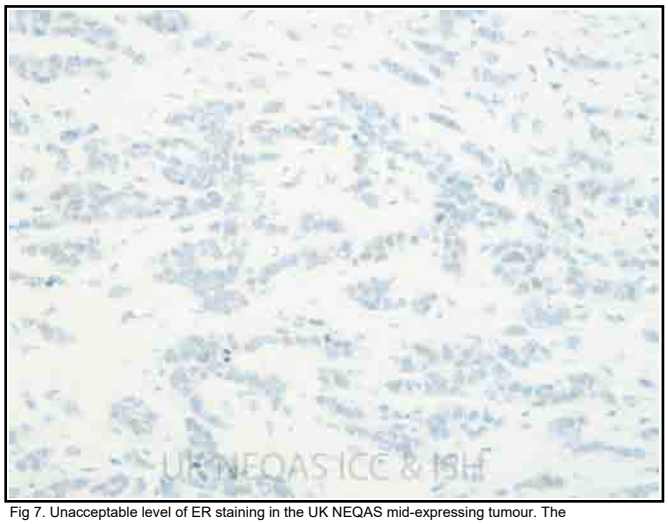


Fig 7. Unacceptable level of ER staining in the UK NEQAS mid-expressing tumour. The staining is far too weak, with many nuclei expected to stain not showing any positivity. Staining was carried out with the Dako EP1 antibody using a low pH retrieval protocol. The EP1 clone requires a high pH buffer retrieval to be used.

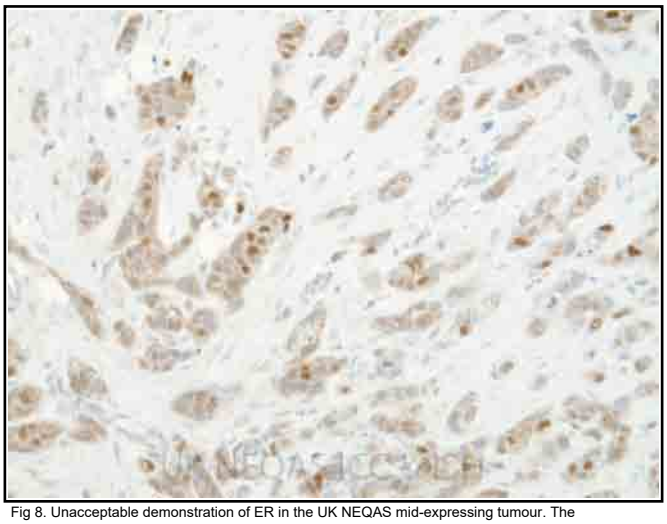


Fig 8. Unacceptable demonstration of ER in the UK NEQAS mid-expressing tumour. The example shows inappropriate cytoplasmic staining, causing the section to be uninterpretable. The section was stained with the Dako 1D5 antibody on the Autostainer with pre-treatment in the PT Link.

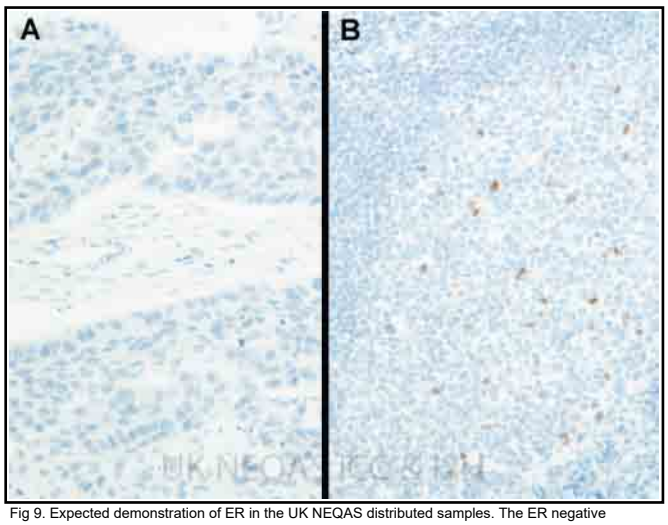


Fig 9. Expected demonstration of ER in the UK NEQAS distributed samples. The ER negative tumour (A) remains unstained, and as expected a small percentage of lymphocytes are staining positive in the tonsil section (B). (Same protocol as Fig 2).

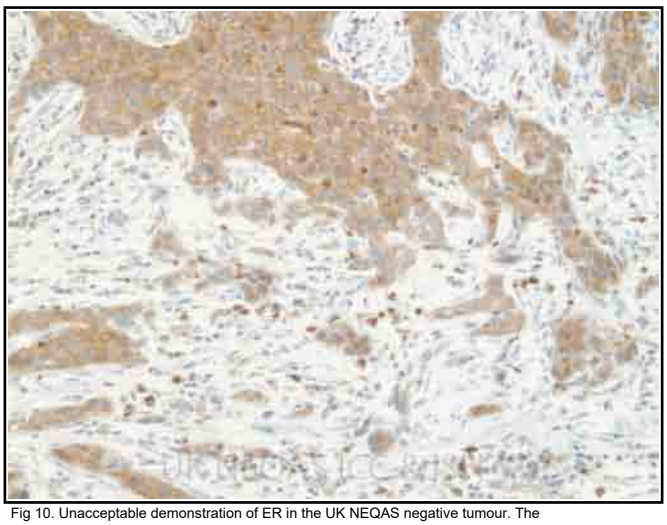


Fig 10. Unacceptable demonstration of ER in the UK NEQAS negative tumour. The inappropriate cytoplasmic staining is excessive, and therefore the section is uninterpretable. (Same protocol as Fig 8).

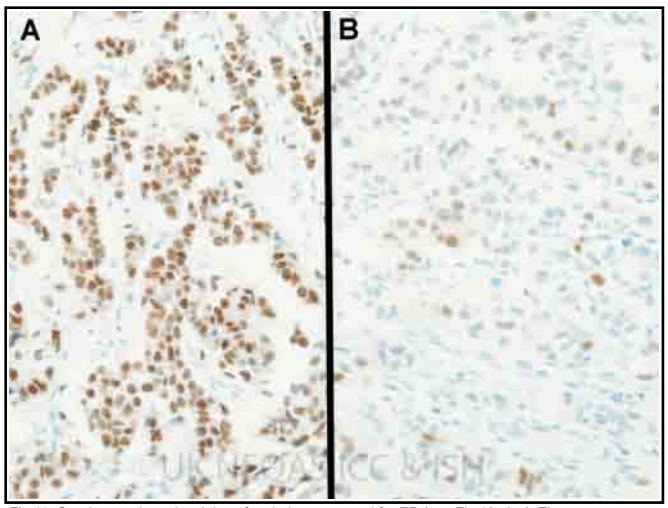


Fig 11. Good example and staining of an in-house control for ER (see Fig 12 also). The multi-block section contains high- and mid-expressing tumours shown in this image (A & B respectively). Stained using the Ventana SP1 pre-diluted antibody on the Benchmark XT, CC1 standard retrieval.

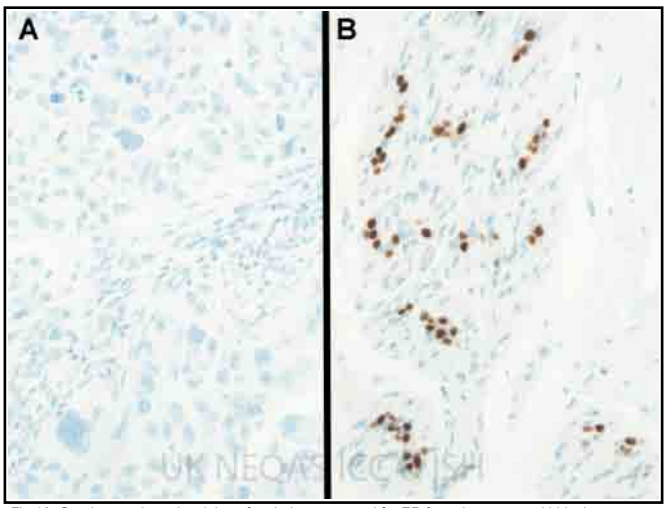
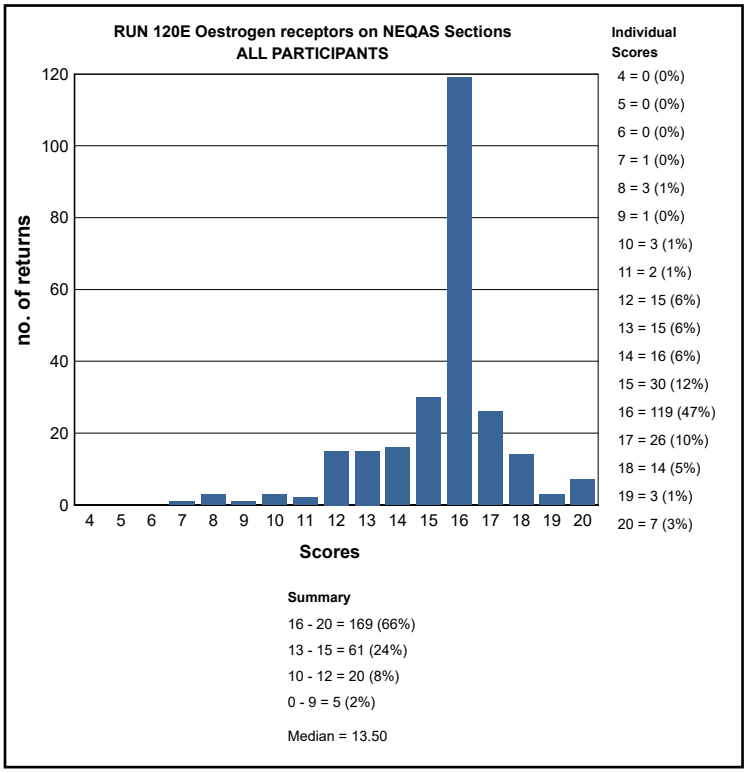
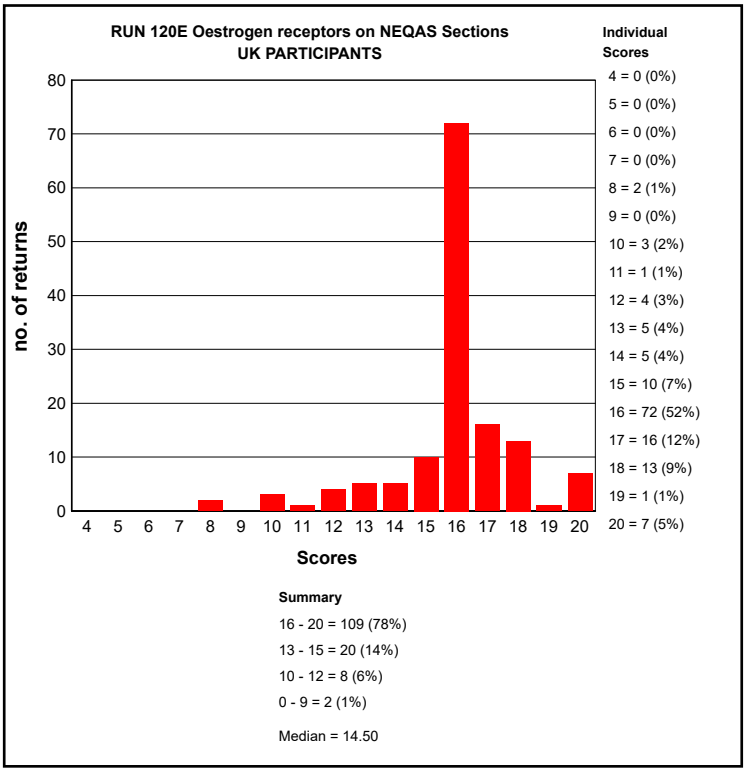
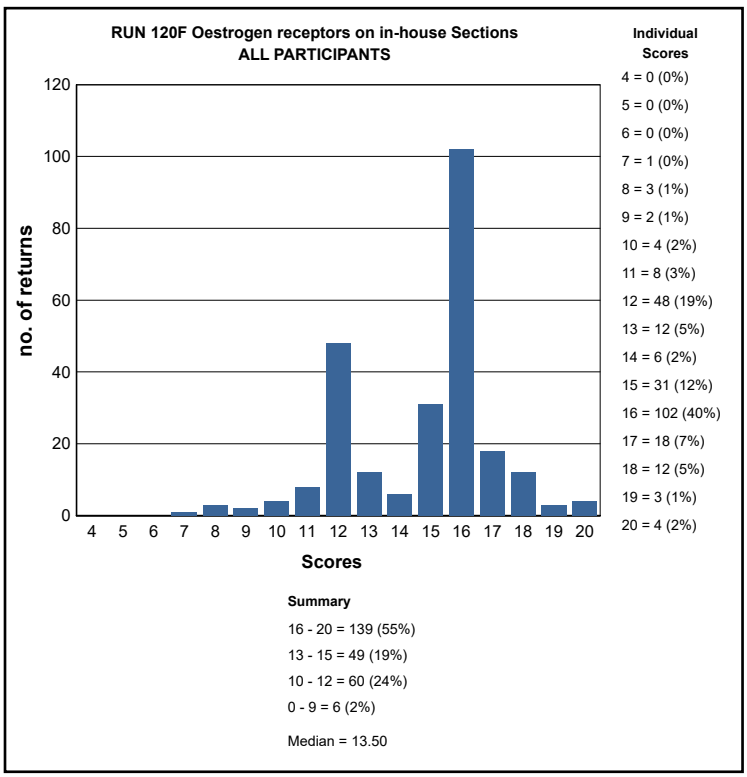


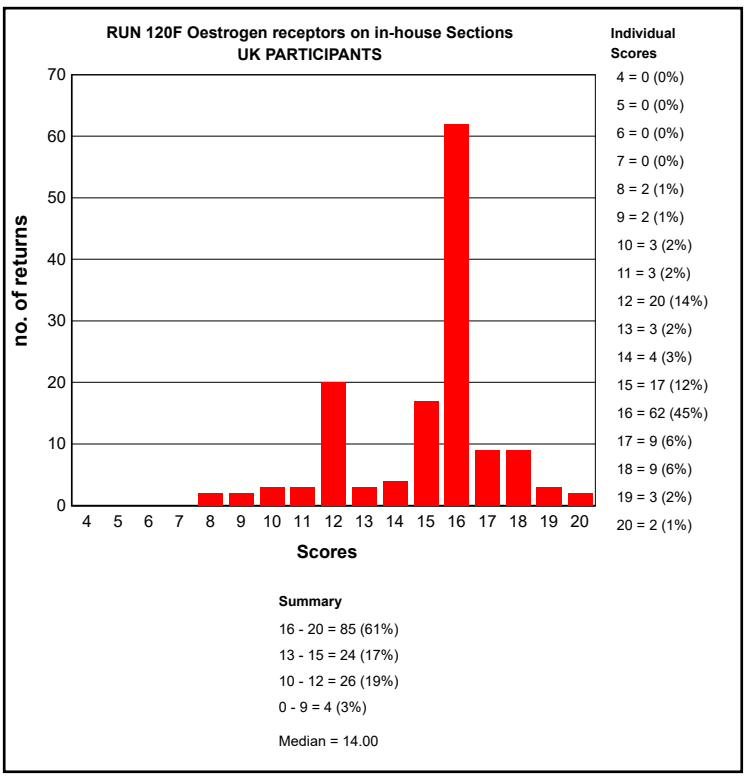
Fig 12. Good example and staining of an in-house control for ER from the same multi-block control shown in Fig 11. (A) negative expressing tumour, and (B) demonstrates ER expression in the normal glands, which act as an internal section control.

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: 120

Primary Antibody : Oestrogen receptors

Antibody Details	N	%
Dako M7047 ER (1D5)	1	100
Thermo Fisher/ Neomarkers RM 9101-S (SP1)	11	73
Leica/Novocastra NCL-ER-6F11 (6F11)	12	67
Vector VP-E613/4 (6F11)	1	0
Ventana 250- 2596 ER (6F11)	1	100
Ventana 760-2132 (6F11)	1	100
Ventana 790-4324 (SP1)	80	96
Leica Bond PA0151 (6F11)	4	50
Dako M3634 (SP1)	5	100
Dako RTU IR151 (SP1)	1	100
Ventana 790-4325 (SP1)	43	98
Leica/Novocastra NCL-L-ER- 6F11	20	80
Leica/Novocastra RTU-ER-6F11	1	100
Leica/Novocastra NCL-ER-6F11/2	9	89
Dako IS151 Dako Autostainer (SP1)	1	100
Dako SK310 pharmDX kit (Link) (1D5 + ER-2-123)	1	100
Dako (EP1) RTU FLEX IR084	16	94
Dako (EP1) M3643	25	92
Dako FLEX (1D5) IR/IS657	2	50
Other	5	100
Dako (EP1) RTU Auto Plus IS084	1	100
Cell Marque 249-R (SP1)	3	67

Breast Steroid Hormone Receptor Run: 120

Automation	Oestrogen receptors N	%
Dako Autostainer	1	0
Dako Autostainer Link 48	18	89
Dako Autostainer Plus Link	3	100
Dako Omnis	9	100
LabVision Autostainer	2	50
Leica Bond Max	16	81
Leica Bond-III	47	85
None (Manual)	3	67
Ventana Benchmark GX	7	100
Ventana Benchmark ULTRA	108	94
Ventana Benchmark XT	39	90

Breast Steroid Hormone Receptor Run: 120

Heat Mediated Retrieval	Oestrogen receptors N	%
Biocare Decloaking Chamber	1	100
Dako Omnis	9	100
Dako PTLink	19	89
Lab vision PT Module	2	50
Leica ER1 20 mins	11	91
Leica ER1 30 mins	14	86
Leica ER1 40 mins	7	43
Leica ER2 10 mins	2	100
Leica ER2 20 mins	26	88
Leica ER2 30 mins	2	100
Microwave	1	0
Other	2	100
Pressure Cooker	1	100
Ventana CC1 16mins	1	100
Ventana CC1 20mins	1	100
Ventana CC1 24mins	4	100
Ventana CC1 32mins	7	57
Ventana CC1 36mins	23	96
Ventana CC1 40mins	3	100
Ventana CC1 48mins	2	50
Ventana CC1 52mins	8	100
Ventana CC1 56mins	2	100
Ventana CC1 64mins	55	98
Ventana CC1 76mins	2	50
Ventana CC1 88mins	1	100
Ventana CC1 8mins	1	100
Ventana CC1 extended	4	100
Ventana CC1 mild	14	100
Ventana CC1 standard	25	92
Ventana CC2 mild	1	0
Water bath 95-98 OC	1	0

Breast Steroid Hormone Receptor Run: 120

Enzyme Mediated Retrieval	Oestrogen receptors N	%
AS PER KIT	8	75
NOT APPLICABLE	169	92

Breast Steroid Hormone Receptor Run: 120

Detection	Oestrogen receptors N	%
AS PER KIT	12	83
BioGenex SS Polymer (QD 430-XAKE)	1	0
Dako EnVision FLEX (K8000/10)	5	100
Dako EnVision FLEX+ (K8002/12)	16	88
Dako Envision HRP/DAB (K5007)	1	100
Dako REAL HRP/DAB (K5001)	1	0
LabVision UltraVision LP HRP (TL 125 HLJ)	1	0
Leica Bond Polymer Refine (DS9800)	57	86
None	1	100
NOT APPLICABLE	2	100
Other	3	67
Ventana iView system (760-091)	3	100
Ventana OptiView (760-700) + Amp. (7/860-099)	1	100
Ventana OptiView Kit (760-700)	19	84
Ventana UltraView Kit (760-500)	126	96

Breast Steroid Hormone Receptor Run: 120

Chromogen	Oestrogen receptors N	%
AS PER KIT	18	89
BioGenex liquid DBA (HK-124-7K)	1	0
Dako DAB K3468	1	0
DAKO DAB+	1	100
Dako EnVision Plus kits	3	100
Dako FLEX DAB	21	90
Dako REAL EnVision K5007 DAB	2	100
Dako REAL K5001 DAB	1	0
LabVision (TA-125-HD)	1	100
Leica Bond Polymer Refine kit (DS9800)	57	86
Other	3	100
Ventana DAB	14	86
Ventana iview	3	100
Ventana Ultraview DAB	127	94

BEST METHODS

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

Oestrogen receptors - Method 1

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

Primary Antibody: Dako (EP1) M3643 , 15 Mins Dilution 1: 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)

Oestrogen receptors - Method 2

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

Primary Antibody: Dako (EP1) M3643 , 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 17/20 (UK NEQAS Slide) and 16/20 (In House slide) using this method.

Primary Antibody: Leica/Novocastra NCL-ER-6F11 (6F11) , 12 Mins, 24 ºC Dilution 1: 40
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)

Oestrogen receptors - Method 4

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

Primary Antibody:	Ventana 790-4324 (SP1) , 60 Mins
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 standard
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

The Breast HER2 ICC Module Run 120

Suzanne Parry

Antigen Assessed:	HER2
Sections Circulated:	4 breast cancer cell lines of varying levels of HER2 expression.
Number of Registered Participants:	243
Number of Participants this Run	215 (88%)

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 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 staining	Faint barely perceptible incomplete membrane staining in >10% of cells
D: 0	No staining in the '0' control cell line	No staining in the '0' control cell line

Validation of Distributed UK NEQAS ICC & ISH Cell Lines

IHC Validation using: Leica Oracle, Ventana Pathway 4B5 and Dako HercepTest

Updated Assessment and Scoring Procedure

UK NEQAS Specific Membrane Scoring Algorithm:

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

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

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

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

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

Score and Interpretation	Interpretation
16-20/20: Excellent	Overall the staining is at the expected level for each of the samples.
13-15/20: Acceptable	Some slight technical issues noted by some of the assessors, but overall the staining is suitable for interpretation
10-12/20: Borderline	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 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. UK NEQAS will also accept commercially prepared cell lines to demonstrate the varying HER2 expression levels when used alongside at least one piece of the participants' own in-house tissue sample. While commercial cell lines can be very useful on-slide controls to gauge the varying HER2 expression levels and ensure the test has worked, it is also important for laboratories and UK NEQAS to see that the pre-analytic factors are compatible with the laboratory HER2 method.

Assessment Summary:

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

	Pass Rates Run 120:	
	NEQAS	**In-House**
Acceptable	75% (N=162)	69% (N=145)
Borderline	17% (N=36)	27% (N=57)
Unacceptable	8% (N=17)	4% (N=8)

The weak 1+ membrane staining in the UK NEQAS cell lines which the assessors observed in the previous Run 119, was not as prevalent on this current Run 120. The majority of participants where able to demonstrate some form of weak, incomplete membrane staining within the adjacent cells in the 1+ cell line. Damaged cell morphology on the 3+ cell line was observed in a handful of laboratories, even when using a standardised assay. One conclusion was that some participants may be placing their slides directly onto a flat hotplate after they have cut their in-house controls onto the slide. The direct heat can damage the delicate membranes, and also causing them to lift from the slide. UK NEQAS recommends to directly follow the instructions for cutting and drying of in-house controls onto the bottom of the distributed slides.

Excessive or weak counterstain was noted on some of the participants' slides. This can make interpretation difficult, particularly on cell lines. If the counterstain is too strong, it can mask the membrane staining. Conversely, if the counterstain is too weak, the 1+ and 0 negative cell lines in particular, are difficult to detect. Also, when there is weak counterstain, any background or cytoplasmic blush can obscure the true membrane staining.

As with previous assessment runs the most popular antibody assay was the Ventana 4B5, used by 76% (n=164) of participants and showed an overall acceptable pass rate of 87%. 9 laboratories employed the Dako HercepTest, with 67% of participants achieving an acceptable pass rate. 15 laboratories are using the Leica Oracle assay kit with an acceptable pass rate of 53%. 21 laboratories used laboratory devised tests (LDTs), incorporating a variety of antibodies (most commonly the Dako polyclonal, A0485), and with a selection of pre-treatment methods and staining platforms. These laboratories showed an acceptable pass rate of 19%. The vast difference in pass rates between those laboratories using a standardised assay (83%) versus those using LDTs (19%) highlights our recommendation to employ a standardised HER2 assay if possible. All data is based on the methodology information submitted by participants, but unfortunately not all methodology was provided by all participants.

References

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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.
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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 assays.

Selected Images showing Optimal and Sub-optimal immunostaining

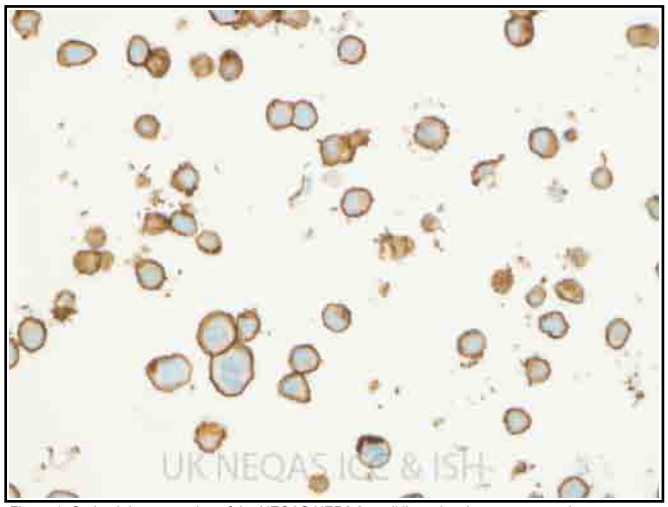


Figure 1: Optimal demonstration of the NEQAS HER2 3+ cell line, showing strong complete membrane staining. The sections was stained using the Ventana 4B5 assay on the Benchmark ULTRA, CC1 for 36 minutes.

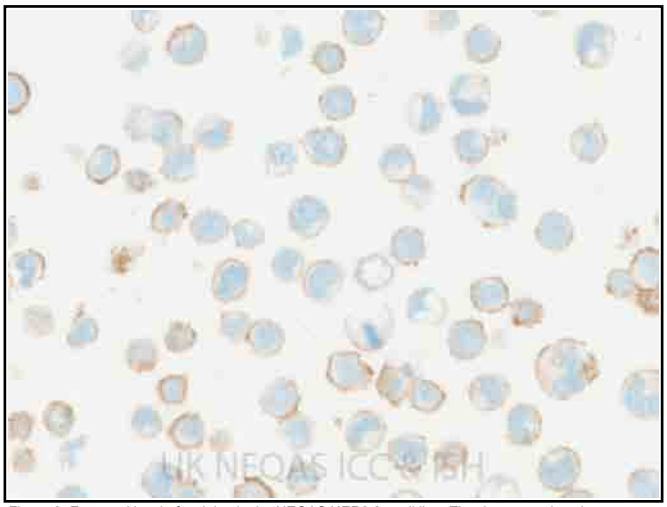


Figure 2: Expected level of staining in the NEQAS HER2 2+ cell line. The demonstration shows weak to moderate complete membrane staining, which is specific and clear of background staining. Stained using the Ventana 4B5 assay on the Benchmark XT, CC1 mild antigen retrieval.

Figure 3: Good demonstration of the NEQAS 1+ cell line. The example shows the distinctive brush border staining with specific delicate partial membrane staining between the cells. (Same protocol as Fig 1).

Figure 4: Expected demonstration of the negative NEQAS cell line which is free of any non-specific staining. (Same protocol as Fig 1).

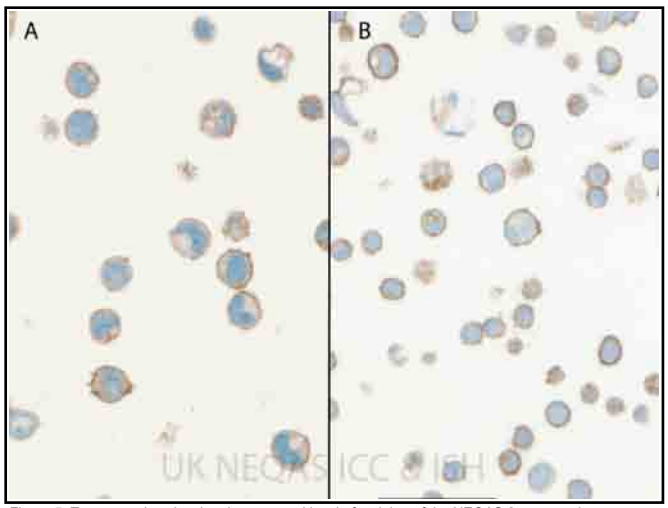


Figure 5: Two examples showing the expected level of staining of the NEQAS 2+ expressing cell line. Both sections show complete moderate intensity membrane staining. (A) stained with the Leica Oracle assay, and (B) stained with the Dako HerCepTest.

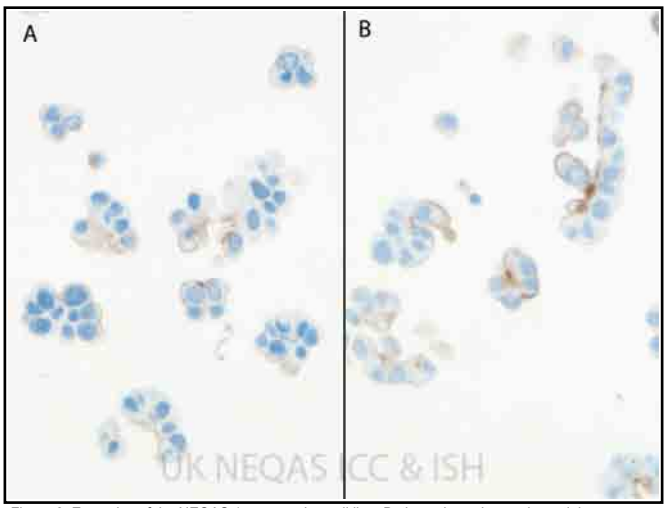


Figure 6: Examples of the NEQAS 1+ expressing cell line. Both sections show only partial membrane staining. (A) stained with the Leica Oracle assay, and (B) stained with the Dako HerCepTest.

Selected Images showing Optimal and Sub-optimal {@stainingtext}

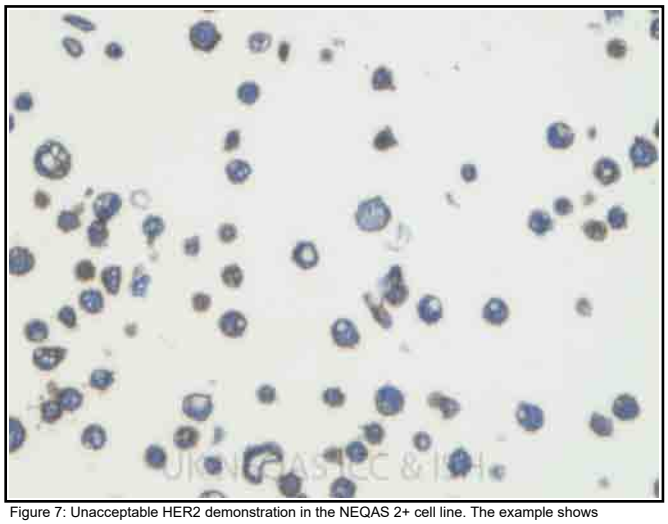


Figure 7: Unacceptable HER2 demonstration in the NEQAS 2+ cell line. The example shows morphology damage and excessive counterstain, and therefore the membranes are uninterpretable. The damage is caused by excessive antigen retrieval: Stained with the HercepTest, with pH9 retrieval buffer. pH 6 retrieval is recommended for this assay.

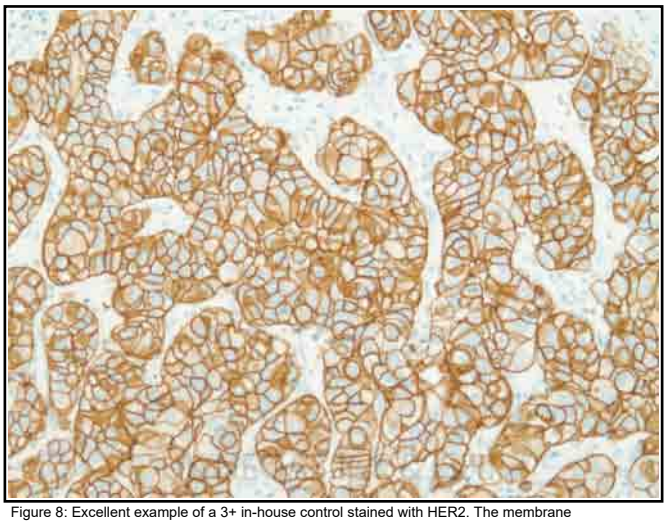


Figure 8: Excellent example of a 3+ in-house control stained with HER2. The membrane staining in the tumour is strong and well-localised, while the background stroma remains clean. Stained with the Ventana 4B4 assay.

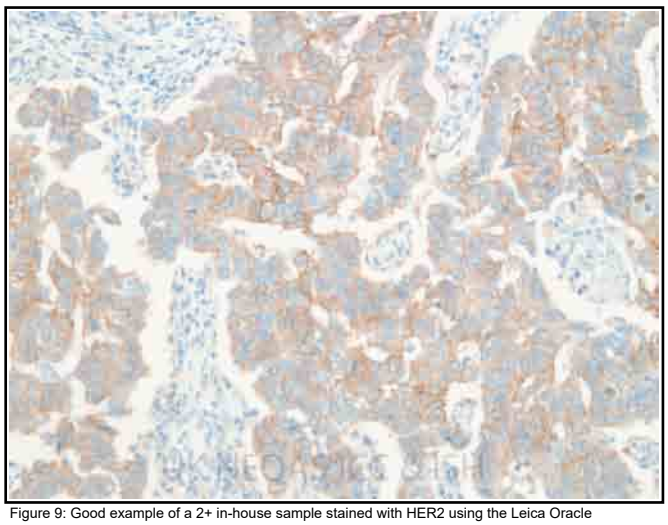


Figure 9: Good example of a 2+ in-house sample stained with HER2 using the Leica Oracle assay. The sample shows some focal areas of 3+ staining, however, using the current guidelines was considered to be of 2+ expression level.

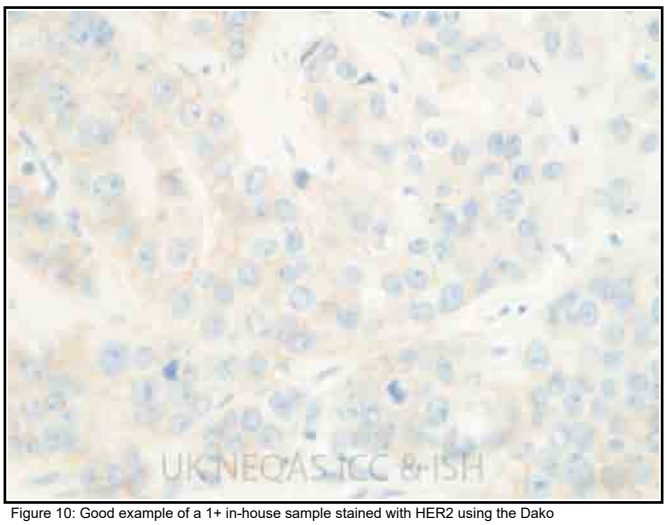
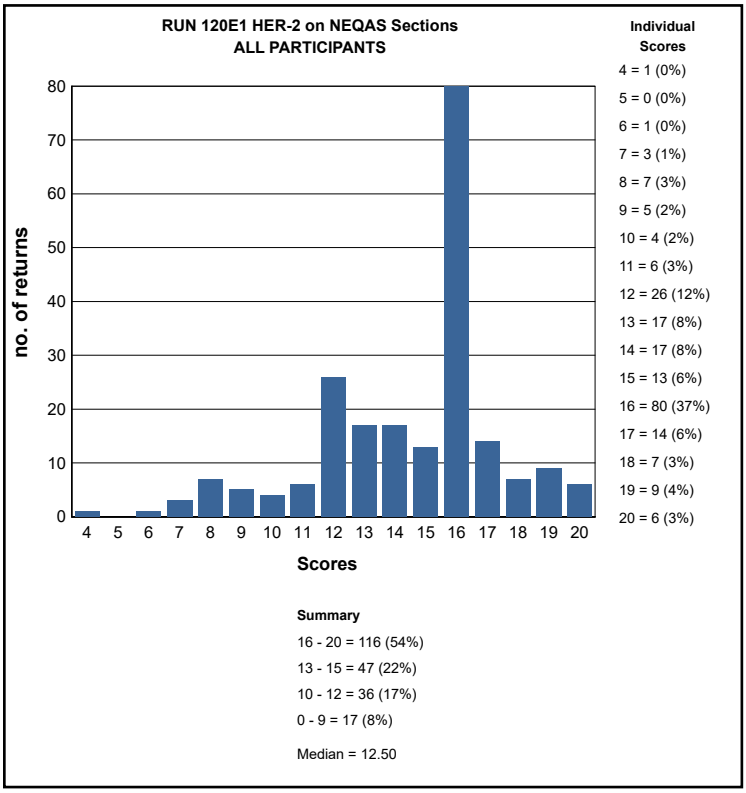
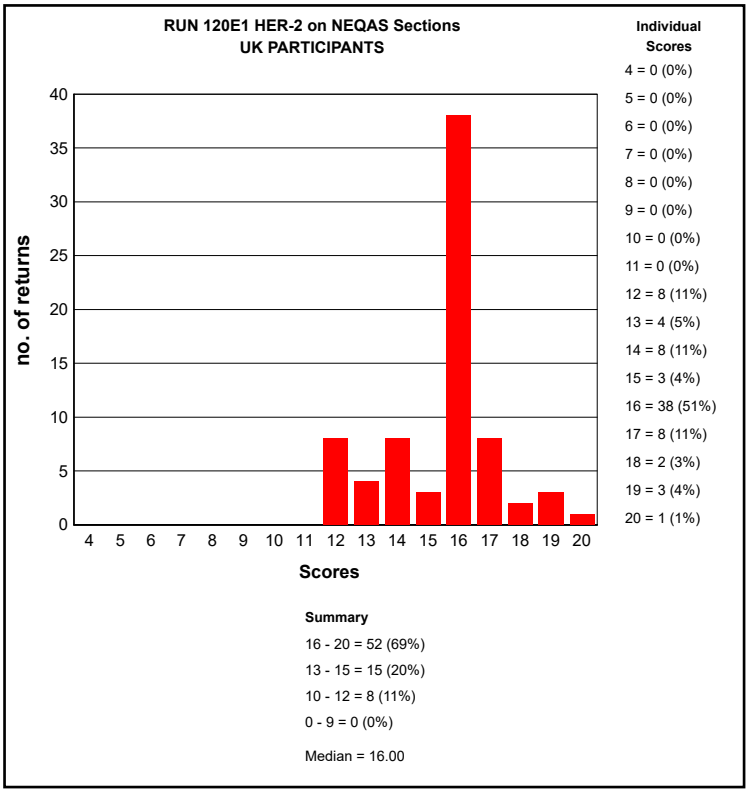
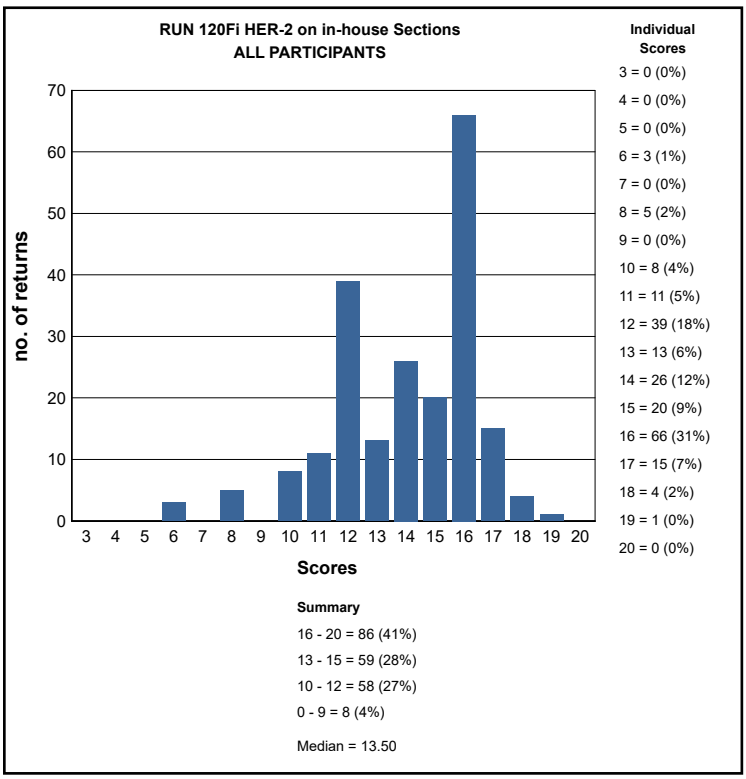


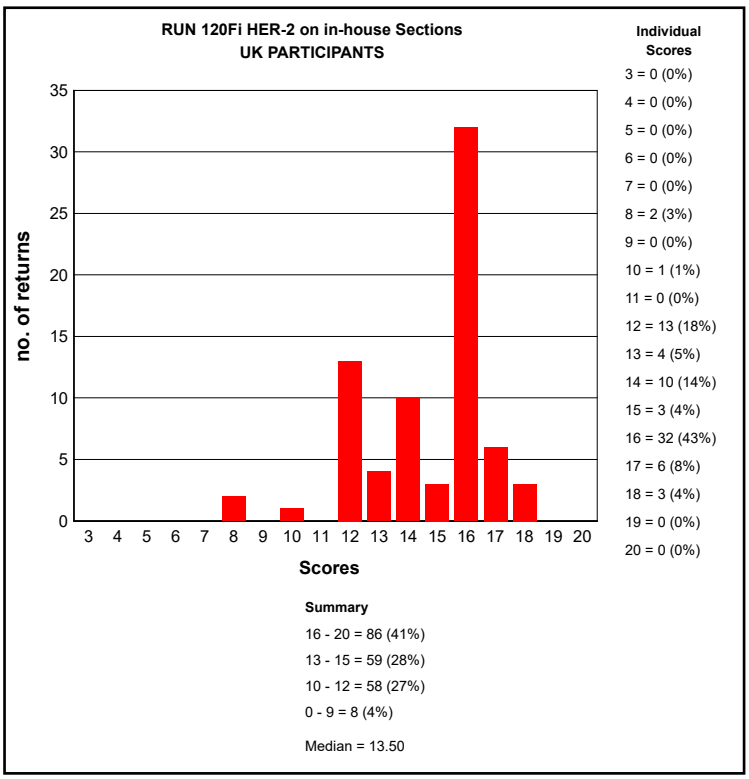
Figure 10: Good example of a 1+ in-house sample stained with HER2 using the Dako HerCepTest.

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: 120

Primary Antibody	N	%
Dako HercepTest K5204 (poly)	2	0
Dako HercepTest K5205 (poly)	1	0
Dako HercepTest K5207 (poly)	1	100
Dako A0485 C-erB-2 (poly)	15	27
Neomarkers MS-730-P	1	0
Ventana Pathway 790-100 (4B5)	5	100
Leica Oracle HER2 Bond IHC (CB11)	15	53
Dako Link HercepTest SK001 (poly)	5	100
BioGenex (EP1045Y) rb mono	1	0
Ventana Confirm 790-4493 (4B5)	39	74
Ventana Pathway 790-2991 (4B5)	121	90
Novocastra NCL-L-CB11 (CB11)	2	0
Other	2	0

Breast HER2 ICC Run: 120

Automation	N	%
Dako Autostainer	1	0
Dako Autostainer Link 48	9	67
Dako Autostainer Plus Link	3	67
Dako Omnis	1	0
LabVision Autostainer	1	0
Leica Bond Max	10	10
Leica Bond-III	16	69
None (Manual)	2	0
Other	1	0
Ventana Benchmark GX	7	86
Ventana Benchmark ULTRA	110	86
Ventana Benchmark XT	49	82

Breast HER2 ICC Run: 120

Heat Mediated Retrieval	N	%
Biocare Decloaking Chamber	1	0
Dako Omnis	2	0
Dako PTLink	12	58
Lab vision PT Module	1	0
Leica ER1 10 mins	1	100
Leica ER1 20 mins	6	0
Leica ER1 25 mins	14	64
Leica ER1 30 mins	2	0
Leica ER2 30 mins	1	100
Microwave	1	0
Other	2	50
Ventana CC1 16mins	2	100
Ventana CC1 20mins	1	100
Ventana CC1 24mins	2	100
Ventana CC1 32mins	11	91
Ventana CC1 36mins	60	88
Ventana CC1 40mins	1	100
Ventana CC1 48mins	1	0
Ventana CC1 52mins	3	100
Ventana CC1 56mins	4	75
Ventana CC1 64mins	11	91
Ventana CC1 76mins	1	0
Ventana CC1 8mins	3	100
Ventana CC1 mild	56	84
Ventana CC1 standard	10	60

Breast HER2 ICC Run: 120

Detection	N	%
AS PER KIT	19	63
BioGenex SS Polymer (QD 420-YIKE)	1	0
Dako HerCep Test (K5204)	1	0
Dako EnVision FLEX+ (K8002/12)	6	33
Dako Envision HRP/DAB (K5007)	1	0
Dako HerCep Test Autor (SK001)	3	100
LabVision UltraVision LP HRP (TL 125 HLJ)	1	0
Leica Bond Polymer Refine (DS9800)	12	42
Ventana iView system (760-091)	5	100
Ventana OptiView Kit (760-700)	11	82
Ventana UltraView Kit (760-500)	144	84

Breast HER2 ICC Run: 120

Enzyme Retrieval	N	%
AS PER KIT	8	63
NOT APPLICABLE	122	77
Ventana Protease	1	100

Breast HER2 ICC Run: 120

Chromogen	N	%
AS PER KIT	33	76
BioGenex liquid DBA (HK-124-7K)	1	0
DAKO DAB+	2	50
Dako DAB+ Liquid (K3468)	1	0
Dako FLEX DAB	8	50
Dako REAL EnVision K5007 DAB	2	0
LabVision (TA-125-HD)	1	0
Leica Bond Polymer Refine kit (DS9800)	11	45
Other	2	100
Ventana DAB	7	86
Ventana iview	2	100
Ventana Ultraview DAB	140	83

BEST METHODS

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

HER-2 - Method 1

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

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

HER-2 - Method 2

Participant scored 16/20 (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
Detection:	AS PER KIT Prediluted

HER-2 - Method 3

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

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

HER-2 - Method 4

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

Primary Antibody:	Ventana Pathway 790-2991 (4B5) , 4 Mins, 37 ºC
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.6
HMAR:	Ventana CC1 mild, PH: 8.5
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB, 37 ºC., Time 1: 8 Mins, Time 2: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC

The Gastric HER2 ICC Module Run 120

Suzanne Parry

Antigen Assessed:	**HER2 for IHC**
The NEQAS distributed sections consisted of a composite slide containing 4 surgical /resections of intestinal gastric carcinoma:	The expected expression levels of each tissue sample were: **A.** 3+ **B**. 2+ or 1+ depending on the block or serial section received **C**. 1+ or 2+ depending on the block or serial section received **D**. 0
Number of Registered Participants	66
Number of Participants this Run	62 (94%)

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

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

Validation of Distributed Samples

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

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

Table 2: HER2 IHC staining and & ISH results

Section Label From slide Label End	Staining Pattern with IHC	HER2 status by ISH
A	**3+**	Amplified
B	**2+**	Amplified
C	**2/1+**	Amplified
D	**0**	Non-amplified

Assessment Procedure

Assessment is carried out around a multi-header microscope with each slide being assessed by 4 independent assessors and a microscope lead. Each of the NEQAS samples (A-D) (as well as in-house samples) are individually assessed. Each assessor provides a membrane interpretation (see table 3), and then an individual score out of 5 (see table 4) based on interpretability of the membrane staining and technical quality. The four assessors scores are then combined to give an overall score out of 20 (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.
0 (negative)	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: (Figures 1, 2, 3 & 4)

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

Features Of Suboptimal or Unacceptable Staining: (Figures 5 & 6)

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

Additional Comment:

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

Pass Rates

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

NEQAS Pass Rates Run 120:	
Acceptable	70% (N=43)
Borderline	13% (N=8)
Unacceptable	18% (N=11)

The pass rate for this module has remained at a reasonably constant level for the past number of assessment Runs. However, 11 laboratories (18%) obtained an unacceptable result due to weak staining. In the clinical setting, false negative results may lead to eligible patients not being put forward for Herceptin therapy. Equally concerning, was the overstaining by 2 laboratories that also failed the assessment. Again, in a clinical setting, this could lead to a patient being 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 laboratories (88%) are using the Ventana 4B5 clone IHC assay, and with the recommended standardised protocols for their particular automated systems; CC1 mild antigen retrieval on the Ventana XT, or 36 minutes antigen retrieval with CC1 on the Ventana ULTRA machines. The Ventana 4B5 users showed an average acceptable pass rate of 85%. 3 laboratories used the Dako Hercept test, which showed an acceptable pass rate of 67%.

There are a few laboratories, however, not using the recommended protocols for their respective assays, which may have the potential to induce weaker or stronger membrane staining than expected. A few laboratories are also using lab-devised techniques (LDTs). Such techniques require much more stringent initial validation when introducing the test to the laboratory. While all assays and protocols require continual monitoring and quality checks, LDTs, will have many more variables to verify and quality control.

All participating laboratories also submitted in-house control material for assessment. The results for these are summarised below:

In-House Pass Rates Run 120:	
Acceptable	76% (N=47)
Borderline	23% (N=14)
Unacceptable	2% (N=1)

The overall pass rates on the in-house controls were higher than on the NEQAS samples, with more laboratories receiving borderline passes rather than a fail. The majority of these borderline passes were given because the laboratories did not submit ideal composite control material, consisting of 3+, 2+ and 1+/0 HER2 expressing gastric/breast control tumour samples. These laboratories were therefore given a maximum score of 12/20. Other laboratories were marked down for poor tissue quality or fixation. Another consideration was that what laboratories had interpreted to be a 2+ control was not actually a true 2+ as interpreted by the expert assessment team. Laboratories were given then advised to include a 2+ control and given feedback to review their interpretation. Recommendations for control tissue are described below.

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 laboratories 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

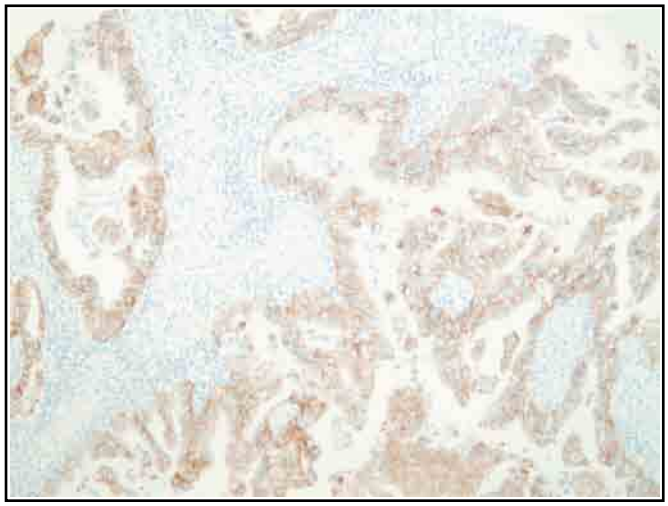


Figure 1. Excellent example of expected HER2 3+ staining of the UK NEQAS distributed gastric tumour sample A, showing the expected level of membrane staining X10. Ventana Pathway (4B5), Ventana CC1 mild, Ventana Benchmark XT.

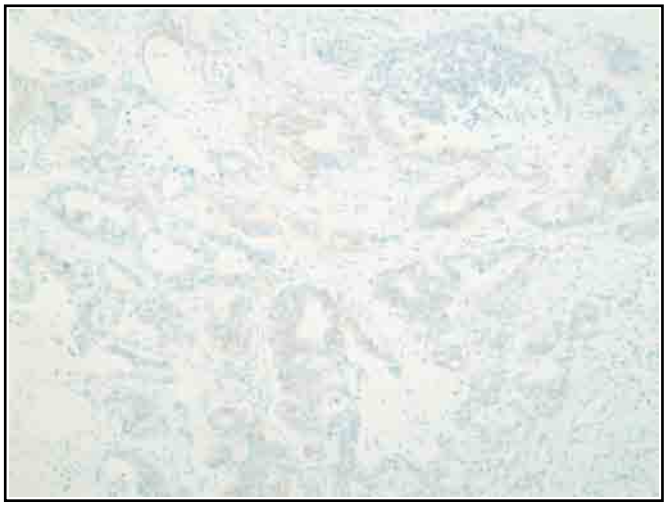


Figure 2. Expected HER2 2+ staining of the UK NEQAS distributed gastric tumour sample B, showing the expected level of membrane staining X10. Ventana Pathway (4B5), Ventana CC1 mild, Ventana Benchmark XT.

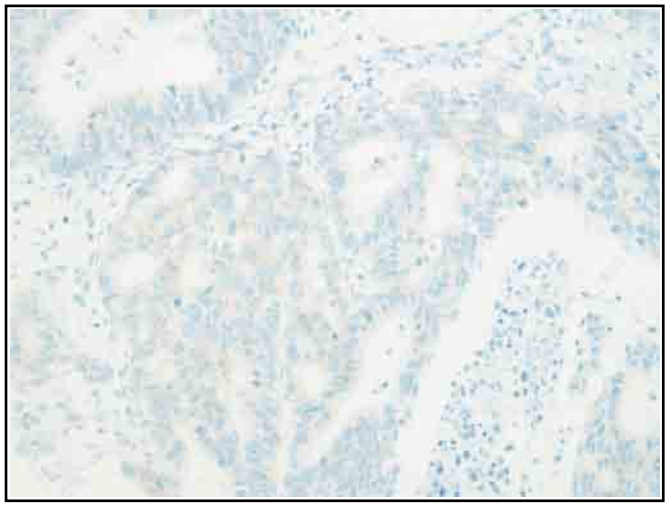


Figure 3. Expected HER2 1+ staining of the UK NEQAS distributed gastric tumour sample C, showing the expected level of membrane staining X20. Ventana Pathway (4B5), Ventana CC1 mild, Ventana Benchmark XT.

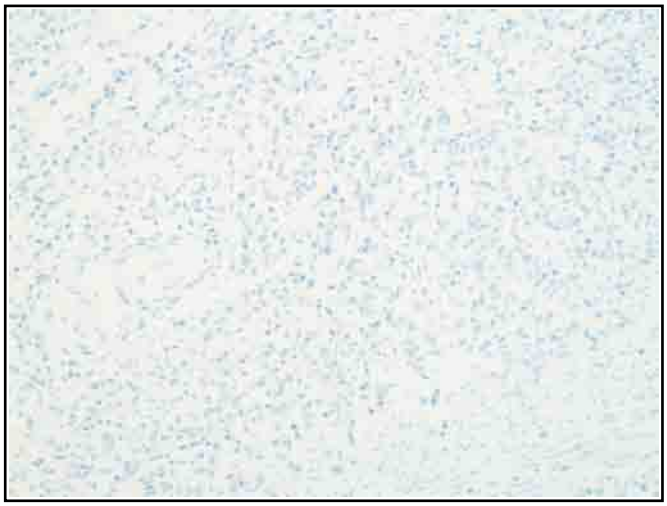


Figure 4. Expected HER2 0 staining of the UK NEQAS distributed gastric tumour sample D, showing the expected level of complete absence of membrane staining X40. Ventana Pathway (4B5), Ventana CC1 mild, Ventana Benchmark XT.

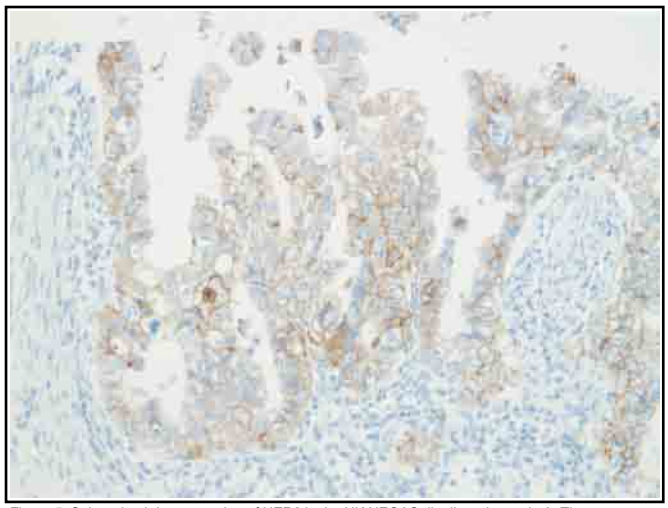


Figure 5. Sub-optimal demonstration of HER2 in the UK NEQAS distributed sample A. The expected level of staining should be 3+, but the staining in the example is much weaker than is acceptable.

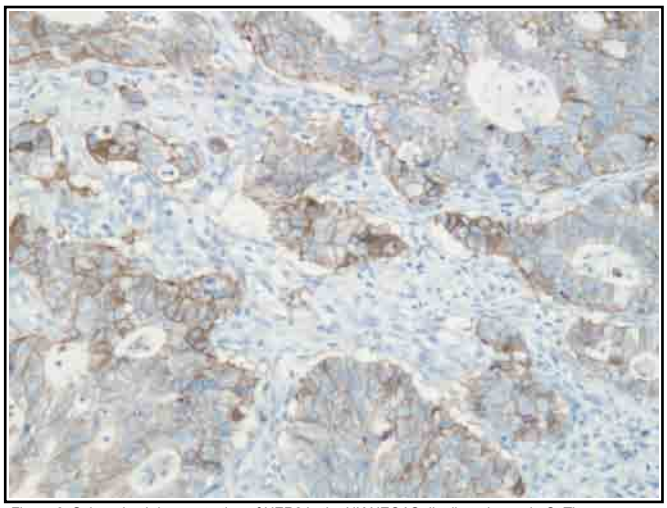
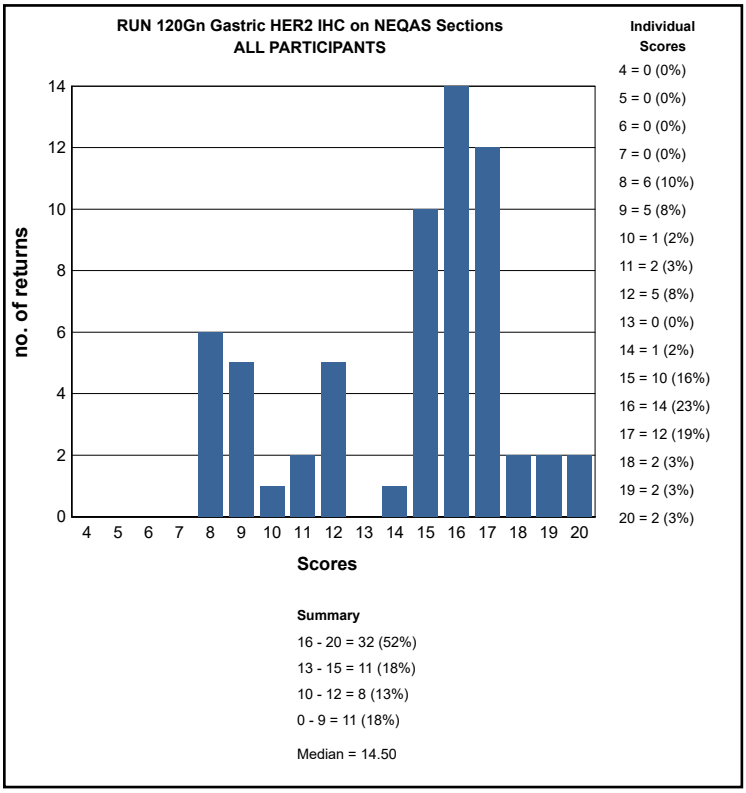
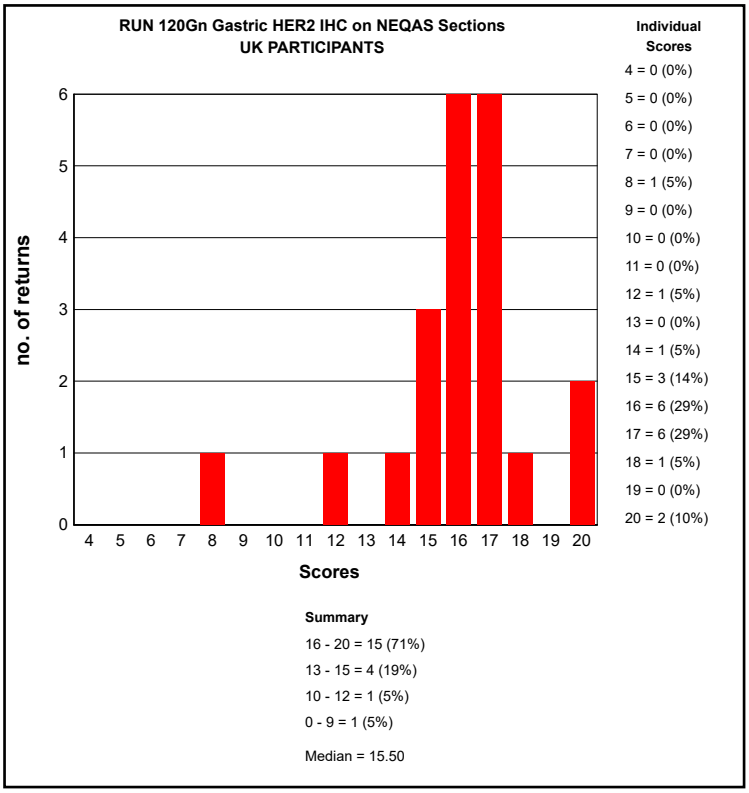
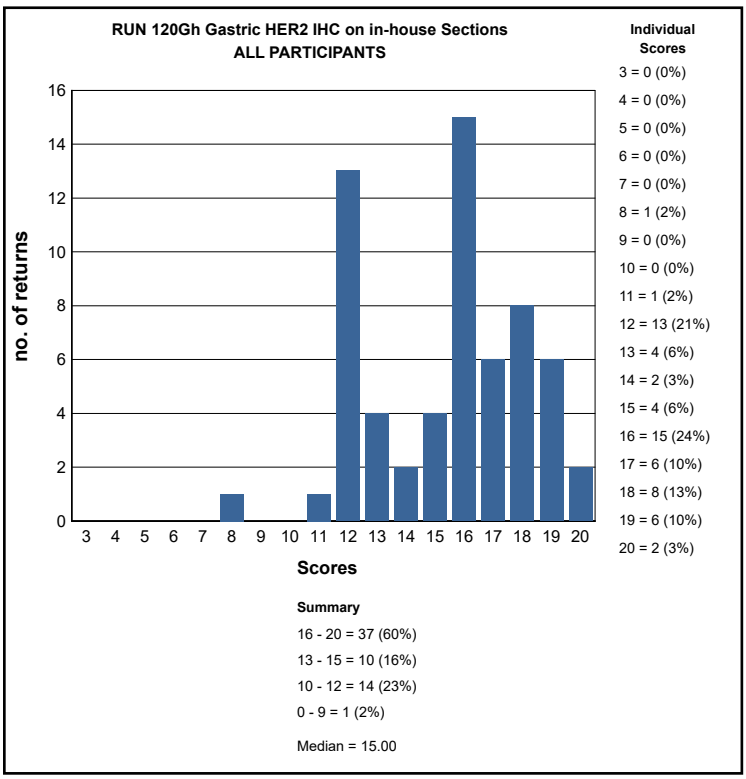


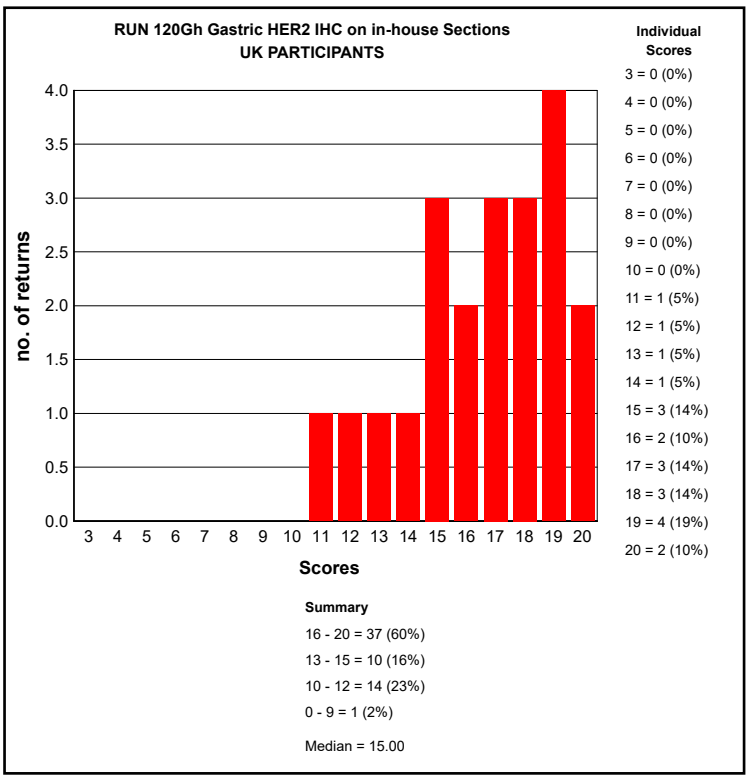
Figure 6. Sub-optimal demonstration of HER2 in the UK NEQAS distributed sample C. The expected level of staining should be 1+, but the staining in the example is considerably stronger than is acceptable. Immunologic (RM9103-S), Lab vision PT Module (Citrate buffer pH6.7), Teecan Freedom Evoware.

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: 120

Primary Antibody	N	%
Dako A0485 C-erB-2 (poly)	2	50
Dako Link HercepTest SK001 (poly)	2	100
Ventana Pathway 790-100 (4B5)	2	100
Ventana Pathway 790-2991 (4B5)	39	74
Other	1	0
Ventana Confirm 790-4493 (4B5)	15	60

Gastric HER2 ICC Run: 120

Automation	N	%
Dako Autostainer Link 48	1	100
Dako Autostainer Plus Link	1	100
Dako Omnis	2	50
Other	1	0
Ventana Benchmark GX	2	100
Ventana Benchmark ULTRA	34	68
Ventana Benchmark XT	20	75

Gastric HER2 ICC Run: 120

Heat Mediated Retrieval	N	%
Dako Omnis	2	50
Dako PTLink	2	100
Lab vision PT Module	1	0
Ventana CC1 16mins	2	50
Ventana CC1 24mins	1	0
Ventana CC1 32mins	7	71
Ventana CC1 36mins	15	73
Ventana CC1 52mins	2	50
Ventana CC1 56mins	2	100
Ventana CC1 64mins	5	100
Ventana CC1 mild	16	63
Ventana CC1 standard	6	83

Gastric HER2 ICC Run: 120

Detection	N	%
AS PER KIT	5	80
Dako EnVision FLEX (K8000/10)	1	0
Dako EnVision FLEX+ (K8002/12)	1	100
Dako HerCep Test Autor (SK001)	1	100
Power Vision DPVB999 HRP	1	0
Ventana iView system (760-091)	1	100
Ventana OptiView Kit (760-700)	5	40
Ventana UltraView Kit (760-500)	45	73

Gastric HER2 ICC Run: 120

Enzyme Retrieval	N	%
AS PER KIT	3	67
NOT APPLICABLE	31	71

Gastric HER2 ICC Run: 120

Chromogen	N	%
AS PER KIT	10	100
Dako DAB+ Liquid (K3468)	1	0
Dako FLEX DAB	3	67
Other	1	100
Ventana DAB	3	33
Ventana iview	1	100
Ventana Ultraview DAB	42	67

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 19/20 (In House slide) using this method.

Primary Antibody:	Ventana Pathway 790-2991 (4B5) , 16 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 mild
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

Gastric HER2 IHC - Method 2

Participant scored 16/20 (UK NEQAS Slide) and 18/20 (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 Prediluted

Gastric HER2 IHC - Method 3

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

Primary Antibody:	Ventana Confirm 790-4493 (4B5)
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 36mins
EAR:	
Chromogen:	AS PER KIT
Detection:	AS PER KIT

David Blythe and Suzanne Parry

	Gold Standard	Second Antibody
Antigens Assessed:	BCL-6	PAX-5
Tissue Sections circulated:	Diffuse large B-cell lymphoma (LN) and reactive tonsil	Diffuse large B-cell lymphoma (LN) and reactive tonsil
Number of Registered Participants:	207	
Number of Participants this Run	202 (98%)	

Introduction

Gold Standard: BCL-6

BCL-6 (B-Cell CLL/Lymphoma 6, zinc finger protein 51) is a 95 kDa zinc-finger transcription factor. It is required for germinal centre formation and is involved in the differentiation of normal germinal centre B-cells. BCL-6 acts as a sequence-specific repressor of transcription and is a suppressor of p53 expression[1]. As an immunohistochemical marker, BCL-6 is used alongside a panel of other lymphoma markers to determine lymphomas of B-cell lymphoma, Burkitt's lymphoma and lymphocyte predominant Hodgkin's lymphoma[2,3,4]. BCL-6 expression is absent in acute lymphatic leukaemia and mantle cell lymphoma.

Features of Optimal Immunostaining (Figures 1, 3 & 4):

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

Features of Sub-optimal Immunostaining (Figures 2, 5 & 6):

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

References:

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

Second Antigen: PAX-5

PAX5 is a B-cell-specific activator protein (BSAP). BSAP is also known as paired box protein 5 (PAX5), a transcription factor expressed on B-cells. BSAP is a member of the *PAX* gene family which encodes transcriptions factors that are significantly involved in B-cell development, differentiation, cell migration and proliferation. PAX5 is expressed as a nuclear marker in B-lineage cells. It is expressed across the differentiation range of B-cells, from precursor B cells to early plasma cells (Jensen *et al*., 2007). The utility of PAX5 immunohistochemistry (IHC) is primarily used to aid in the diagnosis of undifferentiated malignant neoplasms. PAX5 is expressed in the majority of mature and precursor B-cell non-Hodgkin lymphoma/ leukaemia cases, and classic Hodgkin lymphoma cases (Reed-Sternberg cells express PAX5). However, PAX5 is not expressed in multiple myeloma and solitary plasmacytomas, making it very useful in such differentiation (Desouki *et al*., 2010).

References:

1. Desouki, M., Post, G., Cherry, D. and Lazarchick, J. (2010). PAX-5: A Valuable Immunohistochemical Marker in the Differential Diagnosis of Lymphoid Neoplasms. *Clinical Medicine & Research*, 8(2), pp.84-88.
2. Jensen, K., Higgins, J., Montgomery, K., Kaygusuz, G., van de Rijn, M. and Natkunam, Y. (2007). The utility of PAX5 immunohistochemistry in the diagnosis of undifferentiated malignant neoplasms. *Modern Pathology*, 20(8), pp.871-877.

Assessment Summary:

BCL-6

202 laboratories submitted slides for the BCL-6 assessment, and all but 1 laboratory submitted their in-house control sections for this run. The results are summarised in table on following page, with 86% of participants achieving an acceptable result for their NEQAS submission, 10% received a borderline score with only 4% achieving unacceptable. The in-house results were similar. The reason for failure was either due to weak/very weak staining in the normal lymphocytes and tumour cells. Comments for the in-house material noted varying levels of background staining. The most popular clones used in this run were Ventana (1GI191E/A8), Leica (LN22) and Dako (PG-B6p), and used by 57, 48 and 48 of participants respectively. The acceptable pass rate of the in-house was 83% compared to the NEQAS of 86%. 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 control that measures the sensitivity of your assay such as a tonsil and a positive tumour control (i.e. DLBCL).

NEQAS and In-house Pass Rates Run 120 BCL-6		
	NEQAS	In-house
Acceptable	86% (N=173)	83% (N= 167)
Borderline	10% (N=21)	15% (N=31)
Unacceptable	4% (N=8)	1% (N=3)

PAX-5

191 laboratories submitted both the NEQAS and in house sections for this assessment. The pass rates were 83% for the NEQAS sections and 90% for the in-house sections. The Borderline rates were 14% on the NEQAS section and 8% on the in-house sections. The unacceptable rates were 3% on the NEQAS sections and 2% on the in-house sections (summarised in the table below).

NEQAS and In-house Pass Rates Run 120 PAX-5		
	NEQAS	In-house
Acceptable	83% (N=158)	90% (N= 171)
Borderline	14% (N=27)	8% (N=16)
Unacceptable	3% (N=6)	2% (N=4)

It was noted that the predominant reason for laboratories receiving a borderline pass or fail, was due to weak or very weak demonstration PAX-5 in the B-cells, or for poor localisation. The most commonly used antibody clone for PAX-5 was the SP34, sourced from different suppliers, such as Invitrogen, Ventana and CellMarque. This was used by 56 participants, and showed an acceptable pass rate of 88%. Several laboratories did not stock PAX-5, and were therefore asked to stain with CD79a instead.

Selected Images showing Optimal and Sub-optimal immunostaining

Figure 1: Optimal example of BCL-6 on the NEQAS tonsil, showing strong to moderate nuclear staining in the germinal centre B-cells. Stained with the Dako PG-B6p antibody, 1:20, on the Omnis, with 30 minutes antigen retrieval.

Figure 2: Suboptimal demonstration of the BCL-6 antigen on the NEQAS tonsil (compare to Fig 1). The germinal centre B-cells show very weak staining which may be due to insufficient antigen retrieval using the water bath method. The Cell Marque GI191E/A8 antibody was used at a dilution of 1:100.

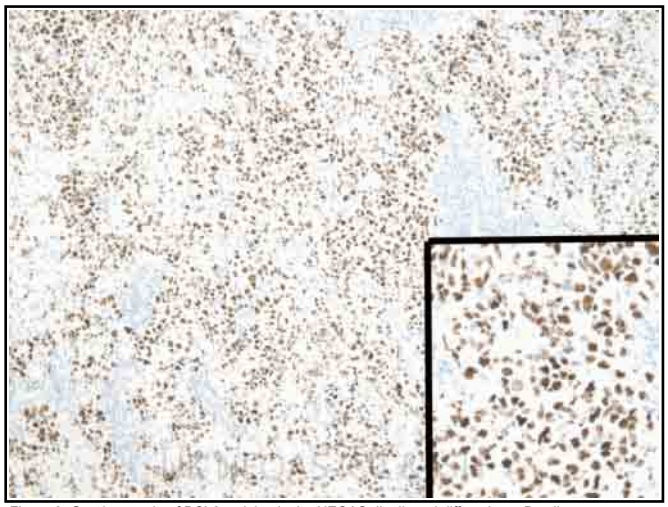


Figure 3: Good example of BCL6 staining in the NEQAS distributed diffuse large B-cell lymphoma (DLBCL). The neoplastic cells show distinct nuclear staining. (Same protocol as Fig 1).

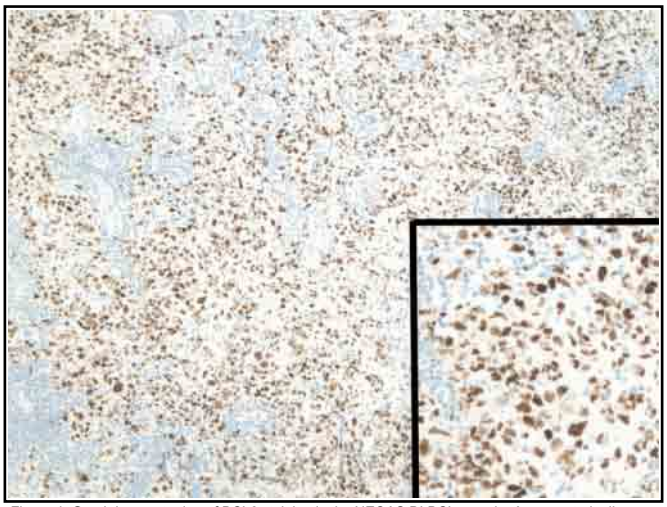


Figure 4: Good demonstration of BCL6 staining in the NEQAS DLBCL sample. As expected, all neoplastic cells show a strong nuclear reaction, while the background remains clean. Stained with the Leica LN22 antibody, 1:100, on the Bond III with ER2 antigen retrieval for 20 minutes.

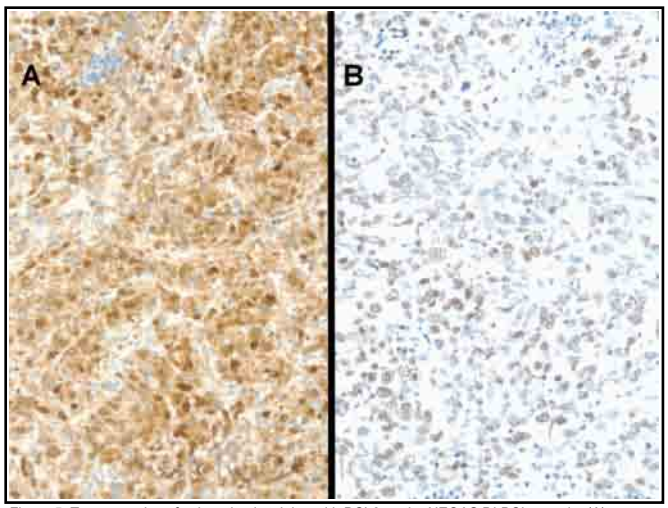


Figure 5: Two examples of sub-optimal staining with BCL6 on the NEQAS DLBCL sample. (A) shows diffuse and background staining. (B) shows a very weak staining pattern.

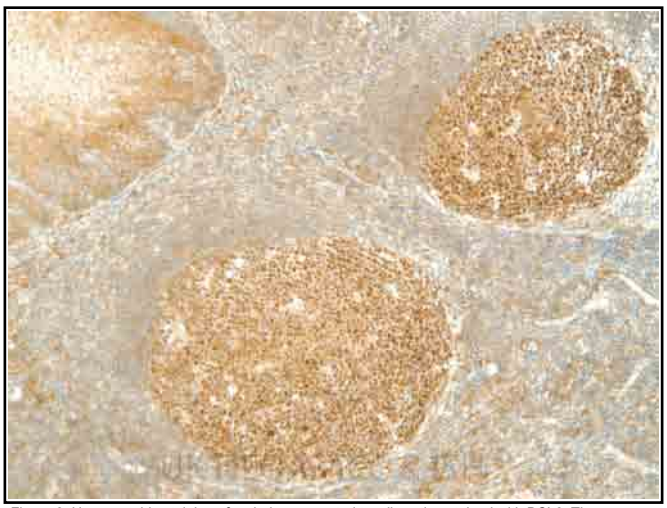


Figure 6: Unacceptable staining of an in-house control tonsil section stained with BCL6. The staining is diffuse and shows excessive background. Same protocol as Fig 5A, using the Cell Marque GI191E/A8 pre-diluted antibody on the Bond Max with ER1 pre-treatment for 10 minutes).

Selected Images showing Optimal and Sub-optimal {@stainingtext}

Figure 7: Excellent demonstration of PAX-5 in the NEQAS tonsil. Even at low power the image clearly shows the strong nuclear staining pattern around the mantle zone and within the germinal centre B-cells. Stained with the Ventana SP34 pre-diluted antibody on the Benchmark ULTRA, CC1 for 64 minutes, and Ultraview detection.

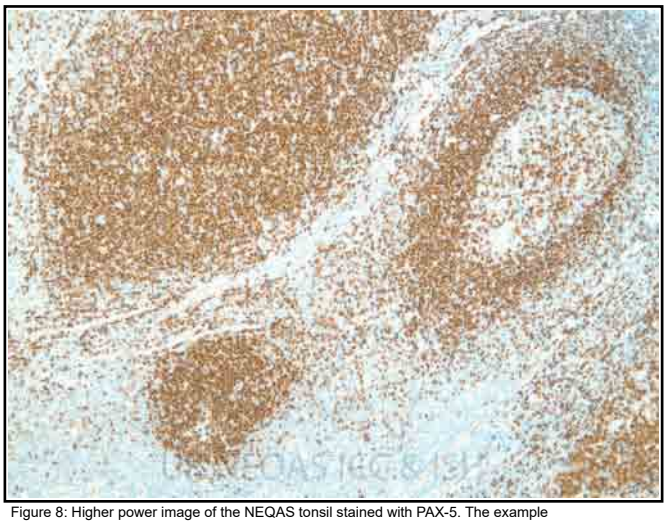


Figure 8: Higher power image of the NEQAS tonsil stained with PAX-5. The example demonstrates the strong nuclear staining and weaker cytoplasmic staining, with a good localisation to the mantle zone and germinal centre B-cells. (Same protocol as Fig 7).

Figure 9: Weak demonstration of the NEQAS tonsil stained with PAX-5. Stained with the Thermo Shandon antibody, 1:25, on the Benchmark ULTRA, CC1 for 32 minutes. Increasing the antigen retrieval time may help to improve the staining.

Figure 10: Excellent demonstration of PAX-5 in the NEQAS DLBCL sample. All the neoplastic cells show strong nuclear staining while the background remains clean. (Same protocol as Fig 7).

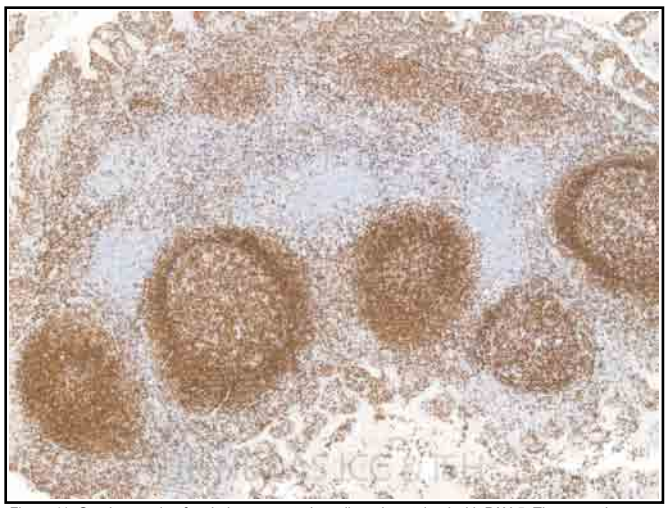
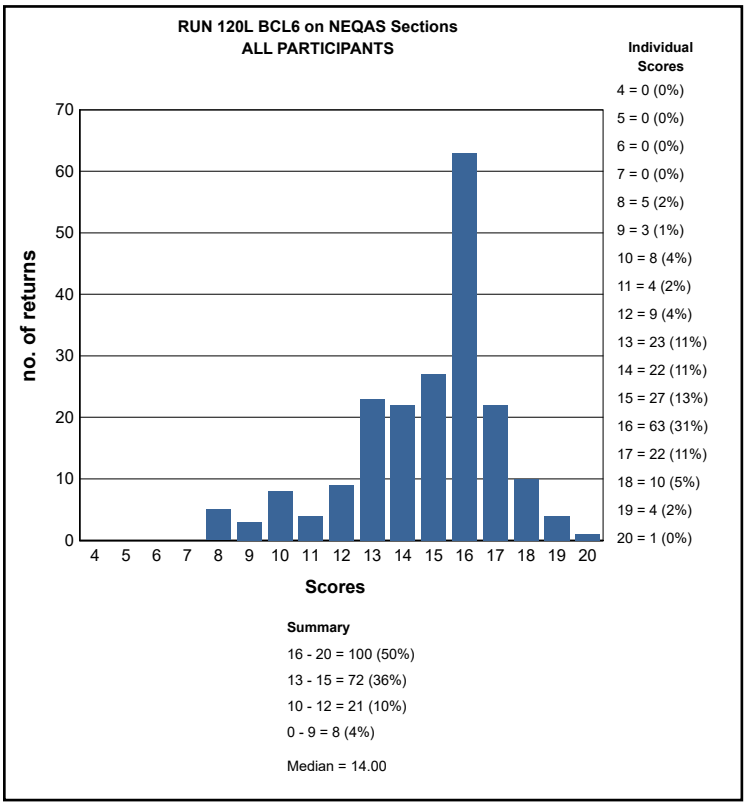
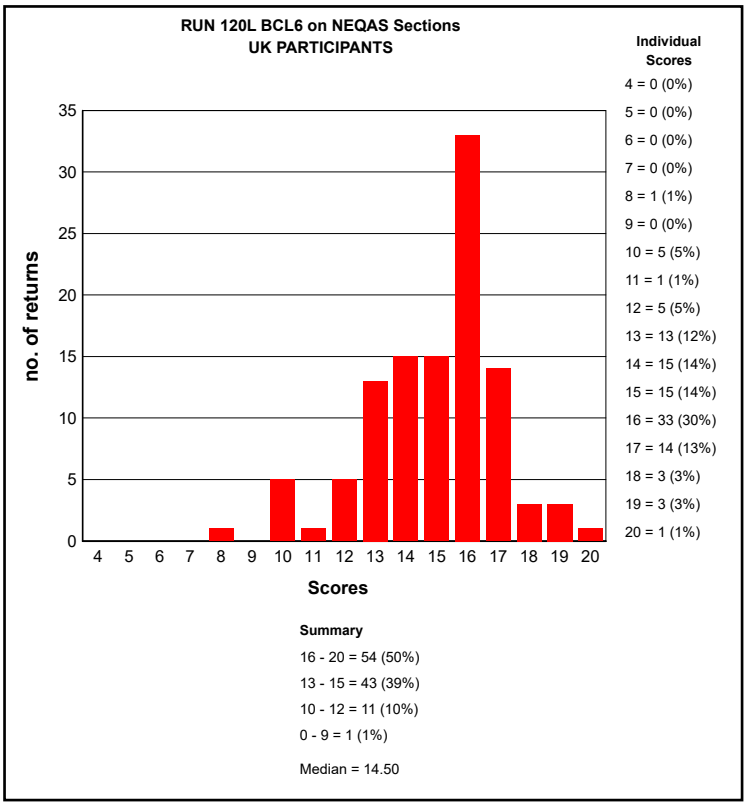


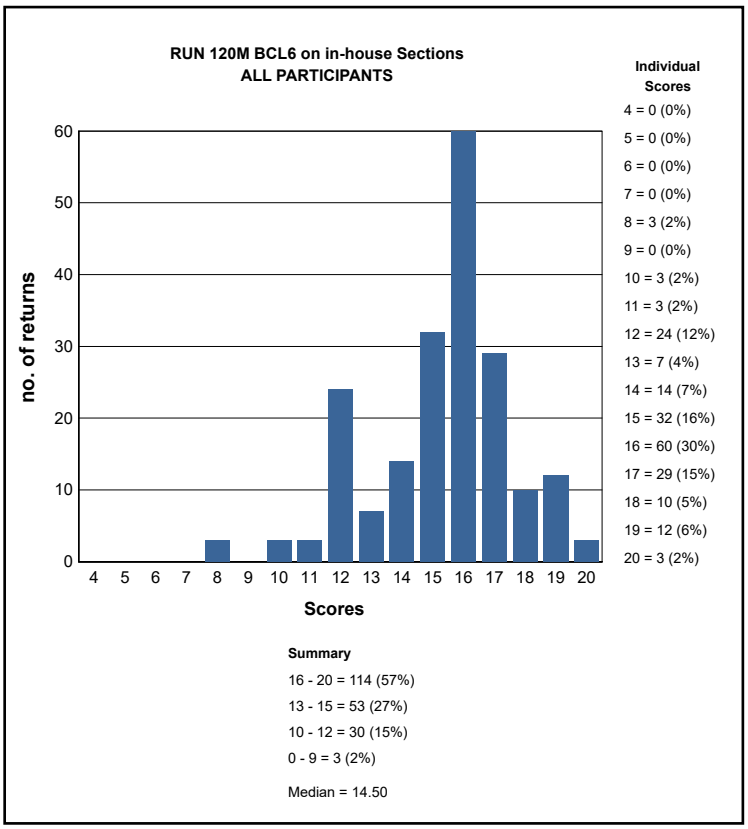
Figure 11: Good example of an in-house control tonsil section stained with PAX-5. The example shows strong and well-localised staining of the B-cells. Stained with the Leica RTU 1EW antibody on the Bond III, ER2 for 30 minutes retrieval.

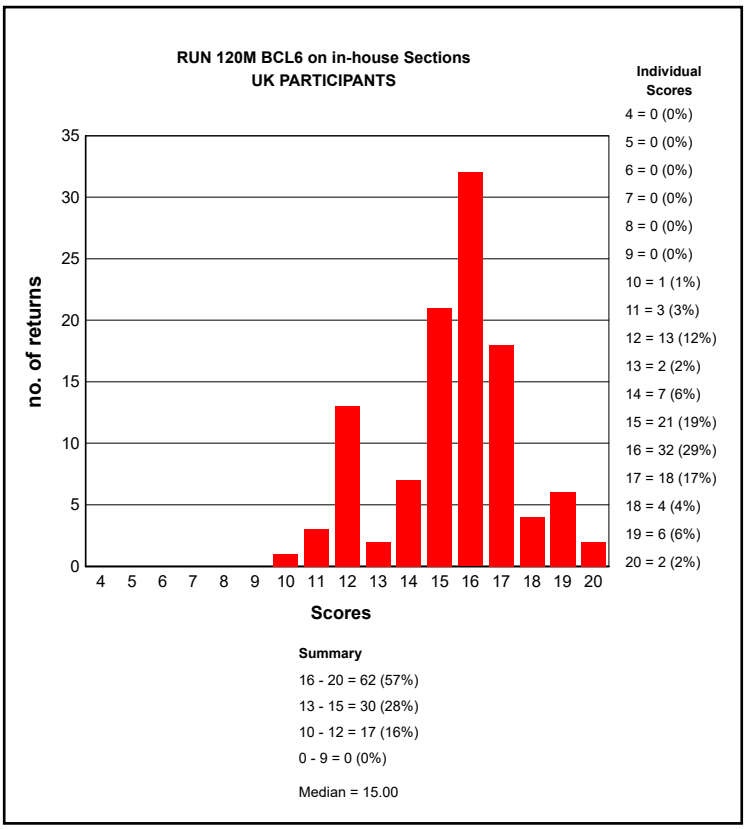
Figure 12. Good demonstration of the CD79a antigen on the NEQAS tonsil sample. Laboratories were offered to stain with CD79a if they do not routinely use the requested marker. The example shows strong membranous staining of the mantle zone B-cells, and a moderate stain in the germinal centre B-cells.

GRAPHICAL REPRESENTATION OF PASS RATES

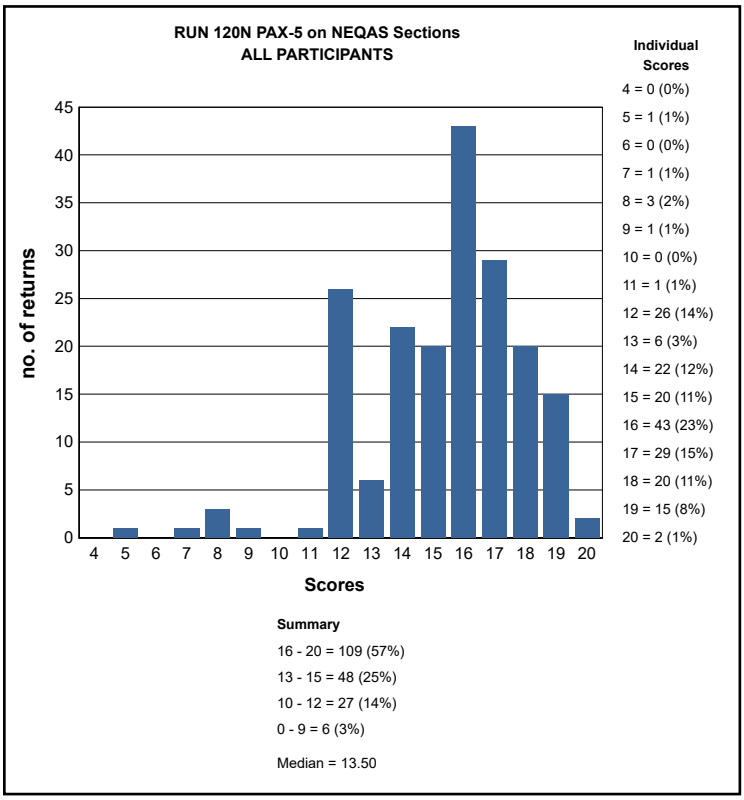


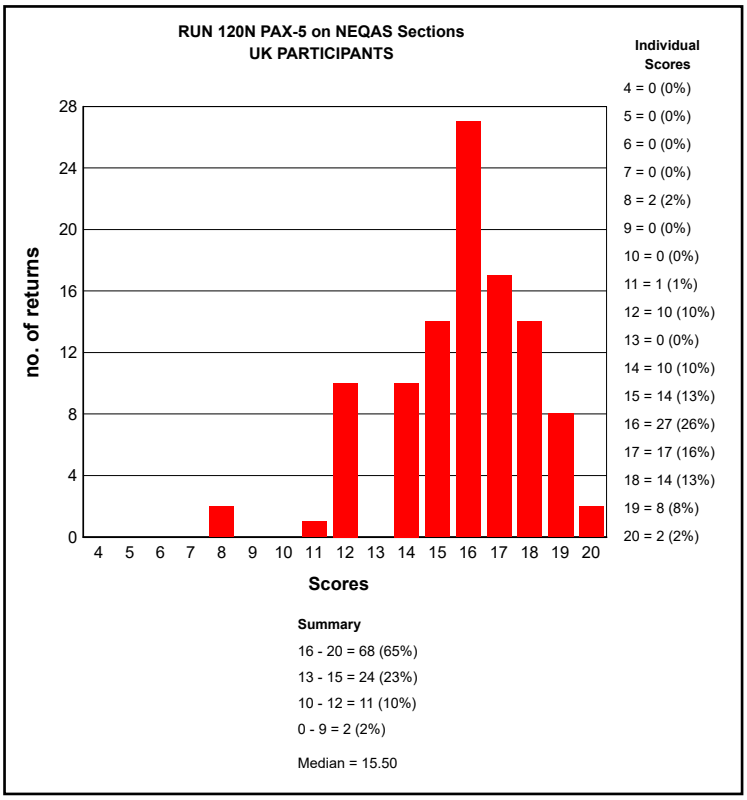


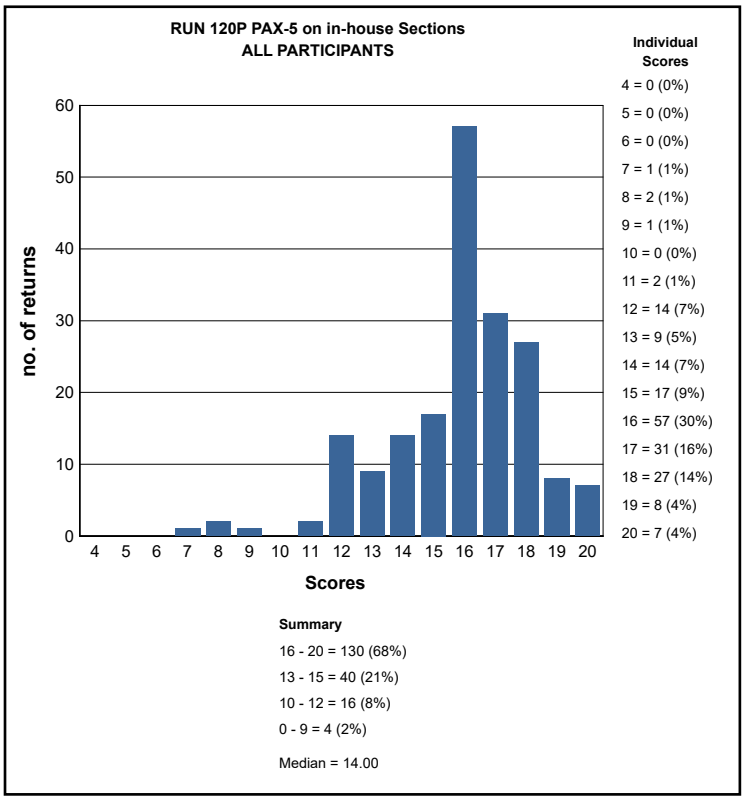


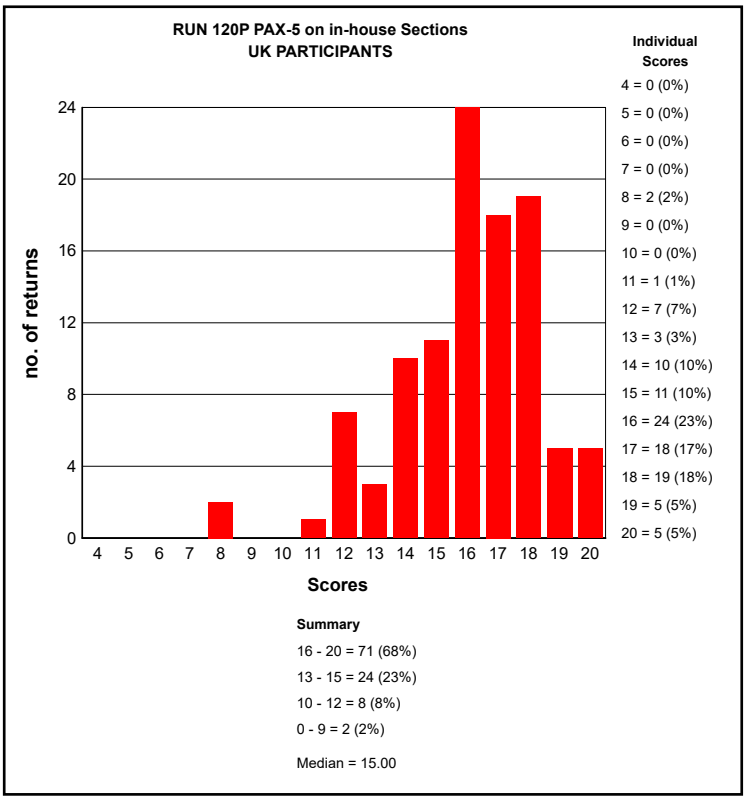


GRAPHICAL REPRESENTATION OF PASS RATES









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: 120

Primary Antibody : BCL6

Antibody Details	N	%
Dako BCL6 M7211 (PG-B6p)	35	77
Novocastra NCL-BCL-6 (P1F6) mm	2	100
Novocastra NCL-L-BCL-6-564 (LN22) mm	48	96
Vector BCL-6 (P1F6) mm	1	100
Other	10	90
Cell marque 227M-94/95/96/97/98	12	58
Dako RTU FLEX Link IR625 (PG-B6p)	6	83
Leica RTU PA0204 (LN22)	21	86
Ventana 760-4241 (1GI191E/A8)	57	89
Dako RTU Omnis (PG-B6p) GA625	7	71

Lymphoma Run: 120

Primary Antibody : PAX-5

Antibody Details	N	%
Abcam (EPR3730(2)) ab109443	1	100
Invitrogen PAX5 (SP34) MA1-39420	1	100
Invitrogen PAX5 (SP34) MA5-16389	1	0
Leica Bond RTU PAX-5 (1EW) PA0552	16	81
Novocastra (1EW) NCL-L-PAX-5	15	67
Dako (DAK-Pax5) M730701-2	14	100
Dako Omnis (DAK-Pax5) GA65061-2	5	60
Dako (DAK-Pax5) Autostainer Plus IS65030-2	4	100
Dako Autostainer Link 48 (DAK-Pax5) IR65061-2	5	100
Other	41	90
Ventana CONFIRM 790-4420 (SP34)	49	84
BD BioSci 610863 (24/Pax-5)	2	50
Leica NCL-L-PAX-5 (1WE) PAX5-L-CE	2	100
CellMarque 312R (SP34)	5	80
CellMarque 312R (EP156)	1	0
Santa Cruz SC13146 (A11)	1	0
A.Menarini MMP-207-PM6 (BC/24) RTU	1	100
Immunologic ILM 0706 (MX017)	1	100

Lymphoma Run: 120

Heat Mediated Retrieval	BCL6 N	BCL6 %	PAX-5 N	PAX-5 %
Dako Omnis	12	75	11	82
Dako PTLink	13	77	11	91
Lab vision PT Module	1	0	1	100
Leica ER1 10 mins	1	0	0	0
Leica ER1 20 mins	0	0	5	80
Leica ER1 30 mins	2	0	9	89
Leica ER2 10 mins	0	0	1	100
Leica ER2 20 mins	36	94	32	91
Leica ER2 30 mins	23	91	4	75
Leica ER2 40 mins	4	100	0	0
Microwave	1	0	1	100
None	0	0	1	0
Ventana CC1 24mins	2	50	3	33
Ventana CC1 32mins	8	88	12	75
Ventana CC1 36mins	1	0	4	75
Ventana CC1 40mins	5	100	6	83
Ventana CC1 48mins	9	89	8	100
Ventana CC1 52mins	1	100	0	0
Ventana CC1 56mins	9	100	3	100
Ventana CC1 64mins	40	93	27	81
Ventana CC1 72mins	3	100	0	0
Ventana CC1 76mins	5	80	2	100
Ventana CC1 88mins	3	100	0	0
Ventana CC1 92mins	4	75	4	25
Ventana CC1 extended	3	100	0	0
Ventana CC1 mild	0	0	2	50
Ventana CC1 standard	10	80	14	100
Ventana CC2 72mins	0	0	1	0
Ventana CC2 80mins	0	0	1	0
Water bath 95-98 OC	2	0	2	50

Lymphoma Run: 120

Enzyme Mediated Retrieval	BCL6 N	BCL6 %	PAX-5 N	PAX-5 %
AS PER KIT	4	75	7	86
Dako Protease (S2019)	1	0	0	0
NOT APPLICABLE	113	85	65	85

Lymphoma Run: 120	BCL6		PAX-5	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	13	92	14	79
BioGenex HRP (HK 519-06K)	1	0	0	0
Dako EnVision FLEX (K8000/10)	5	80	4	100
Dako EnVision FLEX+ (K8002/12)	13	62	9	67
Dako Envision+ HRP mouse K4004/5/6/7	2	100	1	100
Dako REAL HRP/DAB (K5001)	1	0	1	0
Leica Bond Polymer Define (DS9713)	1	0	1	100
Leica Bond Polymer Refine (DS9800)	58	90	42	88
None	0	0	2	100
NOT APPLICABLE	1	100	0	0
Other	4	100	5	100
Power Vision DPVB999 HRP	1	0	1	100
Ventana iView system (760-091)	1	0	1	100
Ventana OptiView (760-700) + Amp. (7/860-099)	11	100	5	40
Ventana OptiView Kit (760-700)	49	92	34	79
Ventana UltraView Kit (760-500)	33	85	31	84

Lymphoma Run: 120	BCL6		PAX-5	
Automation	**N**	**%**	**N**	**%**
Dako Autostainer Link 48	13	69	12	92
Dako Autostainer Plus Link	2	100	1	100
Dako Omnis	12	75	11	82
Leica Bond Max	23	78	14	79
Leica Bond-III	44	95	36	92
Menarini - Intellipath FLX	1	0	1	0
None (Manual)	2	50	0	0
Other	1	0	2	50
Ventana Benchmark GX	3	100	1	100
Ventana Benchmark ULTRA	77	90	70	77
Ventana Benchmark XT	21	86	17	88

Lymphoma Run: 120	BCL6		PAX-5	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	24	96	37	89
BioGenex liquid DBA (HK-124-7K)	1	0	0	0
Dako DAB K3468	0	0	1	100
DAKO DAB+	2	50	1	0
Dako DAB+ Liquid (K3468)	1	0	0	0
Dako EnVision Plus kits	2	50	3	67
Dako FLEX DAB	20	80	15	93
Dako REAL K5001 DAB	1	0	1	0
Leica Bond Polymer Refine kit (DS9800)	60	88	45	89
NOT APPLICABLE	1	100	1	0
Other	9	100	6	50
Ventana DAB	37	86	20	80
Ventana iview	2	100	1	100
Ventana Ultraview DAB	39	85	35	77

BEST METHODS - Gold Standard Antibody

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

BCL6 - Method 1

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

Primary Antibody: Ventana 760-4241 (1GI191E/A8) , 32 Mins, RT ºC Prediluted
Automation: Ventana Benchmark ULTRA
Method: Ventana Optiview
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 64mins, PH: 9
EAR: NOT APPLICABLE
Chromogen: Ventana DAB, RT ºC., Time 1: 8 Mins
Detection: Ventana OptiView (760-700) + Amp. (7/860-099) , 16 Mins, RT ºC Prediluted

BCL6 - Method 2

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

Primary Antibody: Dako BCL6 M7211 (PG-B6p) , 60 Mins Dilution 1: 25
Automation: Ventana Benchmark ULTRA
Method: Ventana Optiview
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 88mins
EAR: NOT APPLICABLE
Chromogen: Other
Detection: Ventana OptiView (760-700) + Amp. (7/860-099)

BCL6 - Method 3

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

Primary Antibody:	Dako BCL6 M7211 (PG-B6p) , 20 Mins Dilution 1: 20
Automation:	Dako Omnis
Method:	Dako FLEX kit
Main Buffer:	Dako Wash Buffer (S3006)
HMAR:	Dako Omnis
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB
Detection:	Dako EnVision FLEX (K8000/10) Prediluted

BCL6 - Method 4

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

Primary Antibody:	Novocastra NCL-L-BCL-6-564 (LN22) mm Dilution 1: 60
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) Prediluted

BEST METHODS - Secondary Antibody

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

PAX-5 - Method 1

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

Primary Antibody:	Ventana CONFIRM 790-4420 (SP34) , 32 Mins, RT ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins, PH: 9
EAR:	
Chromogen:	Ventana DAB, RT ºC., Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 16 Mins, RT ºC Prediluted

PAX-5 - Method 2

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

Primary Antibody:	Dako Autostainer Link 48 (DAK-Pax5) IR65061-2 , 30 Mins, 22 ºC Prediluted
Automation:	Dako Omnis
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis, Buffer: DAKO HIGH PH
EAR:	
Chromogen:	Dako EnVision Plus kits, Time 1: 5 Mins, Time 2: 5 Mins
Detection:	None

PAX-5 - Method 3

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

Primary Antibody:	Abcam (EPR3730(2)) ab109443
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	AS PER KIT

PAX-5 - Method 4

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

Primary Antibody:	Novocastra (1EW) NCL-L-PAX-5 , 60 Mins, 20 ºC Dilution 1: 80
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins, Buffer: EDTA, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800), 20 ºC., Time 1: 8 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, 20 ºC Prediluted

Neil Bilbe

	Gold Standard	Second Antibody
Antigens Assessed:	Ki67	CD34
Tissue Sections circulated:	GBM and meningioma	Haemangioblastoma.
Number of Registered Participants:	59	
Number of Participants this Run	57 (97%)	

Introduction

Gold Standard: Ki67

Antigen Ki-67 is a nuclear protein that is associated with and may be necessary for cellular proliferation. Furthermore it is associated with ribosomal RNA transcription. Inactivation of antigen Ki-67 leads to inhibition of ribosomal RNA synthesis.
Ki-67 is an excellent marker to determine the growth fraction of a given cell population. The fraction of Ki-67-positive tumour cells (the *Ki-67 labelling index*) is often correlated with the clinical course of cancer. The best-studied examples in this context are carcinomas of the prostate, brain, breast and nephroblastoma . For these types of tumours, the prognostic value for survival and tumour recurrence have repeatedly been proven in uni- and multivariate analysis.
Ki-67 and MIB-1 monoclonal antibodies are directed against different epitopes of the same proliferation-related antigen. Ki-67 and MIB-1 may be used on fixed sections. MIB-1 is used in clinical applications to determine the *Ki-67 labelling index*. One of its primary advantages over the original Ki-67 antibody (and the reason why it has essentially supplanted the original antibody for clinical use) is that it can be used on formalin-fixed paraffin-embedded sections, after heat-mediated antigen retrieval.

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

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

References

1. Hsu DW et al, Use of MIB-1 (Ki-67) immunoreactivity in differentiating grade II and grade III gliomas. J Neuropathol Exp Neurol. 1997 Aug;56 (8):857-65.
2. Ralte AM, et al, Clinicopathological features, MIB-1 labeling index and apoptotic index in recurrent astrocytic tumours. Pathol Oncol Res. 2001; 7(4):267-78.
3. S. H Torp. Proliferative activity in human glioblastomas: evaluation of different Ki-67 equivalent antibodies. Mol Pathol 1997;50:198-200.

Second Antigen: CD34

CD34 is a transmembrane glycoprotein and functions as a cell -cell adhesion factor. It is expressed on immature haematopoietic stem/progenitor cells, capillary endothelial cells and embryonic fibroblasts. It can also be found in splenic marginal zones, dendritic interstitial cells around vessels, nerves, hair follicles, muscle cells and sweat glands in various tissues. CD34 labels capillaries in most tissues but is absent in large veins and arteries and is negative in the sinus endothelium of placenta and spleen. CD34 is an excellent indicator of vascular differentiation, regardless of the tumour grade, therefore it is a good marker for vascular tumours (Cerilli *et al*, Leong).

Features Of Optimal Immunostaining
- Strong staining of the vessels and endothelial cells.
- Minimal background staining with no non-specific staining

Features Of Sub-optimal Immunostaining
- Weak, patchy or negative staining of the vessels and endothelial cells.
- Non-specific staining or excessive background staining.

References

1. Cerilli LA and Wick MR. Immunohistology of soft tissue and osseous neoplasms. In: Diagnostic Immunohistochemistry. Dabbs DJ. (Editor). Churchill Livingstone, Philadelphia 2002; 71
2. Leong AS-Y, Cooper K, Leong FJW-M. CD34. In: Manual of Diagnostic Antibodies for Immunohistology (1st Ed.). Oxford University Press, Oxford 1999; 83-84

Assessment Summary:
57 out of 59 registered participants submitted slides. A total of 232 sections were assessed: **G=57, H=57, J=57 K=57**

The overall pass rate was 95.6% (218 slides), borderline 3.5% (8), and the failed slides rate was 0.9% (2). The average score for all slides was 17/20, with the in-house slides (**H & K**) doing marginally better than the NEQAS slides (**G & J**).

1) Summary Table - All Slides (Ki67 & CD34)

Slide	Antigen	Pass	Borderline	Fail
G (NEQAS)	Ki67 (57)	88% (50)	9% (5)	4% (2)
H (In-House)	Ki67 (57)	98% (56)	2% (1)	0% (0)
J (NEQAS)	CD34(57)	96% (55)	4% (2)	0% (0)
K (In-House)	CD34(57)	100% (57)	0% (0)	0% (0)
Total (Average)	228	88%	10%	2%

Ki 67 (**G & H**)
The NEQAS (**G**) pass rate of 88% is back to a similar level as Run 118 (83%) and the best so far for this EQA year. A piece of GBM was used for this run, alongside the meningioma sample, as against the neuroendocrine tumour used for Run 119. Only two slides (4%) failed the assessment (see Report Image Fig. 3). Both were far too weak for reliable diagnostic use. Each used the Dako (M7240) MIB-1: diluted 1:50 and 1:300; one using a familiar protocol: Leica ER2 20 mins; Leica Bond Polymer Refine (DS9800); Leica Bond-III; and the other a less conventional method: *Lab vision PT Module, pH 6.7, citrate; RTU Power Vision DPVB999 HRP; Teecan Freedom Evoware.

Five slides (**G**) were assessed as borderline (9%); three employed the Dako MIB-1, one Ventana RTU (30-9) 790-4286, and one NeoMarkers/Thermo Sci (SP6) RM 9106. The commonest problem was the lack of sufficient staining in the meningioma sample.

Interestingly, as with Run 119, the four slides which obtained scores of $^{20}/_{20}$ on the NEQAS slides (**G**), employed different primaries and protocols: Leica/Novocastra (MM1) NCL-Ki67-CE; Dako FLEX RTU (MIB1) IR626; Biocare CRM325 (SP6); Ventana RTU (30-9) 790-428 (see the Best Methods below for further details).

*Only one slide (2%) obtained a borderline for the in-house Ki 67 (**H**), the same participant who failed their NEQAS (**G**), see protocol above.

CD34 (**J & K**)
The overall results for CD34 were better than for the Ki 67, with 96% passing ($^{55}/_{57}$) on the NEQAS sample (**J**); the other two slides (4%) were assessed as borderline. One primarily had weak staining (see Report Image Fig. 11), and one a more uneven staining pattern. Both used a Dako QBend10 primary: a Flex RTU, IR1632; with no RT; Dako FLEX kit; on the Dako Autostainer Link 48 (uneven); and the other employed the Dako M7165, diluted 1:50; CC1 32 mins; Ventana UltraView Kit (760-500); on a Ventana Benchmark XT (weak pattern).

All slides submitted for the in-house CD34 controls (**K**), passed the assessment, averaging $^{17}/_{20}$. with eight achieving $^{20}/_{20}$ (14%).

CD34 was last requested back in May 2103 for Run 102, when 89% of participants achieved a pass for their NEQAS slide (**J**).

Selected Images showing Optimal and Sub-optimal immunostaining

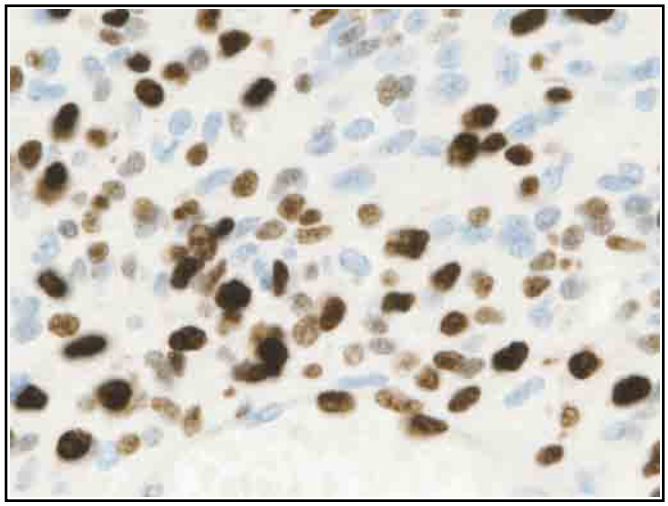


Fig 1. Optimal demonstration of Ki 67 on the NEQAS glioblastoma sample. Tumour nuclei are nicely seen against a clean, and clear background. RTU Leica/Novocastra (MM1) NCL-Ki 67, 15 mins; Leica ER2 20 mins; Leica Bond Polymer Refine (DS9800), 8 mins, on a Leica Bond-III.

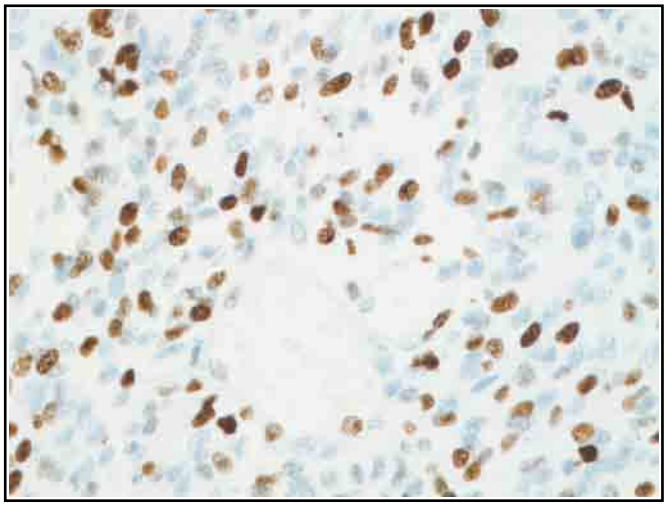


Fig 2. Sub-optimal demonstration of Ki 67 on the NEQAS glioblastoma sample. Staining is weak and the occasional tumour nuclei are missing, although still of a suitable level for diagnostic use. RTU Gennova AP10244C (SP6), 20 mins; pH6 Dako Omnis PT 30 mins; Dako EnVision FLEX+ (K8002/12), 20 mins; on the Dako Omnis platform.

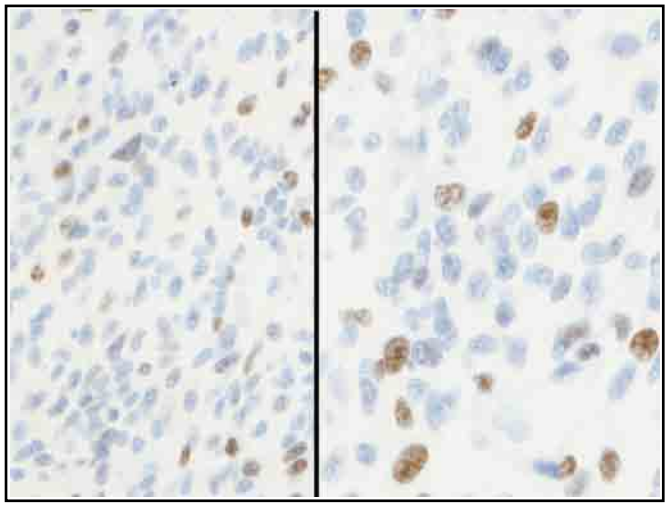


Fig 3. Poor demonstration (failed) of Ki 67 on the NEQAS glioblastoma sample. Staining is far too weak, and even at high power (R) many tumour cells are clearly missing. Dako M7240 (MIB1), 1:50, 15 mins; Leica ER2 20 mins; Leica Bond Polymer Refine (DS9800); on a Leica Bond-III.

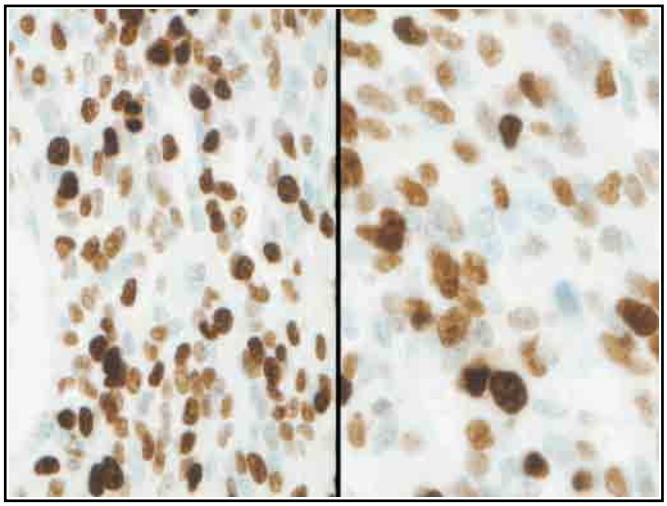


Fig 4. Good demonstration of Ki 67 on the NEQAS glioblastoma sample. Tumour cells are clearly seen, and there is only minimal background staining. Gennova AP10244C (SP6), 1:200, 72 mins; Ventana CC1 standard; RTU Ventana UltraView Kit (760-500), 10 mins; on a Ventana Benchmark XT.

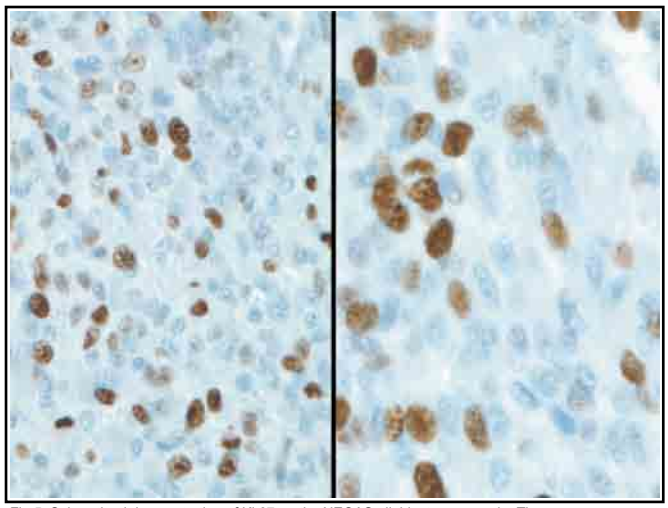


Fig 5. Sub-optimal demonstration of Ki 67 on the NEQAS glioblastoma sample. The counterstain is excessive, which masks some of the nuclei; and there is some evidence of morphological damage, albeit it minor. Dako M7240 (MIB1), 1:50, 60 mins; Ventana CC1 mild; Ventana UltraView Kit (760-500); on a Ventana Benchmark XT.

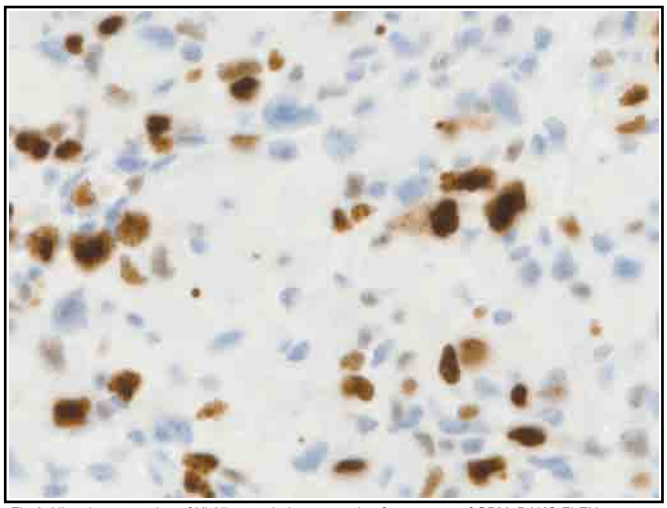


Fig 6. Nice demonstration of Ki 67 on an in-house section from a case of GBM. DAKO FLEX RTU Omnis (MIB1) GA626, 20 mins; Dako Omnis Env TRS High pH, 30 mins; Dako EnVision FLEX (K8000/10), 20 mins; on the Dako Omnis platform.

Selected Images showing Optimal and Sub-optimal {@stainingtext}

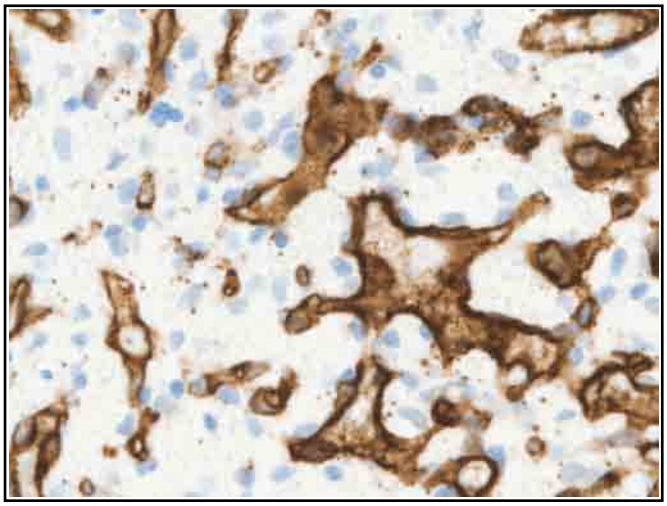


Fig 7. Optimal demonstration of CD34 on the NEQAS haemangioblastoma sample. The vessel endothelia are nicely stained, but there is some granulation seen. Leica Bond RTU PA0212 (QBend10), 15 mins; Leica ER2 30 mins; RTU Leica Bond Polymer Refine (DS9800), 8 mins; on a Leica Bond-III.

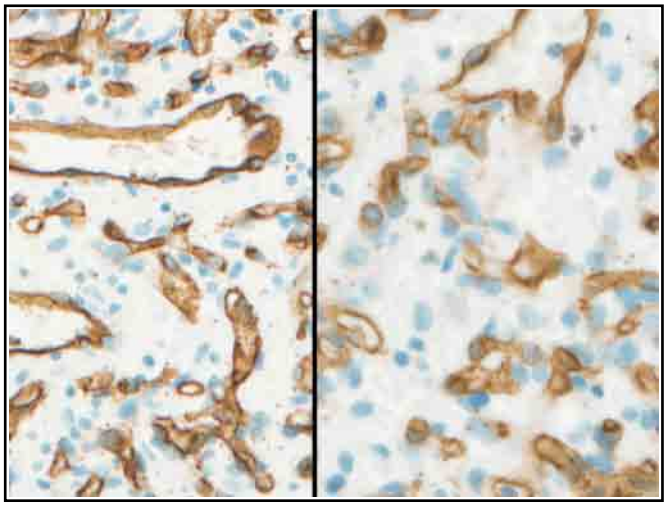


Fig 8. Nice demonstration of CD34 on the NEQAS haemangioblastoma sample, both at low (L) and high power (R). There is minimal background staining. Leica NCL-L-END (QBend), 1:25, 32 mins; Ventana CC1 mild; second layer of Ventana UltraView Kit (760-500); on the Ventana Benchmark XT.

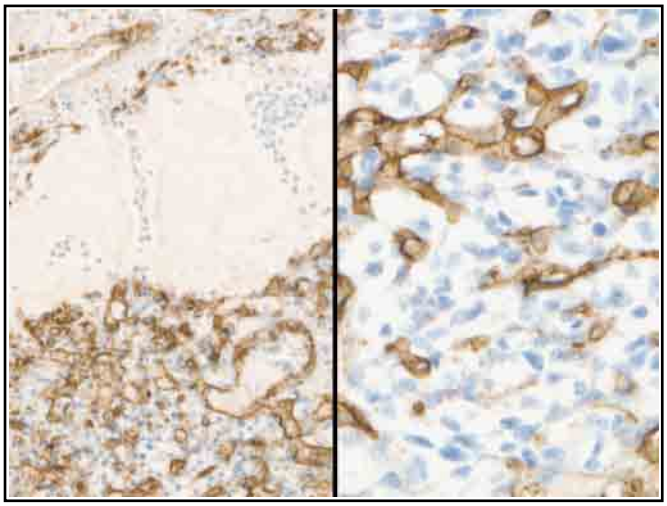


Fig 9. Sub-optimal demonstration of CD34 on the NEQAS haemangioblastoma sample. The endothelium are nicely stained (R), but some of the necrotic areas have a brown hue (L). Leica Bond RTU PA0212 (QBend10), 15 mins; Leica ER1 20 mins; RTU Leica Bond Polymer Refine (DS9800), 8 mins; on a Leica Bond-III.

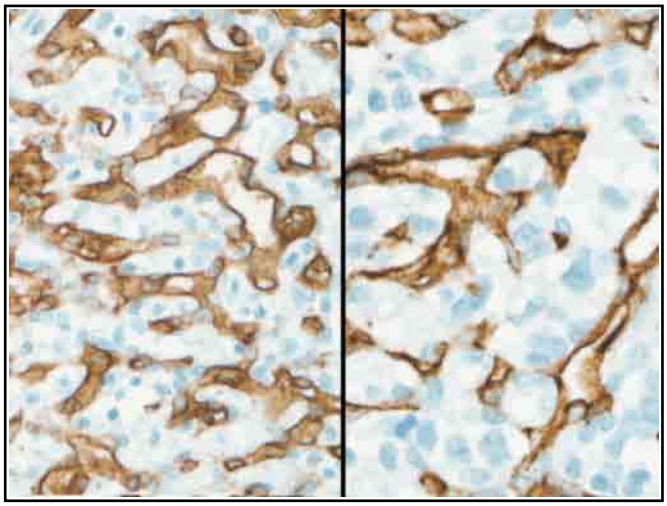


Fig 10. Excellent demonstration of CD34 on the NEQAS haemangioblastoma sample. Staining is both clean and precise. RTU Ventana 790-2927 (QBend10), 8 mins; Ventana CC1 32 mins; RTU Ventana OptiView Kit (760-700), 8 mins; on the Ventana Benchmark ULTRA.

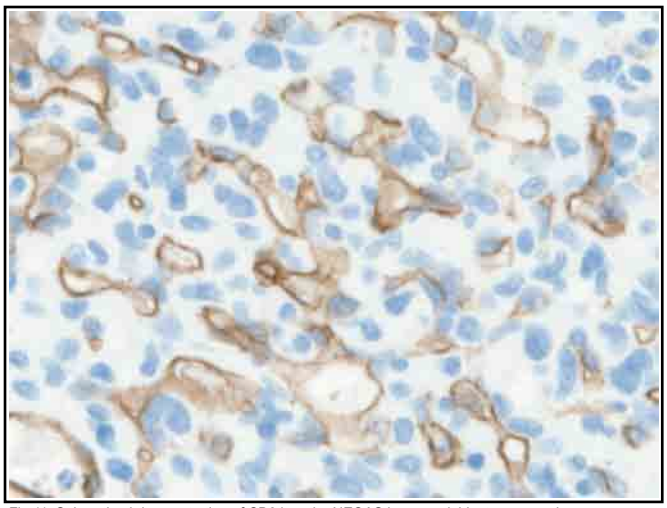


Fig 11. Sub-optimal demonstration of CD34 on the NEQAS haemangioblastoma sample. Staining is a little weak and therefore the slide attained a borderline score. Dako M7165 (QBend10), 1:50, 32 mins; Ventana CC1 32mins; Ventana UltraView Kit (760-500), on the Ventana Benchmark XT.

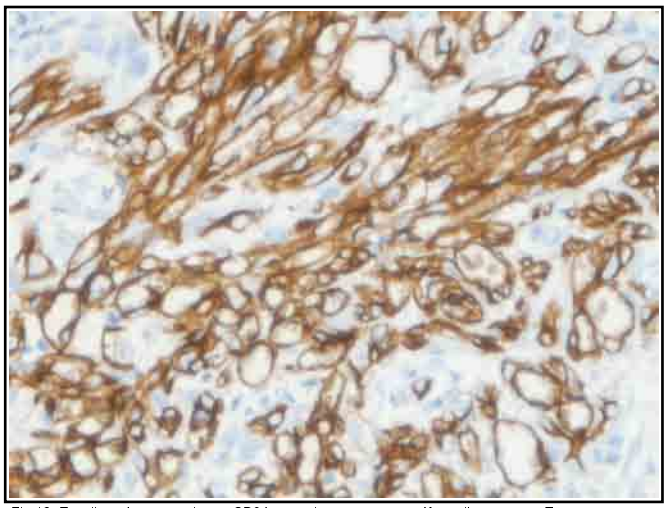
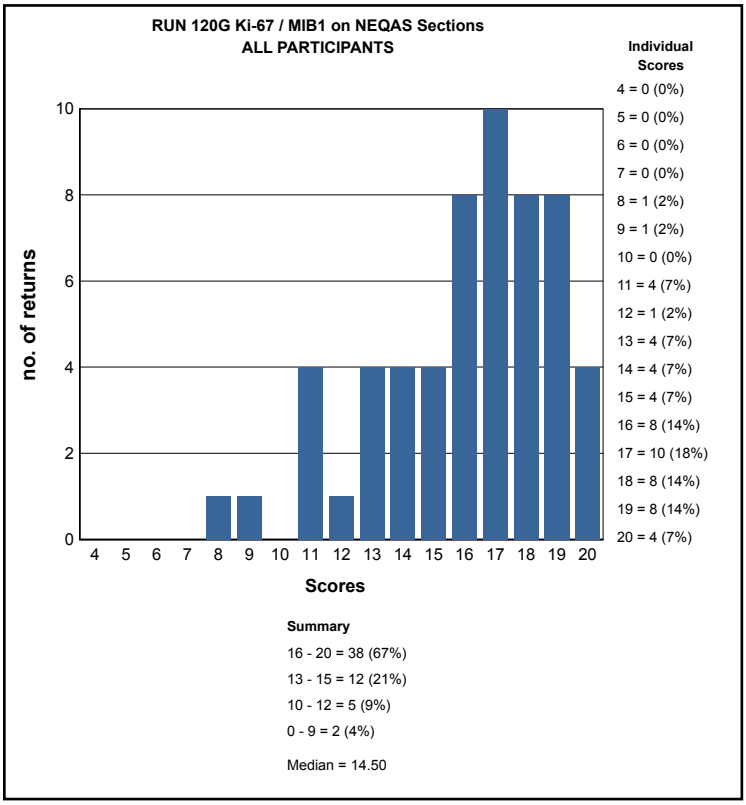
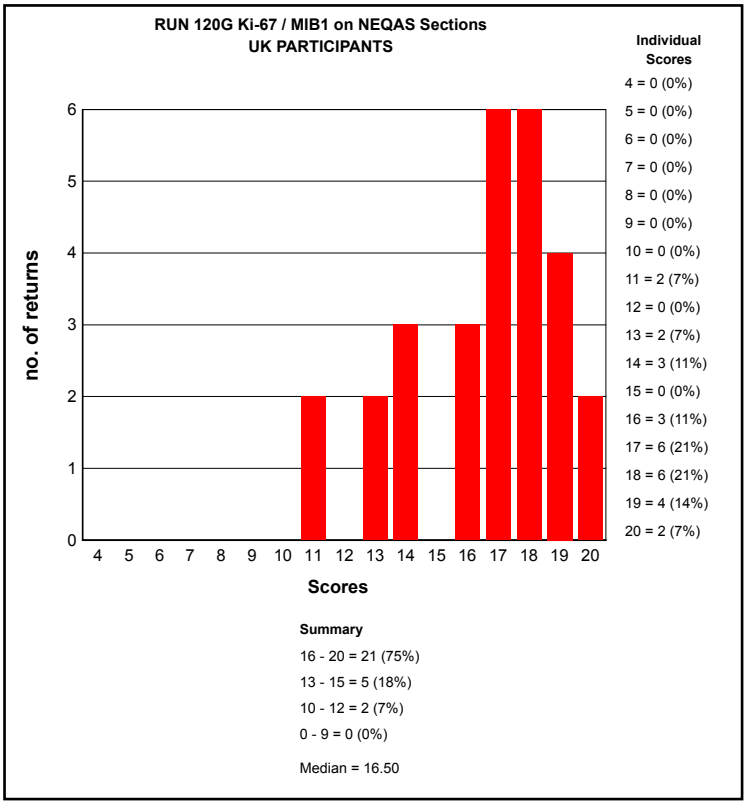
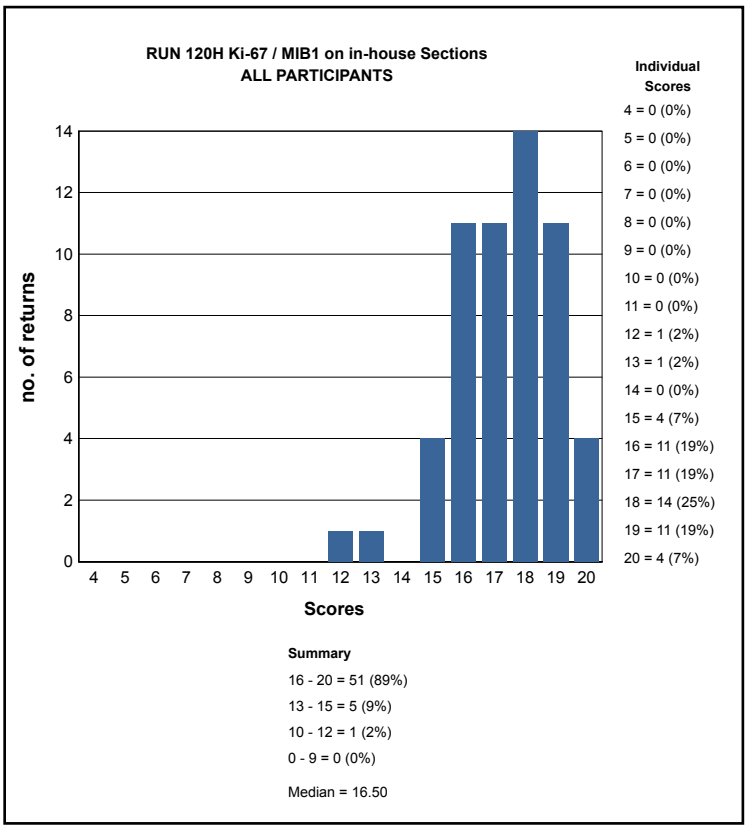


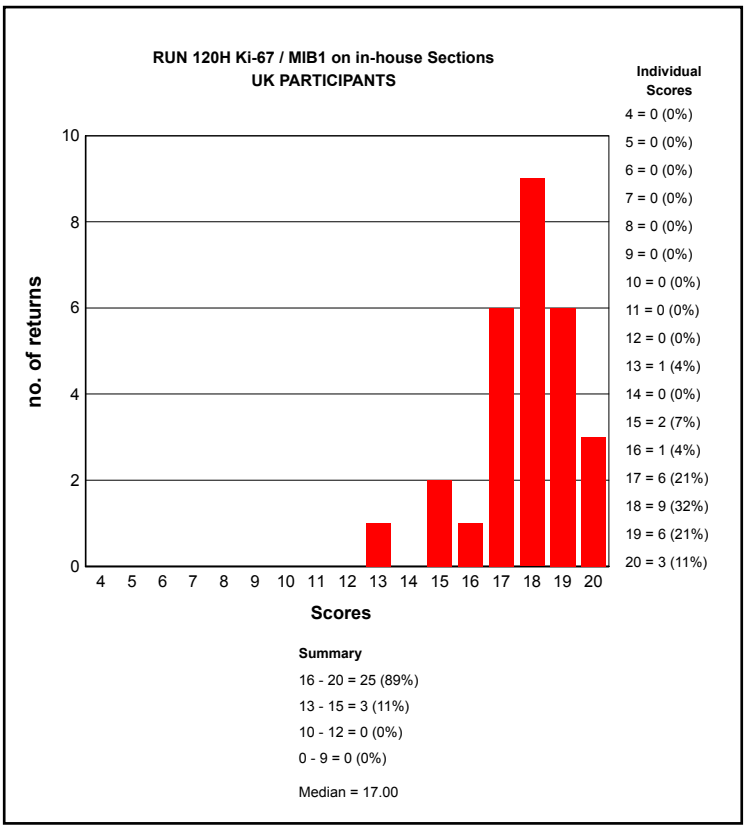
Fig 12. Excellent demonstration of CD34 on an in-house case of Kaposi's sarcoma. The tumour is highly vascular, showing typically irregular blood vessel pattern. Dako M7165 (QBend10), 1:50, 30 mins; Dako PTLink, pH6, 45 mins; RTU Dako EnVision FLEX+ (K8002/12), 30 mins; on the Dako Autostainer Plus Link.

GRAPHICAL REPRESENTATION OF PASS RATES

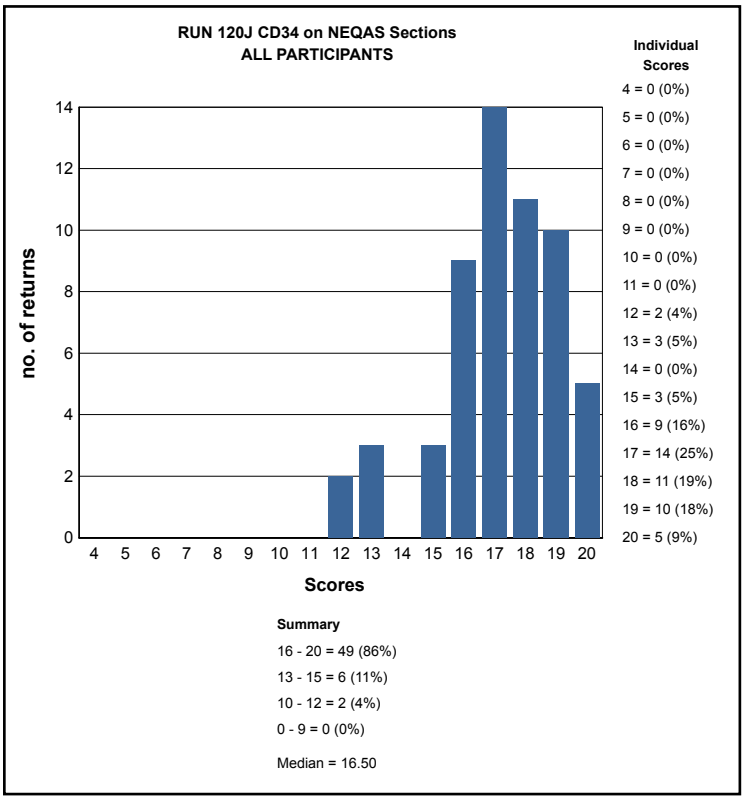


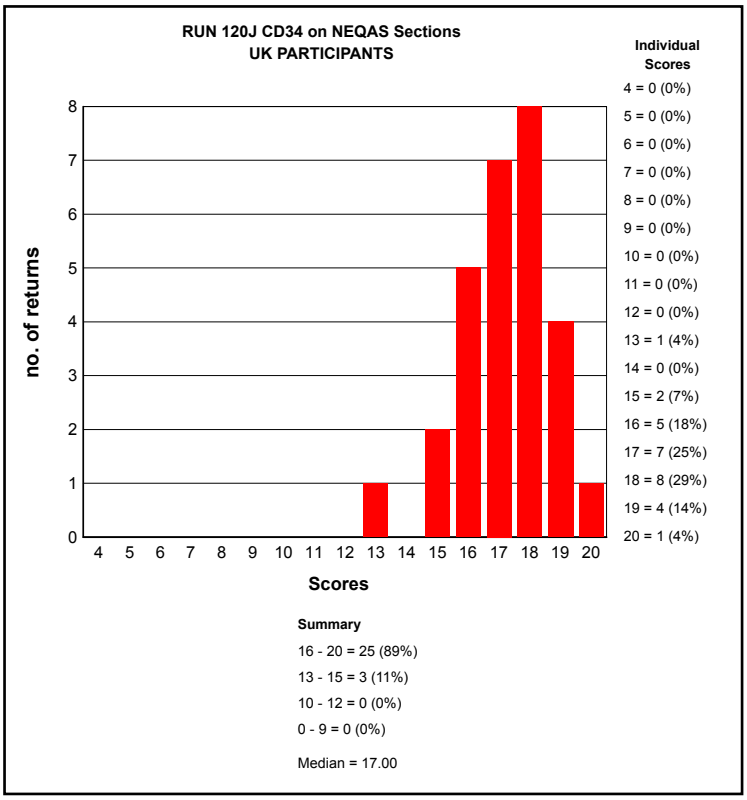


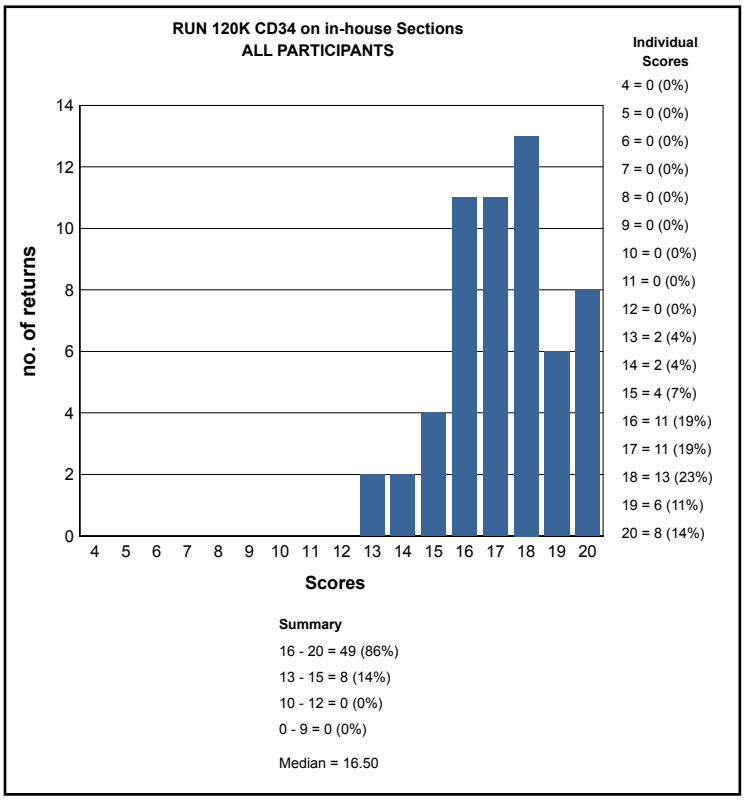


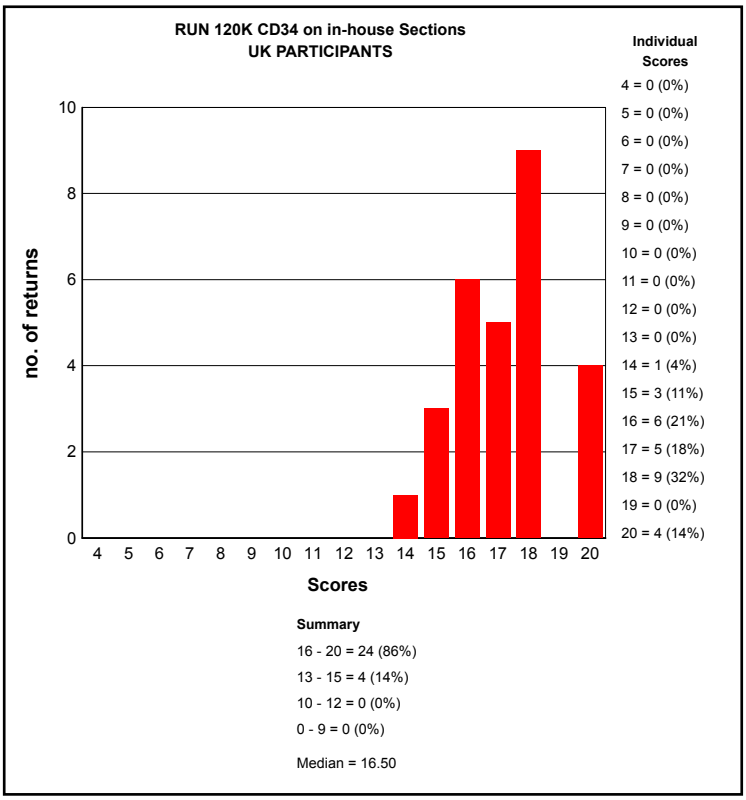


GRAPHICAL REPRESENTATION OF PASS RATES









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: 120

Primary Antibody : Ki-67 / MIB1

Antibody Details	N	%
Dako M7240 (MIB1)	30	83
NeoMarkers/Thermo Sci (SP6) RM 9106	1	0
Leica/Novocastra (MM1) NCL-Ki67-CE	1	100
Leica/Novocastra RTU (MM1) PA0118	2	100
Leica/Novocastra RTU (K2) PA0230	3	100
Ventana RTU (30-9) 790-4286	8	88
Dako FLEX RTU (MIB1) IR626	2	100
DAKO FLEX RTU Omnis (MIB1) GA626	6	100
Gennova AP10244C (SP6)	2	100
Biocare CRM325 (SP6)	1	100

Neuropathology Run: 120

Primary Antibody : CD34

Antibody Details	N	%
Dako M7165 (QBend10)	21	95
LabVision MS363P (QBend10)	1	100
Leica Bond RTU PA0212 (QBend10)	8	100
Ventana 790-2927 (QBend10)	10	100
Other	2	100
Dako Flex RTU IR632/IS632 (QBend10)	6	83
Cell Marque 134M/760-2620/CMC33 (G Bend)	1	100
Leica NCL-END (QBend)	2	100
Leica NCL-L-END (QBend)	4	100
Dako Omnis RTU GA632	1	100

Neuropathology Run: 120

Heat Mediated Retrieval	CD34 N	CD34 %	Ki-67 / MIB1 N	Ki-67 / MIB1 %
_Leica BondMax ER2	1	100	0	0
Dako Omnis	7	100	8	100
Dako PTLink	5	100	3	100
Lab vision PT Module	1	100	1	0
Leica ER1 10 mins	1	100	0	0
Leica ER1 20 mins	2	100	0	0
Leica ER1 30 mins	1	100	0	0
Leica ER2 20 mins	7	100	11	73
Leica ER2 30 mins	3	100	2	100
Leica ER2 40 mins	0	0	2	100
Microwave	1	100	0	0
None	3	67	0	0
Other	0	0	2	100
Ventana CC1 16mins	1	100	0	0
Ventana CC1 24mins	1	100	1	0
Ventana CC1 32mins	5	80	4	50
Ventana CC1 36mins	0	0	1	100
Ventana CC1 48mins	0	0	2	100
Ventana CC1 52mins	1	100	0	0
Ventana CC1 56mins	1	100	1	100
Ventana CC1 64mins	3	100	10	100
Ventana CC1 8mins	4	100	0	0
Ventana CC1 extended	0	0	1	100
Ventana CC1 mild	3	100	3	100
Ventana CC1 standard	2	100	1	100
Ventana CC2 64mins	0	0	2	100
Water bath 95-98 OC	0	0	1	100

Neuropathology Run: 120

Enzyme Mediated Retrieval	CD34 N	CD34 %	Ki-67 / MIB1 N	Ki-67 / MIB1 %
AS PER KIT	1	100	0	0
NOT APPLICABLE	28	93	36	83
VBS Bond Enzyme 1	1	100	0	0
Ventana Protease	1	100	0	0

Neuropathology Run: 120	CD34		Ki-67 / MIB1	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	9	89	8	75
Dako EnVision FLEX (K8000/10)	2	100	4	100
Dako EnVision FLEX+ (K8002/12)	5	100	4	100
Dako Envision HRP/DAB (K5007)	1	100	1	100
Leica Bond Polymer Refine (DS9800)	12	100	14	86
Other	2	100	1	100
Power Vision DPVB999 HRP	1	100	1	0
Ventana OptiView (760-700) + Amp. (7/860-099)	1	100	1	100
Ventana OptiView Kit (760-700)	10	100	9	89
Ventana UltraView Kit (760-500)	12	92	13	92

Neuropathology Run: 120	CD34		Ki-67 / MIB1	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	9	89	10	80
Dako DAB K3468	1	100	0	0
DAKO DAB+	1	100	1	100
Dako DAB+ Liquid (K3468)	0	0	1	0
Dako DAB+ REAL Detection (K5001)	1	100	1	100
Dako EnVision Plus kits	2	100	1	100
Dako FLEX DAB	7	100	7	100
Leica Bond Polymer Refine kit (DS9800)	15	100	15	87
Other	1	100	1	100
Ventana DAB	6	100	5	80
Ventana Ultraview DAB	13	92	14	93

Neuropathology Run: 120	CD34		Ki-67 / MIB1	
Automation	**N**	**%**	**N**	**%**
Dako Autostainer Link 48	4	75	3	100
Dako Autostainer Plus Link	1	100	0	0
Dako Omnis	6	100	8	100
Leica Bond Max	6	100	4	75
Leica Bond-III	11	100	11	82
None (Manual)	1	100	2	100
Other	1	100	1	0
Ventana Benchmark ULTRA	20	100	22	91
Ventana Benchmark XT	6	83	5	80

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 19/20 (In House slide) using this method.

Primary Antibody: Leica/Novocastra (MM1) NCL-Ki67-CE , 15 Mins, 25 ºC Prediluted
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 20 mins
EAR:
Chromogen: Leica Bond Polymer Refine kit (DS9800)
Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins, 25 ºC

Ki-67 / MIB1 - Method 2

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

Primary Antibody: Dako FLEX RTU (MIB1) IR626
Automation: Dako Autostainer Link 48
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako PTLink
EAR: NOT APPLICABLE
Chromogen: Dako FLEX DAB
Detection: Dako EnVision FLEX+ (K8002/12)

Ki-67 / MIB1 - Method 3

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

Primary Antibody:	Dako M7240 (MIB1) , 15 Mins, RT ºC 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) , 8 Mins, RT ºC Prediluted

Ki-67 / MIB1 - Method 4

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

Primary Antibody:	Ventana RTU (30-9) 790-4286 , 16 Mins, 36 ºC
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500) , 36 ºC Prediluted

BEST METHODS - Secondary Antibody

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

CD34 - Method 1

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

Primary Antibody:	Leica Bond RTU PA0212 (QBend10) , 15 Mins Prediluted
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), Time 1: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins Prediluted

CD34 - Method 2

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

Primary Antibody:	Dako M7165 (QBend10) , 30 Mins, 20 ºC Dilution 1: 60
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins, Buffer: EDTA, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800), 20 ºC., Time 1: 8 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, 20 ºC Prediluted

CD34 - Method 3

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

Primary Antibody:	Ventana 790-2927 (QBend10) , 36 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.4
HMAR:	Ventana CC1 24mins, Buffer: Ultra CC1 (Cat. 950-224), PH: 8
EAR:	
Chromogen:	Ventana Ultraview DAB, PH: 7, 36 ºC., Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 36 ºC Prediluted

CD34 - Method 4

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

Primary Antibody:	Dako Flex RTU IR632/IS632 (QBend10)
Automation:	Dako Omnis
Method:	Envision
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis
EAR:	NOT APPLICABLE
Chromogen:	DAKO DAB+
Detection:	Dako EnVision FLEX+ (K8002/12)

Neil Bilbe and Irena Srebotnik Kirbis

	Gold Standard	Second Antibody
Antigens Assessed:	Ki67	CK
Sample circulated; cytospins and cell block sections:	The same sample was used for both sets of slides R & T: Human squamous carcinoma cell line, plus an effusion with mesothelial cells, macrophages and red and white blood cells.	
Number of Registered Participants:	87: 67 Cell block (77%), 20 Cytospins (23%).	
Number of Participants this Run	87 (100%)	

Introduction

Gold Antigen: Ki67

Antigen Ki-67 is a nuclear protein that is associated with and may be necessary for cellular proliferation. Furthermore it is associated with ribosomal RNA transcription. Inactivation of antigen Ki-67 leads to inhibition of ribosomal RNA synthesis.
Ki-67 is an excellent marker to determine the growth fraction of a given cell population. The fraction of Ki-67-positive tumour cells (the *Ki-67 labelling index*) is often correlated with the clinical course of cancer. The best-studied examples in this context are carcinomas of the prostate, brain and the breast and nephroblastoma . For these types of tumours, the prognostic value for survival and tumour recurrence have repeatedly been proven in uni- and multivariate analysis.
Ki-67 and MIB-1 monoclonal antibodies are directed against different epitopes of the same proliferation related antigen. MIB-1 is used in clinical applications to determine the Ki-67 labelling index. Both Ki-67 and MIB-1 may be used on fixed sections.

References
1. P A Hall, et al. The prognostic value of Ki67 immunostaining in non-Hodgkin's lymphoma. J Pathol 1988; 154:223-35
2. D C Brown, et al. Proliferation in non-Hodgkin's lymphoma: a comparison of Ki67 staining on fine needle aspiration and cryostat sections. J Clin Pathol 1990;43:325-328

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

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

Second Antigen: 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.

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:

All participants (87) submitted at least two slides. Two labs did not submit any in-house control slides (**S & U**), one lab did not return a control for Ki67 (**S**). This resulted in 345 slides for the assessors to score: **R = 87, S = 85, T = 87, U = 86.**

Assessment Outcomes:

The overall pass rate was 92% (317 slides), 6% borderline (20), and a 2% failure rate (8).
The average score for all slides was 16/20.

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

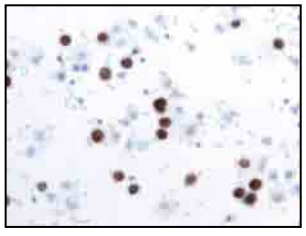


Fig.1 Ki67 NEQAS Cytospin

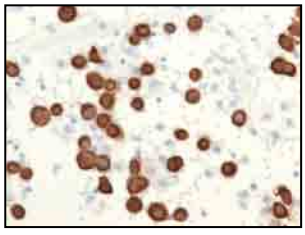


Fig.2 CK NEQAS Cytospin

(1) Summary Table - All Slides:

Slide	Antigen	Pass	Borderline	Fail
R (NEQAS)	Ki67 (87)	90% (78)	7% (6)	3% (3)
S (In-House)	Ki67 (85)	95% (81)	5% (4)	0% (0)
T (NEQAS)	CK (87)	91% (79)	6% (5)	3% (3)
U (In-House)	CK (86)	92% (79)	6% (5)	2% (2)
Average (Total)	345	92% (317)	6% (20)	2% (8)

Ki67 (R & S)

The assessment outcome of a 90% pass rate for the NEQAS Ki67 slides (**R**), was in line with those for Run 118 (92%) and Run 119 (91%).

For Run 120 there were three failed slides; two were on cytospins (see Report Images Fig. 3 and Fig. 6),and one was on a cell block sample. The protocol on the cell block employed the Leica/Novocastra RTU (K2) PA0230 clone; with a retrieval of Leica ER1 for 10 mins; Refine (DS9800) kit; on a Bond III. The staining was both weak and uneven. A total of 10 labs used this same primary, with only seven (70%) achieving a score of > $^{12}/_{20}$.

Both the participants who failed on a cytospin sample, used the Dako M7240 (MIB-1) marker (43 users, 88% pass rate), diluted 1:200 (Fig. 3); and 1:100 (Fig. 6); without retrieval. The first used a similar platform to the failed cell block slide (Refine kit, Leica Bond III), the second was stained on a Ventana Benchmark XT, with the Ventana UltraView DAB kit.

There were five slides which scored $^{20}/_{20}$, four were cell blocks and one was on a cytospin sample (See Images Report Fig 4).

(2) Best Performing Methods Ki67: NEQAS Samples (R)

The protocols employed on the cell block slides are shown in the table below (2) and show very similar methodologies, bar one lab which used a Ventana RTU antibody, as against the other three who used the Dako MIB-1 marker. The participant who performed Ki 67 on a cytospin was the sole user of a Biosystems Cell Marque antibody (RM-9106-S1).

Sample	Antibody	Dilution	Antigen Retrieval	Platform
CS	Biosystems Cell Marque (RM-9106-S1)	100	Leica ER2 20 mins	Leica Bond-III
CB	Dako 7240 (MIB-1)	100	Ventana CC1 32mins	Ventana Benchmark ULTRA
CB	Dako 7240 (MIB-1)	50	Ventana CC1 64mins	Ventana Benchmark ULTRA
CB	Ventana RTU (30-9) 790-4286	RTU	Ventana CC1 standard	Ventana Benchmark GX
CB	Dako 7240 (MIB-1)	50	Ventana CC1 32mins	Ventana Benchmark XT

For the in-house controls (**S**), 19 participants (23%) submitted a sample, other than a FFPE section, which is the same level of labs requesting cytospin preparations of 23% (20 labs).

- Cytospins (11) -13%. 55% of cytospin participants
- Smears (5) - 6%. 25% of cytospin participants
- LBC (3) - 4%. 15% of cytospin participants

N.B. Four labs (5%) did not disclose their sample type.

CK (T & U)

The participants performed to a similar standard with the NEQAS CK samples (**T**) as with the Ki67 NEQAS samples (**R**), with $^{79}/_{87}$ labs passing the assessment (91%).

Three slides failed the assessment (3%); two of these were the result of a non-pan Cytokeratin marker being employed (CK7), which resulted in a greatly reduced level of positivity, compared to the original validation slides.

The single failed slide that used a pan CK marker was on a cell block section. This participant used a Dako AE1/AE3 M3515; 1:300; with a RTU UV AP Red Detection Kit; on a Ventana Benchmark XT. The participant had stated that no retrieval was carried out on the NEQAS sample, even though they had entered Ventana CC1 standard in the HMAR field. Therefore it is difficult to discern if they had retrieved the NEQAS section, had incorrectly entered their protocol, or only retrieved their in-house section, which was also a cell block sample, albeit with a very scanty cell population.

Interestingly, the other 13 labs using this marker all passed the assessment.

(3) Best Performing Methods CK: NEQAS Samples (T):

All the best methods were performed on cell block samples.

Sample	Antibody	Dilution	Antigen Retrieval	Platform
CB	Dako M0821(MNF116)	50	Ventana Protease 1 (760-2018)	Ventana Benchmark ULTRA
CB	Dako M0821(MNF116)	1	Ventana Protease 1 (760-2018)	Ventana Benchmark XT
CB	Dako FLEX RTU IR053 (AE1/AE3)	RTU	Dako PTLink	Dako Autostainer Link 48
CB	Ventana 760 2595 AE1/AE3/PCK26	RTU	Ventana CC1 36mins	Ventana Benchmark ULTRA
CB	Dako M3515 (AE1/AE3)	100	Ventana CC1 32mins	Ventana Benchmark XT

Once again there is a variety of markers and protocols, but with a bias for Dako primary antibodies ($^4/_5$), and a predisposition for Ventana HMAR, and automation systems, again ($^4/_5$) see the table above (3).
N.B. The pass rates for CK have remained relatively consistent over the past few years. For Runs 114 -117, where it was the Gold antibody, the average pass rate was 90%, with a range of between 87% - 94%.
The number of labs who submitted an in-house sample (**U**) other than tissue sections, was slightly less than for the Ki67 slides (**S**), with 16 participants (19%) doing so for their CK controls.

Cell Block v Cytospins

(4) Summary Table - Average Scores NEQAS Samples:

Letter	Antigen	Sample	Average
R	Ki67	Cytospin	15
R	Ki67	Cell block	16
T	CK	Cytospin	15
T	CK	Cell block	16

The outcomes for the cell block (CB) and cytospin (CS) samples does not vary significantly from run to run, with average scores usually varying between 14 and 17. The one constant is that the average scores for cell blocks, are usually higher than those for the cytospin submissions. An example is the current run, where the cell blocks averaged $^{16}/_{20}$ and the cytospins $^{15}/_{20}$ for both antigens (see table 4 above).

(5) Summary Table NEQAS cell block and cytospin samples

Slide	Antigen	Pass	Borderline	Fail
R (Cell block)	Ki67 (67)	93% (62)	4% (3)	3% (2)
R (Cytospin)	Ki67 (20)	80% (16)	10% (2)	10% (2)
T (Cell block)	CK (67)	93% (62)	4% (3)	3% (2)
T (Cytospin)	CK (20)	85% (17)	10% (2)	5% (1)
Average (Total)	174	90% (157)	6% (10)	4% (7)

This table further illustrates that the cell block samples (blue font) for the *NEQAS* **(R & T)** samples out perform the cytospins (red font).

(6) Best Performing Methods CK: NEQAS Cytospin slides (T):

The maximum score achieved by a cytospin participant for the CK (**T**) was $^{18}/_{20}$, which three labs attained (an example can be seen in Report Images Fig 11), unlike for the Ki67 (**R**) where four cytospin preparations were scored either $^{19}/_{20}$ (3) or $^{20}/_{20}$ (1).

None of the three cytospin slides had applied any retrieval, according to their data entry details. The protocols were:

Sample	Antibody	Dilution	Detection	Platform
CS	Dako M0821(MNF116)	50	Ventana UltraView Kit (760-500)	Ventana Benchmark ULTRA
CS	Dako M0821(MNF116)	400	Leica Bond Polymer Refine (DS9800)	Leica Bond-III
CS	Dako M3515 (AE1/AE3)	2000	Ventana OptiView Kit (760-700)	Ventana Benchmark GX

Selected Images showing Optimal and Sub-optimal immunostaining

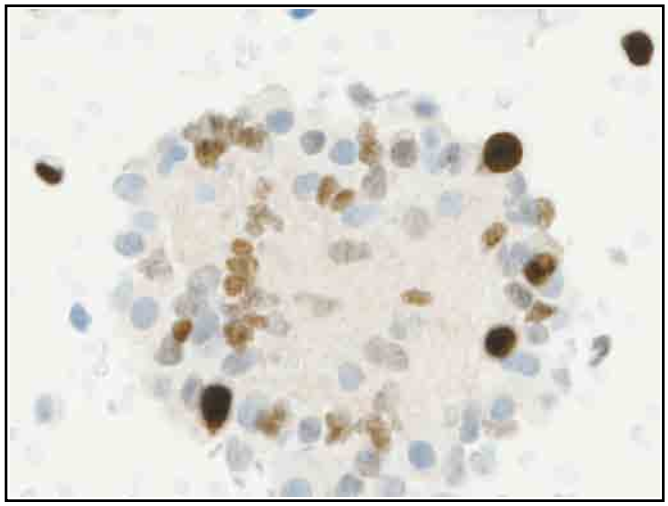


Fig 1. Sub-optimal Ki 67 demonstration in a NEQAS cell block section. Staining could be stronger and there is a slight background hue, but still adequate for diagnostic interpretation. Leica/Novocastra RTU (MM1) PA0118, 15 mins, Leica ER2 20 mins, Leica Bond Polymer Refine (DS9800), on a Leica Bond-III.

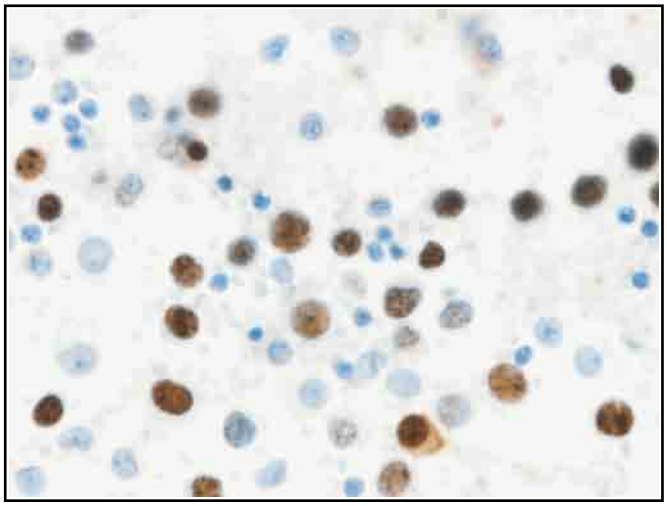


Fig 2. Nice Ki 67 demonstration in a NEQAS cytospin; staining is clean and precise; counterstain is of ideal intensity. Dako 7240 (MIB-1), Bond Polymer Refine, on the Bond-III. Participant submitted a cytospin in-house sample and entered a retrieval method of Leica ER 15 mins, but stated HMAR not used on NEQAS sample.

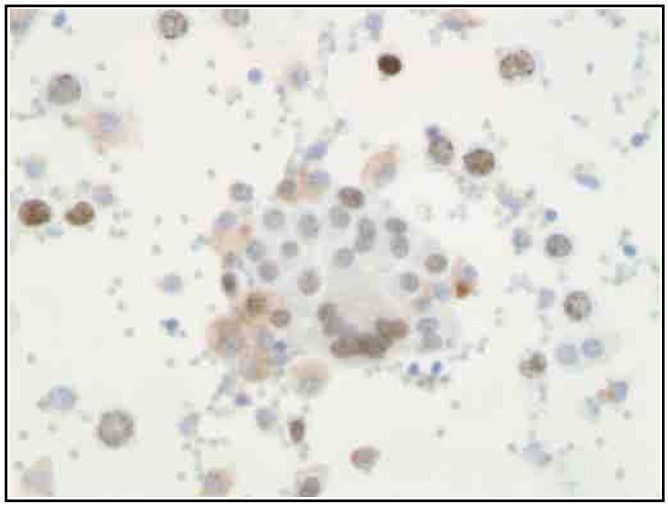


Fig 3. Poor Ki 67 demonstration in a NEQAS cytospin. Nuclei are far too weak, and there is some inappropriate staining. Slide failed the assessment. Dako 7240 (MIB-1), 1:200, without retrieval, a second layer of Leica Bond Polymer Refine (DS9800), on a Leica Bond-III (compare with Fig 2 above).

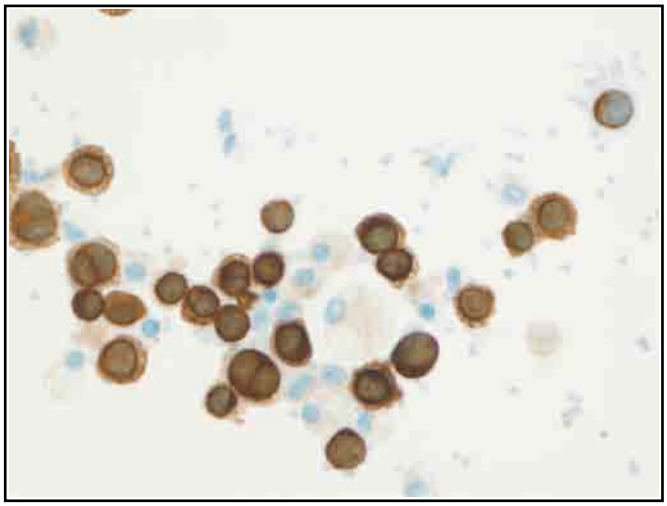


Fig 4. Excellent Ki 67 demonstration in a NEQAS cytospin. The nuclei are nicely contrasted against the negatively staining components. Biosystems Cell Marque (RM-9106-S1), 1:100, 15 mins; with Leica ER2 20 mins; RTU Leica Bond Polymer Refine (DS9800), 8mins; on the Leica Bond-III.

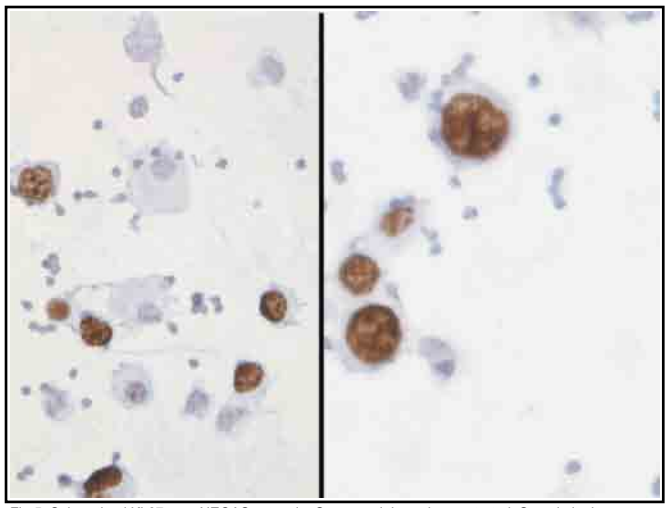


Fig 5. Sub-optimal Ki 67 on a NEQAS cytospin. Some nuclei are demonstrated. Sample looks to have been heat treated*; counterstain is not the correct hue. Borderline assessment outcome. Dako 7240 (MIB-1), 30 mins; Dako Envision+ HRP mouse K4004/5/6/7, 30 mins, *no RT indicated, on a Dako Autostainer Link 48.

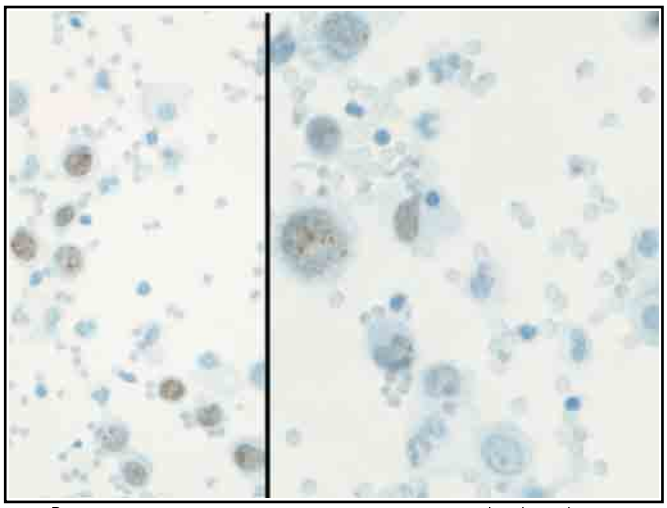


Fig 6. Poor Ki 67 demonstration in a NEQAS cytospin. Staining is very weak, with only the occasional nuclei seen. The slide failed the assessment. Dako 7240 (MIB-1), 1:100, without any retrieval, using a Ventana UltraView DAB kit on the Ventana Benchmark XT.

Selected Images showing Optimal and Sub-optimal {@stainingtext}

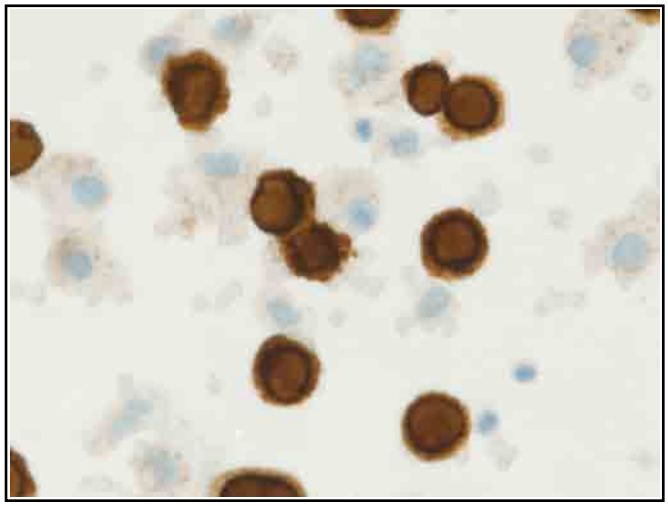


Fig 7. Sub-optimal cytokeratin on a NEQAS cytospin. Tumour cells are nicely demonstrated but there is some non-specific staining, probably due to the pre-treatment. RTU Ventana 760 2595 AE1/AE3/PCK26, 16 mins; Ventana Protease; RTU Ventana UltraView Kit (760-500), 12 mins; on a Ventana Benchmark ULTRA.

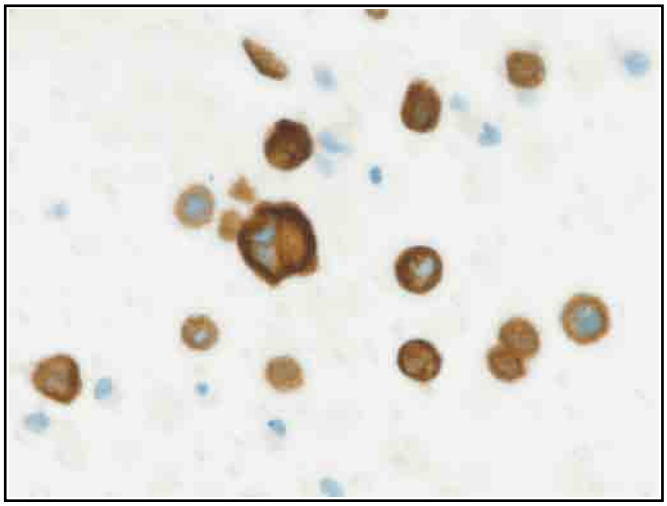


Fig 8. Nice cytokeratin demonstration in a NEQAS cell block sample. Slide is clean and staining is precise. RTU Ventana 760 2135 (AE1/AE3/PCK26), 4 mins; Ventana Protease 1 (760-2018), 8 mins; RTU Ventana OptiView Kit (760-700), 8 mins, on a Ventana Benchmark ULTRA.

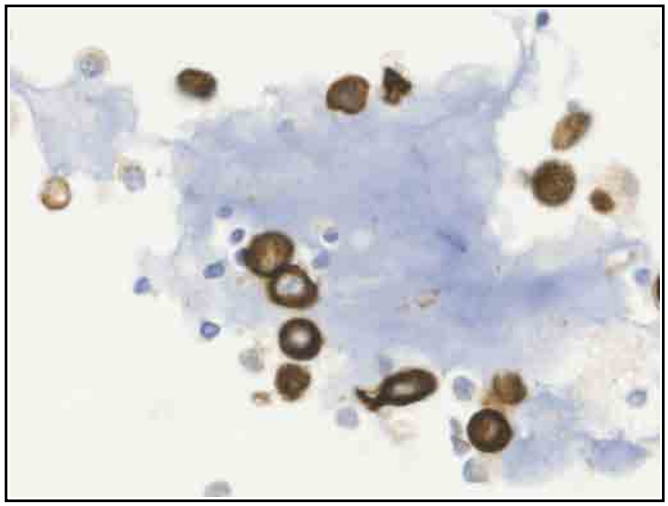


Fig 9. Sub-optimal cytokeratin on a NEQAS cell block. Staining is dirty, with some non-specific reactions, in addition the counterstain is excessive. Borderline assessment score. Dako M0821(MNF116); Leica ER1 10 mins; Leica Bond Polymer Refine (DS9800); on a Dako Autostainer Plus.

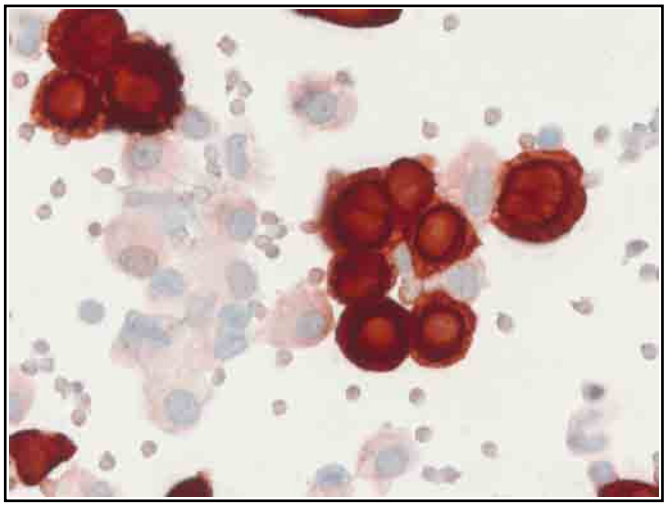


Fig 10. Sub-optimal cytokeratin on a NEQAS cytospin. Positive cells are nicely seen, but there is some bleeding of the chromogen, slide still adequate for diagnostic use. Dako M0821(MNF116), 1:400, 30 mins; no retrieval; with the RTU Leica Bond Polymer Refine kit (DS9800),8 mins; on a Leica Bond Max.

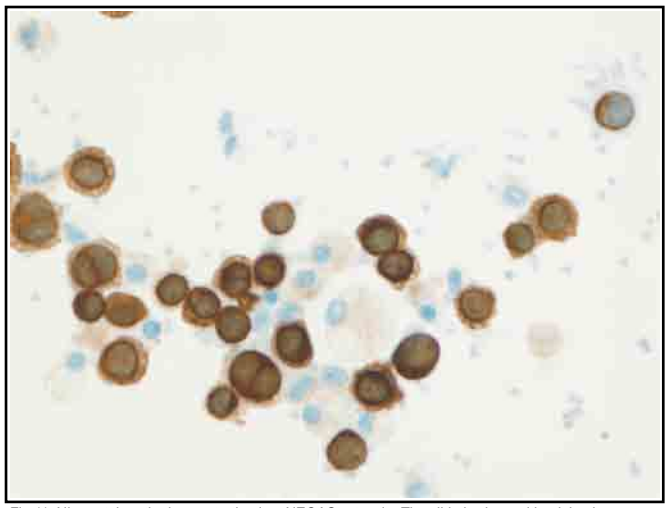


Fig 11. Nice cytokeratin demonstration in a NEQAS cytospin. The slide is clean with minimal background reactions. Tumour cells are easily seen, even at lower powers. Dako M0821(MNF116), 1:400, 15 mins; no retrieval; with the RTU Leica Bond Polymer Refine (DS9800), 8 mins; on a Leica Bond-III.

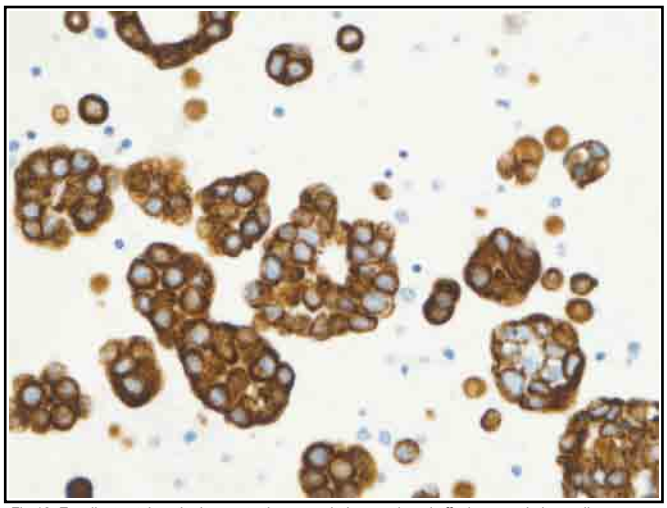
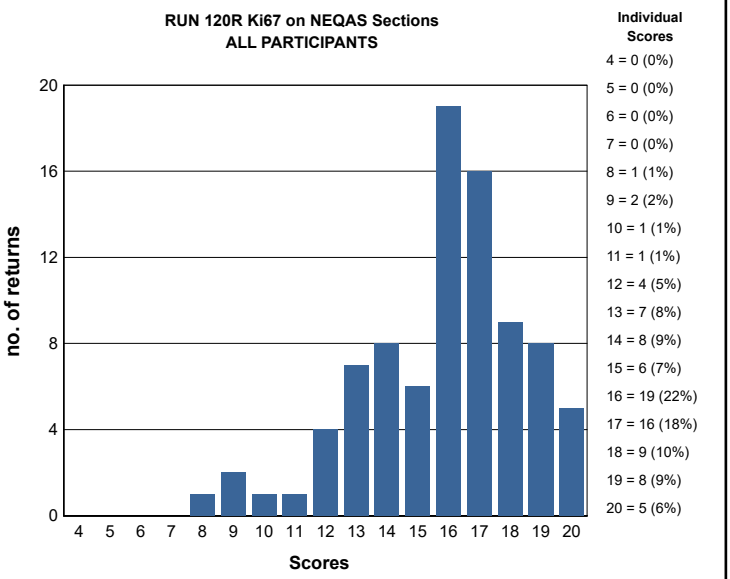


Fig 12. Excellent cytokeratin demonstration on an in-house pleural effusion sample in a cell block. All components are appropriately and optimally stained. RTU Dako M0821(MNF116), 8 mins; Ventana Protease 1 (760-2018), 4 mins @ RT; RTU Ventana OptiView Kit (760-700), 8 mins; on the Ventana Benchmark ULTRA.

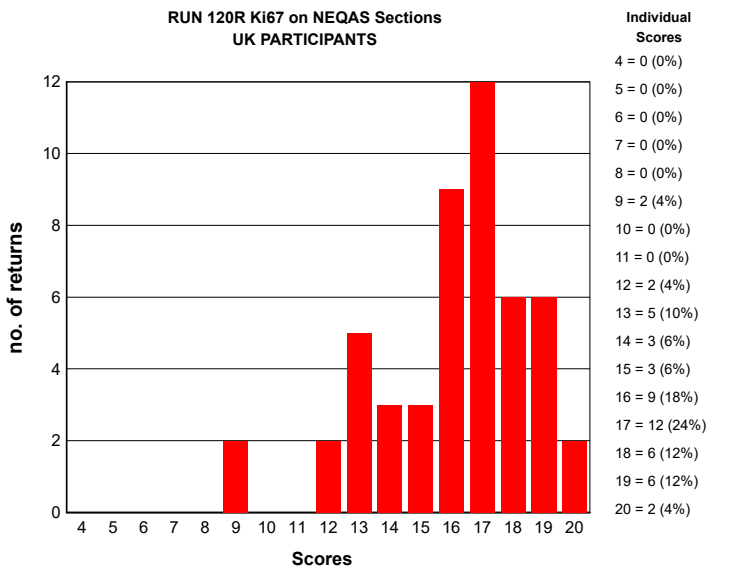
GRAPHICAL REPRESENTATION OF PASS RATES



Summary

16 - 20 = 57 (66%)
13 - 15 = 21 (24%)
10 - 12 = 6 (7%)
0 - 9 = 3 (3%)

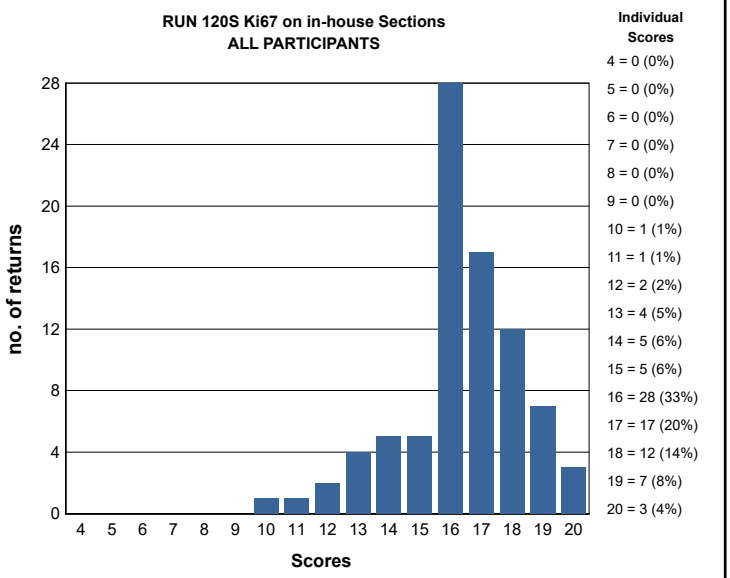
Median = 14.00



Summary

16 - 20 = 35 (70%)
13 - 15 = 11 (22%)
10 - 12 = 2 (4%)
0 - 9 = 2 (4%)

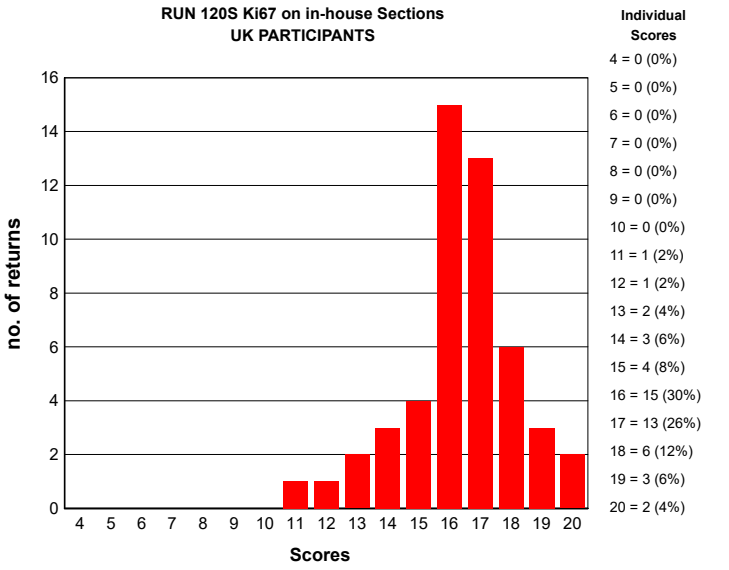
Median = 15.50



Summary

16 - 20 = 67 (79%)
13 - 15 = 14 (16%)
10 - 12 = 4 (5%)
0 - 9 = 0 (0%)

Median = 15.00

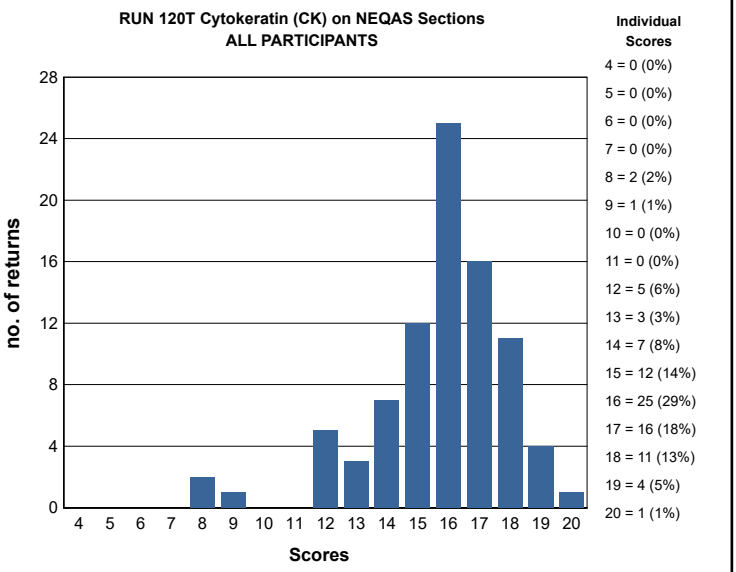


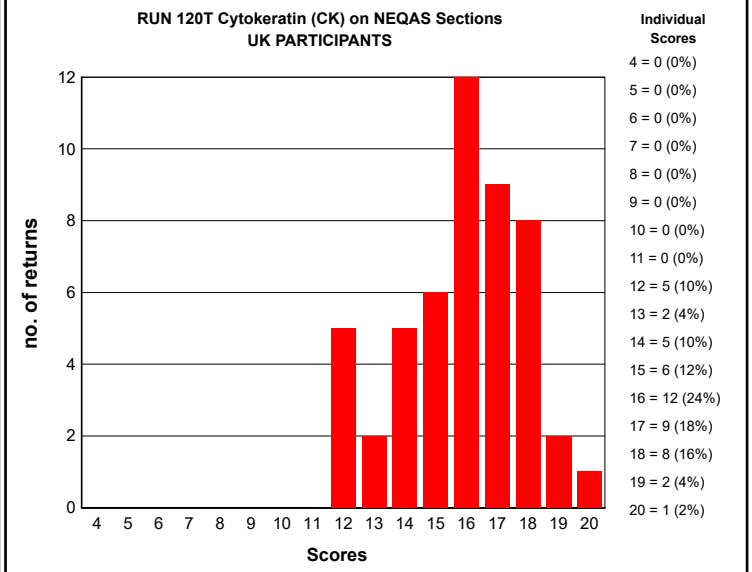
Summary

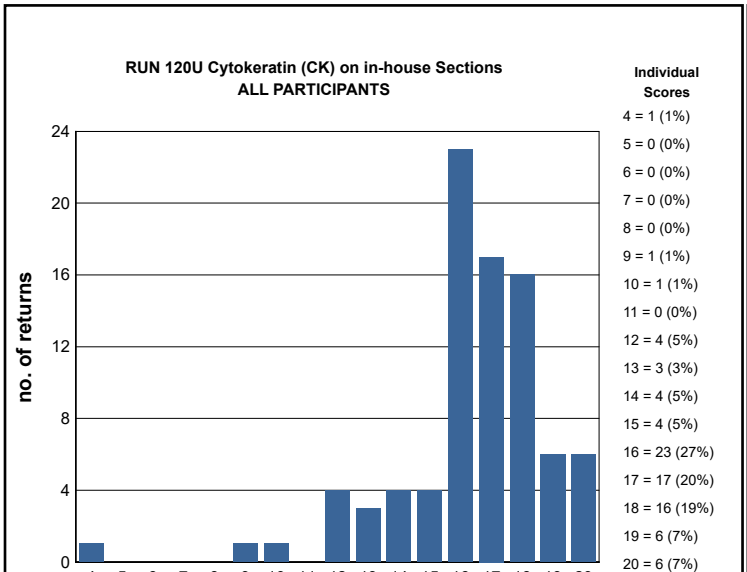
16 - 20 = 39 (78%)
13 - 15 = 9 (18%)
10 - 12 = 2 (4%)
0 - 9 = 0 (0%)

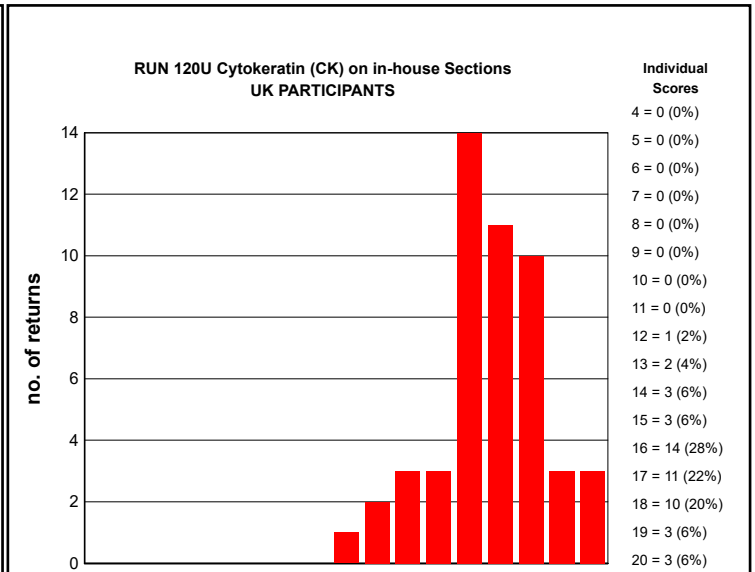
Median = 15.50

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: 120

Primary Antibody : Ki67

Antibody Details	N	%
Dako 7240 (MIB-1)	43	88
Dako FLEX RTU IR626 (MIB-1)	5	100
Neomarkers/Thermo Sci (SP6) RM 9106	1	100
Leica/Novocastra RTU (MM1) PA0118	1	100
Leica/Novocastra RTU (K2) PA0230	10	70
Ventana RTU (30-9) 790-4286	23	96
Other	3	100

Cytology Run: 120

Primary Antibody : Cytokeratin (CK)

Antibody Details	N	%
Becton Dickinson 349205 (CAM5.2)	3	67
BioGenex MU071-UC (clones AE1/AE3)	1	100
Dako M3515 (AE1/AE3)	14	93
Dako M0821(MNF116)	30	93
Leica/Novocastra RTU PA0909 (AE1/AE3)	3	100
Leica/Novocastra NCL-L-AE1/AE3	4	100
Vector VP-C419 (AE1/AE3)	1	100
Ventana 760 2135 (AE1/AE3/PCK26)	4	100
Ventana 760 2595 AE1/AE3/PCK26	10	90
Other	3	0
Cell Marque 313M- (AE1/AE3)	1	100
Leica/Novocastra NCL-AE1/AE3	2	100
Biomedicals BMA-T-1302	1	100
Dako FLEX RTU IR053 (AE1/AE3)	4	100
Ventana 790-4555 (CAM 5.2)	1	100
Leica/Novacastra NCL- L-CK5/6/8/18 (Multi 5D3/LP34	1	100
Dako Omnis FLEX GA053 (AE1/AE3)	2	100

Cytology Run: 120

Primary Antibody : Ki67

Antigen Retrieval	N	%
YES	33	38
NO	54	62
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	33	
Not Specified	0	

Cytology Run: 120

Primary Antibody : Cytokeratin (CK)

Antigen Retrieval	N	%
YES	25	29
NO	62	71
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	25	
Not Specified	0	

Cytology Run: 120

Heat Mediated Retrieval

Cytology Run: 120

Heat Mediated Retrieval

Cytology Run: 120

Enzyme Mediated Retrieval

Cytology Run: 120

Enzyme Mediated Retrieval

Cytology Run: 120

Detection	Cytokeratin (CK)		Ki67	
	N	%	N	%
AS PER KIT	3	100	6	83
Dako EnVision FLEX (K8000/10)	2	100	2	100
Dako EnVision FLEX+ (K8002/12)	7	100	6	100
Dako Envision+ HRP mouse K4004/5/6/7	1	100	1	0
Leica Bond Polymer AP Red Detection (DS9305)	1	0	0	0
Leica Bond Polymer Refine (DS9800)	21	86	19	84
Other	3	67	3	100
Power Vision DPVB999 HRP	0	0	1	100
Ventana iView system (760-091)	4	100	3	100
Ventana OptiView (760-700) + Amp. (7/860-099)	1	100	1	100
Ventana OptiView Kit (760-700)	18	100	17	88
Ventana UltraView Kit (760-500)	22	86	25	100

Cytology Run: 120

Automation	Cytokeratin (CK)		Ki67	
	N	%	N	%
BioGenex GenoMX 6000i	0	0	1	100
Dako Autostainer Link 48	8	100	8	88
Dako Autostainer plus	1	0	0	0
Dako Autostainer Plus Link	1	100	1	100
Dako Omnis	3	100	3	100
Leica Bond Max	9	100	6	83
Leica Bond-III	14	79	16	75
Other	0	0	1	100
Ventana Benchmark GX	2	100	3	100
Ventana Benchmark ULTRA	34	94	36	94
Ventana Benchmark XT	13	85	11	91

Cytology Run: 120

Chromogen	Cytokeratin (CK)		Ki67	
	N	%	N	%
AS PER KIT	8	100	10	80
DAKO DAB+	1	100	1	0
Dako DAB+ Liquid (K3468)	0	0	1	100
Dako FLEX DAB	9	100	8	100
Leica Bond Polymer Refine kit (DS9800)	22	86	19	79
Other	4	50	5	100
Ventana DAB	15	100	15	93
Ventana iview	4	100	4	75
Ventana Ultraview DAB	22	86	23	100

BEST METHODS - Gold Standard Antibody

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

Ki67 - Method 1

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

Primary Antibody:	Dako 7240 (MIB-1) , 16 Mins, RT ºC Dilution 1: 100
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 32mins, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB, RT ºC., Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 16 Mins, RT ºC Prediluted

Ki67 - Method 2

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

Primary Antibody:	Dako 7240 (MIB-1) , 30 Mins, 23 ºC Dilution 1: 80
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer, PH: 7.6
HMAR:	Dako PTLink, Buffer: ENVISION TR SOLUTION, PH: 9
EAR:	
Chromogen:	Dako FLEX DAB, 23 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 35 Mins, 23 ºC Prediluted

Ki67 - Method 3

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

Primary Antibody:	Dako FLEX RTU IR626 (MIB-1)
Automation:	Dako Autostainer Plus Link
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink
EAR:	
Chromogen:	Dako FLEX DAB
Detection:	Dako EnVision FLEX+ (K8002/12)

Ki67 - Method 4

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

Primary Antibody:	Ventana RTU (30-9) 790-4286 , 16 Mins, 20 ºC Dilution 1: 1
Automation:	Ventana Benchmark GX
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 standard
EAR:	
Chromogen:	Ventana Ultraview DAB, Time 1: 16 Mins, Time 2: 4 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 20 ºC

BEST METHODS - Secondary Antibody

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

Cytokeratin (CK) - Method 1

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

Primary Antibody:	Dako M3515 (AE1/AE3) , 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

Cytokeratin (CK) - Method 2

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

Primary Antibody:	Dako M0821(MNF116) , 60 Mins Dilution 1: 1:50
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	
EAR:	Ventana Protease 1 (760-2018), 37 ºC. Digestion Time NEQAS: 8 Mins. In-House: 8 Mins
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500) Prediluted

Cytokeratin (CK) - Method 3

Participant scored 19/20 (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 UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 36mins
EAR:	Other, 36 ºC. Digestion Time NEQAS: 4 Mins. In-House: 4 Mins
Chromogen:	Ventana DAB
Detection:	Ventana UltraView Kit (760-500) , 36 ºC Prediluted

Cytokeratin (CK) - Method 4

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

Primary Antibody:	Dako M3515 (AE1/AE3) , 32 Mins, 37 ºC Dilution 1: 100
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 32mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500) Prediluted

The Alimentary Tract Module: GIST Run 120

Neil Bilbe

	First Antibody	Second Antibody
Antigens Assessed:	CD117	DOG-1
Tissue Sections circulated:	Normal Appendix, GIST and Desmoid tumours.	
Number of Registered Participants:	121	
Number of Participants this Run	121 (100%)	

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: **(Figs 1 & 4)**
- 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: **(Figs 2, 3, 5, & 6)**
- Weak and/or patchy staining of the tumour cells of the GIST
- Little or no staining of the mast cells
- Excessive background or non specific staining
- Staining of the desmoid tumour

Second Antibody: DOG-1

Discovered on GIST 1 (DOG-1) antibody was initially described in 2004[4] and is beginning to be recognized as a more specific marker of GISTs than CD117 [4,5,6]. A recent study has shown DOG-1 sensitivity of 99% and found to be strong and easier to interpret than CD117[6]. The groups also showed that seven CD117 negative GIST cases were found to be DOG-1 positive, and six of the tumours had PDGFRA mutations. Furthermore, DOG-1 is not expressed in Kaposi sarcomas of the GI tract which have been shown to stain for CD117[7].

In more cases, combined CD117 and DOG-1 immunohistochemistry has been suggested as a sufficient panel to confirm diagnosis, with CD117 and DOG-1 negative cases tested further with a panel of antibodies including SMA, Desmin, S100 and molecular analysis, should be considered[6] .

Features of Optimal Immunostaining (Figs 7, 8 & 11)
- Good localisation of DOG-1 to cells of the GIST
- Good localisation of DOG-1- to interstitial cells of Cajal
- No staining of desmoid tumour

Features of Sub-optimal Immunostaining (Figs 9, 10 &12)
- Weak and/or patchy staining of the tumour cells of the GIST
- Excessive background or non specific staining
- Staining of the desmoid tumour
- Staining of the mast cells (Negative with DOG-1 though)

Tissue Distribution and Assessment Procedure

Composite slides comprising of a GIST, appendix and desmoid tumour were distributed to all participants for them to stain with the requested antibodies CD117 and DOG-1 using their routine protocol. The circulated tissue was sequential sections from the same tissue block used for both the requested antibodies.

Assessment Summary:

121 participants are registered on the Alimentary Tract: GIST module. All labs submitted at least one slide. Three labs did not submit any slides for DOG-1. A single lab did not submit any in-house controls for either CD117 (W) or DOG-1 (Wb). 8 labs used an alternative antibody for DOG-1 which was given as SMA, resulting in 476 slides: **V = 121, W = 120, Vb = 118, Wb = 117**

Overall: Pass Rate 86% (409), Borderline 13% (60), Fail1% (7)

CD117

Results from the **CD117** assessment showed an overall acceptable pass rate of 89%. This in line with previous Runs 117, 118, & 119 where the pass rates were 75%, 84%, and 91% respectively.

The main reason for sub-optimal/failed marks (two slides) was due to very weak staining of the GIST, cells of Cajal and mast cells. There were 11 borderline slides (9%), which had a variety of issues: weak staining (Fig 3), poor counterstain (Fig 5), background or inappropriate staining (Fig 6).

Three in-house slides (**W**) were scored < $^{10}/_{20;}$ Apart from weak staining, two of these also submitted inappropriate controls, i.e. not a multi-block with both GIST and appendix/colon.

The most popular CD117 antibody of choice still remains the Dako polyclonal (A4502), used by 84 (69%) of participants, and showed an acceptable pass rate of 90% in this assessment run, the same as for Run 119.

NEQAS Pass Rates Run 119 v 120 CD117 (V)		
Run no	Run 119 (N=118)	Run 120 (N =121)
Acceptable	91% (N=107)	89% (N=108)
Borderline	6% (N=7)	9% (N=11)
Unacceptable	3% (N=4)	2% (N=2)

DOG-1

The results were vastly improved from Run 118, with a pass rate of 91% on the NEQAS tissue (**Vb**), as against 79% previously. There was only a single failed slide (six in Run 118). This lab used the RTU Ventana (SP31) 760-4590; Ventana CC1 32mins; Ventana UltraView Kit (760-500); on the Benchmark XT. Apart from weak staining of the GIST sample, mast cells were also inappropriately stained with DOG-1. This clone also has a tendency to stain lymphocytes, albeit usually only weakly (Fig 9). Interestingly, this participant also failed both their NEQAS CD117 (**V**) and their DOG-1 in-house (**Wb**) slides (only failure). The most popular antibody is Leica NCL-L-DOG-1 (K9) c. 40%.

SMA summary

The average score for the 8 NEQAS SMA slides (**Vb**) was $^{16}/_{20}$ compared to $^{17}/_{20}$ for those submitting a DOG-1 stain. There was a 75% pass rate (6 SMA users) and 25% obtained a borderline mark (2 SMA users), the range of marks were 11-1964

References

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8. Novelli M, Rossi S, Rodriguez-Justo M, Taniere P, Seddon B, Toffolatti L, Sartor C, Hogendoorn PC, Sciot R, Van Glabbeke M, Verweij J, Blay JY, Hohenberger P, Flanagan A, Dei Tos AP. DOG1 and CD117 are the antibodies of choice in the diagnosis of gastrointestinal stromal tumours. Histopathology 2010, 57 (2):259-270.
9. Parfitt JR, Rodriguez-Justo M, Feakins R, Novelli MR. Gastrointestinal Kaposi's sarcoma: CD117 expression and the potential for misdiagnosis as gastrointestinal stromal tumour. Histopathology 2008; 52; 816–823.

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Selected Images showing Optimal and Sub-optimal immunostaining

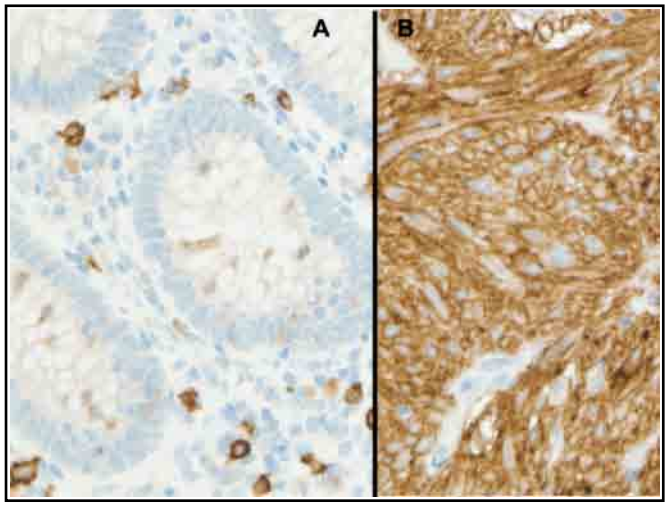


Fig 1. Excellent demonstration of CD117 on the NEQAS appendix (A), and an in-house GIST (B). Staining is clean and crisp in both sections. Dako A4502 polyclonal, 1:200, 15 mins, with Leica ER2 for 20 mins, second layer of the Leica Bond Polymer Refine (DS9800) kit, on the Leica Bond-III.

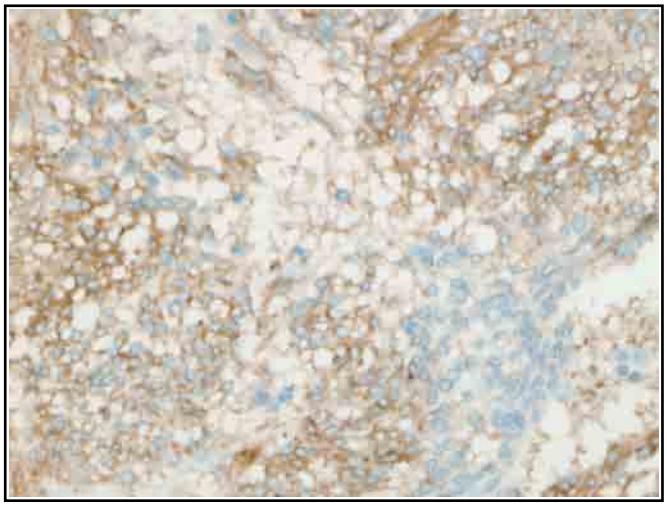


Fig 2. Poor demonstration of CD117 on the NEQAS GIST sample. The staining is weak, and sponge-like in appearance. This slide failed the assessment. Leica/Novocastra NCL-L-CD117 (T595), 1:25, 40 mins; Ventana CC1 64mins, on a Ventana Benchmark ULTRA, with the RTU Ventana OptiView Kit.

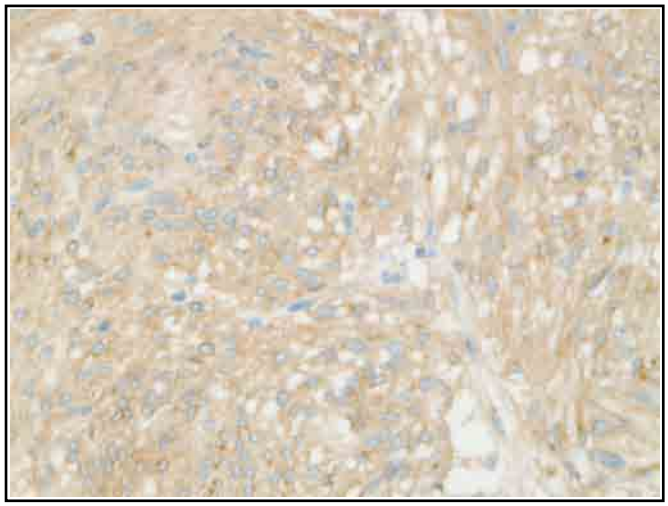


Fig 3. Sub-optimal demonstration of CD117 on the NEQAS GIST. Staining is weak, resulting in a borderline outcome. Dako A4502 polyclonal, 1:600, 25 mins, with Leica ER1 30 mins, on the Leica Bond-III, and the Leica Bond Polymer Refine (DS9800). Primary antibody is too dilute at 1:600.

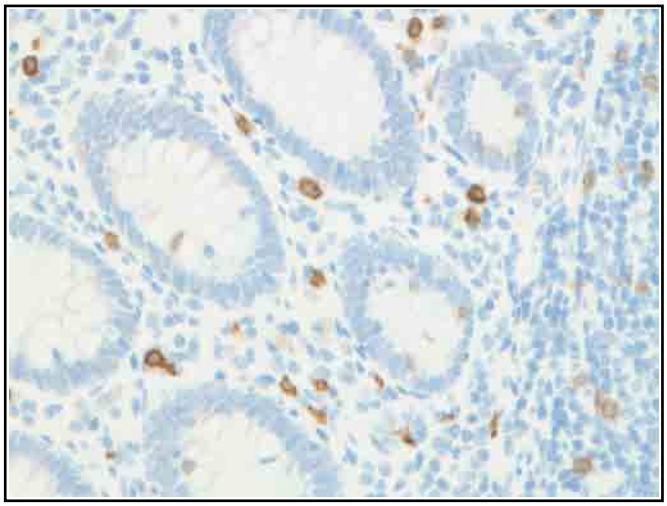


Fig 4. Excellent demonstration of CD117 on the NEQAS appendix sample. Staining is clean and precise, there is no background and the counterstain intensity is ideal. Cell Marque 117R/S-xx (YR145), 1:50, 60 mins, Leica ER1 30 mins, RTU Leica Bond Polymer Refine (DS9800), on a Leica Bond Max.

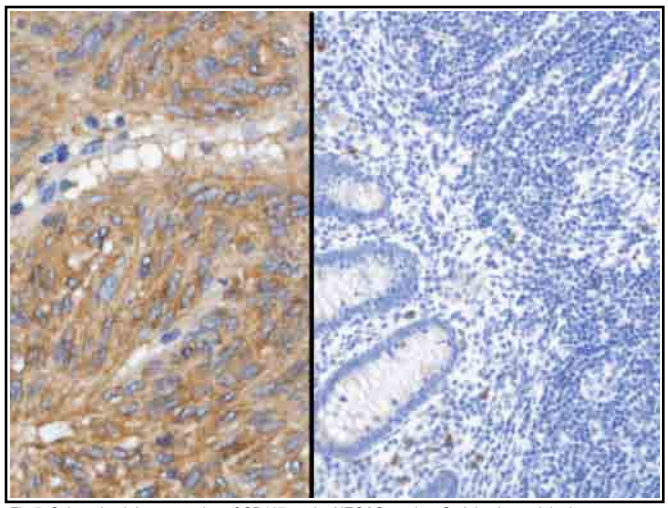


Fig 5. Sub-optimal demonstration of CD117 on the NEQAS section. Staining is weak in the GIST sample (L), but too weak in the appendix (R), haematoxylin intensity is excessive. Dako polyclonal, no dilution given, Leica ER2 10 mins, with the Leica BondMax Refine KIT, on the Leica Bond-III.

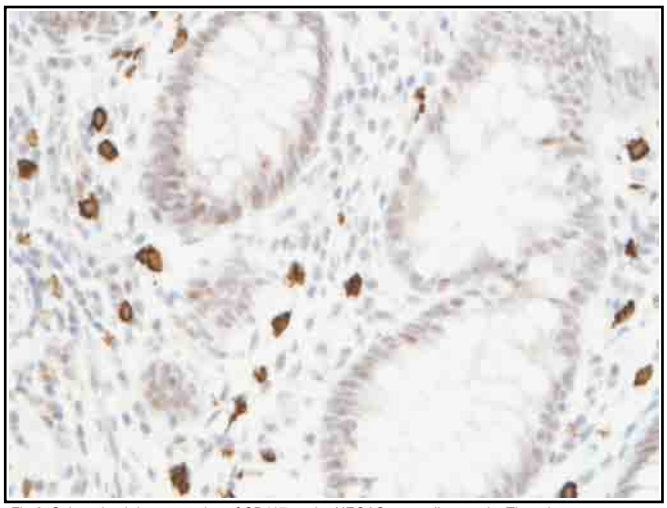


Fig 6. Sub-optimal demonstration of CD117 on the NEQAS appendix sample. There is pronounced background, and non-specific staining. Slides achieved a low pass at assessment. Dako polyclonal, 1:100, with Ventana CC2 mild, with the Ventana UltraView Kit (760-500), on the Ventana Benchmark XT.

Selected Images showing Optimal and Sub-optimal {@stainingtext}

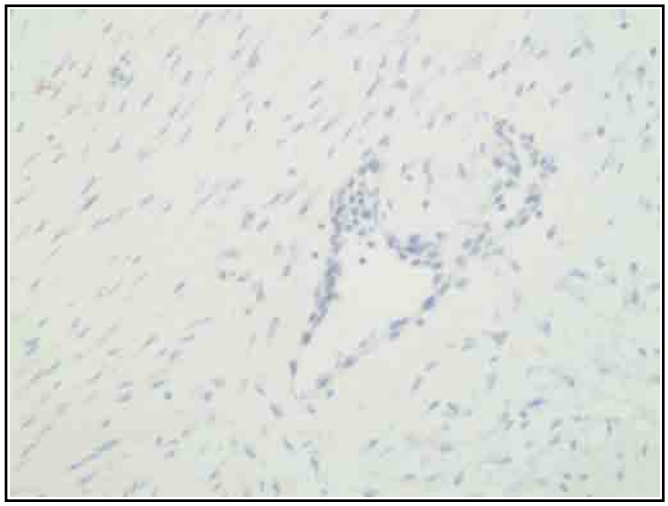


Fig 7. Excellent demonstration of DOG-1 showing the expected negative result in the Desmoid sample, and a corresponding optimally stained GIST sample (see Fig 8 below). The tissue is completely clean with a nice haematoxylin counterstain. See protocol details below.

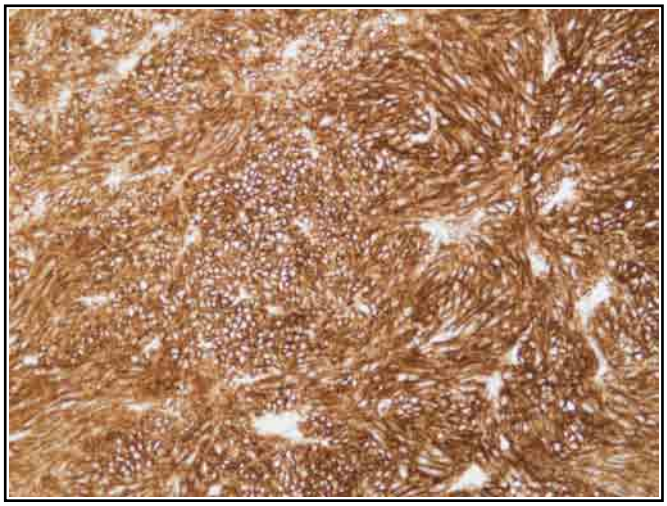


Fig 8. Excellent demonstration of DOG-1 on the NEQAS GIST portion of the section. Staining is crisp and precise without being excessively heavy. Leica NCL-L-DOG-1 (K9), 1:200, 15 mins, at RT, with Leica ER2 20 mins, RTU Leica Bond Polymer Refine (DS9800), on the Leica Bond Max.

Fig 9. Sub-optimal demonstration of DOG-1 on the NEQAS appendix; there is pronounced non-specific staining in the lymphocytes. Slide scored as borderline. RTU Ventana (SP31) 760-4590, 32 mins, Ventana CC1 56 mins, pre-diluted Ventana OptiView Kit (760-700), on the Ventana Benchmark ULTRA.

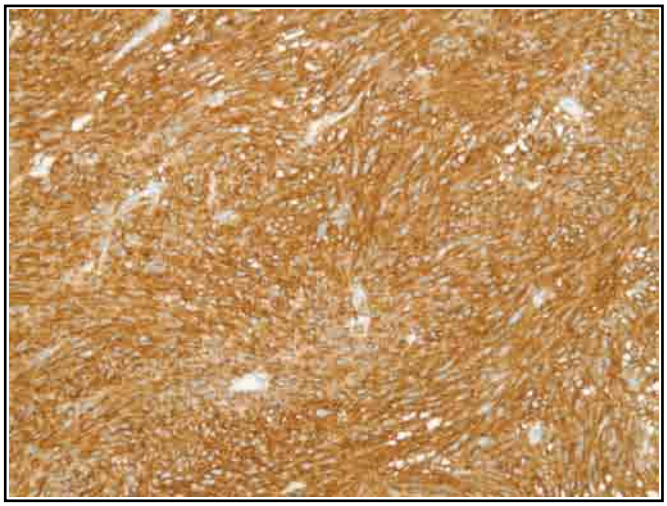


Fig 10. Sub-optimal demonstration of DOG-1 on the NEQAS GIST, staining could be stronger, given intensity achieved by other methods (see Fig 11). Ventana (SP31) 760-4590, RTU, 12 mins, using Ventana CC1 64 mins, secondary layer of Ventana OptiView Kit (760-700), on a Ventana Benchmark ULTRA.

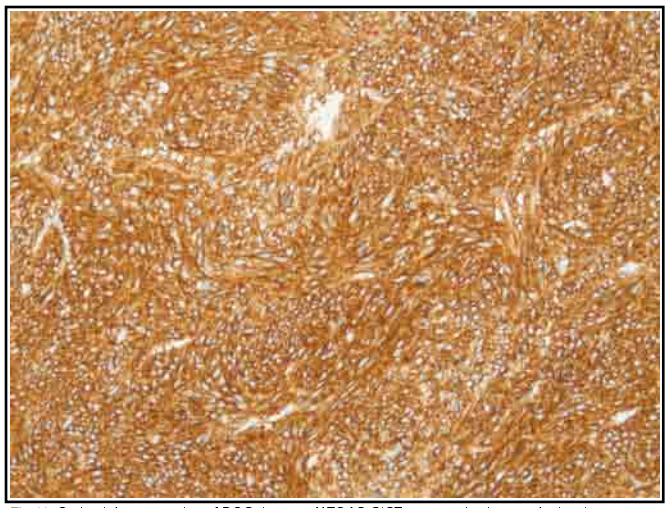


Fig 11. Optimal demonstration of DOG-1 on the NEQAS GIST, staining is clean and crisp. In addition there is a nice contrast with the counterstain. Leica NCL-L-DOG-1 (K9), 20 mins, Dako PTLink, 20 mins, with the Dako FLEX kit, on the Dako Autostainer Link 48.

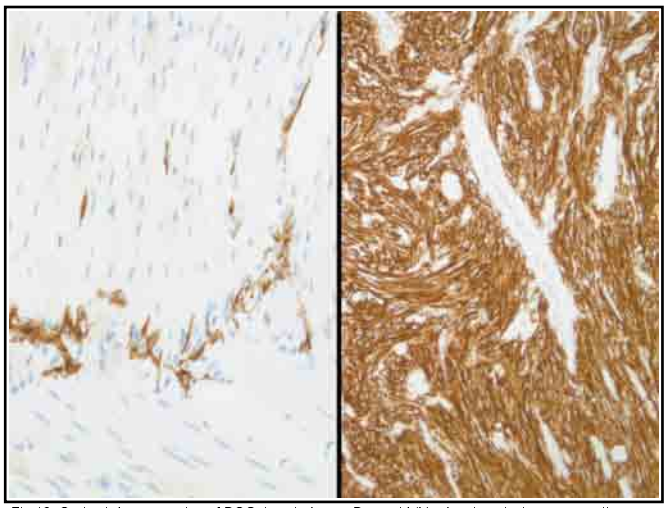
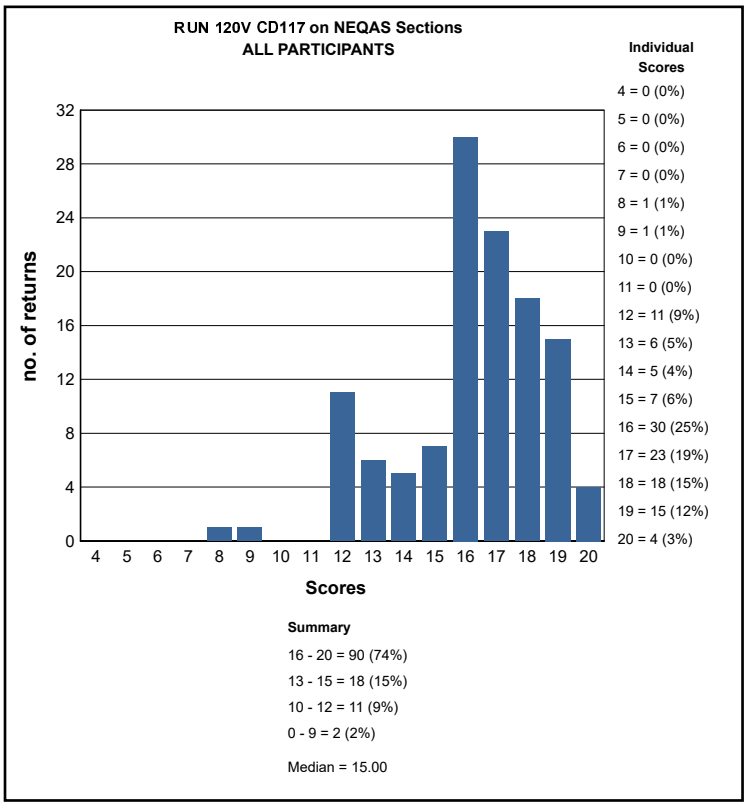
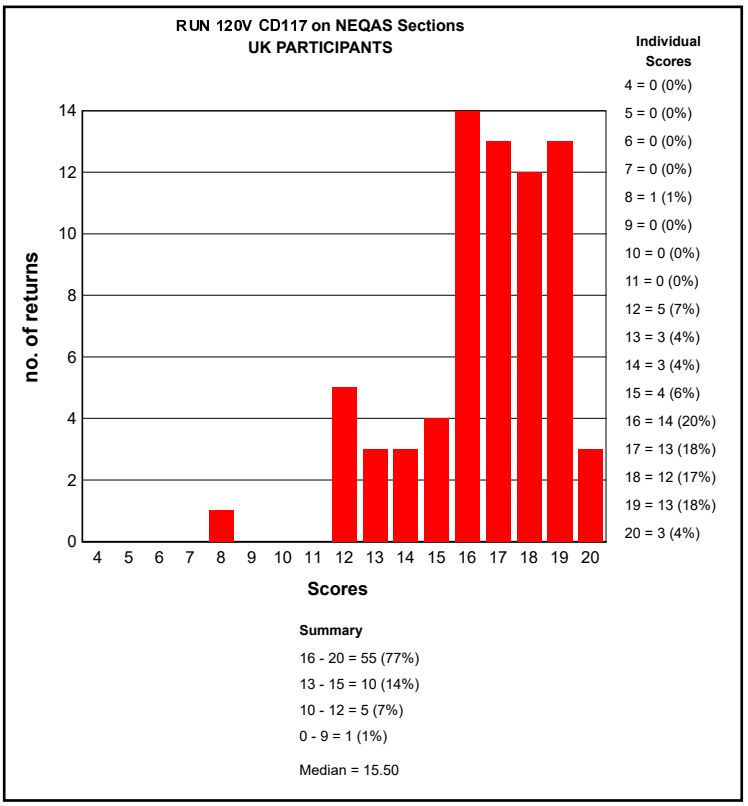
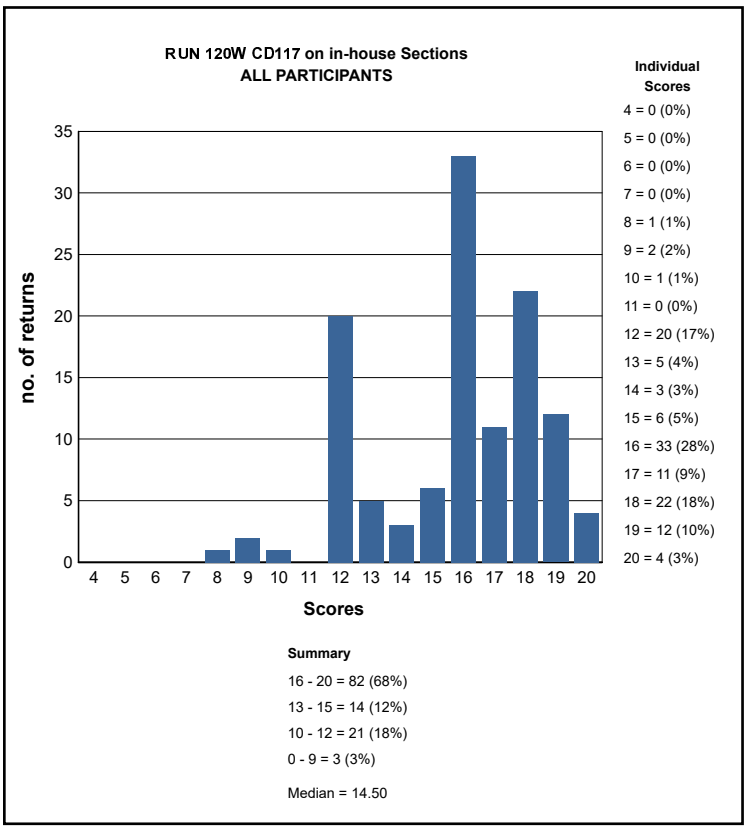


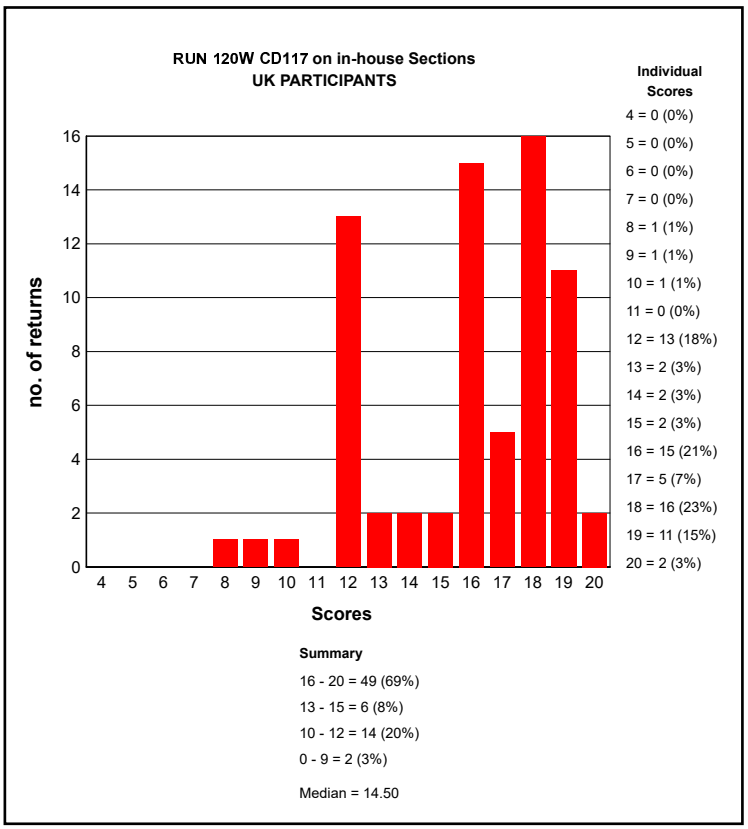
Fig 12. Optimal demonstration of DOG-1 on in-house Desmoid (L), showing nicely stained cells of Cajal, and a GIST sample (R). Leica NCL-L-DOG-1 (K9), 1:100, 30 mins, with the Dako PTLink, pH 9, 20 mins, RTU Dako EnVision FLEX+ (K8002/12) secondary layer, 20 mins, on a Dako Autostainer Link 48.

GRAPHICAL REPRESENTATION OF PASS RATES

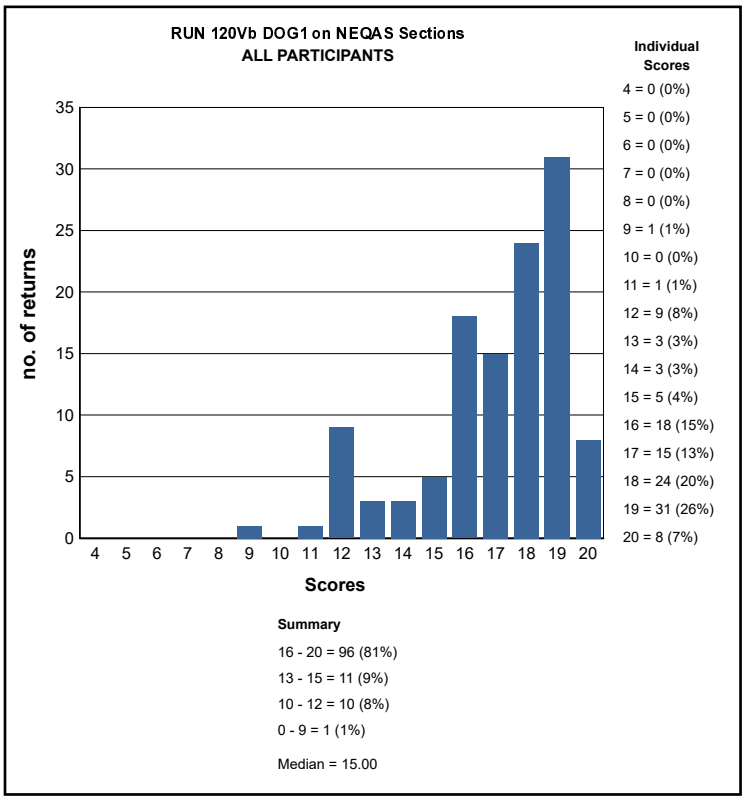


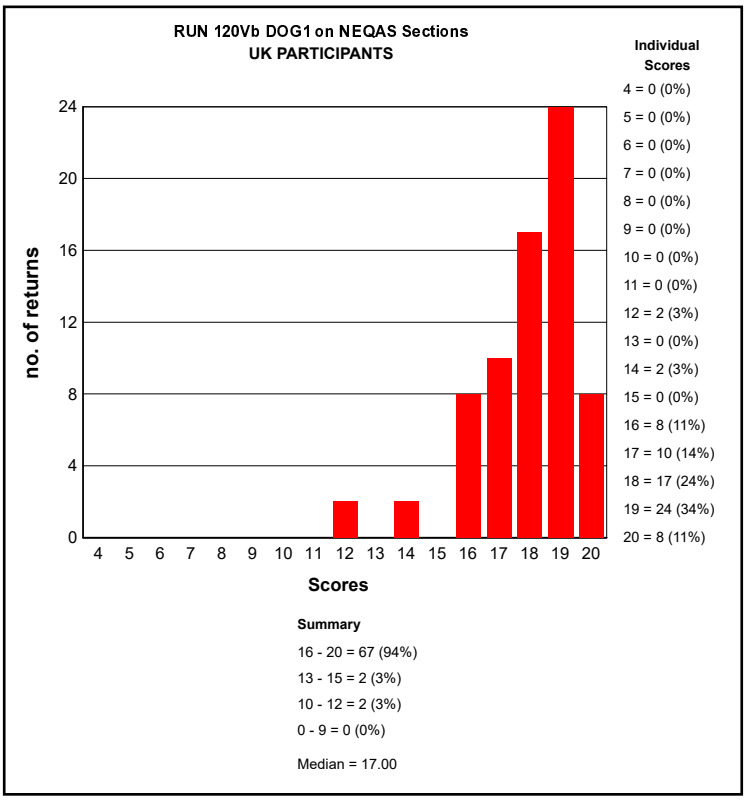


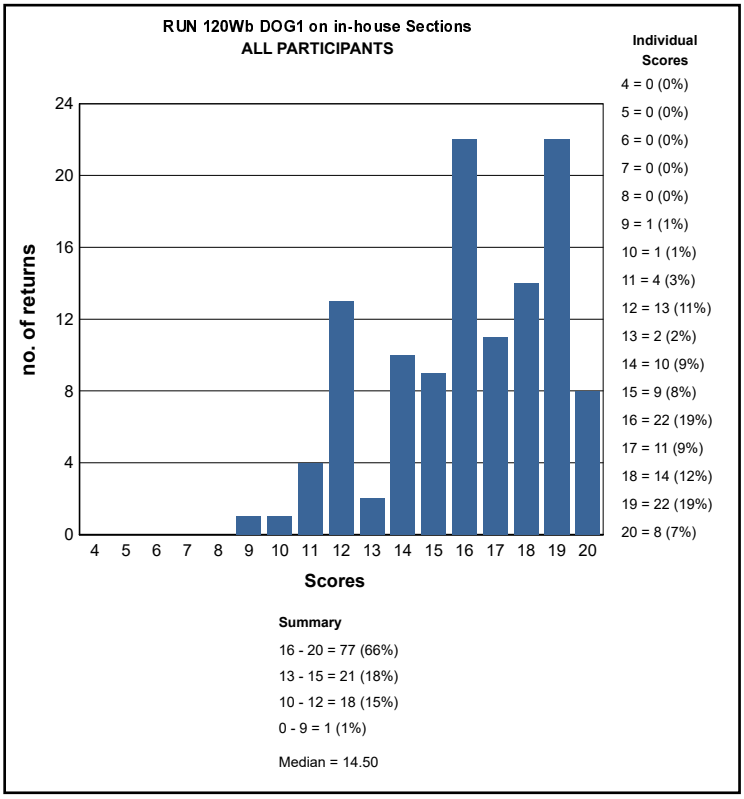


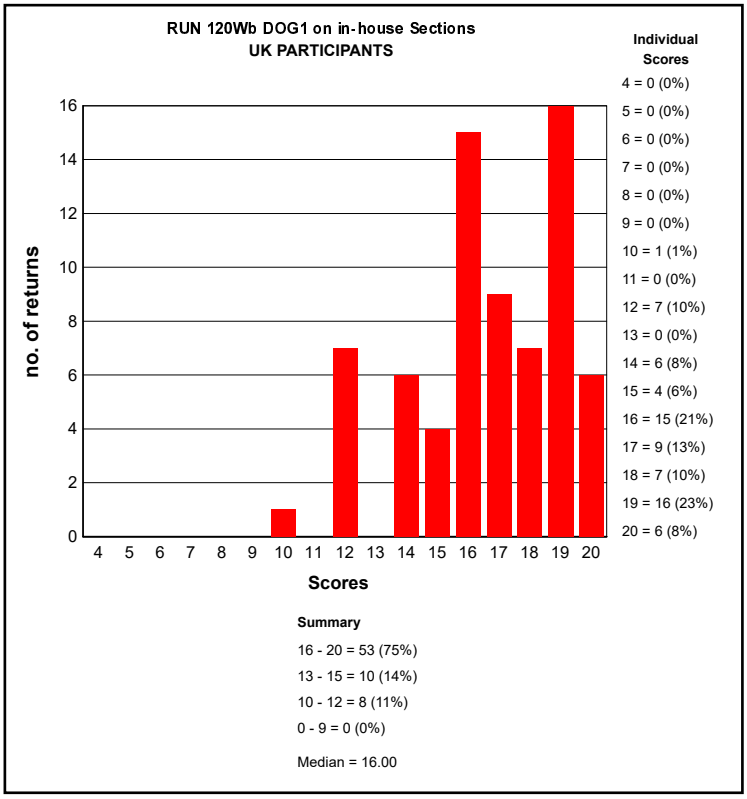


GRAPHICAL REPRESENTATION OF PASS RATES









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: 120

Primary Antibody : CD117

Antibody Details	N	%
Dako A4502 (rb poly)	84	90
Ventana 790-2939 (rb poly)	2	0
Cell Marque 117R/S-xx (YR145)	10	100
Leica/Novocastra NCL-L-CD117 (T595)	1	0
Leica/Novocastra RTU-CD117 (T595)	2	100
Ventana 790-2951 (9.7)	11	82
Epitomics AC-0029 (EP10)	1	100
Leica RTU (EP10) PA0007	9	100

Alimentary Tract Pathology Run: 120

Primary Antibody : DOG1

Antibody Details	N	%
Biocare CM 385 (1.1)	1	100
Cell Marque 244R-14/15/16 (SP31)	1	100
Leica NCL-L-DOG-1 (K9)	46	100
Leica PA0219 (K9)	27	100
Thermo RM-9132-R7 (SP31)	2	100
Other	8	75
Spring Biosciences M3311 (SP31)	1	0
Abcam TMEM16A (ab53212)	1	100
Ventana (SP31) 760-4590	26	73
Menarini MP-385-CM01/1	1	100
Diagnostic Biosystems Mob466 (DOG1.1)	1	100

Alimentary Tract Pathology Run: 120

Heat Mediated Retrieval	CD117 N	CD117 %	DOG1 N	DOG1 %
Dako Omnis	5	80	5	100
Dako PTLink	9	100	9	100
Leica ER1 20 mins	5	100	5	100
Leica ER1 30 mins	4	75	1	100
Leica ER2 10 mins	3	67	1	100
Leica ER2 20 mins	25	100	25	100
Leica ER2 30 mins	5	100	3	100
Leica ER2 40 mins	0	0	2	100
None	2	50	2	50
Other	1	0	0	0
Pressure Cooker	0	0	1	100
Ventana CC1 16mins	1	100	0	0
Ventana CC1 24mins	0	0	2	100
Ventana CC1 32mins	9	100	10	80
Ventana CC1 36mins	5	60	2	100
Ventana CC1 40mins	2	50	1	100
Ventana CC1 48mins	1	100	3	100
Ventana CC1 52mins	1	100	1	100
Ventana CC1 56mins	5	80	2	50
Ventana CC1 64mins	20	90	21	81
Ventana CC1 72mins	2	100	0	0
Ventana CC1 76mins	1	100	1	100
Ventana CC1 88mins	1	100	1	0
Ventana CC1 8mins	0	0	5	60
Ventana CC1 92mins	1	100	0	0
Ventana CC1 mild	5	80	2	100
Ventana CC1 standard	6	83	10	100
Ventana CC2 mild	1	100	1	100

Alimentary Tract Pathology Run: 120

Enzyme Mediated Retrieval	CD117 N	CD117 %	DOG1 N	DOG1 %
AS PER KIT	2	100	2	100
NOT APPLICABLE	90	91	76	93

Alimentary Tract Pathology Run: 120	CD117		DOG1	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	7	86	7	86
Dako EnVision FLEX (K8000/10)	3	67	3	100
Dako EnVision FLEX+ (K8002/12)	5	100	6	100
Dako Envision+ HRP mouse K4004/5/6/7	0	0	1	100
Dako Envision+ HRP rabbit K4008/9/10/11	2	100	0	0
Leica Bond Intense R Detection (DS9263)	0	0	1	100
Leica Bond Polymer Define (DS9713)	0	0	1	100
Leica Bond Polymer Refine (DS9800)	40	98	34	97
Other	2	100	2	100
Ventana iView system (760-091)	2	50	1	100
Ventana OptiView (760-700) + Amp. (7/860-099)	3	100	2	100
Ventana OptiView Kit (760-700)	26	85	25	80
Ventana UltraView Kit (760-500)	29	83	31	90

Alimentary Tract Pathology Run: 120	CD117		DOG1	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	0	0	1	100
Dako Autostainer Link 48	6	100	8	100
Dako Autostainer Plus Link	3	100	1	100
Dako Omnis	6	83	5	100
Leica Bond Max	12	100	11	91
Leica Bond-III	30	93	27	100
Ventana Benchmark GX	2	50	1	100
Ventana Benchmark ULTRA	45	84	48	83
Ventana Benchmark XT	15	87	13	85

Alimentary Tract Pathology Run: 120	CD117		DOG1	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	17	82	13	85
DAKO DAB+	2	50	0	0
Dako EnVision Plus kits	1	100	1	100
Dako FLEX DAB	10	90	10	100
Leica Bond Polymer Refine kit (DS9800)	39	97	36	97
Other	2	100	2	50
Ventana DAB	17	94	20	80
Ventana iview	2	50	1	100
Ventana Ultraview DAB	30	83	32	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: Cell Marque 117R/S-xx (YR145) , 60 Mins, amb ºC Dilution 1: 50
Automation: Leica Bond Max
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER1 30 mins
EAR:
Chromogen: Leica Bond Polymer Refine kit (DS9800), amb ºC., Time 2: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 15 Mins, amb ºC Prediluted

CD117 - Method 2

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

Primary Antibody: Dako A4502 (rb poly) , 60 Mins, 36 ºC Dilution 1: 1:200
Automation: Ventana Benchmark ULTRA
Method: Ventana Optiview
Main Buffer: Ventana reaction buffer (950-300), PH: 7.6
HMAR: Ventana CC1 64mins
EAR: NOT APPLICABLE
Chromogen: Ventana DAB, 36 ºC., Time 1: 8 Mins
Detection: Ventana OptiView Kit (760-700) , 8 Mins, 37 ºC Prediluted

CD117 - Method 3

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

Primary Antibody:	Leica/Novocastra RTU-CD117 (T595) , 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: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, 21 ºC Prediluted

CD117 - Method 4

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

Primary Antibody:	Ventana 790-2951 (9.7) , 32 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB
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

DOG1 - Method 1

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

Primary Antibody:	Ventana (SP31) 760-4590 , 16 Mins, RT ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 32mins, PH: 9
EAR:	
Chromogen:	Ventana DAB, RT ºC., Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 16 Mins, RT ºC Prediluted

DOG1 - Method 2

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

Primary Antibody:	Leica NCL-L-DOG-1 (K9) , 32 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 mild
EAR:	
Chromogen:	Ventana Ultraview DAB, 37 ºC., Time 1: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC Prediluted

DOG1 - Method 3

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

Primary Antibody:	Leica NCL-L-DOG-1 (K9) , 15 Mins, RT ºC Dilution 1: 200
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800), Time 1: 10 Mins, Time 2: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, RT ºC Prediluted

DOG1 - Method 4

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

Primary Antibody:	Leica NCL-L-DOG-1 (K9) , 20 Mins, RT ºC Dilution 1: 50
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX kit
Main Buffer:	AS PER KIT, PH: 7.4
HMAR:	Dako PTLink, PH: 9
EAR:	
Chromogen:	AS PER KIT
Detection:	AS PER KIT

Lynch Syndrome/HNPCC Run 120

Keith Miller and Jamie Hughes

	Gold Standard	Second Antibody
Antigens Assessed:	MLH1	PMS2
Tissue Sections circulated:	Positive and negative colonic tumours plus normal appendix	
Number of Registered Participants:	96	
Number of Participants This Run:	92 (95%)	

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, PMS2. MSH2, & MSH6, and the use of all 4 antibodies is recommended. There is a heterodimeric association between MLH1 & PMS2, and MSH2 & MSH6, 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 **MLH1** (1st Antibody) and **PMS2** (2nd antibody) using their routine protocols. The sections comprised of normal appendix and two colonic tumours (one with loss of MMR protein and one with no loss of MMR). Assessment of the stained slides was carried out around a multi-header microscope, with each slide being assessed by 4 independent assessors and a microscope lead. All samples on the slide were viewed, and then a final overall score out of 5 was given by each individual assessor. These four scores were then combined to give an overall score out of 20.

Features of Optimal Immunostaining: (Figs. 1, 3, 6, 7, 8, 9, 10 & 12)
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.
Tumour without loss of MMR protein: (Figs. 3, 4, 8 & 9)

- Strong nuclear staining in the tumour cells.
- Strong nuclear staining in the lymphocytes and stromal cells.

Tumour with loss of MMR protein: (Figs. 6, 10, 12)

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

Features of Suboptimal Immunostaining: (Figs. 2, 5 & 11)
Appendix: (Figs. 2)

- Weak, uneven, partially missing staining of relevant cells.

Tumour without loss of MMR protein: (Figs. 5)

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

Tumour with loss of MMR protein: (Figs. 11)

- 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:

MLH1
92 laboratories submitted slides for the MLH1 assessment. The results show an increase of 11% in acceptable results from Run 118, as shown in the table below:

MLH1 Pass Rates : NEQAS section		
	Run 118	Run 120
Acceptable	73%(N=66)	84% (N=77)
Borderline	16%(N=14)	11% (N=10)
Unacceptable	11%(N=10)	5% (N=5)

The predominant reasons for a borderline or failed result in run 120 was due to either weak staining or background staining being observed.

The in-house MLH1 had a better result with 93% (N=86) achieving an acceptable result, 5% (N=5) receiving a borderline result and on 1% (N=1) receiving an unacceptable result. Predominant reasons again due to very weak demonstration of antigen and/or excessive background.

The most popular antibody used in this run was Ventana 790-4535 (M1) used by 40 participants with a pass rate of 85%. The second most popular antibody was Dako M3640 (ES05) used by 14 participants with a pass rate of 86%.

The most common automated platform for MLH1 was the Ventana Benchmark Ultra used by 41 participants with a pass rate of 83%. The second most common was the Leica Bond III used by 17 participants with a 88% pass rate.

PMS2
92 laboratories submitted slides for the PMS2 assessment. The results show an increase of 8% in acceptable results from Run 118, as shown in the table below:

PMS2 Pass Rates : NEQAS section		
	Run 118	Run 120
Acceptable	72%(N=33)	79% (N=72)
Borderline	15%(N=7)	12% (N=11)
Unacceptable	13%(N=6)	10% (N=9)

The predominant reasons for a borderline or failed result in run 120 was due to either weak staining or excessive background staining being observed, with some exhibiting cytoplasmic staining.

The in-house PMS2 had an improved result with 89% (N=80) achieving an acceptable result, 8% (N=7) receiving a borderline result and on 3% (N=3) receiving an unacceptable result. Predominant reasons for loss of marks were again due to very weak demonstration of antigen and/or excessive background.

The most popular antibody used in this run was Ventana CONFIRM 760-45331 (EPR3947) used by 40 participants with a pass rate of 75%. The second most popular antibody was Dako M3647 (EP51) used by 19 participants with a pass rate of 89%.

The most common automated platform for MSH2 was the Ventana Benchmark Ultra used by 41 participants with a pass rate of 73%. The second most common was the Leica Bond III used by 18 participants with a 83% pass rate.

In-House Control Tissue Recommendations

A number 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, we would recommend 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 to participants using only appendix. 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.

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1. 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.
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/.
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4. Poulogiannis, G., Frayling, I. and Arends, M. (2010). DNA mismatch repair deficiency in sporadic colorectal cancer and Lynch syndrome. *Histopathology*, 56(2), pp.167-179.
5. Vasen, H., Moslein, G., Alonso, A., Bernstein, I., Bertario, L., Blanco, I., Burn, J., Capella, G., Engel, C., Frayling, I., Friedl, W., Hes, F., Hodgson, S., Mecklin, J., Moller, P., Nagengast, F., Parc, Y., Renkonen-Sinisalo, L., Sampson, J., Stormorken, A. and Wijnen, J. (2007). Guidelines for the clinical management of Lynch syndrome (hereditary non-polyposis cancer). *Journal of Medical Genetics*, 44(6), pp.353-362.
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 MLH1 in the NEQAS distributed appendix, showing strong staining of the basal and lower half of the epithelial crypts, fading towards the luminal surface. The Ventana pre-diluted M1 antibody was used on the Benchmark XT, CC1 pre-treatment for 32 mins with Optiview detection.

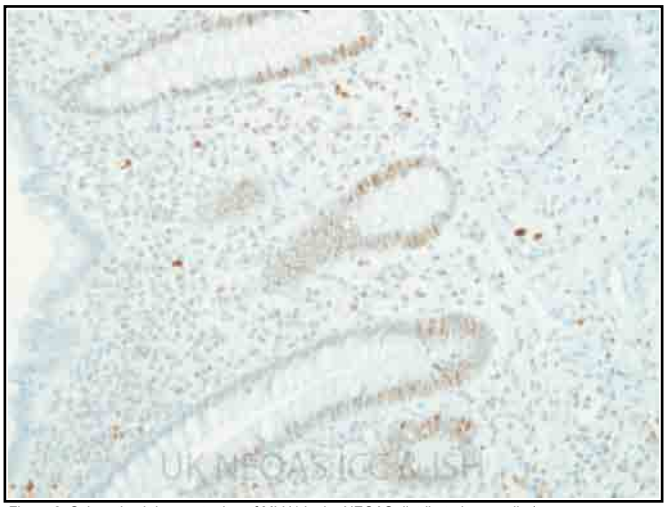


Figure 2: Sub-optimal demonstration of MLH1 in the NEQAS distributed appendix (compare to Fig 1). Not only is the staining weak, but far fewer lymphocytes than expected are demonstrated. Section stained with the Dako ES05 prediluted antibody on the Ventana Benchmark, CC1 retrieval for 64 minutes.

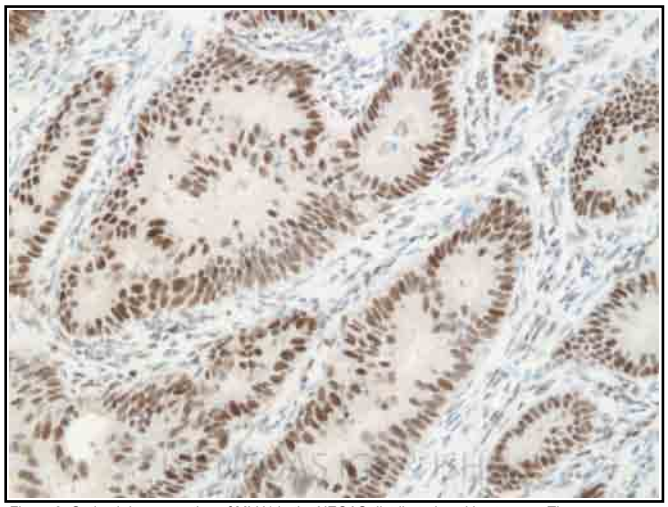


Figure 3: Optimal demonstration of MLH1 in the NEQAS distributed positive tumour. The example shows strong nuclear staining in the tumour cells as well as the intra-tumoral lymphoid and stromal cells. Staining carried out using the Dako ES05 clone on the Leica BondMax, retrieval for 40 minutes with ER2 buffer.

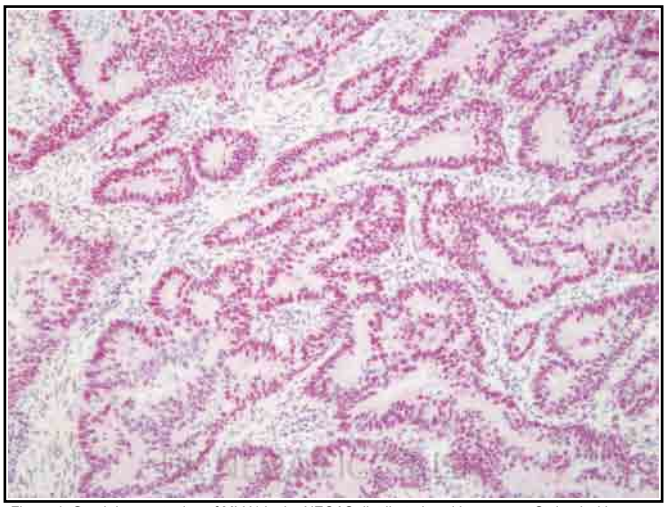


Figure 4: Good demonstration of MLH1 in the NEQAS distributed positive tumour. Stained with the BD Pharmingen G168-15 antibody, 1:25, using the Bond Refine Red Detection on the BondMax, ER2 for 30 minutes.

Figure 5: Unacceptable demonstration of MLH1 in the NEQAS distributed positive tumour (compare to Figs 3&4). The staining is very weak and patchy, with many of the tumour nuclei expected to stain not being demonstrated. (Same protocol as Fig 2).

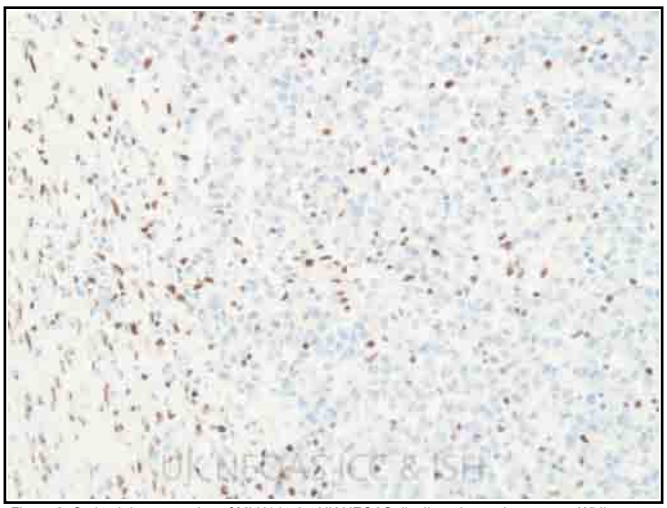


Figure 6: Optimal demonstration of MLH1 in the UK NEQAS distributed negative tumour. Whilst the tumour is negative, the intra-tumoural lymphoid and stromal cells are staining strongly positive, acting as the internal tissue control. (Same protocol as Fig 3).

Selected Images showing Optimal and Sub-optimal {@stainingtext}

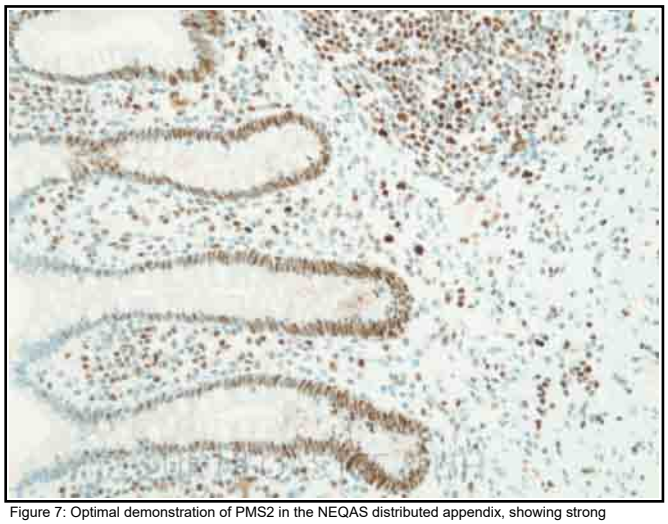


Figure 7: Optimal demonstration of PMS2 in the NEQAS distributed appendix, showing strong staining of the basal and lower half of the epithelial crypts and fading towards the luminal surface. The method used was with the Ventana EPR3947 antibody on the Benchmark ULTRA, CC1 pre-treatment for 64 minutes.

Figure 8: Optimal demonstration of PMS2 in the positive tumour. Not only is the nuclear staining strong in the tumour cells, but the intra-tumoural lymphocytes and stromal cells are also showing strong expression. (same protocol as Fig 7).

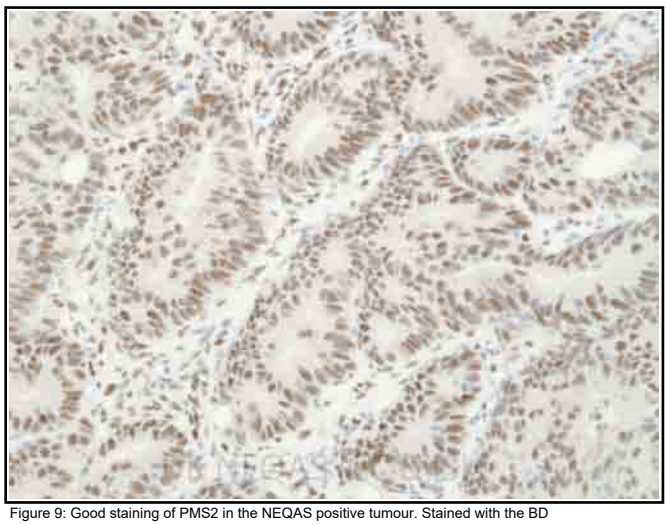


Figure 9: Good staining of PMS2 in the NEQAS positive tumour. Stained with the BD Pharmingen A16-4 antibody on the Leica Bond III with ER2 retrieval for 40 minutes.

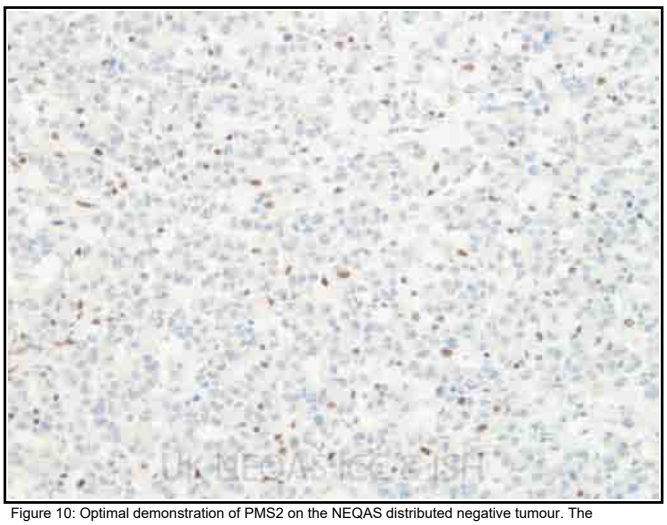


Figure 10: Optimal demonstration of PMS2 on the NEQAS distributed negative tumour. The tumour nuclei remain negative, but the stromal cells and lymphocytes are staining strongly and act as an internal tissue control. (Same protocol as Fig 9).

Figure 11: Sub-optimal demonstration of PMS2 on the NEQAS distributed negative tumour. The image shows inappropriate non-specific background staining. This is most likely caused by the length of the antibody incubation being too long. Stained with the Ventana EPR3947 pre-diluted antibody, on the Benchmark ULTRA with CC1 retrieval 92 minutes. Antibody

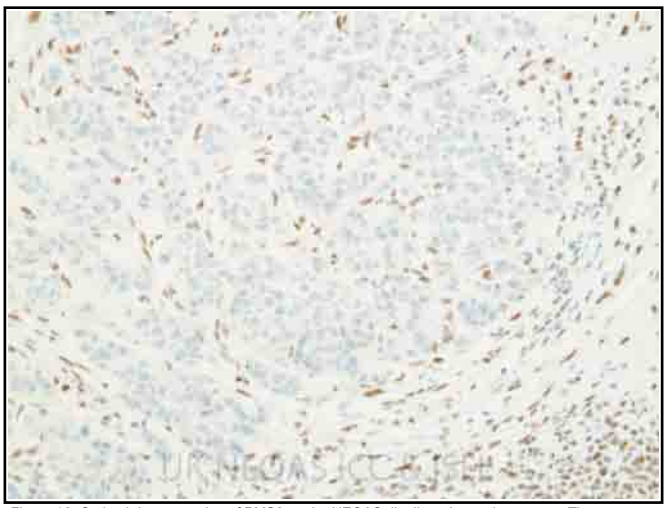
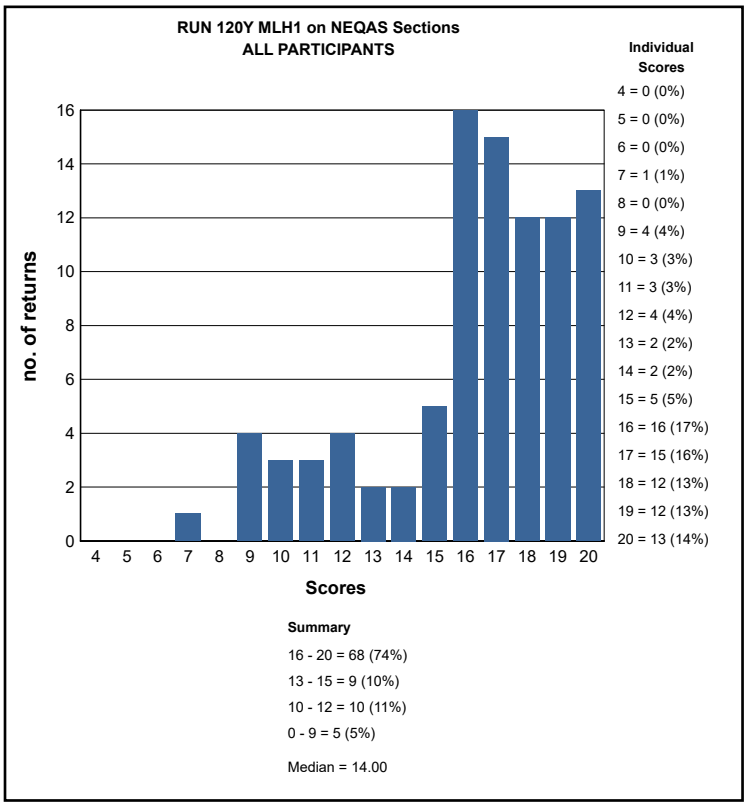
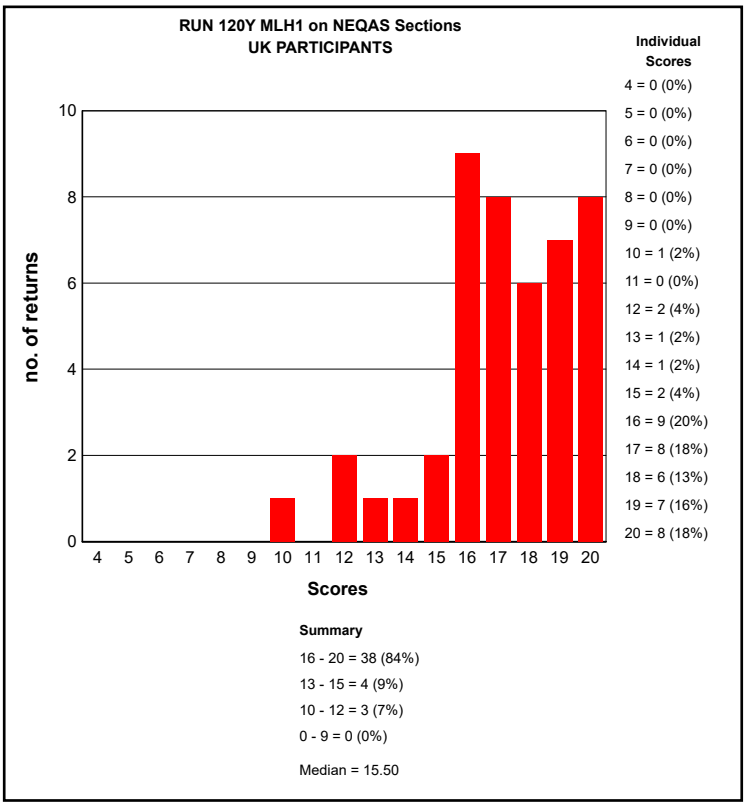
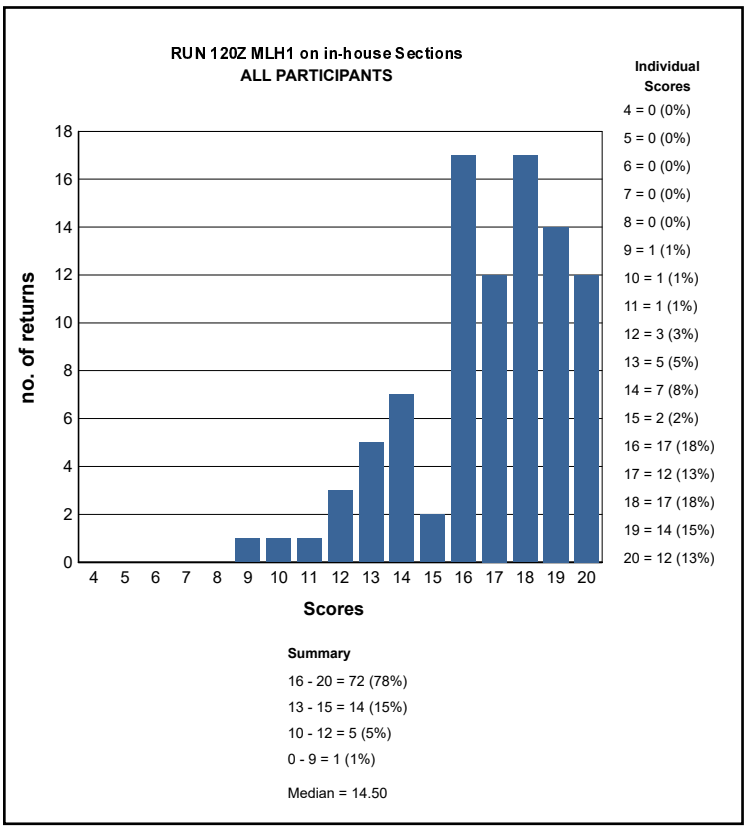


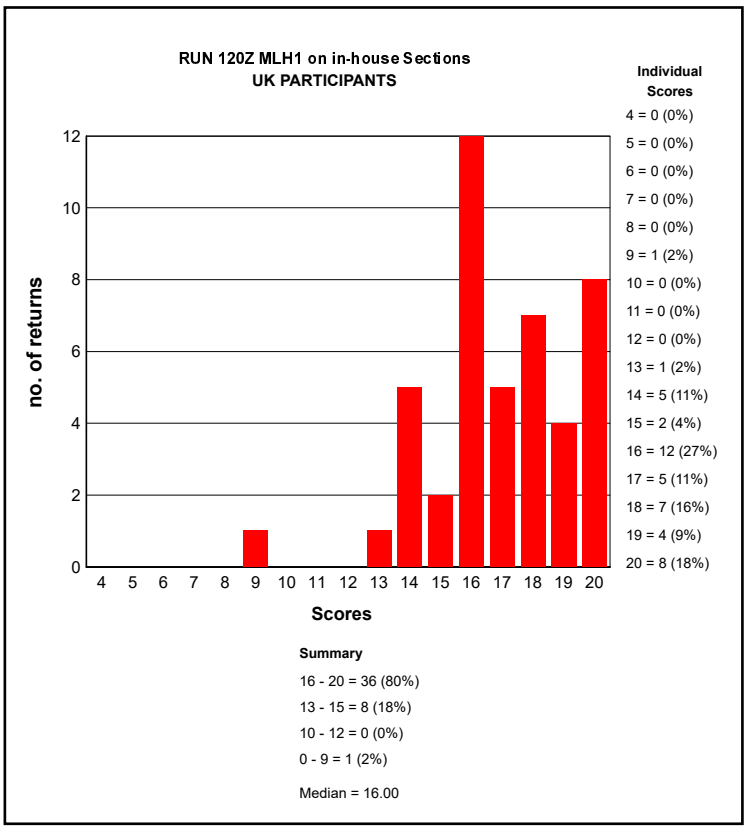
Figure 12: Optimal demonstration of PMS2 on the NEQAS distributed negative tumour. The tumour nuclei remain negative, but the stromal cells and lymphocytes are staining strongly and act as an internal tissue control. Stained with the Cell Marque EPR3947 pre-diluted antibody on the Bond III, ER2 retrieval for 30 minutes.

GRAPHICAL REPRESENTATION OF PASS RATES

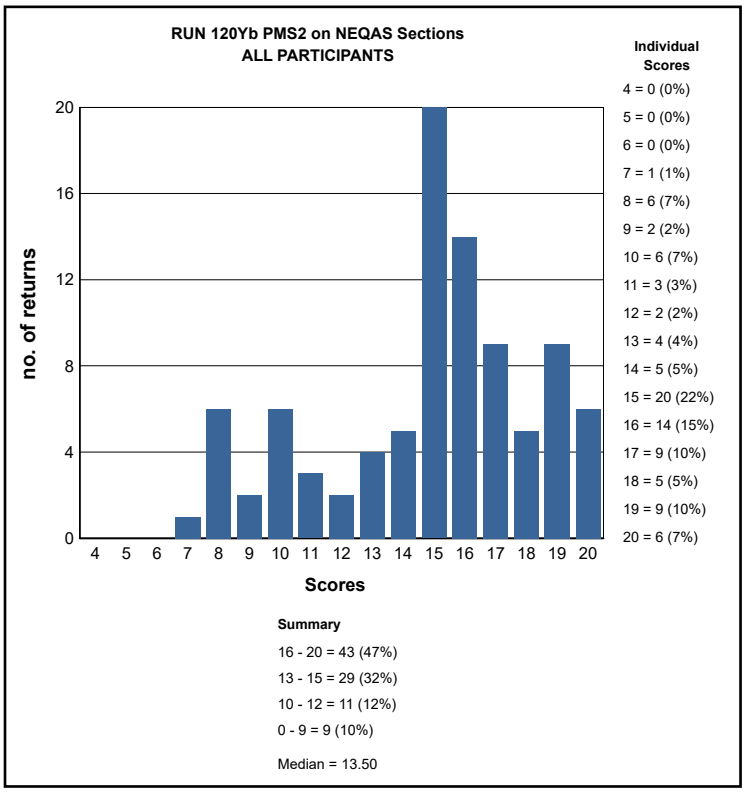


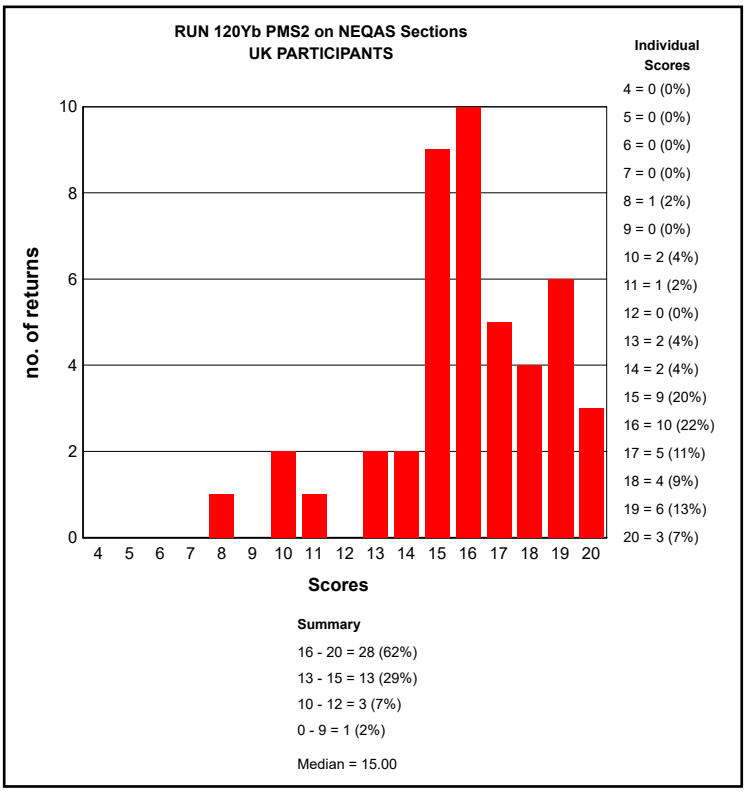


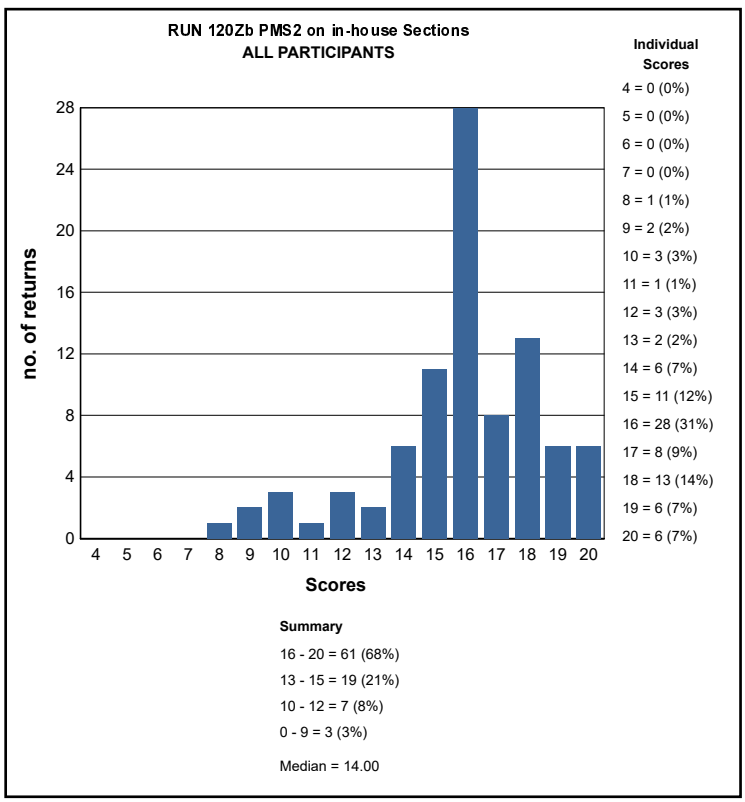


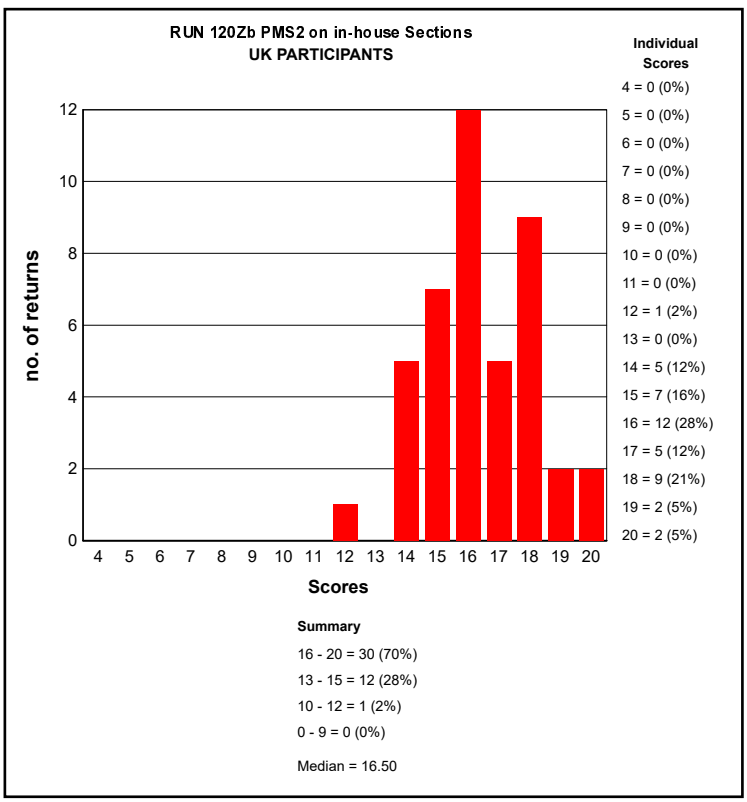


GRAPHICAL REPRESENTATION OF PASS RATES









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: 120

Primary Antibody : MLH1

Antibody Details	N	%
BD Pharmingen (G168-15)	5	80
Biocare medical CM/PM 220 (G168-15)	2	50
Ventana 760-4264 (G168-728)	1	100
BD Pharmingen (G168-728)	1	0
Novocastra NCL-L-MLH1 (ES05)	13	100
Cell Marque CMAx/Cx (G168-728)	1	0
Dako M3640 (ES05)	14	86
Leica Bond RTU PA0610 (ES05)	3	67
Dako Flex RTU IR079/IS079 (ES05)	11	82
Ventana 790-4535 (M1)	40	85

Primary Antibody : PMS2

Antibody Details	N	%
BD Bio/Pharmingen 556415 (A16-4)	8	88
Cell Marque 288R -17/18 (EPR3947)	3	100
Leica/Novoca NCL-L-PMS2 (MOR4G)	4	25
Ventana 760-4531 (EPR3947)	40	75
Cell Marque 288M -16 (MRQ28)	1	100
Dako M3647 (EP51)	19	89
Dako RTU FLEX IR087 (EP51)	13	77
Epitomics AC-0049 (EP51)	1	100
BioSB BSB 2124 (EP15)	2	50

HNPCC Run: 120

Heat Mediated Retrieval	MLH1 N	MLH1 %	PMS2 N	PMS2 %
Biocare Decloaking Chamber	1	0	1	100
Dako Omnis	3	100	4	75
Dako PTLink	9	78	9	100
Lab vision PT Module	1	0	1	100
Leica ER1 20 mins	2	100	0	0
Leica ER1 30 mins	1	100	1	0
Leica ER1 40 mins	1	100	1	100
Leica ER2 20 mins	7	86	6	83
Leica ER2 30 mins	7	100	7	86
Leica ER2 40 mins	7	86	9	89
Other	1	100	1	100
Ventana CC1 32mins	4	75	1	100
Ventana CC1 36mins	2	0	1	0
Ventana CC1 40mins	5	100	1	100
Ventana CC1 48mins	6	67	4	50
Ventana CC1 56mins	4	75	4	75
Ventana CC1 64mins	21	86	10	70
Ventana CC1 72mins	1	100	2	50
Ventana CC1 76mins	0	0	1	0
Ventana CC1 80mins	2	100	4	100
Ventana CC1 88mins	2	100	2	100
Ventana CC1 92mins	1	100	19	74
Ventana CC1 standard	3	100	1	100
Ventana CC2 92mins	0	0	2	50

HNPCC Run: 120

Enzyme Mediated Retrieval	MLH1 N	MLH1 %	PMS2 N	PMS2 %
AS PER KIT	3	100	2	100
NOT APPLICABLE	48	83	52	77

HNPCC Run: 120	MLH1		PMS2	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	5	100	4	75
Dako EnVision FLEX (K8000/10)	3	100	3	67
Dako EnVision FLEX+ (K8002/12)	7	71	8	100
Leica Bond Polymer Define (DS9713)	0	0	1	100
Leica Bond Polymer Refine (DS9800)	23	91	22	82
Other	3	67	3	100
Ventana OptiView (760-700) + Amp. (7/860-099)	16	81	20	75
Ventana OptiView Kit (760-700)	29	86	27	74
Ventana UltraView Kit (760-500)	4	50	2	0

HNPCC Run: 120	MLH1		PMS2	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	0	0	1	100
Dako Autostainer	1	0	1	100
Dako Autostainer Link 48	7	71	7	100
Dako Autostainer plus	1	100	1	100
Dako Autostainer Plus Link	1	100	1	100
Dako Omnis	3	100	4	75
Leica Bond Max	9	100	7	86
Leica Bond-III	17	88	18	83
None (Manual)	1	0	1	100
Ventana Benchmark GX	1	100	1	100
Ventana Benchmark ULTRA	41	83	41	73
Ventana Benchmark XT	9	78	9	56

HNPCC Run: 120	MLH1		PMS2	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	17	94	16	88
Dako EnVision Plus kits	1	100	1	100
Dako FLEX DAB	10	80	11	91
Dako REAL EnVision K5007 DAB	1	0	1	100
Leica Bond Polymer Refine kit (DS9800)	23	91	22	82
Other	6	83	6	50
Ventana DAB	26	81	29	76
Ventana Enhanced Alk. Phos. Red Detection Kit	0	0	1	0
Ventana Ultraview DAB	7	57	5	60

BEST METHODS - Gold Standard Antibody

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

MLH1 - Method 1

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

Primary Antibody: Ventana 790-4535 (M1) , 12 Mins, 37 ºC Prediluted
Automation: Ventana Benchmark XT
Method: Ventana Optiview
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 32mins
EAR:
Chromogen: Ventana DAB, 37 ºC., Time 1: 8 Mins
Detection: Ventana OptiView Kit (760-700) , 8 Mins, 37 ºC Prediluted

MLH1 - Method 2

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

Primary Antibody: Biocare medical CM/PM 220 (G168-15) , 32 Mins, 37 ºC Dilution 1: 1:10
Automation: Ventana Benchmark ULTRA
Method: Ventana Optiview
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 64mins
EAR: NOT APPLICABLE
Chromogen: Ventana DAB, 37 ºC., Time 1: 12 Mins, Time 2: 12 Mins
Detection: Ventana OptiView (760-700) + Amp. (7/860-099) , 12 Mins, 37 ºC Prediluted

MLH1 - Method 3

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

Primary Antibody:	Novocastra NCL-L-MLH1 (ES05) , 12 Mins, 24 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:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

MLH1 - Method 4

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

Primary Antibody:	Dako M3640 (ES05) , 60 Mins, RT ºC Dilution 1: 50
Automation:	Dako Autostainer plus
Method:	Dako FLEX+ kit
Main Buffer:	AS PER KIT, PH: 7.4
HMAR:	Dako PTLink
EAR:	AS PER KIT
Chromogen:	AS PER KIT
Detection:	AS PER KIT

BEST METHODS - Secondary Antibody

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

PMS2 - Method 1

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

Primary Antibody:	Ventana 760-4531 (EPR3947) , 48 Mins, RT ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	
Chromogen:	Ventana DAB, RT ºC., Time 1: 8 Mins
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099) , 16 Mins, RT ºC Prediluted

PMS2 - Method 2

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

Primary Antibody:	BD Bio/Pharmingen 556415 (A16-4) , 20 Mins, 23 ºC Dilution 1: 200
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590), PH: 7.4
HMAR:	Leica ER2 40 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), Time 1: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 15 Mins, 23 ºC Prediluted

PMS2 - Method 3

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

Primary Antibody:	Cell Marque 288R -17/18 (EPR3947) , 12 Mins, 24 ºC Prediluted
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)

PMS2 - Method 4

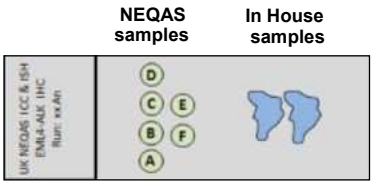
Participant scored 18/20 (UK NEQAS Slide) and 18/20 (In House slide) using this method.

Primary Antibody:	Dako M3647 (EP51) , 60 Mins, 18 ºC Dilution 1: 40
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: 0, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 18 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Other , 15 Mins, 18 ºC Prediluted

Suzanne Parry

Antibody Assessed:	ALK
Samples Circulated:	Composite slide (see table below)
Number of Registered Participants:	71
Number of Participants This Run:	64 (90%)

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



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

Introduction

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

One of the fusion partners of ALK has shown to be echinoderm microtubule associated protein-like 4 (EML4)[5] found in 3-6.7%[6-10] of patients. The EML4-ALK rearrangement was initially assessed using fluorescence in situ hybridisation (FISH). The FISH technique involves the identification of genetic abnormalities using break-apart FISH probes, to identify whether there are genomic rearrangements or deletions between the EML4-ALK fusion pair[3,4]. Brightfield immunohistochemistry (IHC) has also enhanced the possibility of detecting ALK rearrangements[11]. However, IHC for ALK rearrangements can also be challenging due to the relatively low ALK protein concentrations, and therefore requiring more sensitive and/or enhanced detection systems. The publication by Savic and colleagues[12] indicates that certain IHC antibodies perform equally well but still recommend a combined use with FISH testing. More recent publications have indicated ALK IHC to be a better predictor of ALK inhibition outcome[13]. There is also evidence that ALK IHC along with intensity (and H-score) could be used as an affective screening tool[14,15], but this is still a debatable subject requiring further clinical evidence and is quite possibly related to the 'detection system' employed[16].

Assessment Criteria

NEQAS & In-house controls

The NEQAS ICC & ISH quality control samples were placed and orientated as shown in Figure 1 and Table 1. The distributed slide 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
- A more cost-effective EQA, as less reagent is used by the participant

Interpretation criteria incorporating staining intensity

The assessment consists of assessors providing their feedback on whether each of the distributed samples are either ALK IHC positive or negative (+ve'/'-ve'). Alongside this the assessors also provide feedback on the observed intensity of staining, including 3+ (high), 2+ (medium) and 1+ (low) intensity. Assessors are therefore asked to score each of the ALK IHC positive samples as: +ve (3+), +ve (2+) or +ve (1+). This allowas for more informative feedback on the intensity of staining on the ALK positive samples and also reflects the different scoring systems employed by participants.

Assessment scoring guidelines

- A team of four assessors independently, but simultaneously, score all of the returned slides and provide interpretation and feedback on technical issues.
- Each of the assessors then provide an overall score out of 5, with marks summed together to give a final score out of 20.

Table 2: Assessment interpretation

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

Features of Optimal Staining (Figures 1-4)

- Moderate to strong granular cytoplasmic staining of the ganglion and nerve cells in the appendix
- Strong granular cytoplasmic staining of the positive cell line sample
- No staining of the ALK negative cell line sample
- Moderate to strong granular cytoplasmic staining of the

positive tumour tissue samples
- No staining in the negative tumour sample
- No background or inappropriately localised staining

Features of Sub-optimal Staining
- False negative or absence of ALK staining where tumour cells should be staining positive
- Lower expression level than expected
- Weaker staining than expected for the assay/method used
- Non-specific/excessive tyramide staining
- Absence of staining in the appendix ganglion and nerve cells

Results & Discussion

Distributed NEQAS Sample Results

Of the 64 laboratories that participated in the Run 120 assessment, all returned both the NEQAS and their in-house samples for assessment. The results were very similar to the previous assessment, with 77% (N=49) of laboratories receiving an acceptable pass on the NEQAS samples, and a further 16% (N=10) achieved borderline passes. 5 laboratories (8%) failed the assessment.

Most laboratories receiving borderline passes showed the correct clinical result, however, there were factors to highlight that the staining was not optimal, such as the staining was weaker than expected or there was slight background, but still readable. If a laboratory had failed to demonstrate the expected staining expression, which could potentially lead to an incorrect clinical decision, then the slide failed. Similarly, if the sections were difficult to read due to excessive teramide or inappropriate staining, then this cold potentially also lead to misinterpretation and therefore failed the assessment. The appendix control was very helpful at assessment; if a laboratory failed to stain this appropriately, with the expected strong staining of the ganglion and nerve cells, then a laboratory would also fail the assessment or at most receive a borderline pass for slightly weak staining.

The most popular antibody of choice was the Ventana/Roche (D5F3), used by 51 participants and showed an acceptable pass rate of 82%. The Cell Signalling Technology (D5F3) was used by 5 participants, and all of these participants achieved an acceptable pass. Other antibodies included the Novocastra/Leica 5A4 antibody clone, which was also used by 5 laboratories, and showed an acceptable pass rate of 40% on the NEQAS material.

In-house Control Results

Pass rates on the participants in-house tissues were similar to that on the NEQAS samples. However, less laboratories achieved an acceptable pass (67%), and a further 16% achieved a borderline. 5 laboratories (8%) failed the assessment, which was the same as the NEQAS samples. Some of the borderline passes were due to the laboratory not providing an ideal selection of controls, necessary to help gauge the sensitivity of their staining assay. (See section below on ALK IHC control recommendations). Failures on the in-house samples were due to no or uninterpretable staining due to excessive inappropriate teramide or background staining.

ALK IHC Control Recommendations

The UK NEQAS ICC & ISH ALK IHC assessment panel recommend that the ideal control should consist of:
- NSCLC ALK IHC positive tumour: Gauges sensitivity
- NSCLC ALK IHC negative tumour: Gauges specificity
- Appendix is also recommended 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, such as cell lines or xenografts showing at least positive and negative ALK IHC expression may also be used in combination with an in-house tissue sample.
- As best practice, control material should be cut and placed alongside the clinical sample being tested. Not only for NEQAS assessment, but for all ALK IHC testing.

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Selected Images showing Optimal and Sub-optimal immunostaining

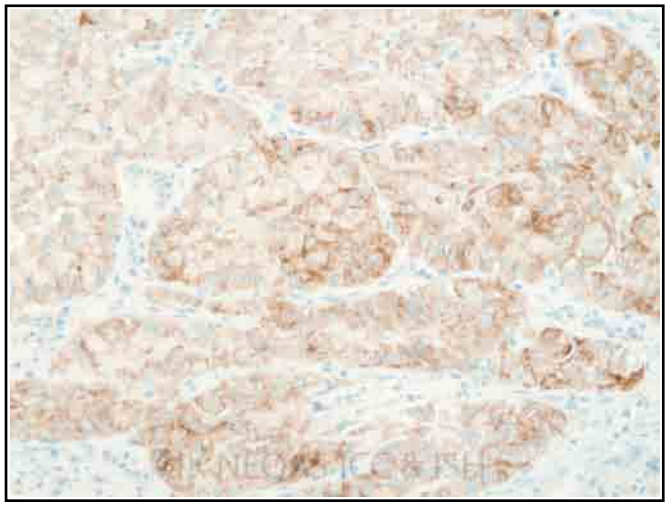


Fig 1. Optimal ALK IHC staining of the UK NEQAS distributed positive tumour (sample A), showing moderate membranous and cytoplasmic staining in the neoplastic cells. Stained with the Ventana D5F3 assay on the Benchmark XT, CC1 extended, Optiview detection with amplification.

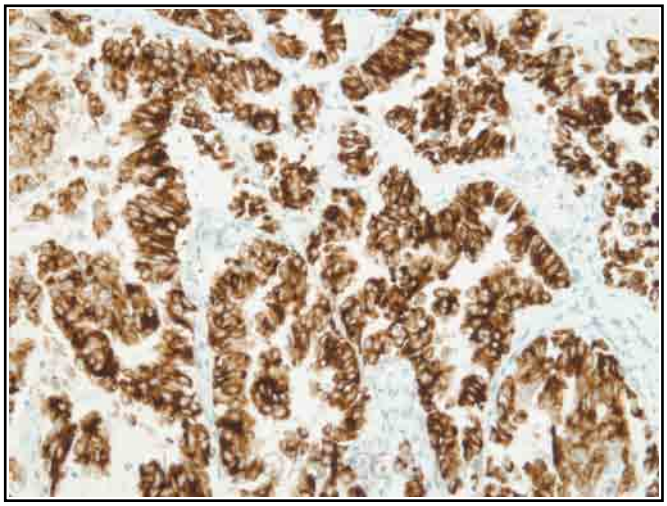


Fig 2. Optimal demonstration of staining in the UK NEQAS distributed positive tumour (sample B). As expected, the tumour shows strong staining in all of the neoplastic cells. Stained using the Venatana D5F3 assay (same protocol as Fig 1).

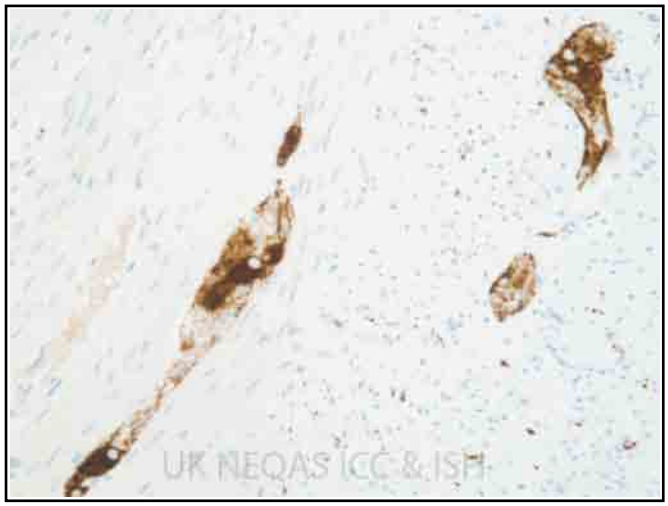


Fig 3. Optimal staining of the UK NEQAS distributed appendix (sample D). The example shows the expected strong positive staining of ganglion cells and axons. Stained using the Ventana D5F3 assay with the recommended protocol.

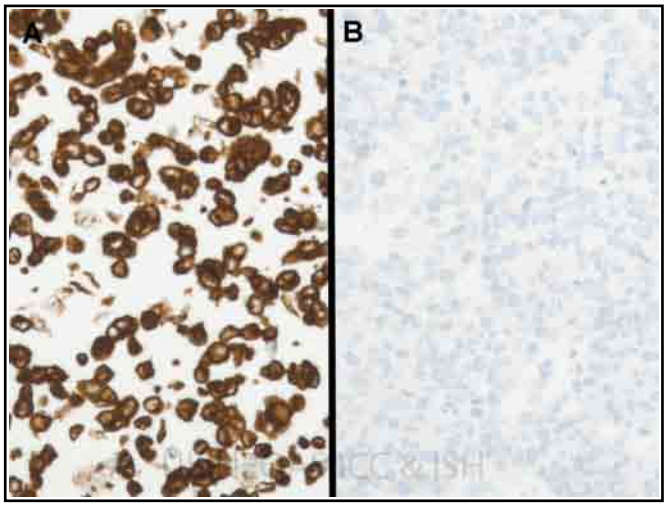


Fig 4. Good examples of ALK IHC staining in the UK NEQAS distributed cell lines: (A) The positive cell line (sample E) shows strong membranous and cytoplasmic staining of the neoplastic cells, and the negative cell line (sample F), shows the expected negative result. Stained with the Ventana D5F3 assay.

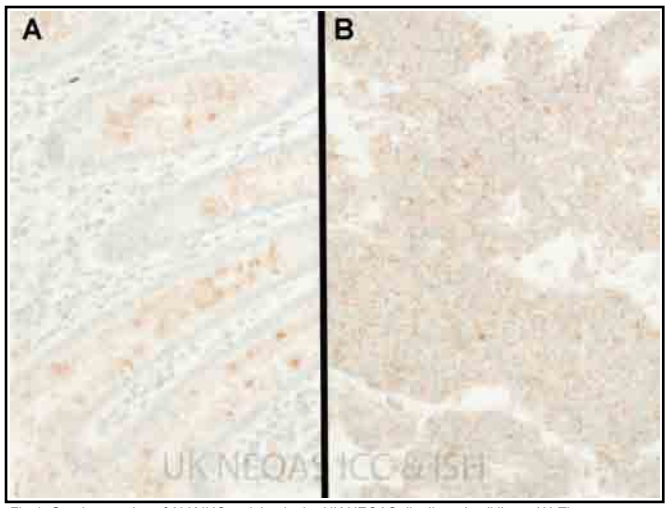


Fig 4. Good examples of ALK IHC staining in the UK NEQAS distributed cell lines: (A) The positive cell line (sample E) shows strong membranous and cytoplasmic staining of the neoplastic cells, and the negative cell line (sample F), shows the expected negative result. Stained with the Ventana D5F3 assay.

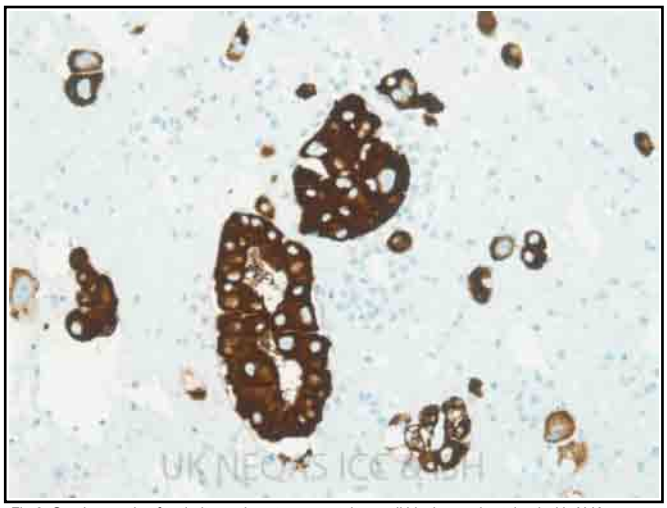
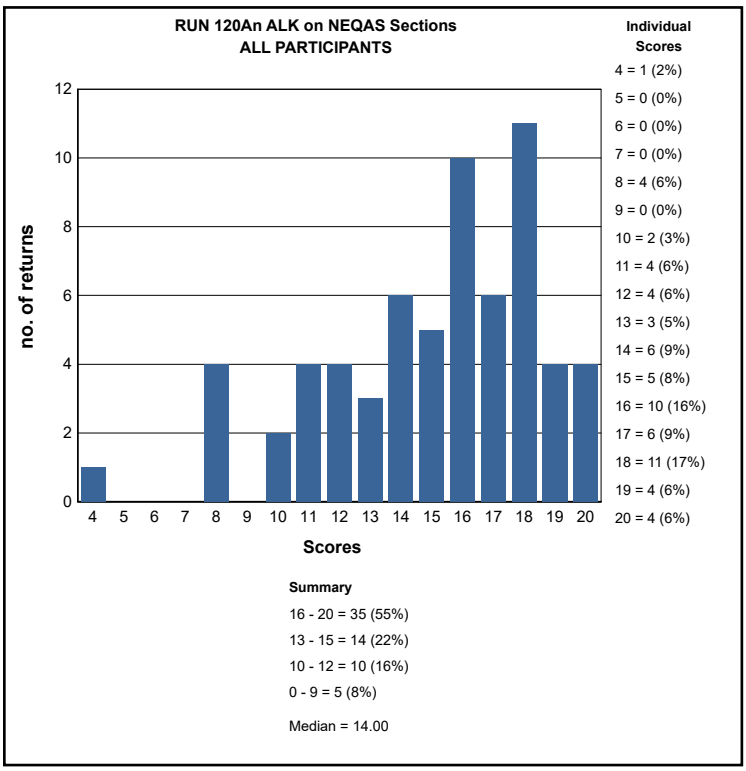
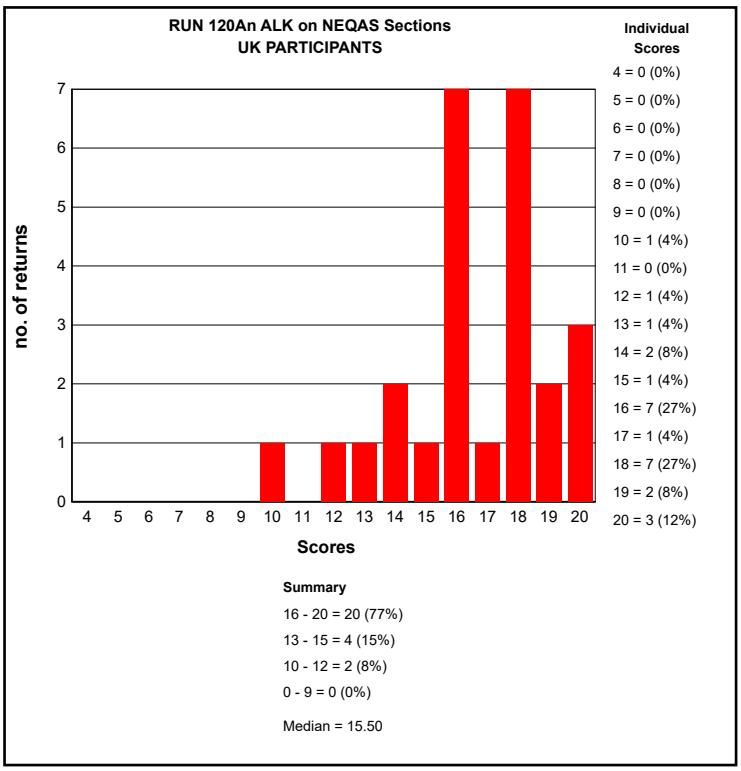
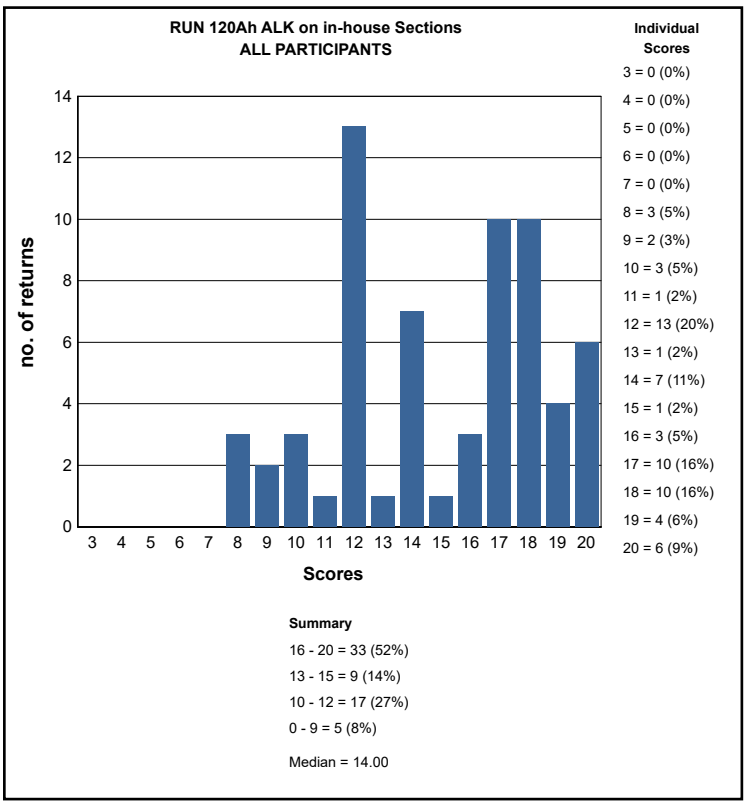


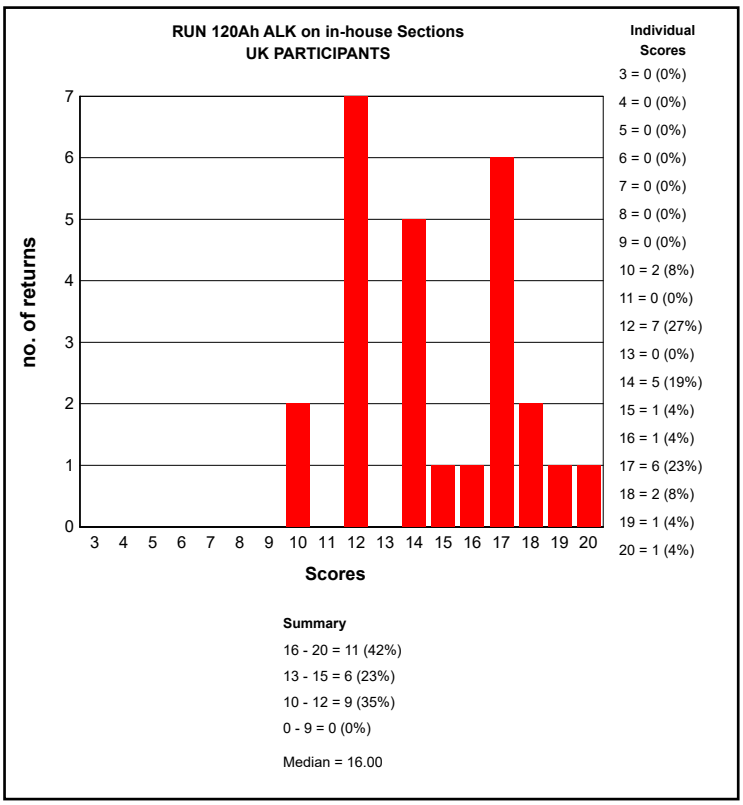
Fig 6. Good example of an in-house lung tumour cytology cell block sample stained with ALK. The example shows strong staining of the tumour cells while the background remains clean. Both examples are stained with the Ventana D5F3 assay.

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: 120

Primary Antibody	N	%
Cell Sign. Tech. (D5F3)	5	100
Novocastra NCL-ALK (5A4)	4	25
Novocastra PA0306 (5A4)	1	100
Other	1	0
Ventana/Roche (D5F3)	51	82

ALK NSCLC Run: 120

Automation	N	%
Dako Autostainer Link 48	1	0
Dako Omnis	1	100
Leica Bond Max	1	0
Leica Bond-III	3	100
Ventana Benchmark GX	3	100
Ventana Benchmark ULTRA	33	79
Ventana Benchmark XT	20	80

ALK NSCLC Run: 120

Heat Mediated Retrieval	N	%
Dako Omnis	1	100
Dako PTLink	1	0
Leica ER2 20 mins	3	67
None	1	100
Other	2	100
Ventana CC1 36mins	1	100
Ventana CC1 64mins	2	100
Ventana CC1 72mins	1	0
Ventana CC1 88mins	2	50
Ventana CC1 92mins	41	78
Ventana CC1 extended	5	100
Ventana CC1 standard	2	100

ALK NSCLC Run: 120

Detection	N	%
Dako EnVision FLEX (K8000/10)	1	0
Dako EnVision FLEX+ (K8002/12)	1	100
Leica Bond Polymer Refine (DS9800)	4	75
Ventana OptiView (760-700) + Amp. (7/860-099)	36	81
Ventana OptiView Kit (760-700)	19	79
Ventana UltraView Kit (760-500)	1	100

ALK NSCLC Run: 120

Enzyme Retrieval	N	%
AS PER KIT	4	100
NOT APPLICABLE	34	74

ALK NSCLC Run: 120

Chromogen	N	%
AS PER KIT	19	63
DAKO DAB+	1	100
Dako EnVision Plus kits	1	0
Leica Bond Polymer Refine kit (DS9800)	4	75
Ventana DAB	34	91
Ventana Ultraview DAB	3	67

BEST METHODS

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

ALK - Method 1

Participant scored 19/20 (UK NEQAS Slide) and 16/20 (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:	Ventana DAB
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099) , 12 Mins

ALK - Method 2

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 XT
Method:	
Main Buffer:	
HMAR:	Ventana CC1 92mins
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099)

The HER2 ISH Module - Interpretive Run 49

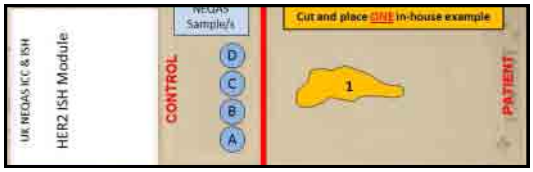
Suzanne Parry

Gene Assessed:	HER2
Sections Circulated:	Composite slide consisting of 4 breast carcinoma sections (see table below)
Number of Registered Participants:	146
Number of Participants This Run	128 (88%)

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+**	Amplified
B	**2+**	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. 70% of participants achieved excellent or acceptable results. 21% received a borderline pass and 9% recieved an unacceptable interpretation result. There were 2 unacceptable results from the UK. The most common brightfield method was the Ventana DDISH with 50 (39%) laboratories using this technique. The most popular FISH metheod was the Pathvysion Vysis Kit with 36 (28%) laboratories using this technique. DDISH had a lower pass rate of 58% compared to 89% for Pathvysion Vysis Kit.

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.

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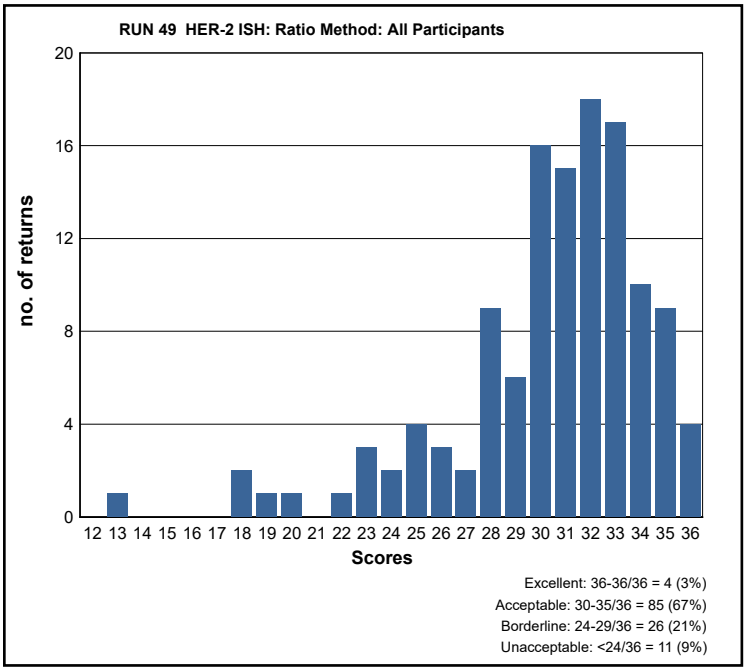
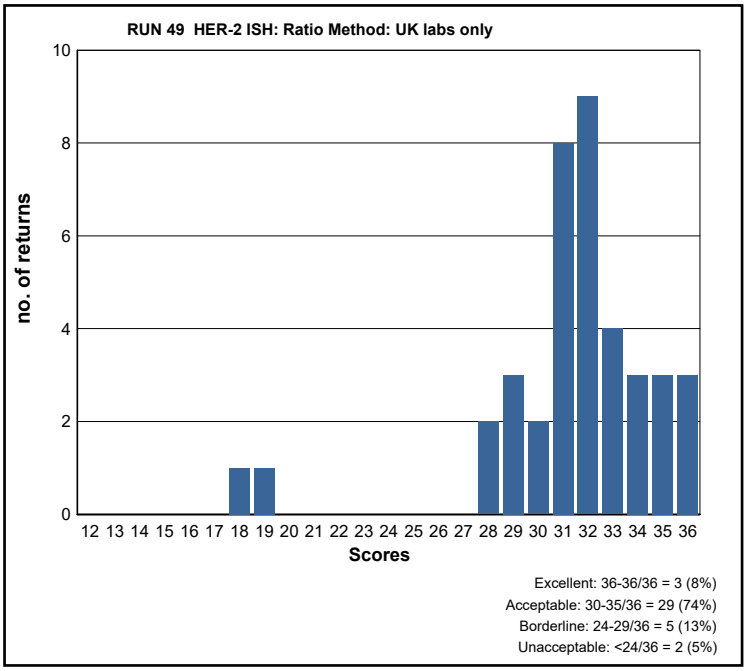
GRAPHICAL REPRESENTATION OF PASS RATES (ratio methods only)





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

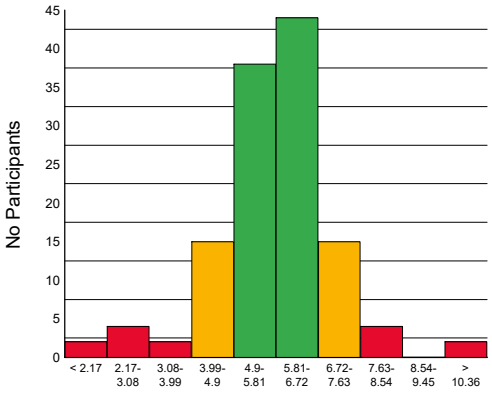
Ratio Method	N	% Pass (score ≥ 30/36)
Ratio: Dako IQFISH pharmDX	11	73%
Ratio: In house FISH	2	50%
Ratio: Kreatech Probes	3	67%
Ratio: Leica HER2 FISH TA9217	8	100%
Ratio: Other - CISH	1	100%
Ratio: Other - FISH	1	0%
Ratio: Pathvysion Vysis Kit	36	89%
Ratio: Ventana BDISH 800-098/505	1	100%
Ratio: Ventana DDISH (780/800-4422)	50	60%
Ratio: Ventana Inform Silver ISH	2	50%
Ratio: Zytovision ZytoDot 2C	1	0%
Ratio: Zytovision ZytoLight	8	63%

Copy Method (Shown Only When Applicable)	N	% Pass (score ≥ 10/12)
Copy No.: Other	1	100%

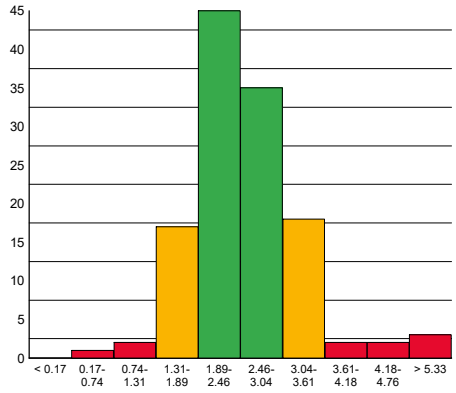
FREQUENCY HISTOGRAMS

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

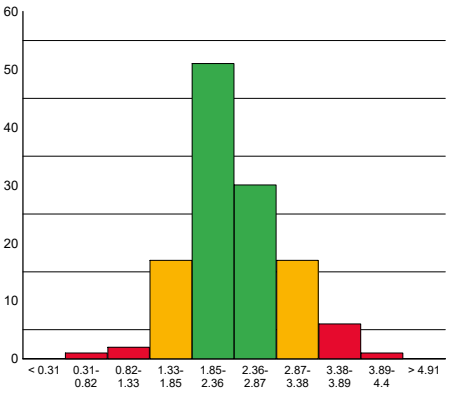
Green = mean +/- 1 Stdev Amber = mean +/- 2 Stdev Red = mean ≥ +/- 3 stdev



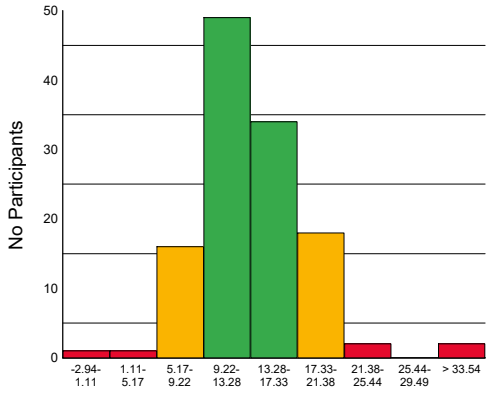
Sample A: Av. HER2 copy



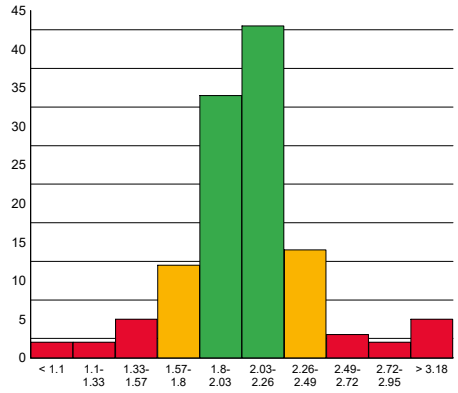
Sample A: Av. Chr17



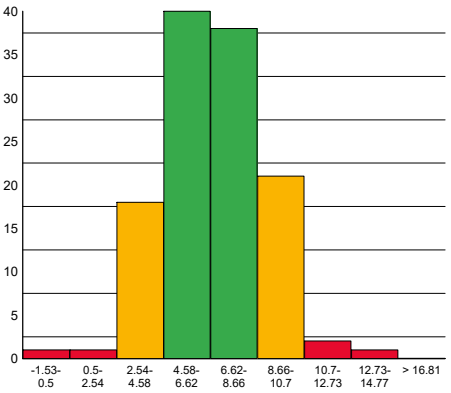
Sample A: Ratio



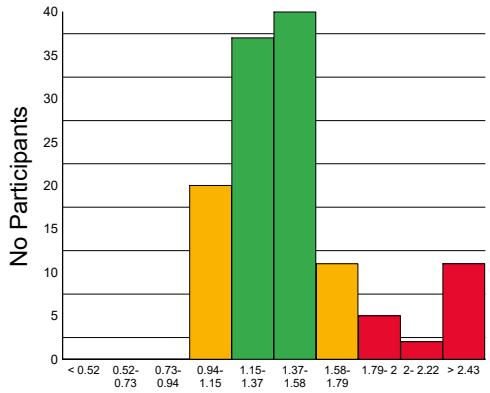
Sample B: Av. HER2 copy



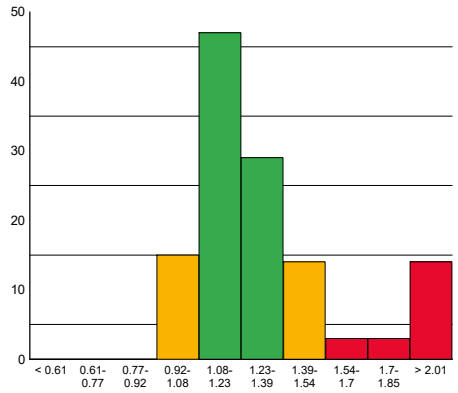
Sample B: Av. Chr17



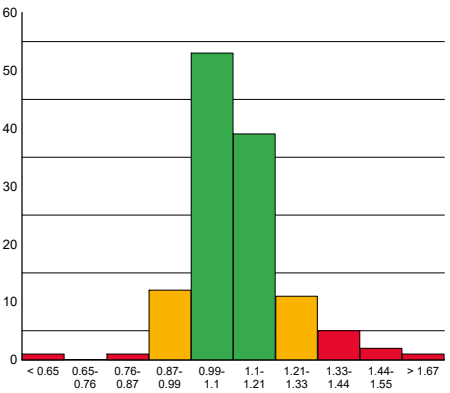
Sample B: Ratio



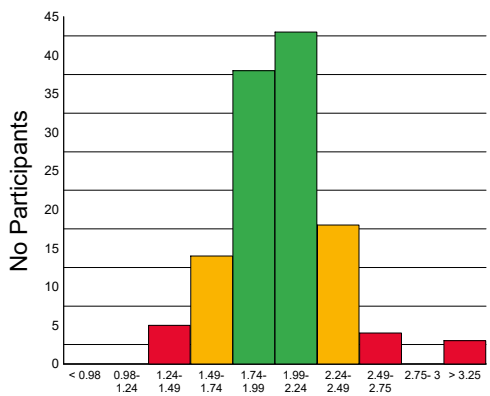
Sample C: Av. HER2 copy



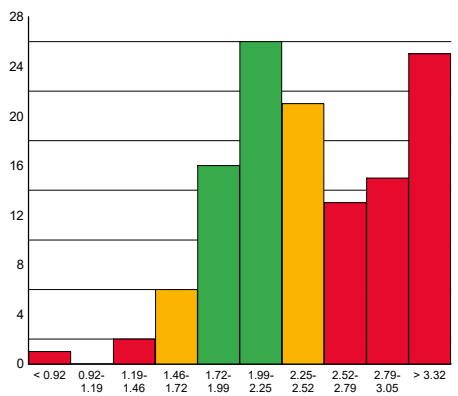
Sample C: Av. Chr17



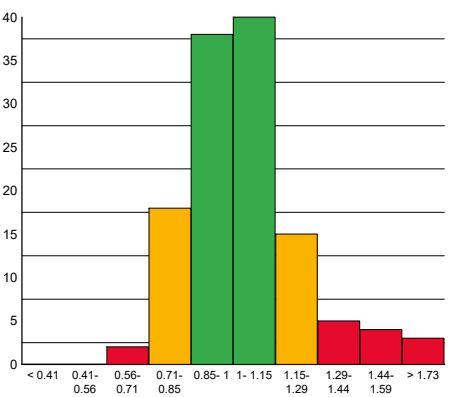
Sample C: Ratio



Sample D: Av. HER2 copy



Sample D: Av. Chr17



Sample D: Ratio

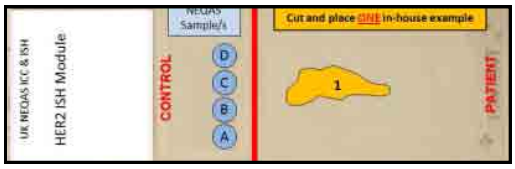
The HER2 ISH Module - Technical Run 49

Suzanne Parry and Jamie Hughes

Gene Assessed:	HER2
Sections circulated:	Composite slide consisting of 4 breast carcinoma sections (see table below)
Number of Registered Participants:	146
Number of Participants Taking Part this Run	123 (84%) (69 FISH & 54 CISH)

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

Sample	HER2 status by IHC	HER2 status by FISH
A	**2+**	Amplified
B	**2+**	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.

An example of an in-house sample is also requested for this module, and participants are required to place them on the distributed NEQAS slides.

Results Summary

CISH Results

Selected images (Figures 1-6) show examples of the acceptable and unacceptable levels of staining for the different methods. The overall results showed lower acceptable pass rates to the previous assessment; only 30% of laboratories achieved an acceptable pass, 35% laboratories a borderline pass, and 35% failed the assessment on the NEQAS material. There were a variety of reasons for failure, which included weak and leaching signals, dust or excessive silver deposit, and several slides also showed a red precipitate over the samples.

The DDISH technique is the most popular CISH method of choice; used by 48 laboratories, and showed an acceptable pass rate of 31%. A further 40% of DDISH users received a borderline score. The numbers of laboratories using other methods was low. Such methods included the Ventana BDISH, Ventana Inform (single probe slides), and the Zytovision ZytoDot 2C.

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.

FISH Results

71 laboratories submitted slides for the FISH assessment. The results show an decrease of 7% in acceptable results from Run 48, as shown in the table below:

There was an deterioration in the current Run 49 compared to the previous Run (48). There was a high level of FISH submissions with weak or no HER2 and/or Cen17 signals. This

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

may have been attributed to technical handling (e.g. cleaning of slides with alcohol before sealing), storage or transport errors (e.g. temperature fluctuations), as it did not appear to be assay specific.
The failure rate increased from 21% (Run 48) to 28% (Run 49). This is reflected in the overall pass rate with an decrease of acceptable pass rates from 79% (Run 48) to 72% (Run 49).
Images of acceptable and unacceptable levels of staining are illustrated in **Figures 7-12**.

HER2 FISH Pass Rates : NEQAS section		
	Run 48	**Run 49**
Acceptable	79%(N=56)	72% (N=50)
Borderline	7%(N=5)	6% (N=4)
Unacceptable	14%(N=10)	22% (N=15)

The Pathvysion Vysis Kit was the most commonly used by laboratories for FISH, used by 49% participants. Pass rates using the Vysis Kit for this Run were 68%, which have declined compared to Run 48 (75%). The second most common assay was the Dako IQFISH used by 14% of participants with a pass rate of 90%, followed by Zytovision (ZytoLight) used by 13% of participants with a pass rate of 89%. Other FISH assays/probes used include; Dako Pharm Dx, Leica HER2 FISH system and Kreatech Probes, all of which performed well overall, although the numbers of laboratories adopting these methods are low.

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 asssay, 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 staining

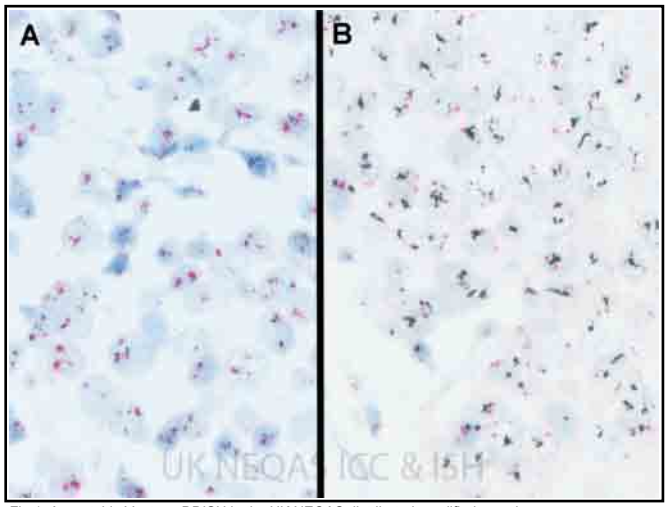


Fig 1. Acceptable Ventana DDISH in the UK NEQAS distributed amplified samples 'A' and 'B'. Both examples show distinct HER2 signals (black) and CEP signals (red).

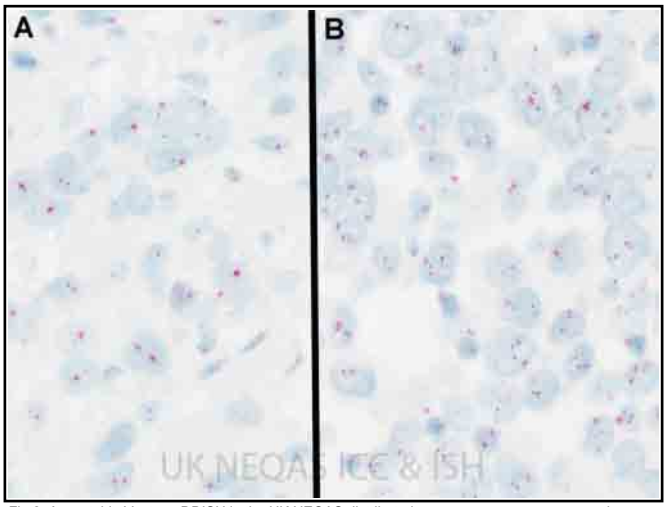


Fig 2. Acceptable Ventana DDISH in the UK NEQAS distributed non-amplified samples 'C' and 'D'. The sections show strong HER2 signals (black) and CEP signals (red) with the expected copy numbers per cell.

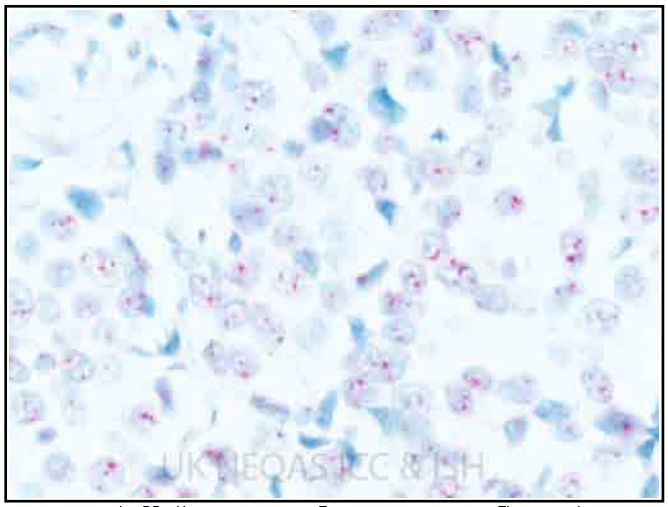


Fig 3. Unacceptable DDISH staining in the UK NEQAS amplified sample 'A'. The image shows no HER2 signals at all, and therefore uninterpretable.

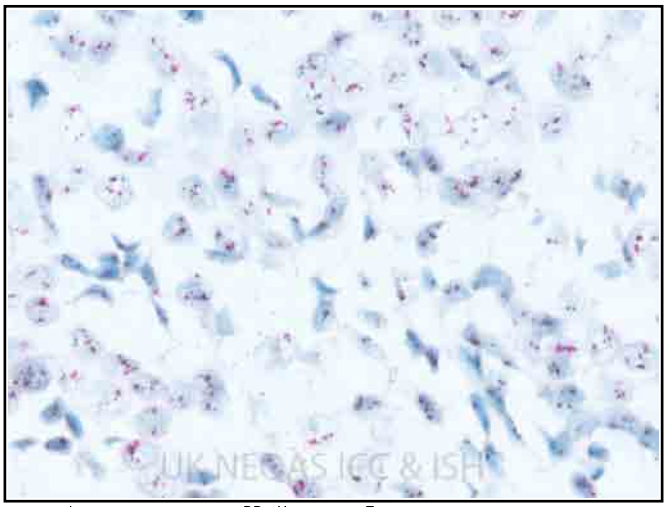


Fig 4. Sub-optimal demonstration of DDISH in the UK NEQAS amplified sample 'A'. Although the HER2 and CEP signals are strong, the section also shows many signals outside of the nuclei.

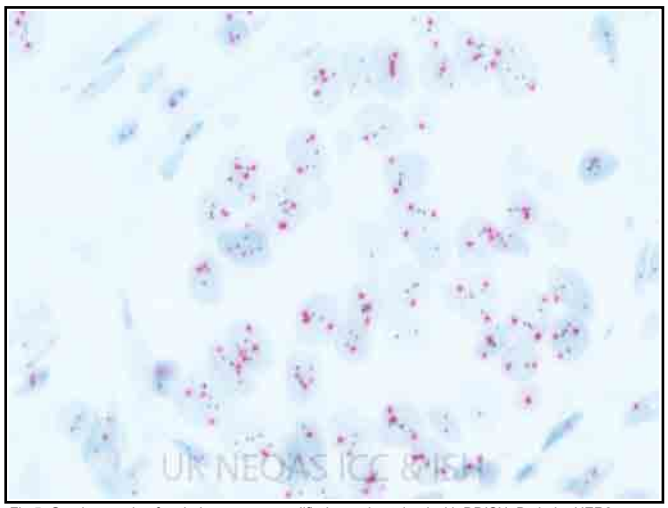


Fig 5. Good example of an in-house non-amplified sample stained with DDISH. Both the HER2 and CEP signals are strong and distinct.

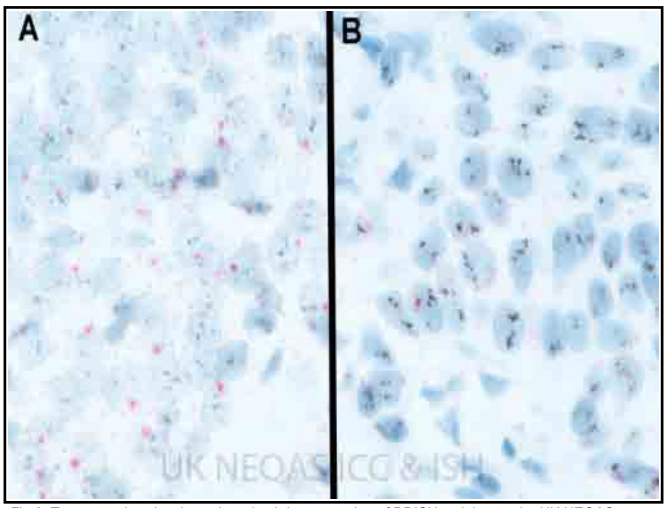


Fig 6. Two examples showing sub-optimal demonstration of DDISH staining on the UK NEQAS samples. Example A shows excessive silver dust deposit over the section. Example B shows leaching of the CEP signals.

Selected Images showing Optimal and Sub-optimal staining

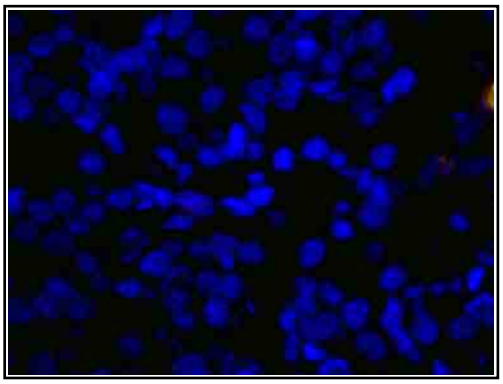


Figure 7: Sub-optimal example of HER2 gene and CEP17 demonstration on core A of the UK NEQAS distributed sample. Sample appears to be under-digested. Pathvysion Vysis Kit; Vysis kit II (32-801210); Protease I at 37°C for 5 mins.

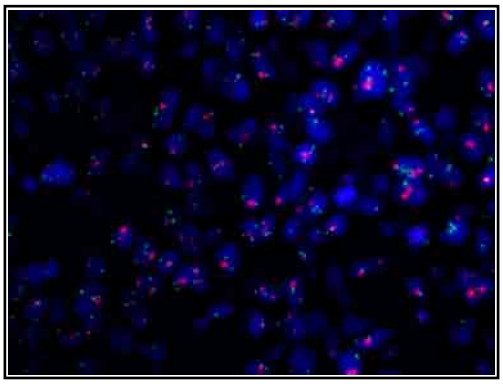


Figure 8: Excellent demonstration of HER2 gene and CEP17 on core B of the UK NEQAS distributed sample. Expected level of gene amplification observed. Pathvysion Vysis Kit; Cytocell Aquarius Tissue Pre-treatment Kit; Cytocell Aquarius enzyme reagent at room temperature for 19 mins.

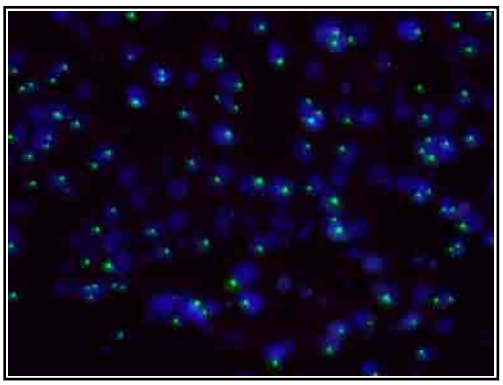


Figure 9: Sub-optimal example of HER2 gene and CEP17 demonstration on core C of the UK NEQAS distributed sample. Non-specific CEP17 and weak HER2 signals observed. Pathvysion Vysis Kit; Protease I at 37°C for 60 mins; Vysis (VP2000).

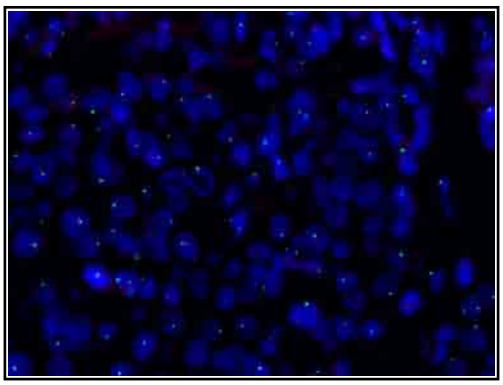


Figure 10: Excellent demonstration of HER2 gene and CEP17 on core C of the UK NEQAS distributed sample. Image shows clear monosomy of both the HER2 gene and CEP17. Method as Figure 2.

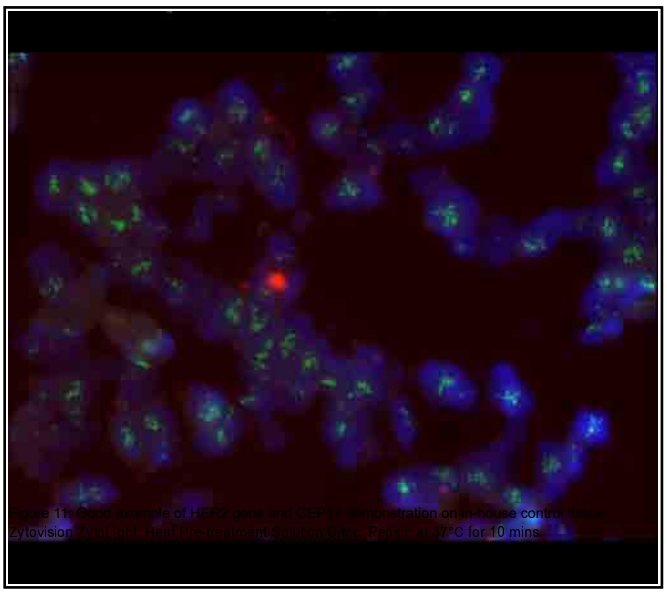


Figure 11: Good example of HER2 gene and CEP17 demonstration on in-house control tissue. Zytovision ZytoLight Heat Pre-treatment Solution Citric; Pepsin at 37°C for 10 mins.

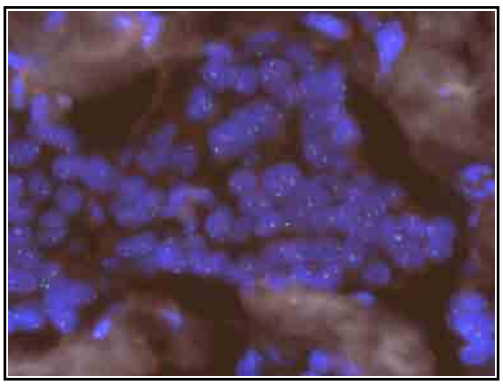
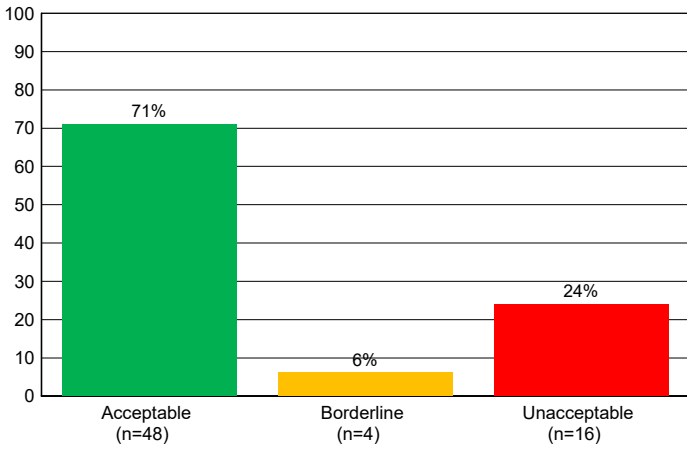


Figure 12: Good example of HER2 gene and CEP17 demonstration on in-house control tissue. Pathvysion Vysis Kit; Protease I 37°C for 35 mins; Vysis (VP2000).

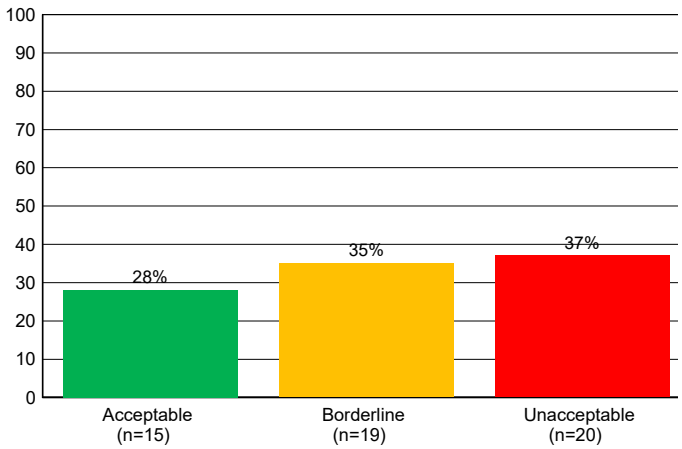
Technical ISH: Pass Rates and Methods

Overall Pass Rates

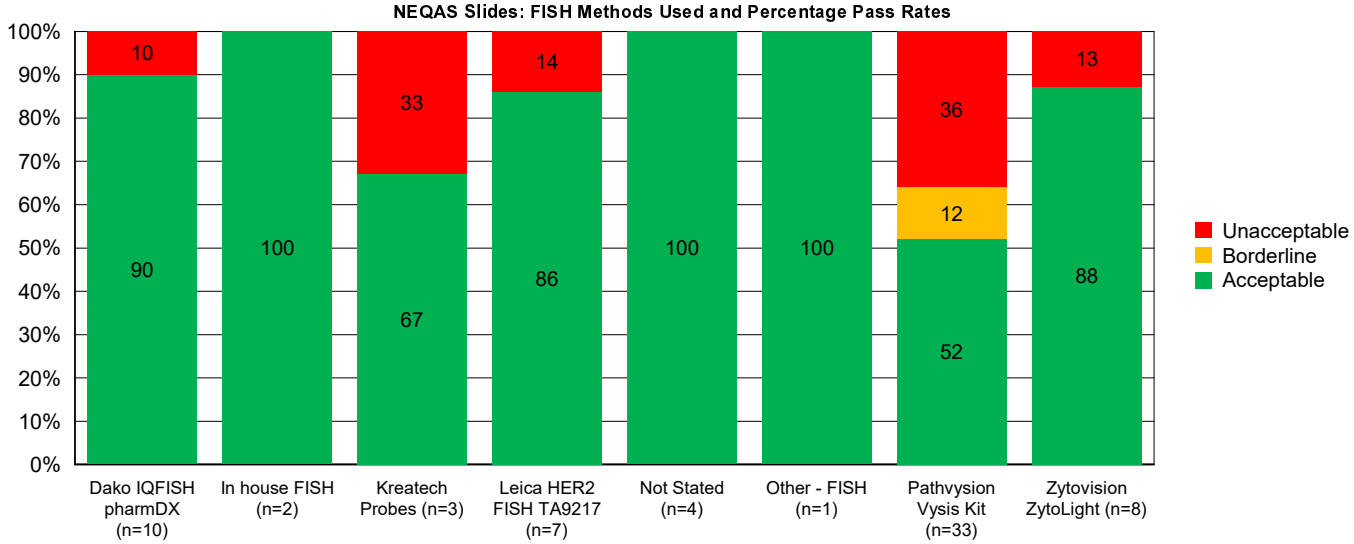
FISH NEQAS slide (n=68)



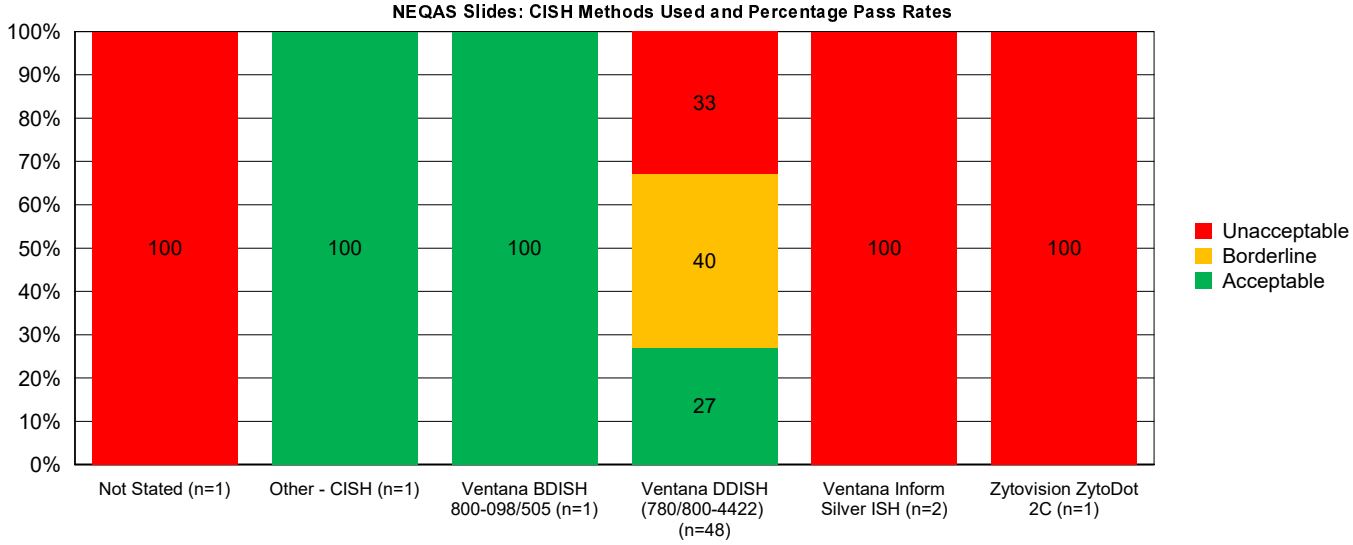
CISH NEQAS Slide (n=54)



NEQAS Slides: FISH Methods Used and Percentage Pass Rates



NEQAS Slides: CISH Methods Used and Percentage Pass Rates



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