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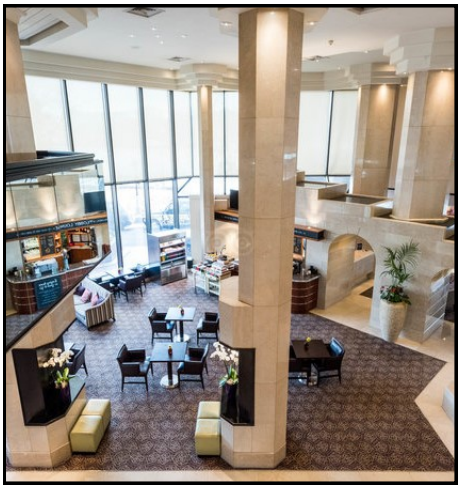
International Quality Expertise

Immunocytochemistry

Improving Immunocytochemistry for Over 25 Years

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

Assessment Dates: 5th July — 21st July 2017


Immunocytochemistry Modules

In situ Hybridisation Modules

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Save The Date

UK NEQAS ICC & ISH 2018: Summer Conference

When: June 22nd - 24th 2018

Where: The Tower Hotel, St. Katherine's Way. London

www.ukneqasiccish.org/conference-2018/

General Information

UK NEQAS
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7833

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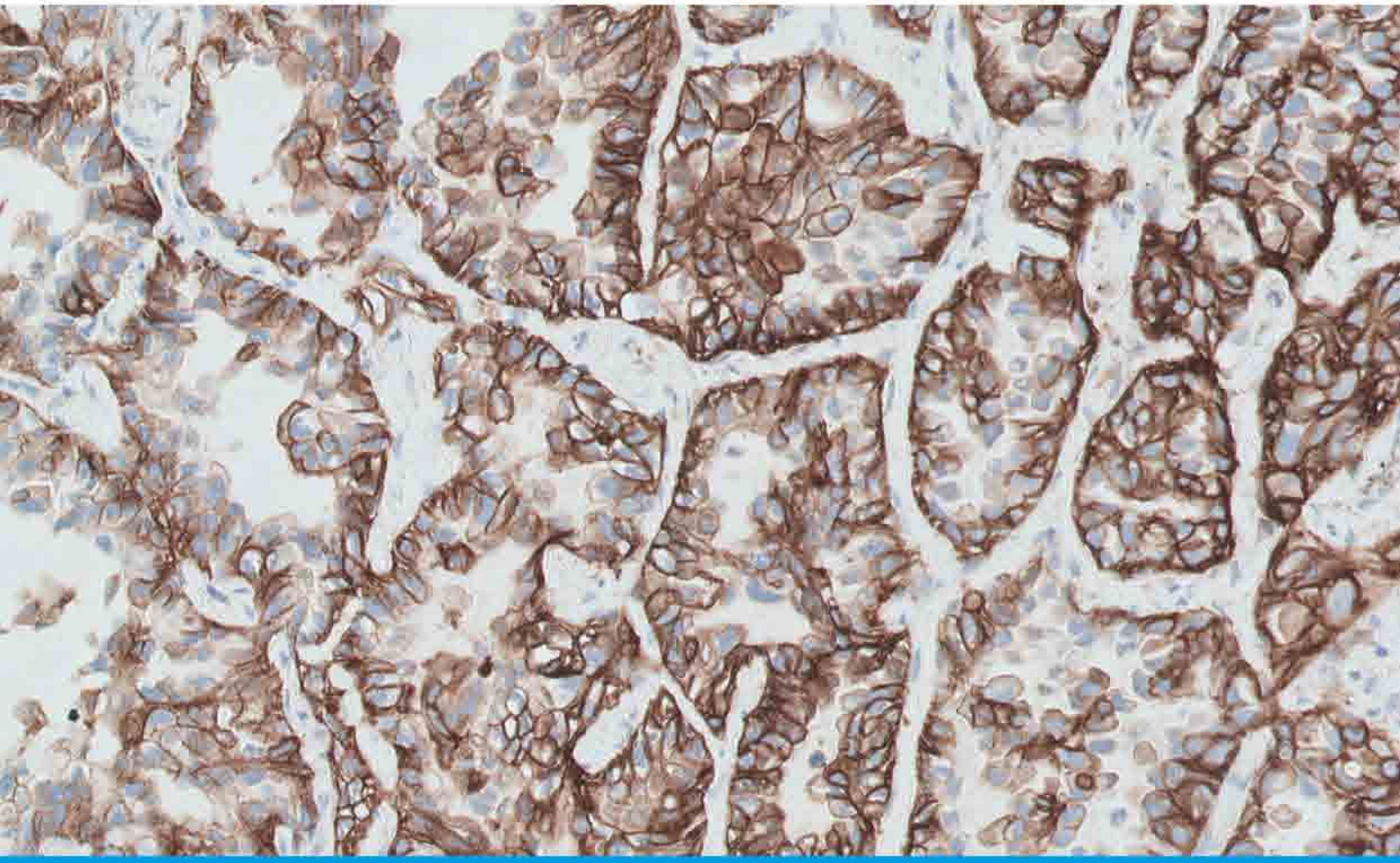


Agilent Technologies

The General Pathology Module Run 118

Gavin Rock and Suzanne Parry

	Gold Standard	Second Antibody
Antigens Assessed:	CD45	Melanoma Markers
Tissue Sections circulated:	Colorectal Cancer and Tonsil	Metastatic Melanoma
Number of Registered Participants:	312	
Number of Participants this Run	299 (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: (Figs 1, 2, 4, 5 & 6)

- 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: (Fig 3)

- 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: Melanoma Markers

HMB45- Normal adult tissues that exhibit positive staining with anti-melanosome **HMB45,** include melanocytes (fetal and a subset of melanocytes containing immature melanosomes), and retinal pigment epithelia (prenatal and infantile). HMB45 is a useful marker to help in the classification of melanomas and melanocytic lesions and to distinguish metastatic amelanotic melanomas from other poorly differentiated tumours of uncertain origin. Those lesions positive for HMB45 include melanocytic atypical hyperplasia, melanocytic neuroectoderm of infancy and angiomyolipoma. Various naevi are also stained by the anti-HMB45 melanoma marker.

Melan-A is expressed in skin, retina and most melanocytes, and is a very useful marker in the identification of melanomas. While a vast variety of other tissues and cancers do not express Melan-A, this marker is highly specific as a melanoma antigen. However, if melanoma is ruled out, Melan A is also helpful in the differential diagnosis of adrenocortical carcinomas.

Features of Optimal Immunostaining: (Figs 7, 8, 9, 10 & 12)

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

Features of Sub-optimal Immunostaining: (Fig 11)

- Weak, uneven or no staining of melanoma tumour cells.
- Background staining of other elements of the tissue.
- Non-specific staining of cell types or components not expected to stain.
- Excessive or very weak nuclear counterstain.

References

1. Miettinen M, Fernandez M, Franssila K, Gatalica Z, Lasota J, Sarlomo-Rikala M. Microphthalmia transcription factor in the immunohistochemical diagnosis of metastatic melanoma: comparison with four other melanoma markers. Am J Surg Pathol. 2001 Feb;25(2):205-11.
2. Jungbluth AA, et al. An anti melan-A monoclonal antibody for the detection of malignant melanoma in paraffin embedded tissues. Am J Surg Pathol 1998;22:595-602.
3. Fetsch PA, Cormier J, Hijazi YM. Immunocytochemical detection of MART-1 in fresh and paraffin-embedded malignant melanomas. J Immunother 1997;20:60-4.

Assessment Summary

CD45

A total of 299 laboratories submitted NEQAS slides for the Run 118 CD45 assessment. Only 3 of these laboratories did not submit their in-house control sections. Of these, 86% of laboratories showed acceptable staining on the NEQAS material, with a further 13% receiving a borderline score of 10 -12/20, and 2% (6 laboratories) failed the assessment. The in-house sections showed slightly higher acceptable pass rates, with 92% of laboratories receiving an acceptable pass, 8% receiving borderline, and only 3 laboratories (1%) failing the assessment.

A breakdown of the Run 118 CD45 results is summarised in the table below:

CD45 Pass Rates Run 118		
	NEQAS	IN-HOUSE
Acceptable	86%(N=255)	92%(N=272)
Borderline	13%(N=38)	8%(N=23)
Unacceptable	2%(N=6)	1%(N=3)

The reason for failure or a lower score was mostly due to weak, or in some cases, barely staining at all. Another reason was for a diffuse staining pattern of the antigen, which often gave the appearance of background staining. These findings were on both the NEQAS and in-house samples.

The most popular clone used in this run was the Dako 2B11+PD7/26 clone. This was used by 167 participants, and showed an acceptable pass rate of 88%. Other popular choices were the Ventana RP2/18 clone, used by 39 participants with a 90% acceptable pass rate, and also the Leica X16/99 antibody, which was used by 26 laboratories, and showed an acceptable pass rate of 69%. For the in-house material, most laboratories only used a piece of tonsil section as their control. Tonsil is certainly an ideal tissue choice to demonstrate the B and T-cell lymphocytes, and will also highlight any non-specific or over-staining if a reaction is seen in the squamous epithelial cells where staining is not expected. However, UK NEQAS ICC & ISH encourages the use of a multi-block to include tonsil alongside a tissue known not to express CD45. This would help to further highlight any false staining or non-specific background.

Melanoma Markers (HMB45 /Melan-A)

297 laboratories submitted slides for the melanoma assessment. Of these, all apart from 1 laboratory also submitted their in-house material. The pass rates on the NEQAS sections was very good, with 92% (N=273) of laboratories receiving an acceptable pass, and a further 20 laboratories (7%) achieving a borderline pass. 4 laboratories (1%) failed the assessment. This was due to very weak or low expression of the antigen. The borderline passes were also mostly due to weak staining.

The in-house results showed very similar pass rates to that of the NEQAS material, with 92% receiving acceptable passes and 5% achieving a borderline score. Slightly more laboratories, N=9 (3%) failed the assessment on their in-house material. Again, weak staining was the cause of failed or borderline passes.

The table below shows a summary of the melanoma pass rates on both the NEQAS and In-house submitted samples:

Melanoma Pass Rates Run 118		
	NEQAS	IN-HOUSE
Acceptable	92% (N=273)	92%(N=272)
Borderline	7% (N=20)	5%(N=15)
Unacceptable	1% (N=4)	3%(N=9)

The methodology data showed that almost twice as many laboratories used the Melan A antibody (N=182) compared to the HMB45 clone (N=98). However, the overall acceptable pass rates for both antibodies was almost identical: 93% of participants using the Melan A antibody received an acceptable pass, while 91% of participants using the HMB45 antibody achieved an acceptable score.

The most popular Melan A antibodies of choice amongst participants were the Dako (A103), used by 59 participants, and the Leica/Novocastra (A103), used by 58 participants. The Ventana MART-1 clone was also a popular choice, used by 50 participants in the current assessment run. For the HMB45 clone, the Dako antibody was used by many participating laboratories (N=51). The Ventana and the Leica/ Novocastra HMB45 antibodies were also popular, used by 27 and 18 participants respectively.

The participants' in-house control sections mostly consisted of a melanoma sample alone. While this is an appropriate control, it is also important to bare in mind that the control should also include some expected negative tissue/elements to help gauge the sensitivity of the melanoma assay.

It was noted that many participants are commonly using a red detection system for their melanoma assay. This is particularly helpful to highlight a pigmented melanocytic lesion. While it is not routine for laboratories to submit more than one slide for the NEQAS assessment, several laboratories did include both red and DAB-chromogen stained sections for additional feedback and comment. For these laboratories, both assays performed equally well.

Selected Images showing Optimal and Sub-optimal Immunostaining

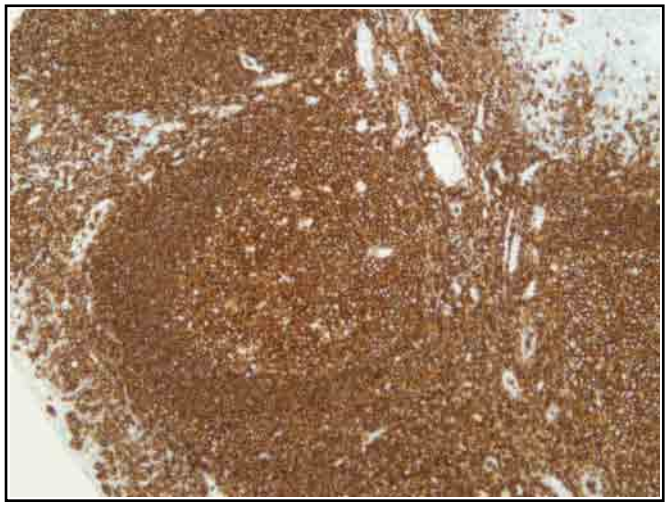


Fig 1. Optimal demonstration of CD45 in the UK NEQAS distributed tonsil. The example shows strong and distinct membranous staining of both B- and T-cells. Stained with the Dako antibody (2B11+PD7/26 clone), 1:180, on the autostainer Link48, pre-treatment in the PT link with high pH buffer.

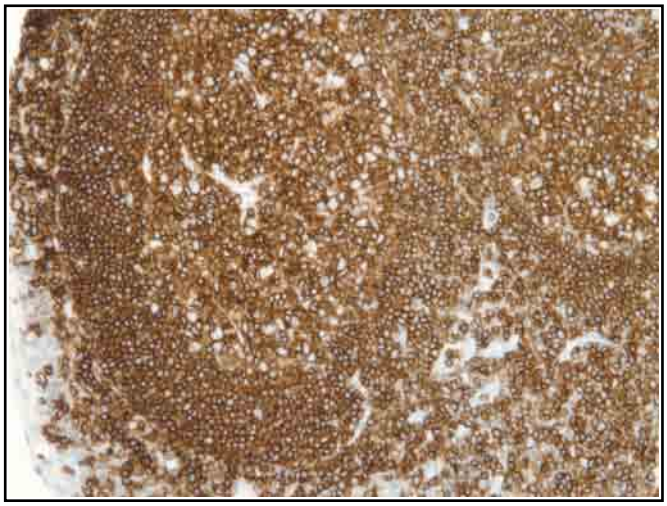


Fig 2. Higher power image of the UK NEQAS tonsil stained with CD45. The example demonstrates the strong crisp membranous staining of the B- and T-cells. Stained with the Ventana RP2/18 RTU clone on the ULTRA, CC1 mild with OptiView detection.

Fig 3. Sub-optimal demonstration of CD45 on the UK NEQAS distributed tonsil (compare to Figs 1&2).The staining is weak and diffuse with many of the lymphocytes expected to stain not demonstrated. Insufficient antigen retrieval is most likely the reason for the poor staining. The Leica RTU X16/99 antibody was used on the Leica BondMax with ER1 for 10 minutes.

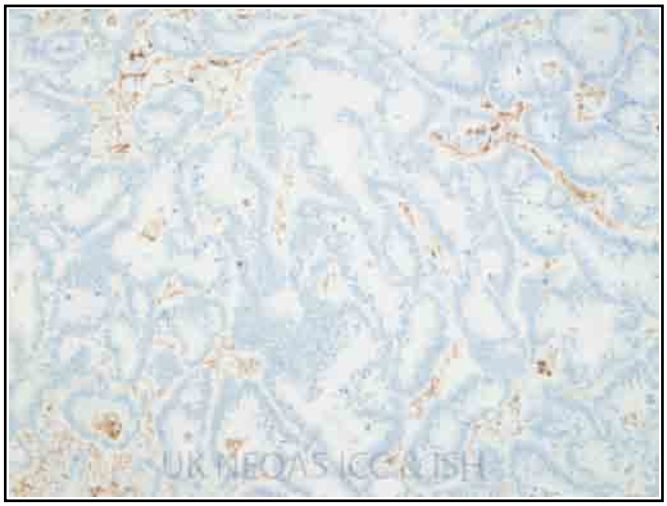


Fig 4. Optimal demonstration of CD45 on the UK NEQAS distributed colorectal carcinoma. As expected the tumour is negative, and only some of the surrounding scattered lymphocytes show strong to moderate distinct staining. (Same protocol as Fig 1).

Fig 5. Good demonstration of CD45 on a participant in-house tonsil section. Even at low power the strong membranous staining pattern can be seen. Stained with the Dako RTU Omnis 2B11+PD7/26 antibody on the Omnis with high pH retrieval.

Fig 6. Low power view of an in-house appendix section stained with CD45. The image demonstrates the strong staining localised to the lymphocytes, while the elements not expected to stain, including the glandular epithelium and crypts remain unstained. (Same protocol as Fig 2).

Selected Images showing Optimal and Sub-optimal Immunostaining

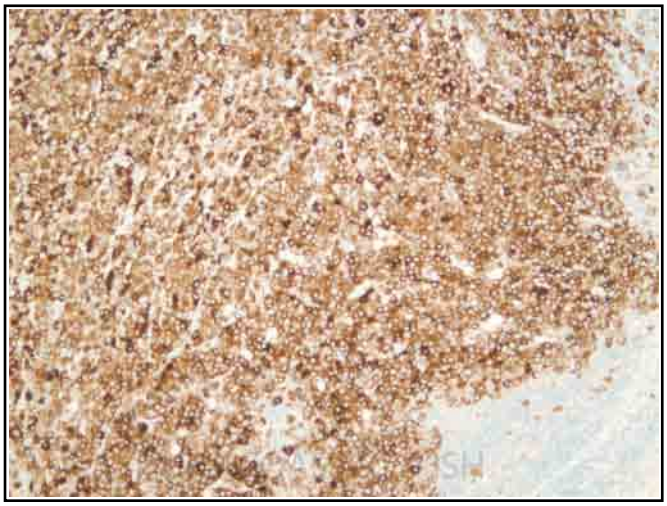


Fig 7. Optimal demonstration of the UK NEQAS distributed metastatic melanoma sample stained with the Dako Melan A (A104) antibody, 1:25. The example shows strong cytoplasmic staining of the neoplastic cells while the background remains clean. Stained on the Ventana ULTRA, CC1 for 64 minutes and OptiView detection.

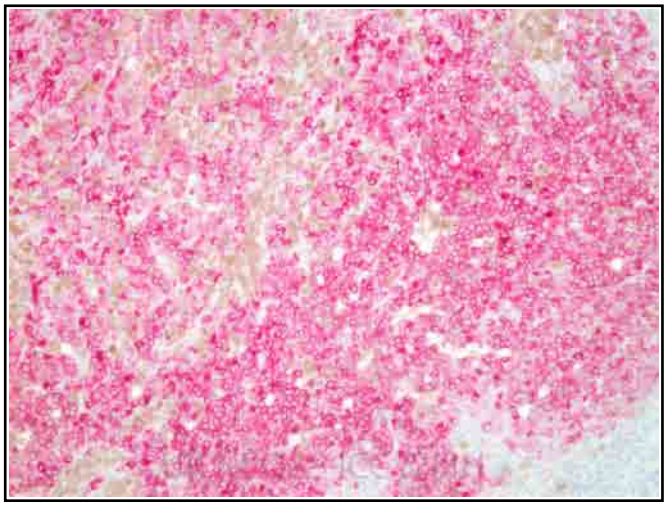


Fig 8. Optimal staining of the UK NEQAS distributed metastatic melanoma sample stained using the Dako Melan A (A104) antibody with Alkaline Phosphatase (AP) Red detection. All of the neoplastic cells show strong cytoplasmic staining. Stained using the Dako Melan A (A104) antibody, 1:100, on the Ventana ULTRA, CC1 standard.

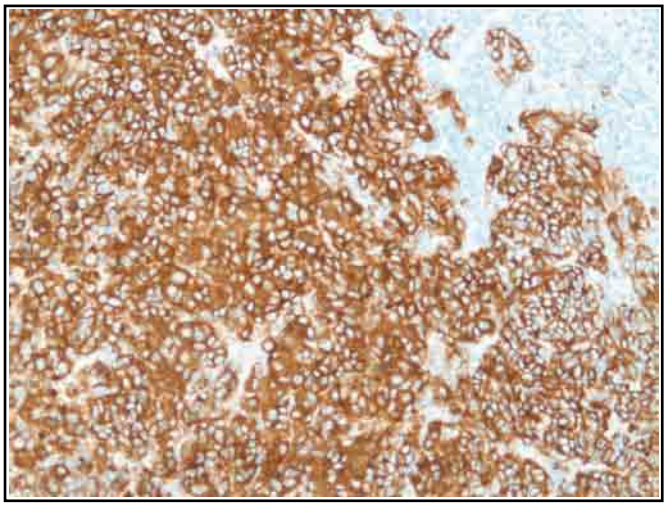


Fig 9. Optimal demonstration on the UK NEQAS metastatic melanoma stained with the HMB45 clone. All neoplastic cells show strong cytoplasmic staining while the background remains clean.

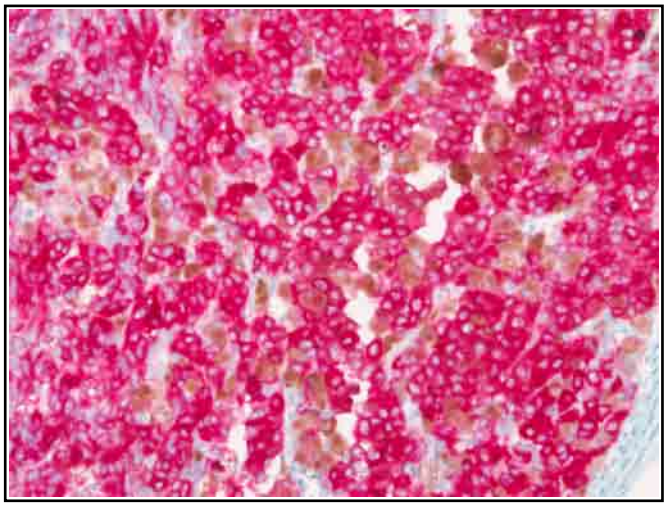


Fig 10. Good example of MART-1/MelanA staining on the UK NEQAS metastatic melanoma sample, showing strong cytoplasmic staining of all tumour cells. Stained using the Ventana prediluted antibody on the Benchmark ULTRA with Alkaline Phosphotase Red detection system.

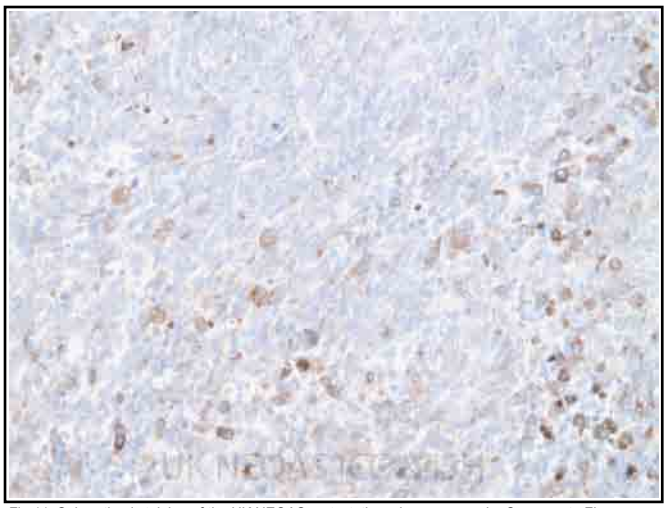


Fig 11. Sub-optimal staining of the UK NEQAS metastatic melanoma sample. Compare to Figs 7-10. The staining is very weak with many of the tumour cells expected to stain not demonstrated. The lack of staining is most likely due to the fact that no antigen retrieval was carried out. Stained with the Dako HMB45 on the Leica BondMax.

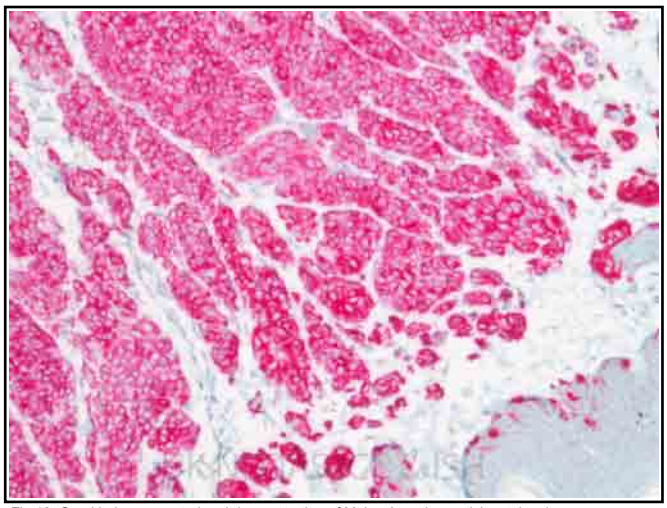
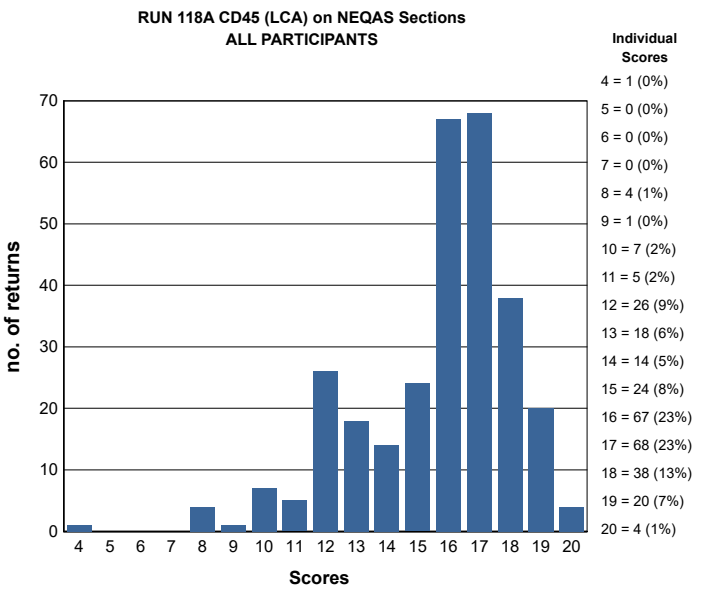
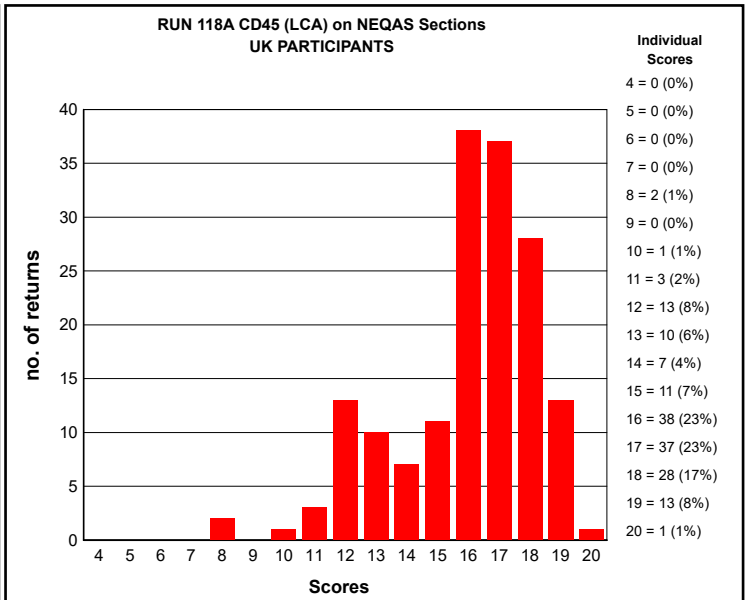
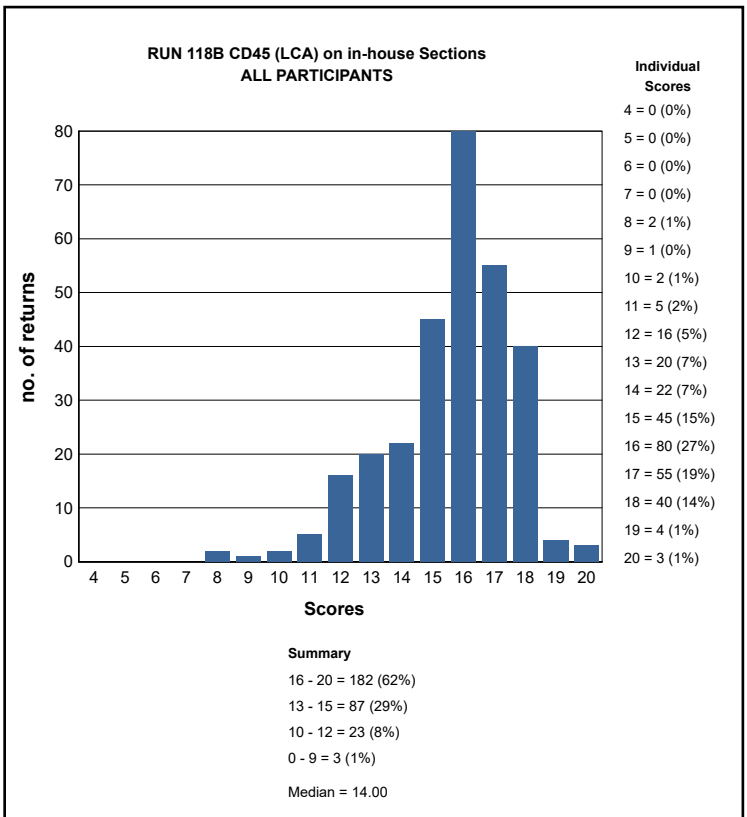


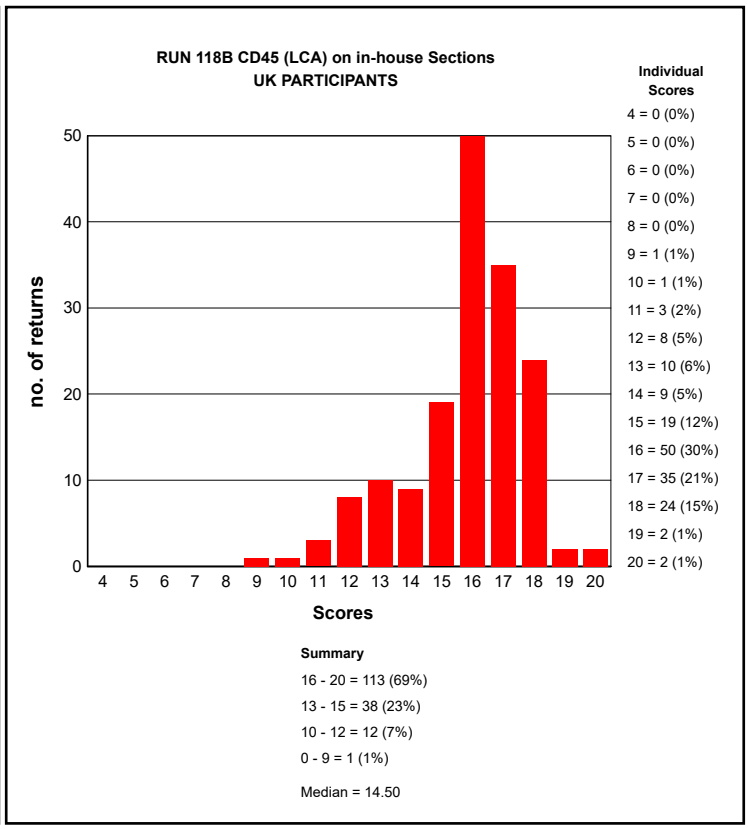
Fig 12. Good in-house control and demonstration of Melan A on the participants' melanoma skin sample, showing strong and distinct cytoplasmic (Same protocol as Fig 10).

GRAPHICAL REPRESENTATION OF PASS RATES

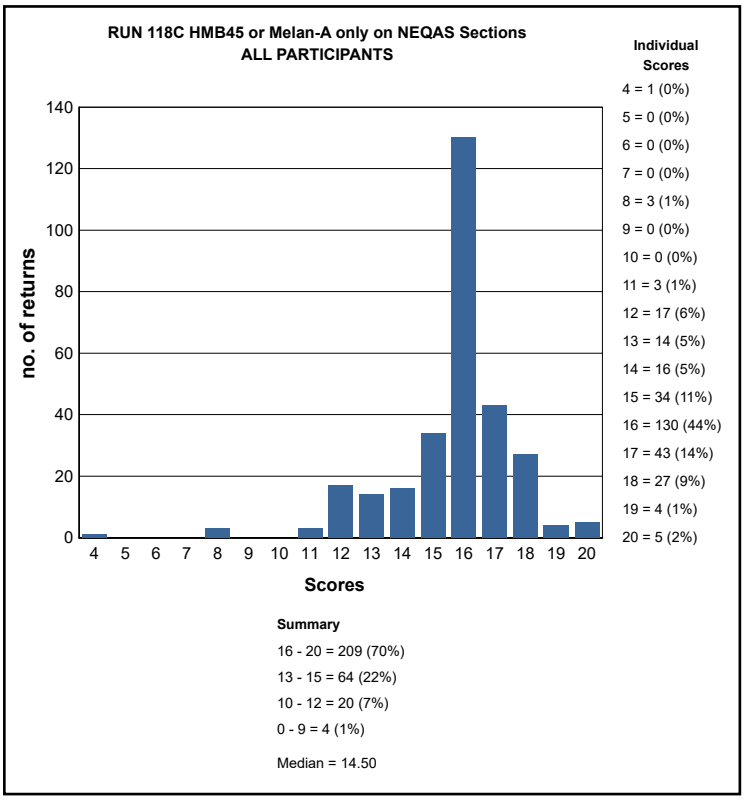


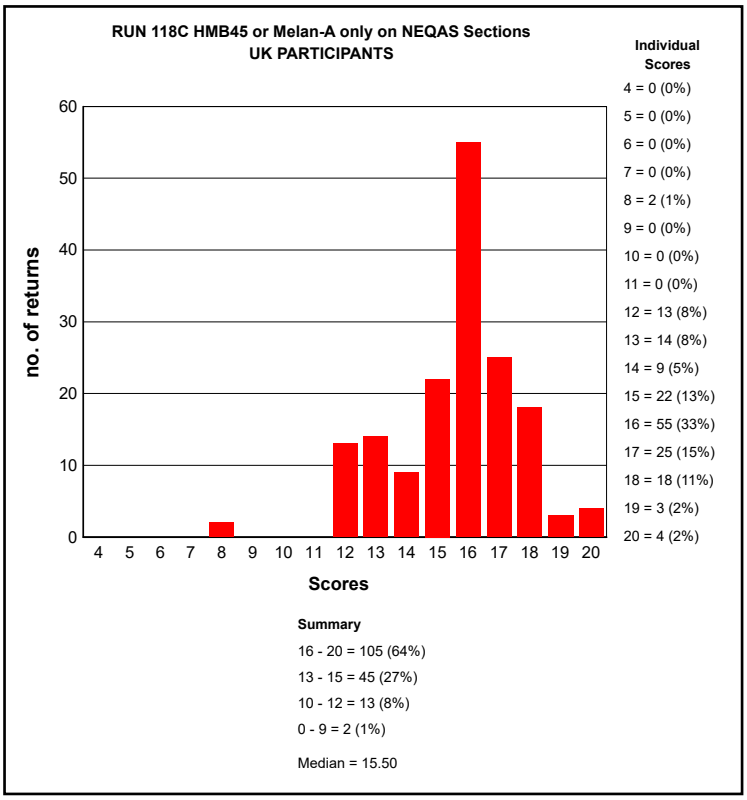


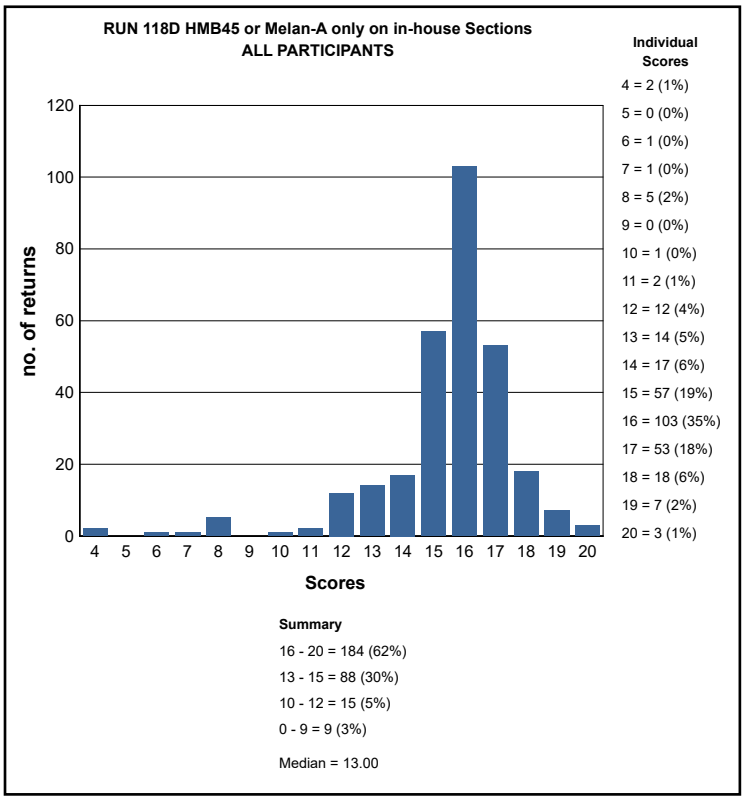


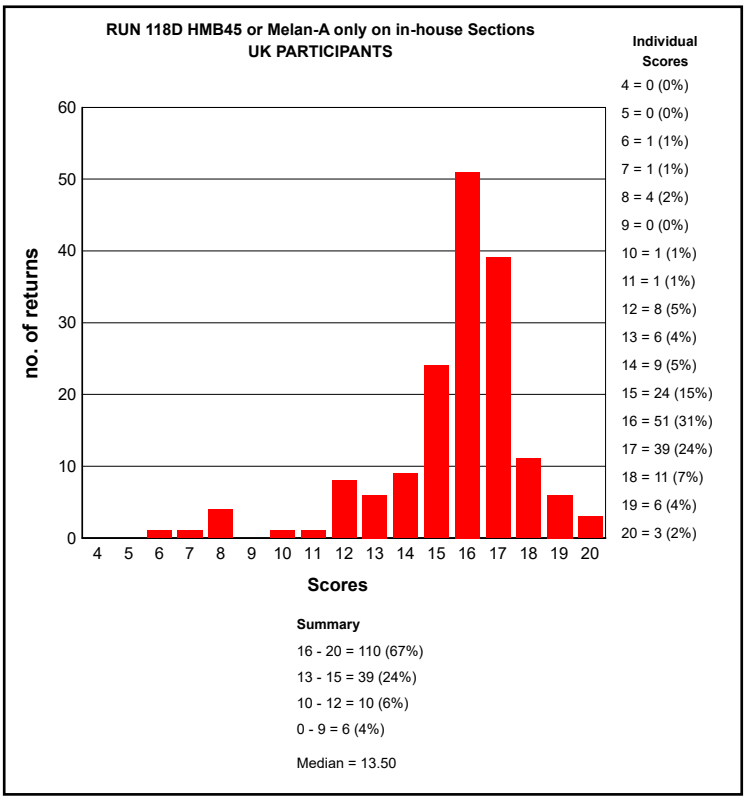


GRAPHICAL REPRESENTATION OF PASS RATES









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

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

General Pathology Run: 118

Primary Antibody : CD45 (LCA)

Antibody Details	N	%
A. Menarini MU0 71UC	1	100
Dako M0701 (clones 2B11+PD7/26)	166	88
Dako M0754 (clone 4KB5) CD45RA	2	100
Dako M0 (clone PD7/26) CD45RB	2	100
Novocastra NCL-LCA	3	67
Ventana 760 2505	39	90
BioGenex AM111-5M	1	0
BioGenex MU371-UC	1	100
Cell Marque clone (PD7/26&2B11)760-4279	3	67
Dako Flex IS751	4	75
Diagnostic BioSystems Mob 040	1	100
Leica Bond PA0042 CD45 (X16/99)	26	69
Ventana Confirm LCA RP2/18	11	73
Ventana 760 4279	14	93
Dako RTU Omnis GA751 (2B11+PD7/26)	9	100
Other	8	88

General Pathology Run: 118

Primary Antibody : HMB45 or Melan-A only

Antibody Details	N	%
Cell Marque CMA710 (HMB45)	1	100
Dako M0634 (HMB45)	27	96
Dako M7196 (A103) Melan-A	59	90
Novocastra NCL-HMB45 (HMB45)	9	100
Novocastra NCL-MELAN A	15	60
Ventana 760 2518 (HMB45)	1	100
Other	12	83
Ventana 790 2990 MART-1/MelanA	50	96
Novocastra NCL-RTU-MelanA (103)	1	100
Novocastra NCL-L-MelanA (103)	18	89
Novocastra PA0233 RTU MelanA (103)	1	100
Dako M0634 Melanosome (HMB45)	13	92
Dako N1545 Melanoaome (HMB45)	2	50
Cell Marque CM281M-9x MelanA (M2-7C10)	2	100
Biogenex AM361-5/10M (MelanA)	1	100
Leica Bond PA0233 (A103) Melan A	14	100
Leica RTU-Melan A (A103)	8	88
Dako RTU Auto Link IR052 (HMB45)	2	50
Cell Marque 282M (HMB45)	1	100
Ventana 790-4366 (HMB45)	26	100
Epitomics AC-0041 (MART-1 EP43)	1	100
Dako RTU Auto Plus IS052 (HMB45)	1	100
Dako RTU Omnis GA052 (HMB45)	4	100
Dako Melan A/MART-1 RTU Auto Link IR633	7	100
Dako Melan A/MART-1 RTU Auto Plus IS633	4	75
Novocastra PA0044 RTU MelanA (A103)	1	100
Novocastra PA0027 RTU HMB45	9	100

General Pathology Run: 118	CD45 (LCA)		HMB45 or Melan-A only	
Heat Mediated Retrieval	**N**	**%**	**N**	**%**
_Leica BondMax ER2	3	67	0	0
_Ventana Benk CC1 (Extended)	0	0	1	100
_Ventana Benk CC1 (Standard)	1	100	0	0
_Ventana Benk XT CC1 (Standard)	1	100	0	0
Biocare Decloaking Chamber	0	0	1	100
Dako Omnis	13	100	13	100
Dako PTLink	29	93	26	92
Lab vision PT Module	2	100	2	100
Leica ER1 10 mins	5	20	4	100
Leica ER1 20 mins	37	73	17	94
Leica ER1 30 mins	14	93	6	67
Leica ER2 10 mins	2	100	1	100
Leica ER2 20 mins	17	94	44	93
Leica ER2 30 mins	4	100	11	100
Microwave	5	60	4	100
None	3	33	15	87
Other	1	100	0	0
Pressure Cooker	3	67	2	100
Pressure Cooker in Microwave Oven	1	100	1	100
Steamer	2	100	2	100
Ventana CC1 16mins	5	100	3	100
Ventana CC1 20mins	0	0	2	100
Ventana CC1 24mins	11	100	4	100
Ventana CC1 32mins	34	97	13	100
Ventana CC1 36mins	12	92	12	83
Ventana CC1 40mins	2	100	6	100
Ventana CC1 44mins	2	100	0	0
Ventana CC1 48mins	2	100	7	100
Ventana CC1 52mins	3	100	1	0
Ventana CC1 56mins	3	100	4	100
Ventana CC1 64mins	20	85	35	89
Ventana CC1 76mins	2	100	4	75
Ventana CC1 8mins	5	20	4	100
Ventana CC1 92mins	0	0	2	100
Ventana CC1 extended	0	0	1	100
Ventana CC1 mild	15	93	6	100
Ventana CC1 standard	11	91	21	76
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	2	100	1	100
Ventana CC2 standard	0	0	1	0
Water bath 68 OC	1	100	1	100
Water bath 95-98 OC	2	0	1	0

General Pathology Run: 118	CD45 (LCA)		HMB45 or Melan-A only	
Enzyme Mediated Retrieval	**N**	**%**	**N**	**%**
AS PER KIT	4	50	9	100
NOT APPLICABLE	138	89	140	91
Other	0	0	1	100
VBS Bond Enzyme 1	1	100	4	100
Ventana Protease 1 (760-2018)	1	100	5	80

General Pathology Run: 118	CD45 (LCA)		HMB45 or Melan-A only	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	20	90	20	95
BioGenex HRP (HK 519-06K)	1	0	1	100
BioGenex SS Polymer (QD 430-XAKE)	1	100	0	0
Dako EnVision FLEX (K8000/10)	12	100	16	100
Dako EnVision FLEX+ (K8002/12)	20	95	17	88
Dako Envision HRP/DAB (K5007)	5	80	7	86
Dako Envision+ HRP mouse K4004/5/6/7	2	100	1	100
Dako Envision+ HRP rabbit K4008/9/10/11	0	0	1	100
Dako REAL (K5005)	0	0	1	100
LabVision UltraVision LP HRP (TL 125 HLJ)	1	100	1	100
Leica Bond Polymer AP Red Detection (DS9305)	0	0	1	100
Leica Bond Polymer Define (DS9713)	0	0	2	50
Leica Bond Polymer Refine (DS9800)	77	78	76	95
MenaPath X-Cell Plus (MP-XCP)	1	100	1	100
None	1	0	0	0
NOT APPLICABLE	1	100	0	0
Novocastra Novolink PDS (RE7-140/150/280/290-K)	2	100	2	100
Other	6	100	18	83
Power Vision DPVB999 HRP	1	0	0	0
Ventana iView system (760-091)	2	100	1	0
Ventana OptiView (760-700) + Amp. (7/860-099)	2	100	5	100
Ventana OptiView Kit (760-700)	61	93	50	100
Ventana UltraView Kit (760-500)	67	84	63	81

General Pathology Run: 118	CD45 (LCA)		HMB45 or Melan-A only	
Automation	**N**	**%**	**N**	**%**
Dako Autostainer	3	100	3	67
Dako Autostainer Link 48	23	96	24	92
Dako Autostainer plus	3	67	3	100
Dako Autostainer Plus Link	2	50	0	0
Dako Omnis	13	100	13	100
LabVision Autostainer	3	100	3	100
Leica Bond Max	42	74	45	96
Leica Bond-III	49	84	48	90
Menarini - Intellipath FLX	1	100	0	0
None (Manual)	10	60	8	88
Shandon Sequenza	1	100	1	100
Ventana Benchmark GX	5	100	5	100
Ventana Benchmark ULTRA	93	87	96	91
Ventana Benchmark XT	43	91	42	86

General Pathology Run: 118	CD45 (LCA)		HMB45 or Melan-A only	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	35	91	29	93
BioGenex liquid DBA (HK-124-7K)	1	0	1	100
Dako DAB K3468	1	100	0	0
DAKO DAB+	1	100	0	0
Dako DAB+ Liquid (K3468)	4	75	4	75
Dako EnVision Plus kits	2	100	2	100
Dako FLEX DAB	32	94	34	94
Dako REAL EnVision K5007 DAB	6	67	6	83
Dako REAL K5001 DAB	0	0	1	100
Leica Bond Polymer Refine kit (DS9800)	78	78	84	94
menapath xcell kit DAB (MP-860)	1	100	0	0
Other	15	93	16	94
Sigma DAB (D5637)	2	50	1	100
Ventana DAB	35	91	32	100
Ventana Enhanced Alk. Phos. Red Detection Kit	0	0	17	82
Ventana iview	3	67	4	75
Ventana Ultraview DAB	72	86	59	85
Vision BioSystems Bond X DAB	1	100	0	0

BEST METHODS - Gold Standard Antibody

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

CD45 (LCA) - Method 1

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

Primary Antibody: Dako M0701 (clones 2B11+PD7/26) , 20 Mins, RT ºC Dilution 1: 150

Automation: Leica Bond-III

Method: Leica BondMAx Refine KIT

Main Buffer: Bond Wash Buffer (AR9590)

HMAR: Leica ER1 30 mins

EAR:

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

Detection: Leica Bond Polymer Refine (DS9800) , 20 Mins, RT 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) , 40 Mins, 20 ºC Dilution 1: 400
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: HIGH PH RETRIEVAL SOLUTION
EAR:	
Chromogen:	Dako FLEX DAB, 20 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 20 Mins, 20 ºC Prediluted

CD45 (LCA) - Method 3

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

Primary Antibody:	Ventana 760 2505 , 16 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.5
HMAR:	Ventana CC1 32mins, PH: 8.25
EAR:	
Chromogen:	Ventana DAB, Time 1: 8 Mins, Time 2: 37 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 37 ºC Prediluted

CD45 (LCA) - Method 4

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

Primary Antibody:	Dako M0701 (clones 2B11+PD7/26) , 32 Mins Dilution 1: 100
Automation:	Ventana Benchmark GX
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	_Ventana Benk CC1 (Standard)
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

BEST METHODS - Secondary Antibody

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

HMB45 or Melan-A only - Method 1

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

Primary Antibody:	Dako M0634 (HMB45) , 20 Mins, RT ºC Dilution 1: 100
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER1 20 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), RT ºC., Time 1: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 20 Mins, RT ºC Prediluted

HMB45 or Melan-A only - Method 2

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

Primary Antibody:	Dako N1545 Melanoaome (HMB45) , 15 Mins, 20 ºC Dilution 1: 50
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER1 20 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

HMB45 or Melan-A only - Method 3

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

Primary Antibody:	Dako M7196 (A103) Melan-A , 40 Mins, 20 ºC Dilution 1: 50
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink
EAR:	
Chromogen:	Dako FLEX DAB, 20 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 20 Mins, 20 ºC Prediluted

HMB45 or Melan-A only - Method 4

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

Primary Antibody:	Ventana 790 2990 MART-1/MelanA , 32 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Enhanced Alk. Phos. Red Detection Kit
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 92mins
EAR:	
Chromogen:	Ventana Enhanced Alk. Phos. Red Detection Kit
Detection:	AS PER KIT

The Breast Hormonal Receptor Module Run 118

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:	263
Number of Participants This Run	258 (98%)

Circulated Tissue

The table below shows the staining characteristics of the tissue sections circulated during Run 118. This composed of three invasive 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	50%	Mid	4-6 (expression level varied depending on the serial section received)
C. IDC	0%	Negative	0
E. Tonsil	≤ 3%	Weak to Medium	0 (Negative)

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

General Guideline Used in The Assessment of Slides:

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

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

In-House Tissue Recommendations:

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

The required composite control should consist of the following samples:

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

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

Introduction

Expression of the 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, 2002). They also play a key-role in proliferative and neoplastic diseases of the breast.

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.*, 1989). 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; moreover, it potentially defines a subpopulation of patients with superior response to tamoxifen; 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. 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, 2000). All these factors lead to the conclusion that correct PR status is becoming increasingly important. Correct staining protocols and validated staining

techniques are therefore vital to avoid false ER and/or PR staining (Rhodes *et al*., 2001; Ibrahim et al., 2008), which can have a direct impact on patient treatment.

Choice of Tissue for Assessments

This assessment consisted of three invasive breast tumours of varying ER expression levels (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 the 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 (Figs 1-6, 9 & 10)

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

Features of Suboptimal Immunostaining (Figs 8 & 9)

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

NEQAS Section Assessment Results

258 laboratories submitted their slides for the ER assessment. The pass rates are slightly higher than the previous ER assessment (Run 117), but in keeping with the pass rates seen over the assessment year.

Please see table below for breakdown of the ER results on the NEQAS material over the last 3 UK NEQAS ICC assessments:

ER NEQAS Pass Rates :			
	Run 116	**Run 117**	**Run 118**
Acceptable	84%(N=228)	81%(N=217)	87%(N=222)
Borderline	10%(N=27)	13%(N=35)	10%(N=26)
Unacceptable	6%(N=15)	6%(N=15)	4%(N=10)

The borderline and failed marks for this assessment were mostly due to weak staining, particularly in the mid-expressing tumour. A few labs failed due to inappropriate false-positive staining in the negative tumour. Excessive staining was also seen in the high-expresser and mid-expressing tumour in some of these slides. It was clear when looking at some of the protocols that the antigen retrieval time was too long, and therefore featuring inappropriate staining.

In-House Tissue Assessment Results

Apart from 2 participants, all laboratories also submitted their in-house controls for assessment. Overall these showed a lower acceptable pass rate of 70% (N=180) compared to the NEQAS sections. The in-house samples showed a higher percentage of borderline passes, 29% (N=73) compared to the NEQAS pass rate. Only 3 laboratories (1%) failed on their in-house material. Many of the borderline passes were not due to poor staining. Instead participants lost marks due to not providing the required in-house material consisting of a high-expresser, mid-expresser and a negative ER tumour (as outlined in the in-house recommendations section of this article).

Methodologies

The most popular antibody was the Ventana SP1 clone, used by 45% (N=117) of participants and showed an acceptable pass rate of 96%. The other 2 most popular clones were the Dako EP1, used by 21% (N=52), and the Leica 6F11, which was used by 20% (N=51). These showed acceptable pass rates of 95% and 64% respectively.

The Ventana SP1 clone is a pre-diluted antibody, and was only seen at the Run 118 assessment to be used on the Ventana automated platforms, for which the antibody was validated. While both the Dako EP1 and Leica 6F11 clones were also originally validated by the vendors for use on their automated platforms, these antibodies are known to also work well on other platforms. Both can be used in the ready-to-use or diluted from a concentrated format. Many laboratories continue to use the antibody which was originally introduced for use on the same company automated platform, and it is important that laboratories follow the guidance provided in the datasheet accompanying the antibody for recommended protocol, particularly the antigen retrieval. Regardless of the method used, laboratories must ensure validation/verification is carried out using appropriate controls and clinical samples with known reactivity.

References

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2. Jennet M. Harvey, Gary M. Clark, C. Kent Osborne, and D. Craig Allred (1999) Estrogen Receptor Status by Immunohistochemistry Is Superior to the Ligand-Binding Assay for Predicting Response to Adjuvant Endocrine Therapy in Breast Cancer J Clin Oncol 17:1474-1481.
3. Robin Leake, Diana Barnes, Sarah Pinder, Ian Ellis, Liz Anderson, Tom Anderson, Ruth Adamson, Tony Rhodes, Keith Miller and Rosemary Walker (2000) J. Clin. Pathol. 2000: 634-635.

Acknowledgments

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

Selected Images showing Optimal and Sub-optimal Immunostaining

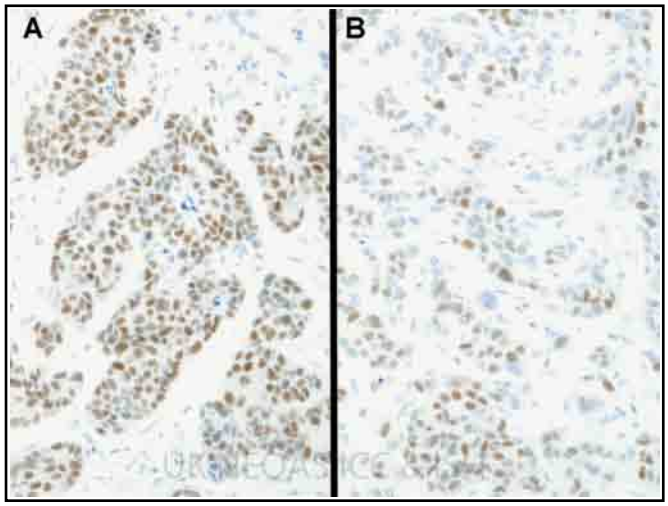


Fig 1. Optimal demonstration of ER in the UK NEQAS high- (A) and mid- (B) expressing tumours. Both sections show the expected level of staining. Stained using the Dako RTU EP1 antibody on the autostainer and pre-treatment in the PT link with high pH retrieval buffer.

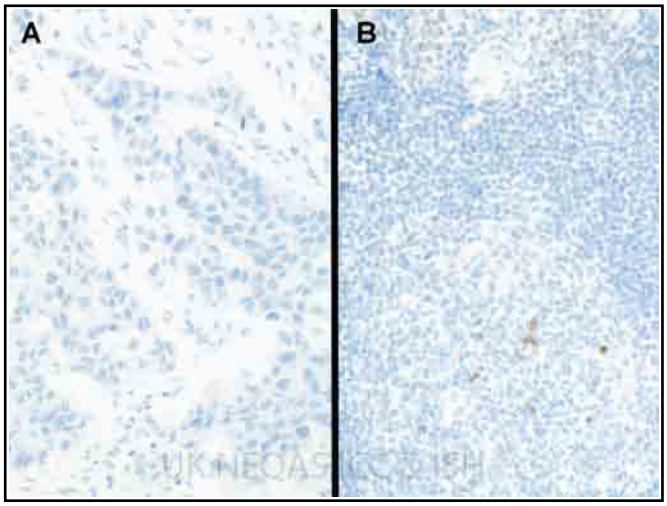


Fig 2. Expected demonstration of ER in the UK NEQAS distributed samples. The ER negative tumour (A) remains unstained, and as expected, a small percentage of lymphocytes are staining positive in the tonsil section (B). (Same protocol as Fig 1).

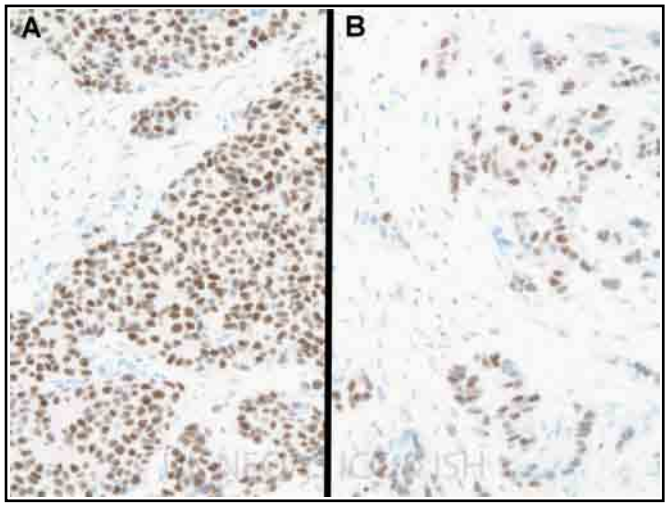


Fig 3. Optimally stained UK NEQAS distributed samples: The high expressing ER tumour (A) shows intense staining in over 95% of neoplastic cells, while the mid-expressing tumour (B) shows varying intensity of positive staining in over 60% of neoplastic cells. Stained using the Dako EP1 antibody, 1:75 on the Dako Omnis.

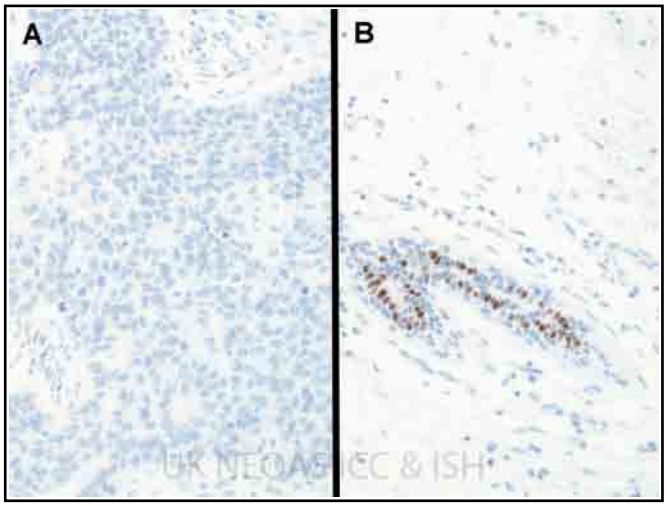


Fig 4. Expected ER demonstration in the UK NEQAS negative distributed sample. As shown in image (A) the negative tumour remains unstained. Section (B) demonstrates a normal gland, which shows the expected proportion of positive cells staining. (Same protocol as Fig 3).

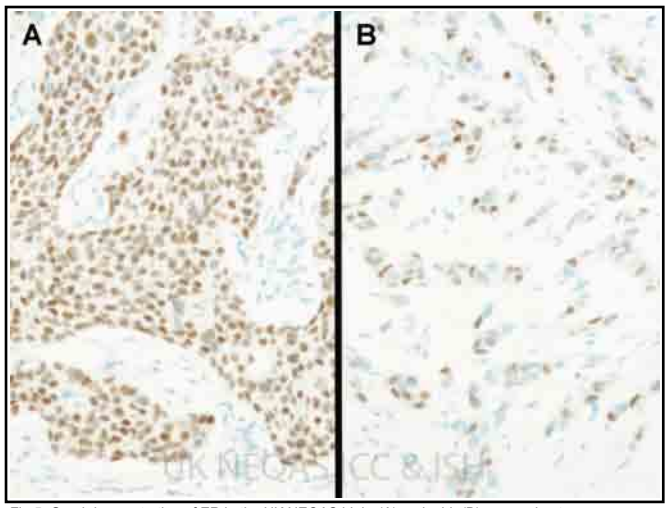


Fig 5. Good demonstration of ER in the UK NEQAS high- (A) and mid- (B) expressing tumours. Both sections show the expected level of staining. Stained with the Ventana SP1 RTU clone on the ULTRA, CC1 standard and UltraView detection.

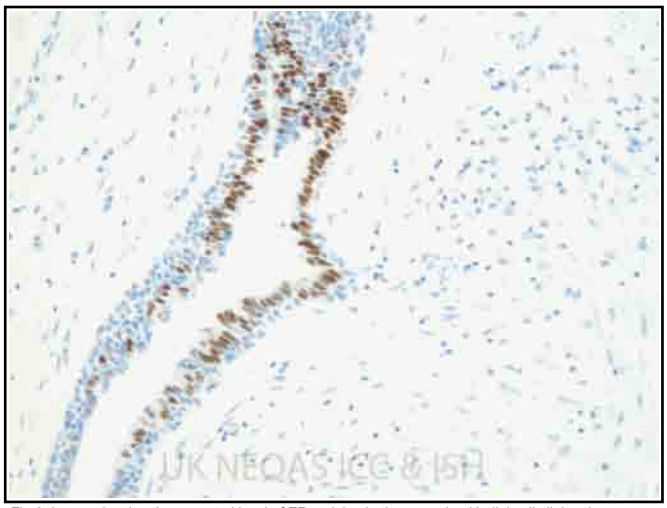


Fig 6. Image showing the expected level of ER staining in the normal epithelial cells lining the breast ductal gland seen within the UK NEQAS distributed tissue. While the cells normal cells show strong nuclear staining, the back ground remains clean. (Same protocol as Fig 1).

Selected Images showing Optimal and Sub-optimal Immunostaining

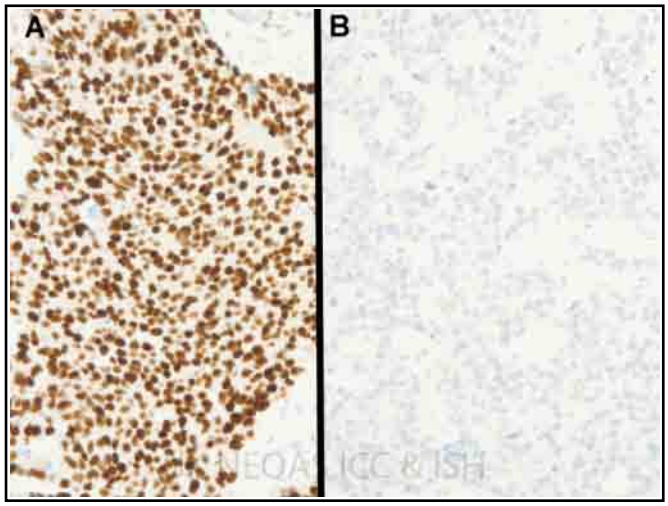


Fig 7. Two examples of sub-optimal ER staining of the UK NEQAS high-expressing tumour. (A) appears over pre-treated and stronger than expected, while the staining in (B) is very weak. (A) Leica 6F11 clone on the Ventana Benchmark with OptiView detection. (B) Using the Dako EP1 clone, but low pH retrieval buffer instead of high pH buffer recommended for this clone.

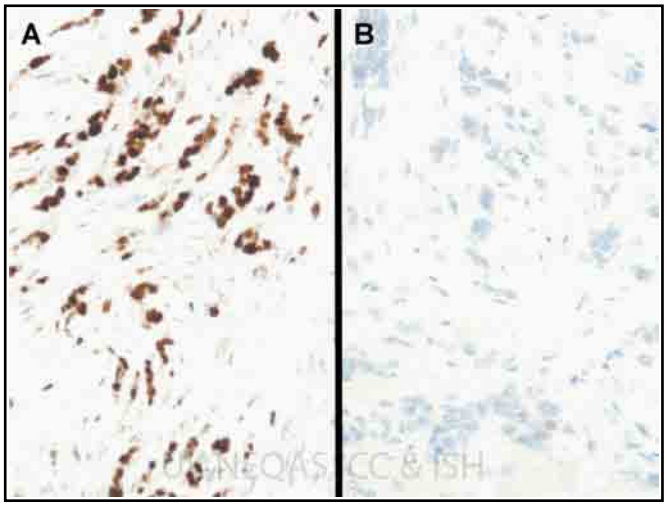


Fig 8. Two examples showing unacceptable ER staining of the UK NEQAS mid-expressing tumour. (A) shows excessive staining, and appears more representative of a high-expressing tumour. The staining in (B) is very weak. (same protocols as Fig 7A & B).

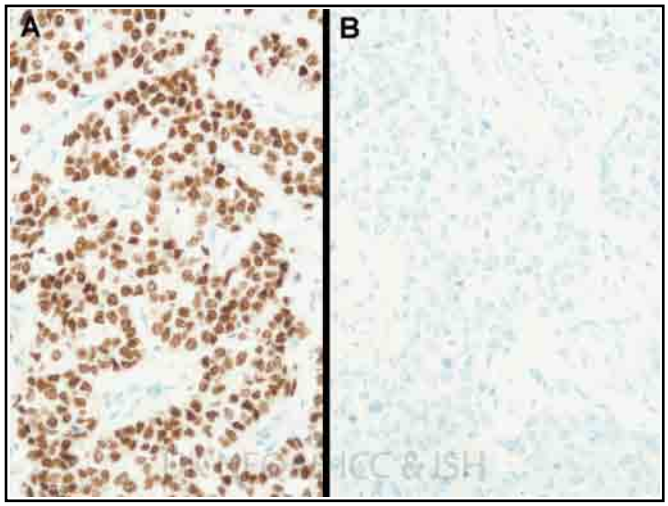


Fig 9. Good example and staining of an in-house control for ER. (See Fig 10 also). The multi-block section contained high- and negative-expressing tumours shown in this image (A & B respectively). Stained using the Ventana SP1 RTU clone on the Benchmark GX.

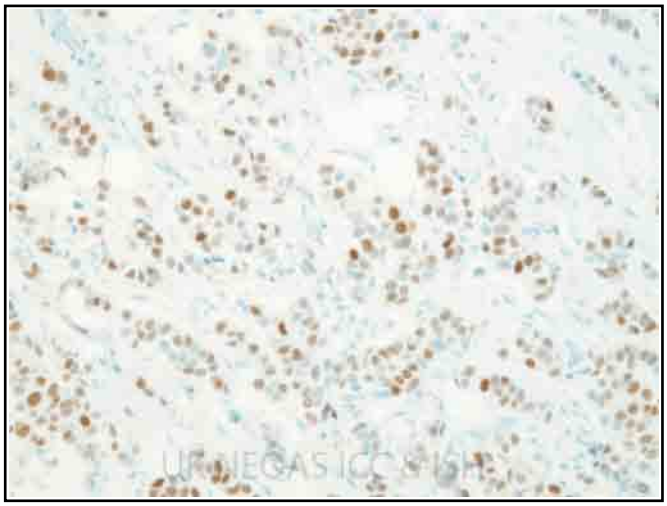
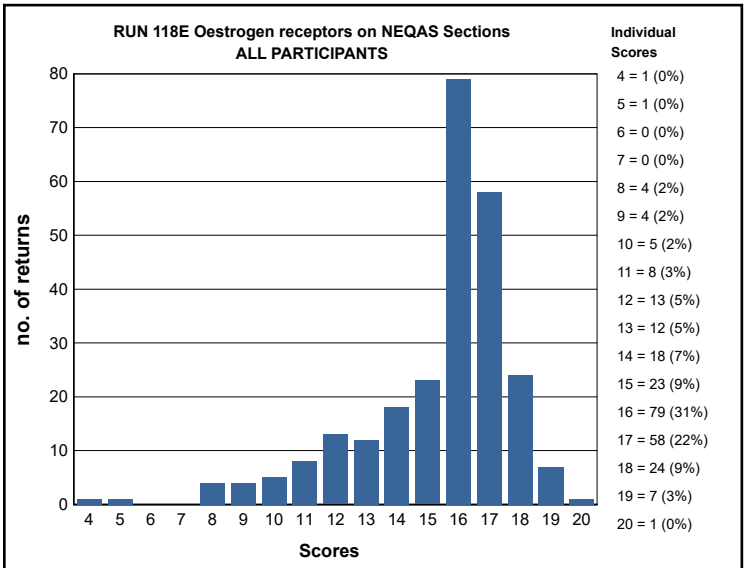
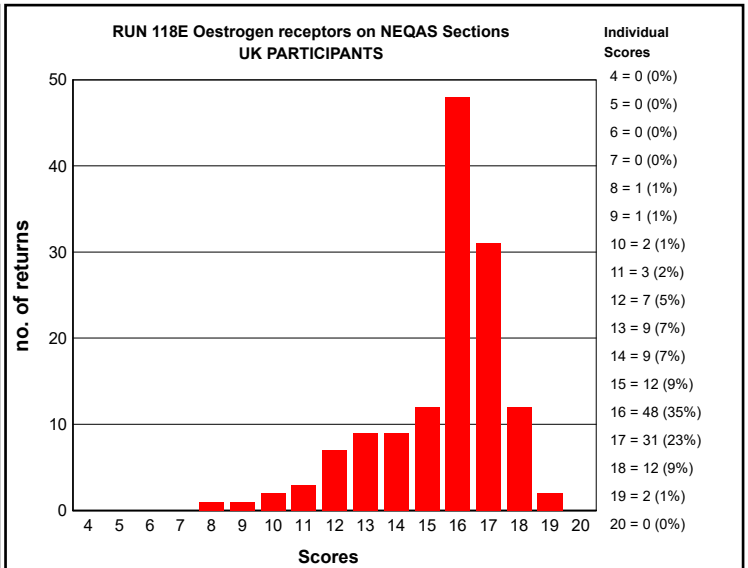
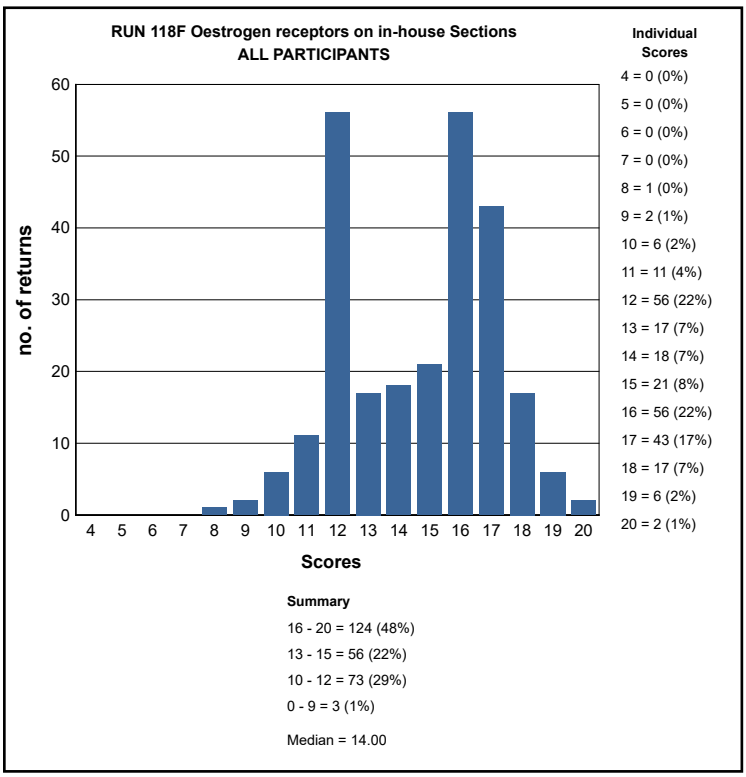


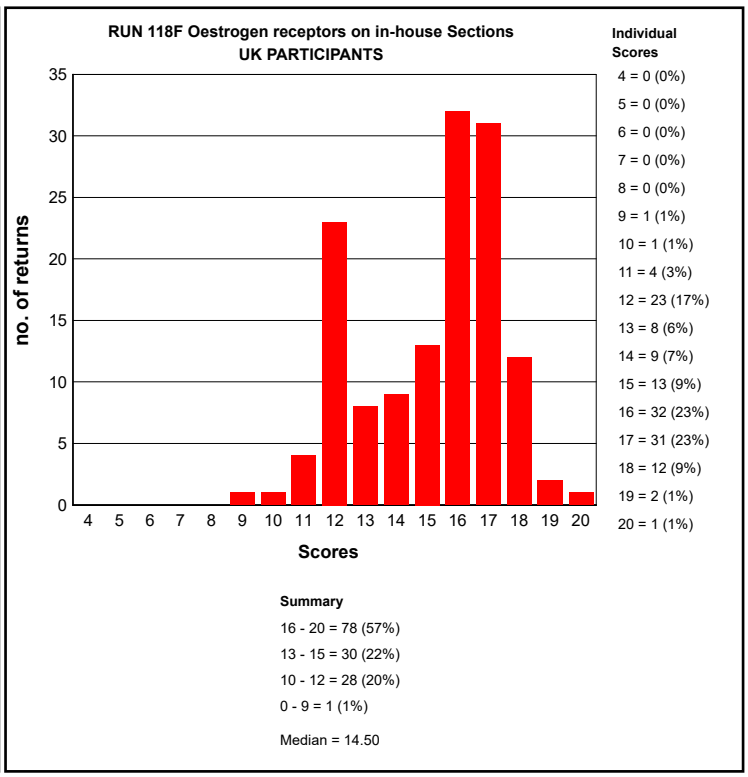
Fig 10. Good example and staining of an in-house mid-expressing tumour control for ER (from the same multi-block control shown in Fig 9). (Same protocol as Fig 9).

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: 118

Primary Antibody : Oestrogen receptors

Antibody Details	N	%
Dako M7047 ER (1D5)	1	100
Thermo Fisher/ Neomarkers RM 9101-S (SP1)	10	100
Leica/Novocastra NCL-ER-6F11 (6F11)	11	45
Ventana 790-4324 (SP1)	79	94
Leica Bond PA0151 (6F11)	4	75
Dako M3634 (SP1)	2	100
Dako N1575 (1D5)	1	100
Dako RTU IR151 (SP1)	1	100
Ventana 790-4325 (SP1)	38	97
Leica/Novocastra NCL-L-ER- 6F11	24	63
Leica/Novocastra RTU-ER-6F11	4	50
Leica/Novocastra NCL-ER-6F11/2	8	88
Dako SK310 pharmDX kit (Link) (1D5 + ER-2-123)	1	100
Dako (EP1) RTU FLEX IR084	21	100
Dako (EP1) M3643	29	86
Dako FLEX (1D5) IR/IS657	1	0
Other	7	86
Dako (EP1) RTU Auto Plus IS084	2	100
Cell Marque 249-R (SP1)	3	67

Breast Steroid Hormone Receptor Run: 118

Automation	Oestrogen receptors N	%
Dako Autostainer	1	100
Dako Autostainer Link 48	20	85
Dako Autostainer Plus Link	2	100
Dako Omnis	9	100
LabVision Autostainer	2	100
Leica Bond Max	19	68
Leica Bond-III	47	72
Menarini - Intellipath FLX	1	100
None (Manual)	5	60
Ventana Benchmark GX	7	86
Ventana Benchmark ULTRA	102	94
Ventana Benchmark XT	40	88

Breast Steroid Hormone Receptor Run: 118

Heat Mediated Retrieval	Oestrogen receptors N	%
_Ventana ULTRA CC1 (36mins)	1	100
Biocare Decloaking Chamber	2	50
Dako Omnis	9	100
Dako PTLink	21	86
Lab vision PT Module	2	100
Leica ER1 20 mins	13	62
Leica ER1 30 mins	12	42
Leica ER1 40 mins	9	44
Leica ER2 10 mins	2	100
Leica ER2 20 mins	27	93
Leica ER2 30 mins	3	100
Microwave	1	0
Other	2	100
Pressure Cooker	2	100
Ventana CC1 16mins	1	100
Ventana CC1 20mins	1	100
Ventana CC1 24mins	4	100
Ventana CC1 32mins	8	50
Ventana CC1 36mins	25	88
Ventana CC1 40mins	3	100
Ventana CC1 48mins	2	50
Ventana CC1 52mins	7	100
Ventana CC1 56mins	2	100
Ventana CC1 64mins	46	100
Ventana CC1 76mins	1	0
Ventana CC1 88mins	1	100
Ventana CC1 92mins	2	50
Ventana CC1 extended	5	100
Ventana CC1 mild	11	91
Ventana CC1 standard	24	96
Ventana CC2 mild	2	100
Water bath 95-98 OC	3	100

Breast Steroid Hormone Receptor Run: 118

Enzyme Mediated Retrieval	Oestrogen receptors N	%
AS PER KIT	7	71
NOT APPLICABLE	166	86
Sigma T7409 Trypsin	1	100
Ventana Protease 1 (760-2018)	1	100

Breast Steroid Hormone Receptor Run: 118

Detection	Oestrogen receptors N	%
AS PER KIT	13	100
BioGenex SS Polymer (QD 430-XAKE)	1	0
Dako EnVision FLEX (K8000/10)	8	100
Dako EnVision FLEX+ (K8002/12)	15	80
Dako Envision HRP/DAB (K5007)	2	100
Dako REAL HRP/DAB (K5001)	1	100
LabVision UltraVision LP HRP (TL 125 HLJ)	1	100
Leica Bond Polymer Refine (DS9800)	60	70
MenaPath X-Cell Plus (MP-XCP)	1	100
None	1	100
NOT APPLICABLE	2	50
Other	4	100
Ventana iView system (760-091)	2	100
Ventana OptiView Kit (760-700)	18	83
Ventana UltraView Kit (760-500)	122	93

Breast Steroid Hormone Receptor Run: 118

Chromogen	Oestrogen receptors N	%
AS PER KIT	16	94
BioGenex liquid DBA (HK-124-7K)	1	0
Dako DAB K3468	1	100
DAKO DAB+	2	100
Dako EnVision Plus kits	3	100
Dako FLEX DAB	21	86
Dako REAL EnVision K5007 DAB	4	75
Dako REAL K5001 DAB	1	100
Leica Bond Polymer Refine kit (DS9800)	60	68
menapath xcell kit DAB (MP-860)	1	100
Other	8	88
Ventana DAB	11	100
Ventana Enhanced Alk. Phos. Red Detection Kit	1	0
Ventana iview	3	100
Ventana Ultraview DAB	121	93

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 18/20 (In House slide) using this method.

Primary Antibody: Ventana 790-4324 (SP1) , 16 Mins, 37 ºC Prediluted
Automation: Ventana Benchmark XT
Method: Ventana UltraView DAB
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 standard
EAR:
Chromogen: Ventana Ultraview DAB
Detection: Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC

Oestrogen receptors - Method 2

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

Primary Antibody: Dako (EP1) RTU FLEX IR084 , 20 Mins, ROOM ºC Prediluted
Automation: Dako Autostainer Link 48
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako PTLink, Buffer: RETRIEVAL SOLUTION HIGH PH
EAR: NOT APPLICABLE
Chromogen: Dako FLEX DAB, ROOM ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection: Dako EnVision FLEX+ (K8002/12) , 20 Mins, ROOM ºC Prediluted

Oestrogen receptors - Method 3

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

Primary Antibody: Thermo Fisher/ Neomarkers RM 9101-S (SP1) , 88 Mins, 37 ºC Dilution 1: 100
Automation: Ventana Benchmark XT
Method: Ventana Optiview
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 88mins, Buffer: cc1
EAR:
Chromogen: AS PER KIT, PH: 37, Time 1: 8 Mins
Detection: Ventana OptiView Kit (760-700) , 60 Mins, 37 ºC Prediluted

Oestrogen receptors - Method 4

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

Primary Antibody:	Ventana 790-4324 (SP1) , 16 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark GX
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 48mins
EAR:	
Chromogen:	AS PER KIT
Detection:	Ventana OptiView Kit (760-700)

The Breast HER2 ICC Module Run 118

Keith Miller and Amy Newman

Antigen Assessed:	HER2
Sections Circulated:	4 Cell lines of varying Breast HER2 Expression Level (see table below)
Number of Registered Participants:	233
Number of Participants this Run	218 (91%)

Specific Guidelines Used in The Assessment of Slides:

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

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

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

Validation of Distributed UK NEQAS ICC & ISH Cell Lines

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

Assessment and Scoring Procedure

UK NEQAS Specific Membrane Scoring Algorithm:

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

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

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

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

Numerical Scoring Criteria

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

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

Introduction

HER2 immunohistochemistry has been routinely used as a predictive marker in breast cancer for more than 10 years. Patients with HER2 positive tumours generally have a poor overall prognosis. However, patients with 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 laboratories 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 & ISH are always willing to assist any laboratory struggling to meet the required standard of HER2 immunostaining, and would be happy to receive requests for assistance via e-mail in the first instance (see front of journal or the UK NEQAS ICC & ISH website for contact details).

In-House Control Tissue Recommendations

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

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

Assessment Summary:

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

	Pass Rates Run 118:	
	NEQAS	**In-House**
Acceptable	73% (N=160)	62% (N=135)
Borderline	17% (N=37)	29% (N=63)
Unacceptable	10% (N=21)	9% (N=19)

As with previous runs the most popular antibody was the Ventana 4B5, used by 68% (n=159) of participants and showed an overall acceptable pass rate of 82%. 10 laboratories employed the Dako HercepTest, with 50% of participants achieving an acceptable pass rate. 15 laboratories are using the Leica Oracle assay kit with an acceptable pass rate of 87%. 28 laboratories used laboratory devised tests (LTDs), incorporating a variety of antibodies (most commonly Dako A0485), pre-treatment methods and staining platforms. These laboratories showed an acceptable pass rate of 21%. 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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2. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med 353:1659-1672, 2005
3. Bartlett JM, Ibrahim M, Jasani B, et al. External quality assurance of HER2 FISH testing: results of a UK NEQAS pilot scheme. J Clin Pathol 2007 60 (7):816-819.
4. Walker RA, Bartlett JM, Dowsett M, Ellis IO, Hanby AM, Jasani B, Miller K, Pinder SE. HER2 testing in the UK: further update to recommendations. J Clin Pathol. 2008 61(7):818-824.
5. Wolff AC, Hammond MEH, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol 2007;25:1–28.
6. Wolff AC, et al. Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/ College of American Pathologists Clinical Practice Guideline Update. J Clin Pathol. 2013; 31 (31):3998-4013.
7. Rakha EA, et al. Updated UK rRecommendations for HER2 assessment in breast cancer. J Clin Pathol. 2015;68:93-99.

Acknowledgments

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

Selected Images showing Optimal and Sub-optimal Immunostaining

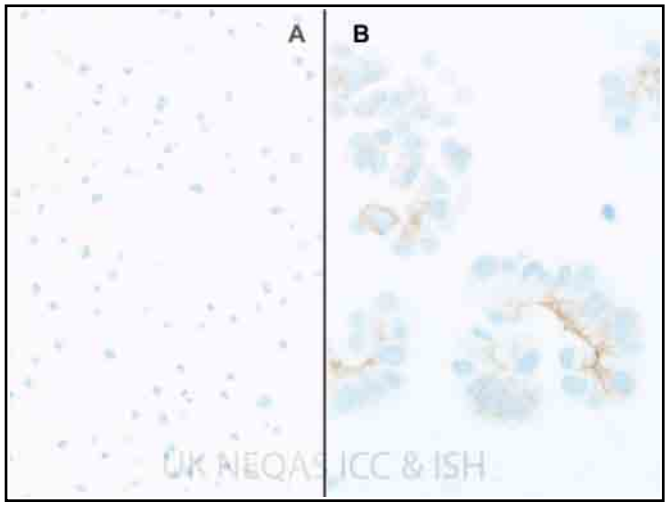


Figure 1: Good demonstration of the negative NEQAS cell lines . The 0 cell line (A) is free of any non-specific staining. The 1+ cell line (B) shows the distinctive brush border staining with specific delicate partial membrane staining between the cells. Method: BioGenex Rb mAb (1/15); Ventana XT (CC1 mild, UltraView Assay).

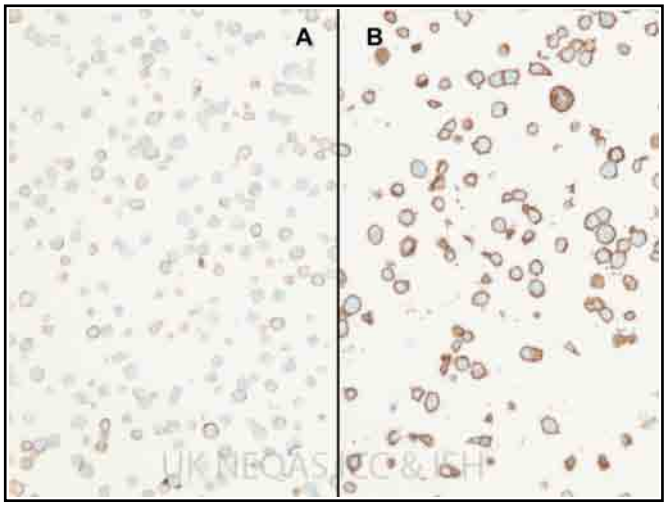


Figure 2: Excellent demonstration of the equivocal and positive NEQAS cell lines. The 2+ cell line (A) is demonstration weak to moderate complete membrane staining. The membrane staining is specific and clear of background staining. The 3+ cell line (B) shows clear, strong membrane staining. Method: As in Figure 1.

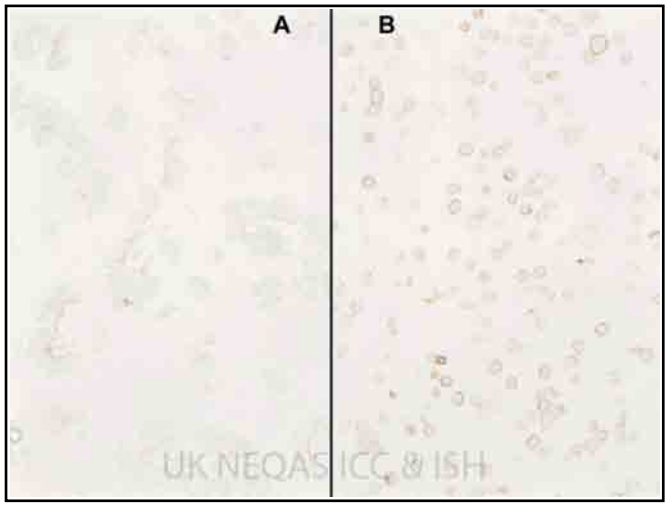


Figure 3: Weak Hematoxylin in the 1+ (A) and 2+ (B) NEQAS cell lines, making interpretation difficult due to the lack of contrast between nuclei and cell membranes. Method: Ventana Pathway (4B5).

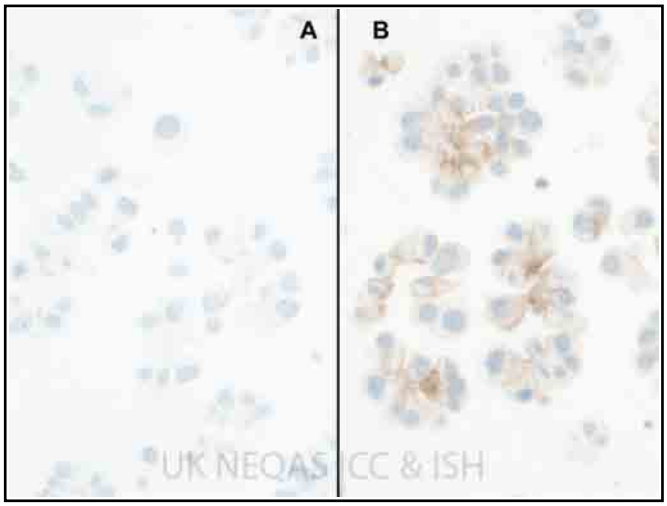


Figure 4: (A) exhibits weak membrane staining in the 1+ NEQAS cell line. There is occasional very weak membrane staining present. Method: Ventana Pathway (4B5); (B) demonstrates overstaining of the 1+ NEQAS cell line. There are distinct cells with moderate complete membrane staining (more indicative of 2+). Method: Dako (A0485)(1:400); Leica BondmaX

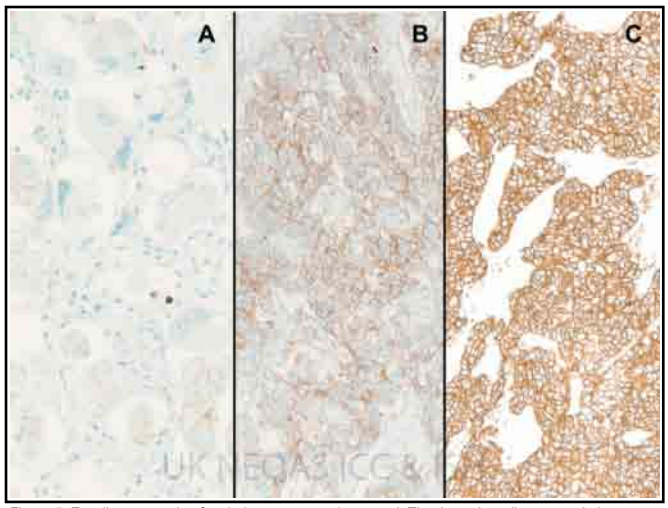
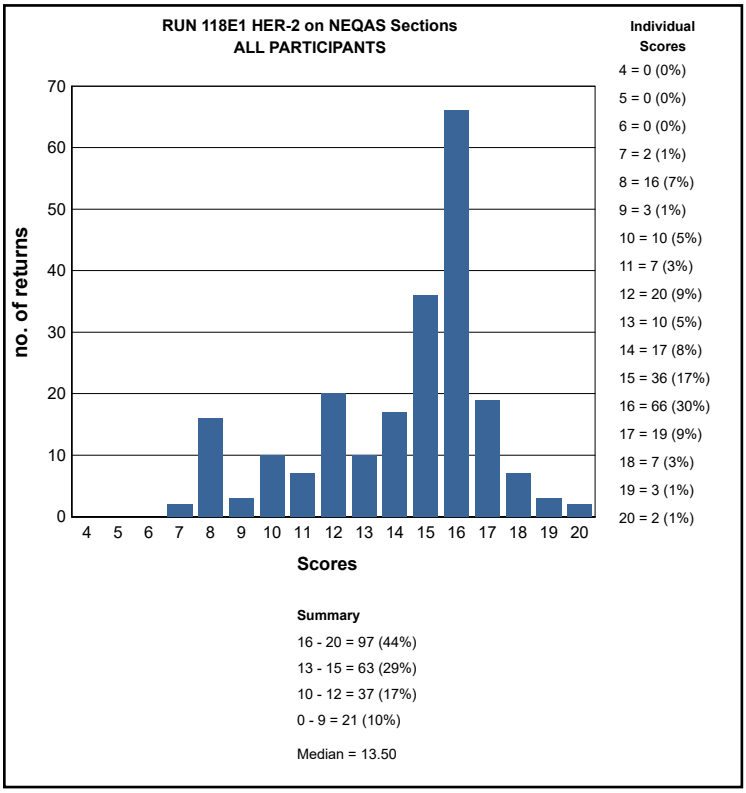
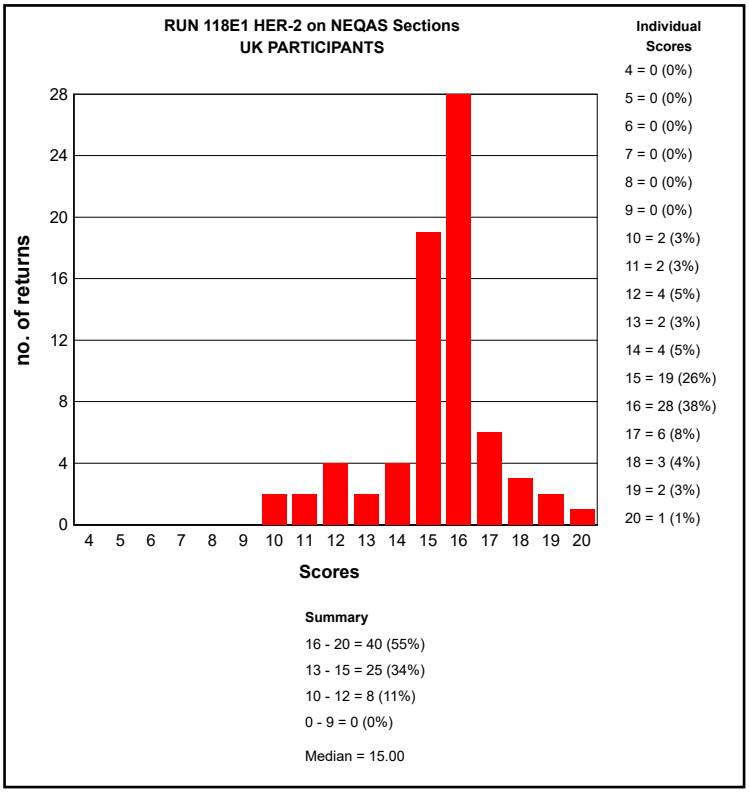


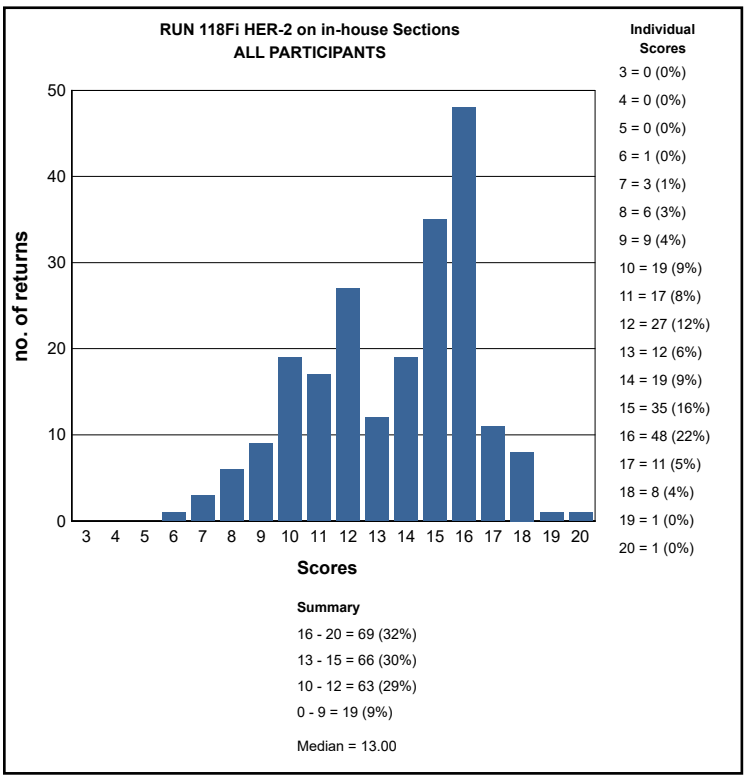
Figure 5: Excellent example of an in-house composite control. The tissue is well preserved, the staining is specific and there is the required demonstration of negative (1+), equivocal and positive HER2 expression: (A) 1+; (B) 2+ and (C) 3+. Method: Ventana Confirm (4B5); Benchmark XT; iView assay.

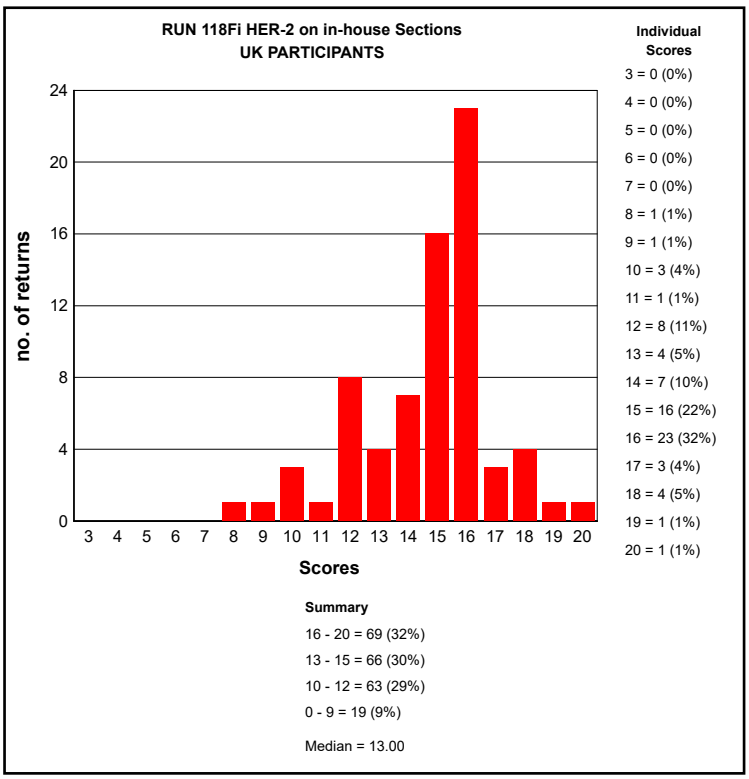
Figure 6: Sub-optimal demonstration of a 2+ in-house control. The tissue quality is poor and appears to have had excessive antigen retrieval. The staining is diffuse and may be artefact. Method: Dako (A0485) (1:600); Leica BondmaX (ER1 20', Bond Polymer Refine).

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: 118

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)	17	24
Cell Marque CMA 601 (CB11)	1	100
Ventana Pathway 790-100 (4B5)	4	75
Novocastra NCL-L-CBE356 (10A7)	1	0
Leica Oracle HER2 Bond IHC (CB11)	15	87
Dako Link HercepTest SK001 (poly)	6	100
BioGenex (EP1045Y) rb mono	1	100
Ventana Confirm 790-4493 (4B5)	39	79
Ventana Pathway 790-2991 (4B5)	120	81
Novocastra NCL-L-CB11 (CB11)	2	0
Other	2	0

Breast HER2 ICC Run: 118

Automation	N	%
Dako Autostainer Link 48	10	40
Dako Autostainer Plus Link	3	67
Dako Omnis	1	100
LabVision Autostainer	1	0
Leica Bond Max	9	22
Leica Bond-III	17	76
None (Manual)	4	0
Ventana Benchmark GX	12	67
Ventana Benchmark ULTRA	100	85
Ventana Benchmark XT	54	76

Breast HER2 ICC Run: 118

Heat Mediated Retrieval	N	%
_Ventana ULTRA CC1 (52mins)	1	100
Dako Omnis	2	50
Dako PTLink	11	55
Lab vision PT Module	2	0
Leica ER1 10 mins	1	100
Leica ER1 20 mins	8	13
Leica ER1 25 mins	13	85
Leica ER1 30 mins	1	0
Leica ER2 30 mins	1	0
Microwave	1	0
None	1	0
Other	3	100
Ventana CC1 16mins	2	100
Ventana CC1 20mins	1	100
Ventana CC1 24mins	3	67
Ventana CC1 32mins	13	77
Ventana CC1 36mins	55	85
Ventana CC1 40mins	1	100
Ventana CC1 48mins	1	100
Ventana CC1 52mins	4	100
Ventana CC1 56mins	6	83
Ventana CC1 64mins	6	67
Ventana CC1 8mins	2	50
Ventana CC1 mild	55	82
Ventana CC1 standard	12	67
Water bath 95-98 OC	4	0

Breast HER2 ICC Run: 118

Detection	N	%
AS PER KIT	17	82
Biocare polymer (M4U534)	1	0
BioGenex SS Polymer (QD 420-YIKE)	1	0
Dako HerCep Test (K5204)	1	0
Dako EnVision FLEX (K8000/10)	2	0
Dako EnVision FLEX+ (K8002/12)	5	20
Dako Envision HRP/DAB (K5007)	1	0
Dako HerCep Test Autor (SK001)	4	100
LabVision UltraVision LP HRP (TL 125 HLJ)	1	0
Leica Bond Polymer Refine (DS9800)	15	40
Other	1	0
Ventana iView system (760-091)	4	100
Ventana OptiView Kit (760-700)	9	89
Ventana UltraView Kit (760-500)	141	80

Breast HER2 ICC Run: 118

Enzyme Retrieval	N	%
AS PER KIT	12	58
NOT APPLICABLE	117	74
Ventana Protease 1 (760-2018)	1	100

Breast HER2 ICC Run: 118

Chromogen	N	%
AS PER KIT	32	81
BioGenex liquid DBA (HK-124-7K)	1	0
Dako DAB+ Liquid (K3468)	1	0
Dako FLEX DAB	10	50
Dako REAL EnVision K5007 DAB	2	0
Leica Bond Polymer Refine kit (DS9800)	14	43
Other	6	67
Ventana DAB	5	60
Ventana iview	4	100
Ventana Ultraview DAB	136	79

BEST METHODS

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

HER-2 - Method 1

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

Primary Antibody:	Ventana Pathway 790-2991 (4B5) , 12 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 36mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB, 37 ºC., Time 1: 4 Mins, Time 2: 4 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC Prediluted

HER-2 - Method 2

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

Primary Antibody:	Dako Link HercepTest SK001 (poly) , 30 Mins, 20-25 ºC Prediluted
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: EPITOPE RETRIEVAL SOLUTION (10X)
EAR:	AS PER KIT
Chromogen:	AS PER KIT, Time 1: 10 Mins
Detection:	

HER-2 - Method 3

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

Primary Antibody:	Ventana Pathway 790-2991 (4B5) , 12 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 mild
EAR:	
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

The Gastric HER2 ICC Module Run 118

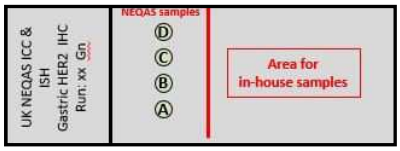
Suzanne Parry

Antigen Assessed:	HER2
Sections Circulated:	Composite slide consisting of 4 surgical intestinal gastric carcinomas with different levels of HER2 expression
Number of Registered Participants:	75
Number of Participants this Run	60 (80%)

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

Sample Position (from left to right on slide)	Expected HER2 IHC Expression Level
A	3+
B	3+ (heterogeneous case, also showing over 50% 2+ expression)
C	1+ or 2+ depending on the serial section received
D	0

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



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

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

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

Validation of Distributed Samples

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

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

Table 2: Showing the HER2 IHC staining and ISH results

Assessment Procedure

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

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

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

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

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

Introduction

The American Society of Clinical Oncology (ASCO), the College of American Pathologists (CAP) and the American Society for Clinical Pathology (ASCP) have issued a **new** joint guideline on HER2 testing for patients with gastroesophageal cancers (GEA) on 14th of Nov 2016. Please see: www.jco.org. This guideline provides specific recommendations for assessment of *HER2* in patients with advanced GEA while addressing pertinent technical issues and clinical implications of the results. Immunohistochemical testing of HER2 status is now routinely used in breast cancer testing and is recognised as a prognostic and predictive marker, generally used alongside breast hormonal receptor markers ER/PR. More recently the Trastuzumab for Gastric Cancer (ToGA) study, which investigated Trastuzumab in HER2 positive advanced gastric cancer (Bang et al., 2010) showed overall median survival of nearly 3 months. Similar to breast cancer, the ToGA trial showed an increased benefit from Trastuzumab treatment for patients showing 3+ IHC and IHC2+/FISH+ expression. The initial development of the HER2 scoring criteria was developed as a precursor to the ToGA trial (Hoffman et al., 2008) with the study group modifying the breast HER2 IHC scoring algorithm to compensate for the incomplete membrane staining and greater tumour heterogeneity seen in gastric cancers. A different scoring system was also established for resection and core biopsies as illustrated in table 1. A more recent article by Rüschoff et al., (2010) has validated the scoring procedure further with a detailed approach to 'stepwise' HER2 IHC scoring in gastric cancers. The article also compared the Ventana 4B5 assay with the HercepTest and indicated "a tendency towards higher sensitivity for 4B5 detecting positive HER2 amplification" but

"there was no difference between both test platforms with respect to the ISH-negative cases, with the one equivocal case testing negative at some sites and as positive at others". Rüschoff and colleagues also used both fluorescent (Dako PharmDX or PathVysion Vysis) and chromogen (Ventana DDISH) ISH techniques to confirm their IHC findings.

Assessment Results

Features Of Acceptable Staining: (Figs 1, 2, 3 & 6a)

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

Features Of Sub-Optimal or Unacceptable Staining: (Figs 4 & 5)

- Weaker or stronger 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
- Membrane 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.

NEQAS Section Assessment Results

59 laboratories submitted their slides for the Gastric HER2 assessment. The pass rates showed a significantly lower acceptable pass rate than the previous Gastric HER2 assessment (Run 117). In parallel, more laboratories received borderline passes on the NEQAS material. 4 laboratories failed the assessment. See table below for a breakdown of the Gastric HER2 results on the NEQAS material over the last 2 UK NEQAS ICC assessments:

Gastric HER2 NEQAS Pass Rates :		
	Run 117	**Run 118**
Acceptable	96% (N=60)	54% (N=59)
Borderline	2% (N=1)	39% (N=23)
Unacceptable	2% (N=1)	7% (N=4)

The borderline passes were mostly due to weaker staining that expected, particularly on cores B and C, which were known amplified cases by ISH. Other participating slides were marked down due to inappropriate, excessive or cytoplasmic staining. Those labs that failed the assessment showed significantly under or over-staining, which in a clinical setting would have led to an incorrect result, and therefore, mismanagement of the patient.

In-House Tissue Assessment Results

Apart from 2 participants, all laboratories also submitted their in-house controls for assessment. Overall these showed a higher acceptable pass rate of 64% (N=36) compared to the NEQAS sections. The in-house borderline pass was similar to that seen on the NEQAS sections at 36% (N=21), and no labs failed on their own in-house samples. See table below for summary of the Gastric HER2 in-house pass rates for run 118:

In-House Pass Rates Run 118:	
Acceptable	64% (N=36)
Borderline	36% (N=21)
Unacceptable	0% (N=0)

Several of the borderline passes were not due to poor staining, but instead participants lost marks due to not providing the required in-house material consisting of a composite control including 3+, 2+ and 0/1+ HER2 expressing tumours.

Methodologies

The majority of labs are using the Ventana 4B5 standardised kit for their gastric HER2 testing: In this particular assessment run the Ventana assay was used by 86% (N=51) of participants with an average acceptable pass rate of 55%. 4 laboratories used the Dako Hercept test and 3 laboratories used laboratory devised methods.

Control Tissue and Recommendations

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

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

References:

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

Acknowledgments

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

Selected Images showing Optimal and Sub-optimal Immunostaining

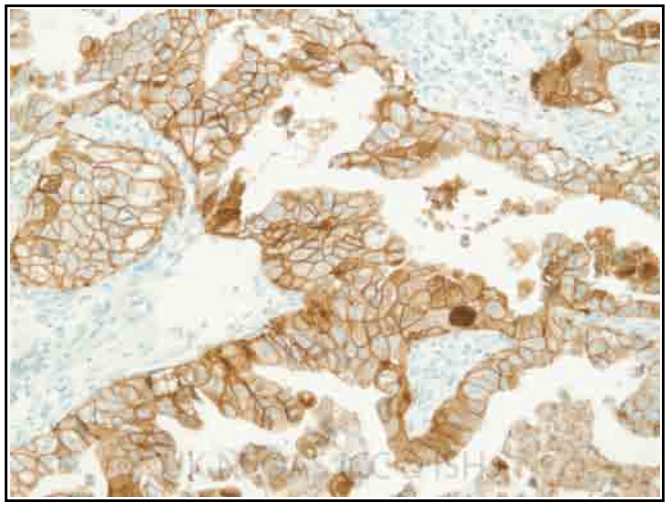


Fig 1. Expected HER2 3+ staining of the UK NEQAS distributed gastric tumour sample A, showing the expected level of complete intense membrane staining. Section stained with Ventana Pathway 4B5 assay on the ULTRA platform.

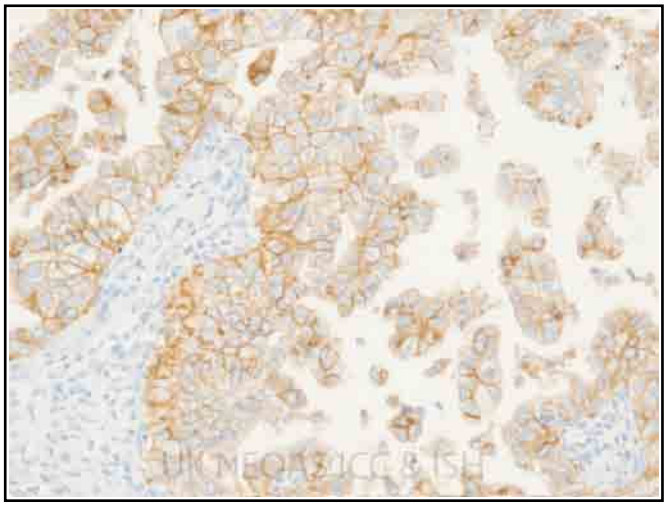


Fig 2. Acceptable HER2 staining on the UK NEQAS distributed 3+ gastric tumour sample A. Although the staining is slightly weaker than seen in the example shown in Fig 1, this is still a 3+ expression level.

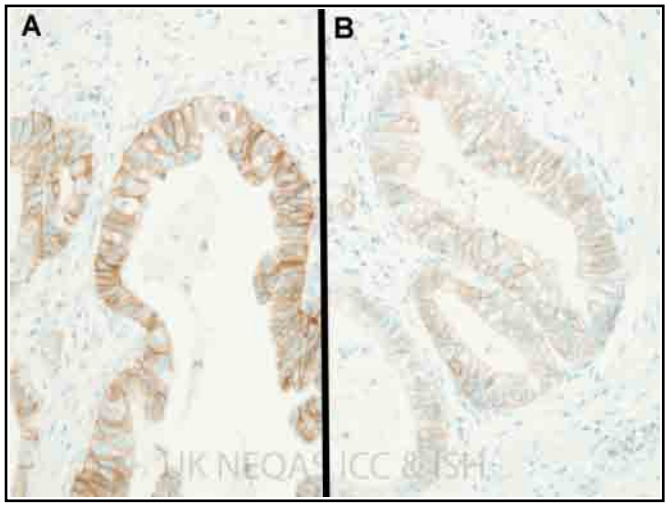


Fig 3. Expected level of staining in the UK NEQAS distributed gastric tumour sample B. The sample showed areas of both 3+ expression (image A), and 2+ expression (image B). Stained using the Ventana Pathway 4B5 assay.

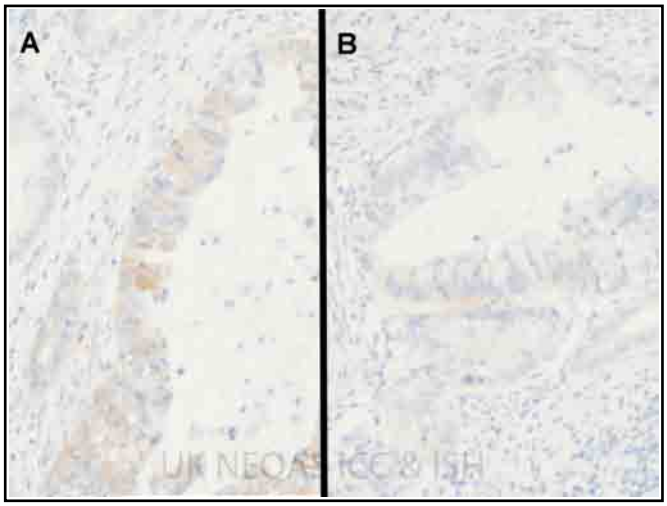


Fig 4. Sub-optimal demonstration of HER2 in the UK NEQAS distributed sample B (compare to Fig 3). The sample should show both 3+ (A) and 2+ (B) staining, but the staining in the example is more representative of 1+ and negative staining. Stained using the Dako HercepTest with pre-treatment in the PT Link.

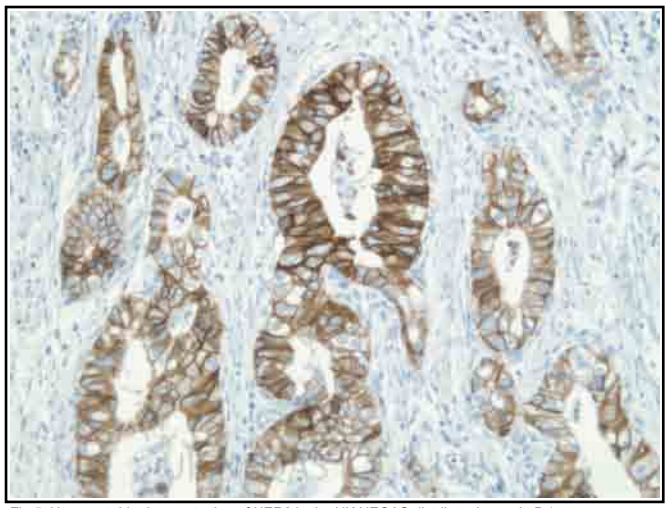


Fig 5. Unacceptable demonstration of HER2 in the UK NEQAS distributed sample B (compare to Fig 3B). This are of the sample should be 2+, but the staining in the example is too high and more representative of 3+ staining. The staining was carried out using a lab-devised method with the Immunologic SP3 clone.

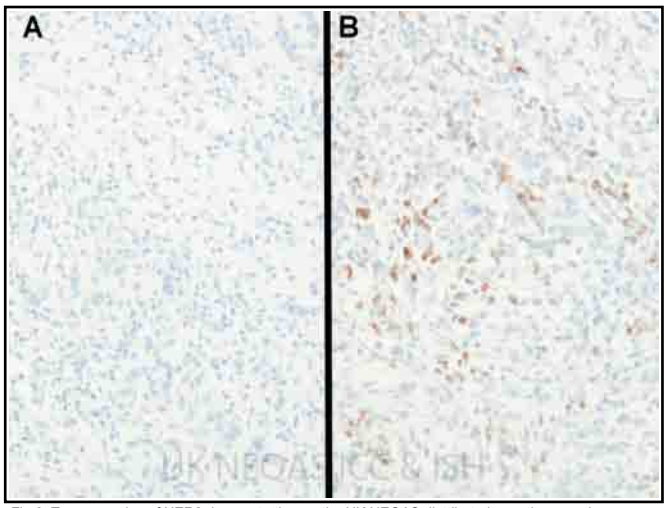
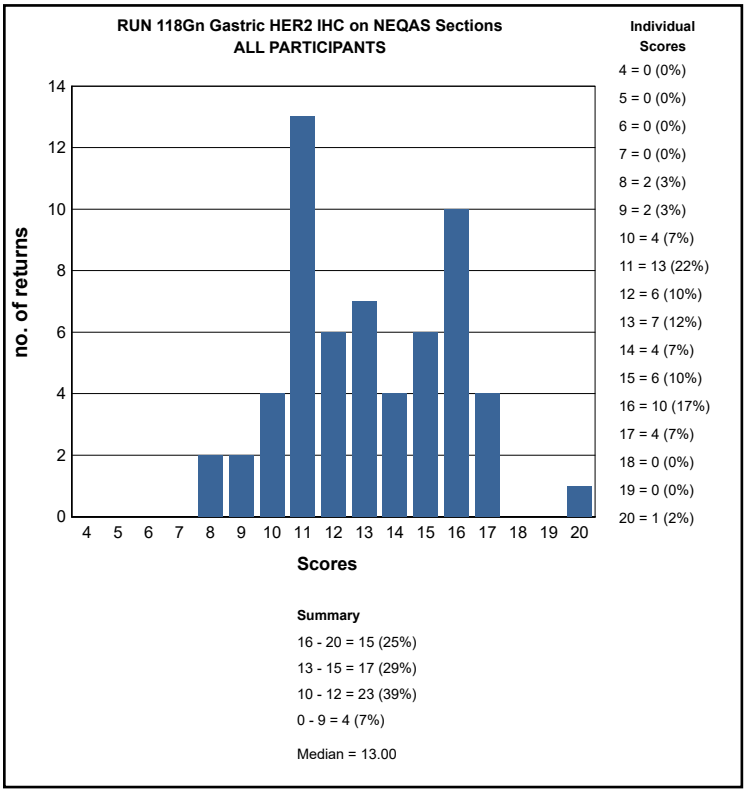
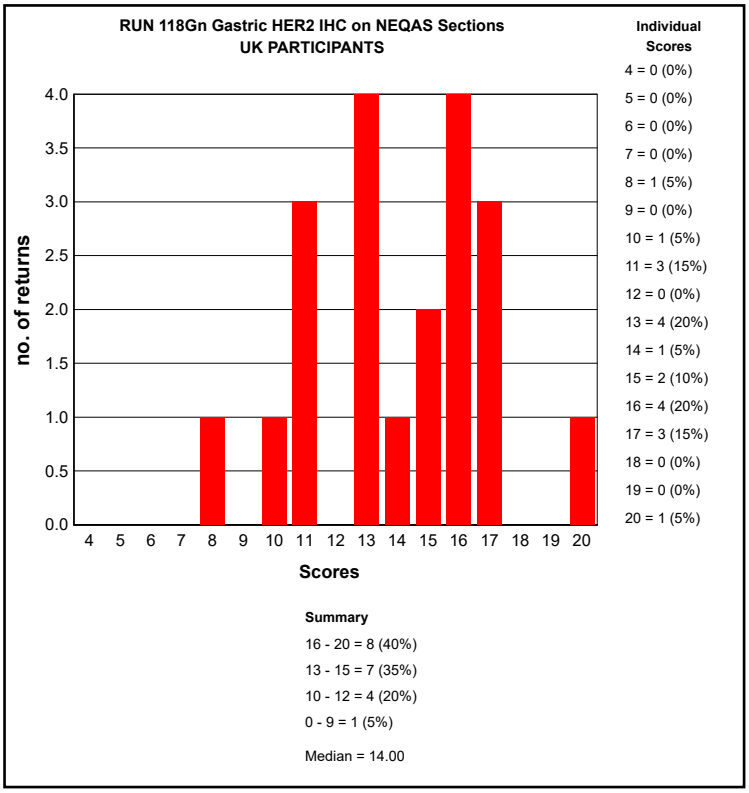
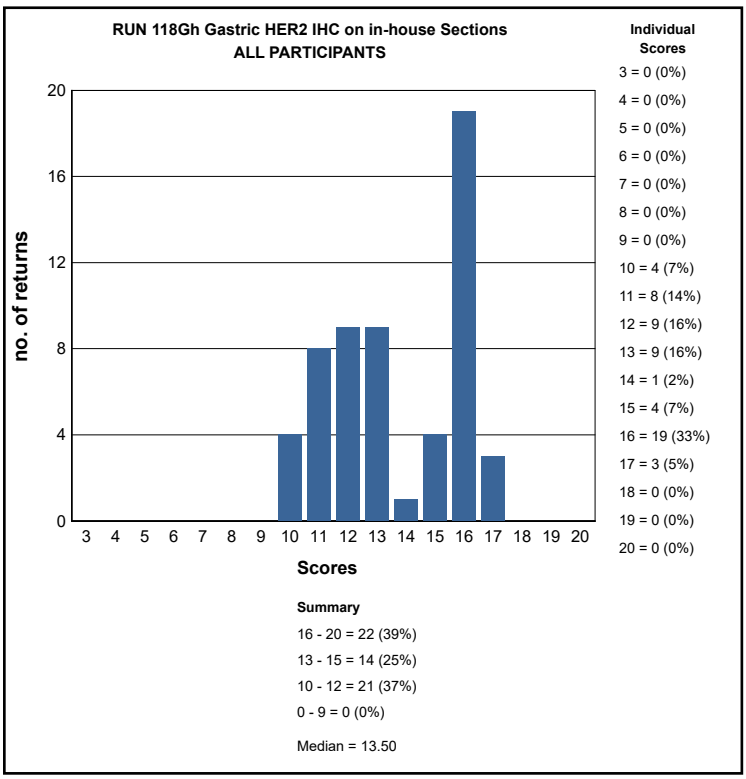


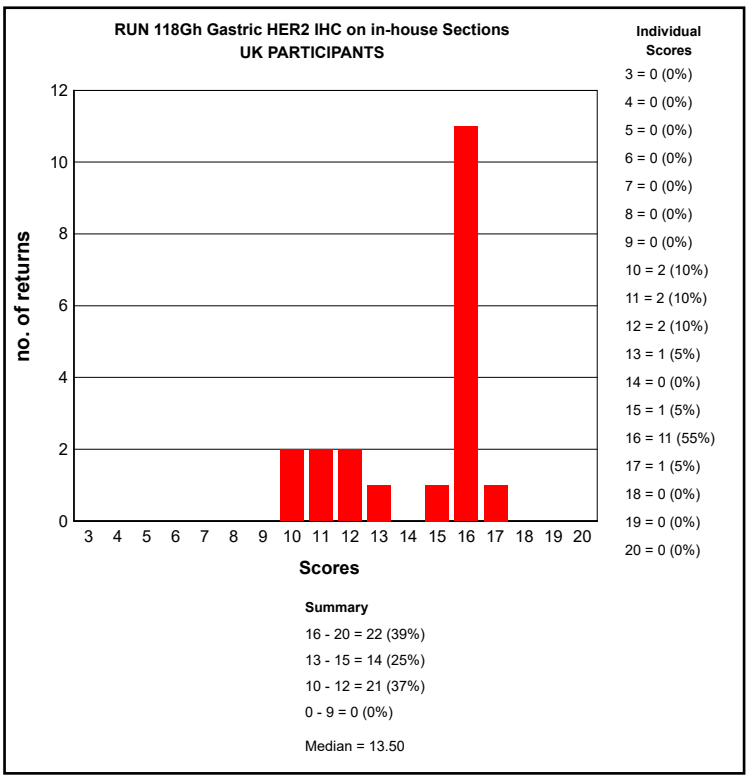
Fig 6. Two examples of HER2 demonstration on the UK NEQAS distributed negative sample. Example A shows the expected HER2 result, while the image in section B shows inappropriate non-specific staining. Both sections stained with the Ventana 4B5 assay.

GRAPHICAL REPRESENTATION OF PASS RATES









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

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

Gastric HER2 ICC Run: 118

Primary Antibody	N	%
Dako A0485 C-erB-2 (poly)	2	50
Dako Link HercepTest SK001 (poly)	4	0
Ventana Pathway 790-100 (4B5)	2	50
Ventana Pathway 790-2991 (4B5)	35	66
Other	1	0
Ventana Confirm 790-4493 (4B5)	14	50

Gastric HER2 ICC Run: 118

Automation	N	%
Dako Autostainer Link 48	2	0
Dako Autostainer Plus Link	2	0
Dako Omnis	2	50
Other	1	0
Ventana Benchmark GX	2	50
Ventana Benchmark ULTRA	31	45
Ventana Benchmark XT	18	89

Gastric HER2 ICC Run: 118

Heat Mediated Retrieval	N	%
Dako Omnis	2	50
Dako PTLink	4	0
Lab vision PT Module	1	0
Ventana CC1 16mins	3	0
Ventana CC1 24mins	1	100
Ventana CC1 32mins	5	80
Ventana CC1 36mins	14	36
Ventana CC1 56mins	2	100
Ventana CC1 64mins	6	50
Ventana CC1 mild	17	76
Ventana CC1 standard	3	100

Gastric HER2 ICC Run: 118

Detection	N	%
AS PER KIT	5	20
Dako EnVision FLEX (K8000/10)	1	0
Dako EnVision FLEX+ (K8002/12)	1	100
Dako HerCep Test Autor (SK001)	3	0
Power Vision DPVB999 HRP	1	0
Ventana iView system (760-091)	2	100
Ventana OptiView Kit (760-700)	5	60
Ventana UltraView Kit (760-500)	39	62

Gastric HER2 ICC Run: 118

Enzyme Retrieval	N	%
AS PER KIT	2	50
NOT APPLICABLE	28	64
Ventana Protease 1 (760-2018)	2	50

Gastric HER2 ICC Run: 118

Chromogen	N	%
AS PER KIT	11	55
DAKO DAB+	1	100
Dako DAB+ Liquid (K3468)	1	0
Dako FLEX DAB	3	0
Other	2	0
Ventana DAB	1	100
Ventana iview	1	100
Ventana Ultraview DAB	38	61

BEST METHODS

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

Gastric HER2 IHC - Method 1

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

Primary Antibody:	Ventana Confirm 790-4493 (4B5) , 32 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 mild
EAR:	
Chromogen:	Ventana Ultraview DAB, Time 1: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC

Gastric HER2 IHC - Method 2

Participant scored 17/20 (UK NEQAS Slide) and 16/20 (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:	
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500) Prediluted

David Blythe and Amy Newman

	Gold Standard	Second Antibody
Antigens Assessed:	BCL-6	CD79a
Tissue Sections circulated:	Diffuse Large B Cell Lymphoma (LN) Reactive Tonsil	Follicular Lymphoma (LN) Reactive Tonsil
Number of Registered Participants:	210	
Number of Participants this Run	200 (95%)	

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 (Figs 1, 3, 5&6):
- Moderate to strong nuclear staining of all germinal centre B-cells in the distributed tonsil and lymph node (follicular lymphoma).
- Clean background with no non-specific staining.

Features of Sub-optimal Immunostaining (Figs 2&4):
- Weak, uneven or partially missing staining of relevant cells.
- Poor/diffuse nuclear localisation.
- High background or non-specific staining of cell types not expected to stain.
- 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: CD79a

The CD79a complex is a disulphide-linked heterodimer that is non-covalently associated with membrane-bound immunoglobulins in B-cells. This complex of polypeptides and immunoglobulins constitute the B-cell antigen receptor. One of the components is designated CD79a which first appears in the pre B-cell stage and persists throughout the development of the B-cells through to the plasma cell stage[1].
CD79a is present on normal and neoplastic B-cells from the early stages of B-cells maturation through to plasma cell differentiation. It plays a major functioning role in antigen triggering which may indicate its high specificity for B-cells[2]. CD79a is useful for aiding in the identification of B-cell tumours of all maturation stages as it has been found to be negative in mature T-cell neoplasms and tumours of non-lymphoid origin[1]. However, approximately 10% of pre-cursor T -cell neoplasms are positive for CD79a expression[3]. Malignant lymphocytic and histiocytic cells of nodular lymphocyte-predominant Hodgkin's lymphoma are positive for CD79a, whereas only 20% of Classical Hodgkin's lymphoma are positive for CD79a.

Features of Optimal Immunostaining (Figs 7, 9, 11 & 12):
• Moderate to strong membrane and cytoplasmic staining of all normal and neoplastic B-cells
- Strong to intense staining of plasma cells
- Clean background

Features of Sub-optimal Immunostaining (Figs 8, 9 & 10):
• Weak, uneven or partially missing staining of relevant cell.
- Poor/diffuse membrane localisation.
- High background or non-specific staining of cell types not expected to stain.

References
1. Mason D Y, Cordell J, Brown M et al., CD79a: a novel marker for B-cell neoplasms in routinely processed tissue samples. Blood 1995; 86(4): 1453-1459.
2. Korkolopoulou P, Cordell J, Jones M et al., The expression of the B-cell marker mb-1(CD79a) in Hodgkin's disease. Histopathology 1994; 14(6): 511-515.
3. Yao X, Teruya-Feldstein J, Faffeld M, Sorbara L, Jaffe ES. Peripheral T-cell lymphoma with aberrant expression of CD79a and CD20: a diagnostic pitfall. Mod Pathol. 2001; 14(2): 105-110.
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Assessment Summary:

BCL-6

200 laboratories submitted slides for the BCL-6 assessment, and all but 1 laboratory submitted their in-house control sections for this run. This was the first run for the assessment year and results were pleasing (Summarised in the table below) with 87% of participants achieving an acceptable result for their NEQAS submission, 11% received a borderline score with only 4% achieving unacceptable. The in-house results where almost identical. 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 Dako (PG-B6p), Ventana (1GI191E/A8) and Leica (LN22) used by 25%, 29% and 31% of participants respectively. The acceptable pass rate for the in-house control was 93% compared to the NEQAS of 78%, this is due to the fact that many laboratories are only using an appendix as an in-house positive control, however NEQAS recommend a composite control to be able to measure the sensitivity of your assay. Unlike other modules at this moment in time, we do not penalise for just using a single control, however best practice would be to use a control that measures the sensitivity of your assay such as a tonsil and a

NEQAS and In-house Pass Rates Run 118 BCL-6		
	NEQAS	In-house
Acceptable	87% (N=172)	88% (N= 175)
Borderline	11% (N=21)	11% (N=21)
Unacceptable	4% (N=7)	2% (N=3)

David Blythe and Suzanne Parry

CD79a

197 laboratories submitted both the NEQAS and in house sections for this assessment. The pass rates were both 89% for the NEQAS sections and the in-house sections. The Borderline/Unacceptable pass rates on the in-house sections were similar to the NEQAS sections.
(summarised in table below):

NEQAS and In-house Pass Rates Run 118 CD79a		
	NEQAS	In-house
Acceptable	89% (N=175)	89% (N= 174)
Borderline	9% (N=18)	10% (N=19)
Unacceptable	2% (N=4)	1% (N=2)

It was noted the predominant reason for laboratories receiving a borderline pass or fail submitting sub-optimal staining of CD79a was due to weak or very weak demonstration of the B-cells or poor localisation. The most commonly used antibody for CD79a with the Dako (JCB117) used by 104 ((52.80%) participants. The other commonest antibodies are Ventana (SP18) and Leica (11E3).

Selected Images showing Optimal and Sub-optimal Immunostaining

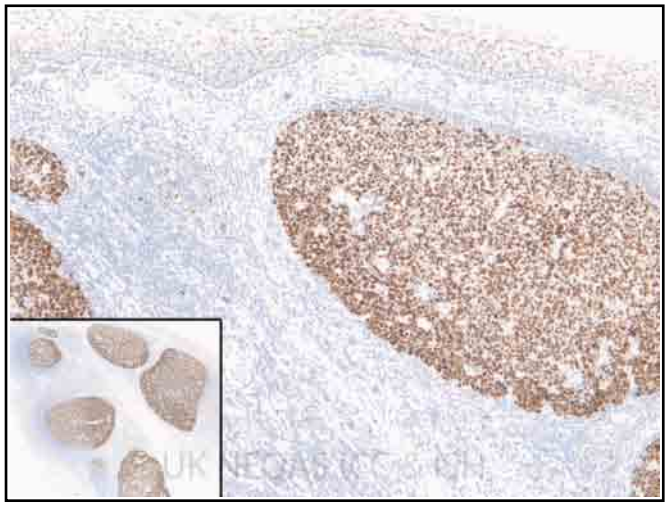


Figure 1: Excellent example of BCL-6 on the NEQAS tonsil. The germinal centre B-cells show strong to moderate nuclear staining. There is weak to moderate staining in the squamous epithelial cells. Methods: NCL-L-BCL-6 (LN22); Leica Bond Max (ER2, 20'; Polymer Refine). Score 20/20

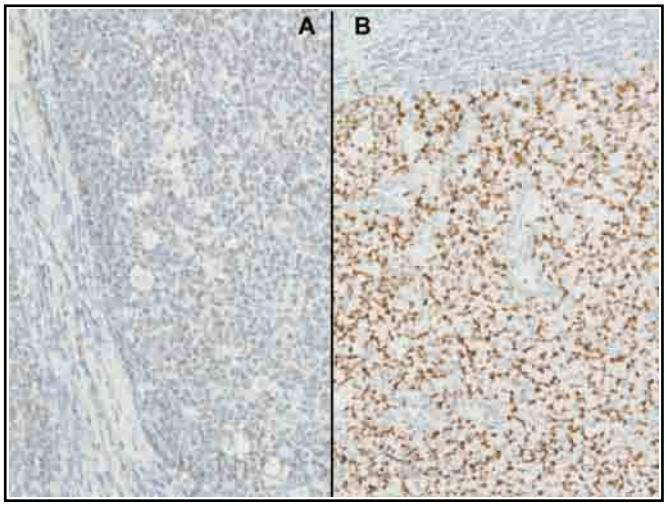


Figure 2: Suboptimal demonstration of the BCL-6 antigen on the NEQAS tonsil. (A) The germinal centre B-cells show very weak staining which may be due to insufficient antigen retrieval using the water bath method. (B) The morphology is damaged. This may be due to excessive antigen retrieval using a pH9.0 Tris-EDTA in a pressure cooker.

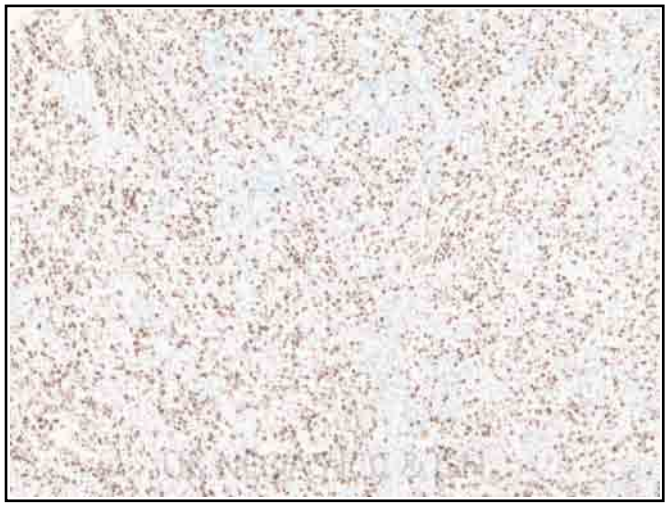


Figure 3: Good example of the NEQAS diffuse large B-cell lymphoma (DLBCL). The neoplastic cells show distinct nuclear staining. Method: Dako BCL-6 (PG-B6p); Omnis; Flex Kit. Score 20/20.

Figure 4: Weak staining in the NEQAS DLBCL. Some of the neoplastic cells are positive, however, the staining is too weak. Method: Cell Marque 227M (1:50); Ventana ULTRA (CC1 40'; OptiView kit). Score 8/20.

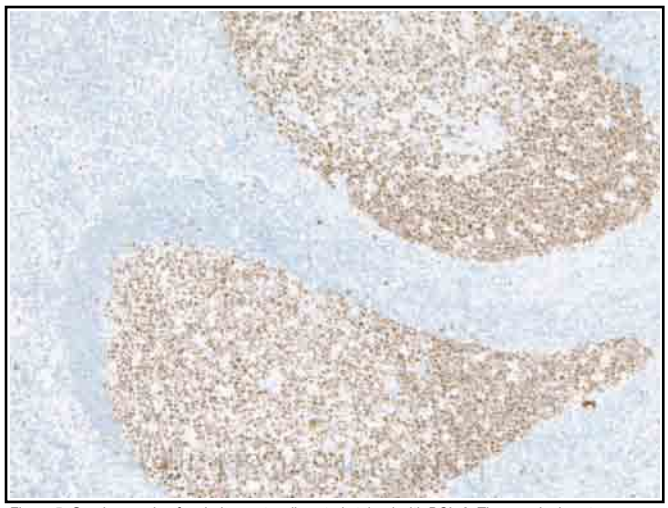


Figure 5: Good example of an in-house tonsil control stained with BCL-6. The germinal centre B-cells are clearly positive and the morphology is well preserved. Method: Identical to Figure 3. Score 20/20.

Figure 6: Excellent in-house tonsil control demonstrating BCL-6. Method: Dako BCL-6 (PG-B6p); Leica Bond Max (ER2 40'; Refine Red detection kit). Score 18/20.

Selected Images showing Optimal and Sub-optimal Immunostaining

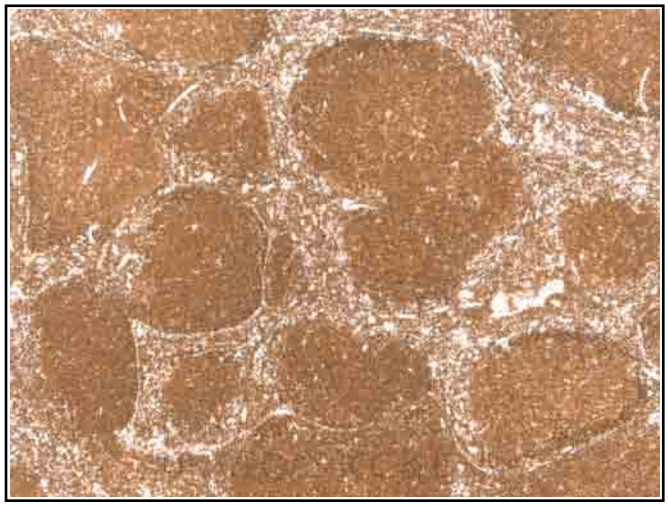


Figure 7: Excellent demonstration of CD79a in the NEQAS follicular lymphoma. The tumour cells are showing a strong reaction. Method: Dako CD79a (JCB117) (1:50, 30'); PT Link; Autostainer plus (EnVision FLEX).

Figure 8: Weak demonstration of the NEQAS follicular lymphoma. There is patchy and weak staining in the tumour membranes. Method: NCL-CD79a-225 (11E3) (1:200, 30'); Leica BondmaX (ER2 20'; Polymer Refine).

Figure 9: Slightly stronger staining in the follicular lymphoma in the germinal centres and mantle zones. However, the tumour in the surrounding inter-follicular areas is still weak. Method: NCL-CD79a- 225 (11E3) (1:100, 15'); Leica Bond III (ER2 10', Polymer Refine).

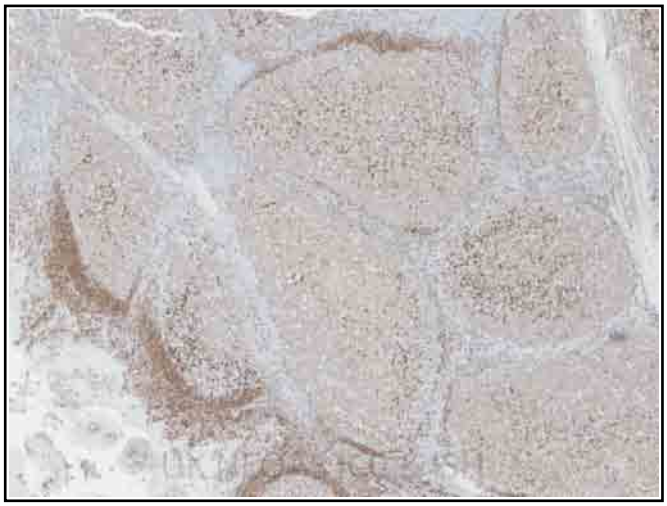


Figure 10: Weak CD79a staining in the NEQAS tonsil. The mantle zones and plasma cells in the germinal centres are strong. However, the B-cells in germinal centres are weak in comparison to Figures 11 & 12. Method: Dako CD79a (JCB117)(1:500, 32'); Ventana ULTRA (CC1 32', OptiView Kit).

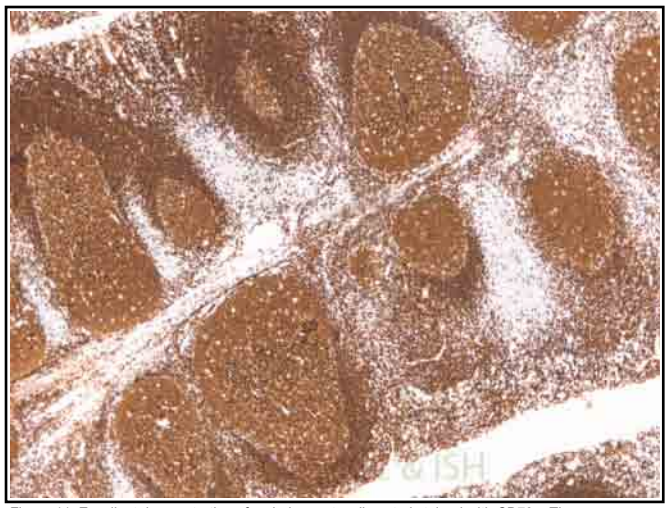


Figure 11: Excellent demonstration of an in-house tonsil control stained with CD79a. The expected components are staining with good contrast between the negative lymphocytes. Method: Dako CD79a (JCB117)(1:25; 30'); PT Link (Ph9.0); Autostainer Link 48 (EnVision FLEX+).

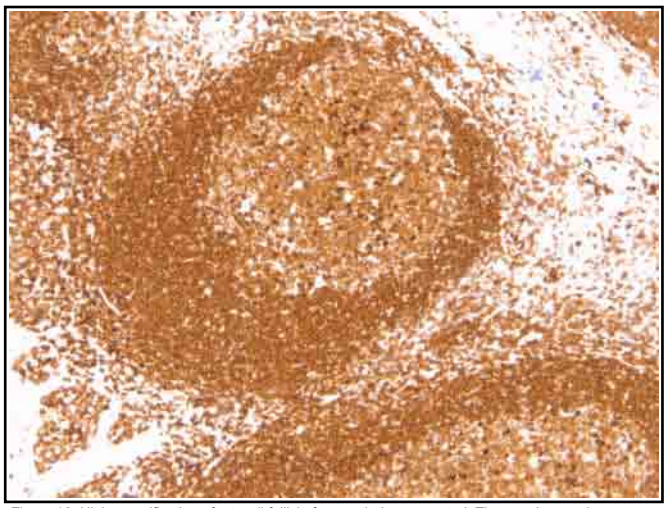
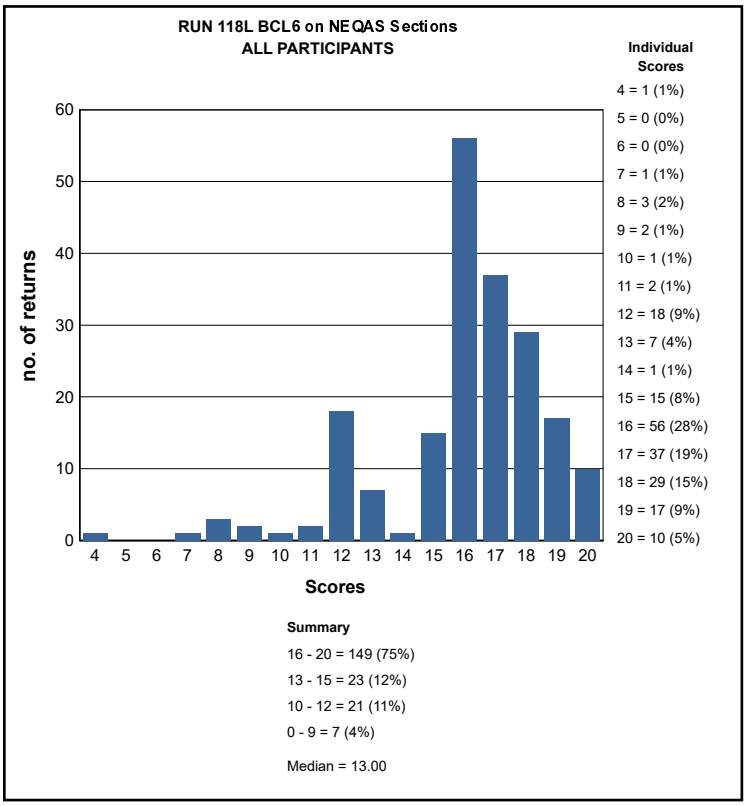
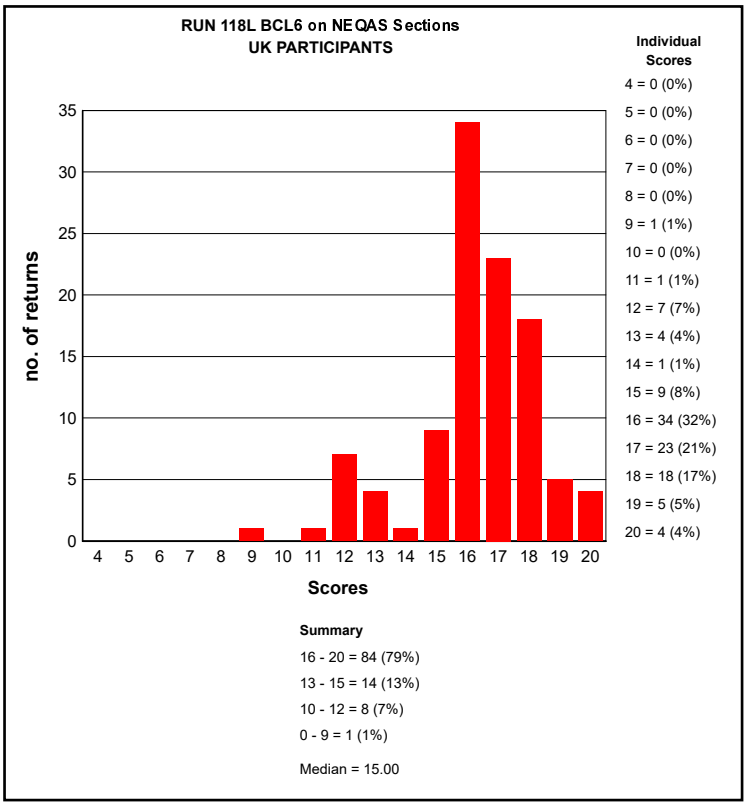
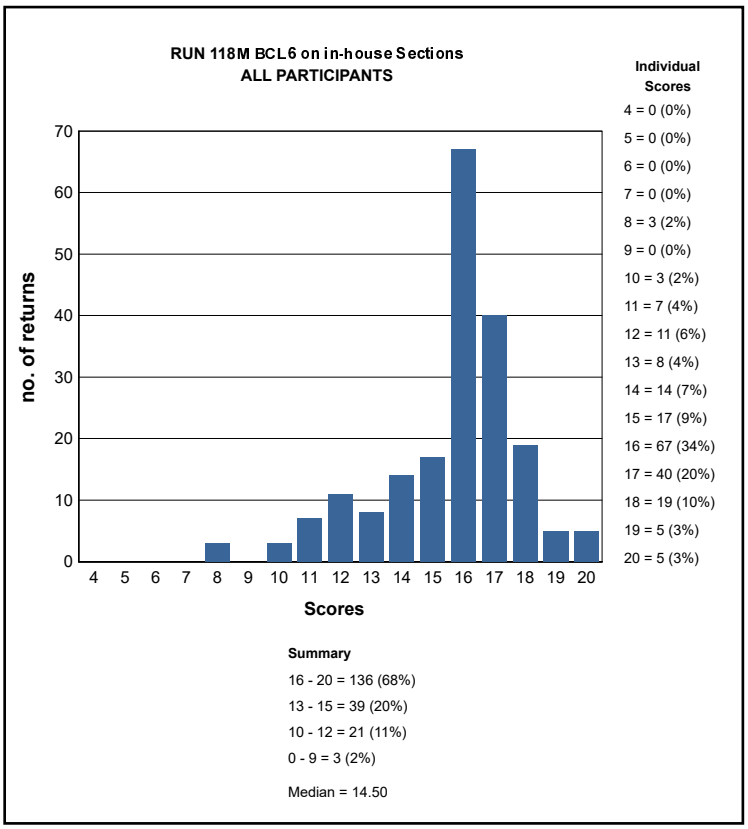


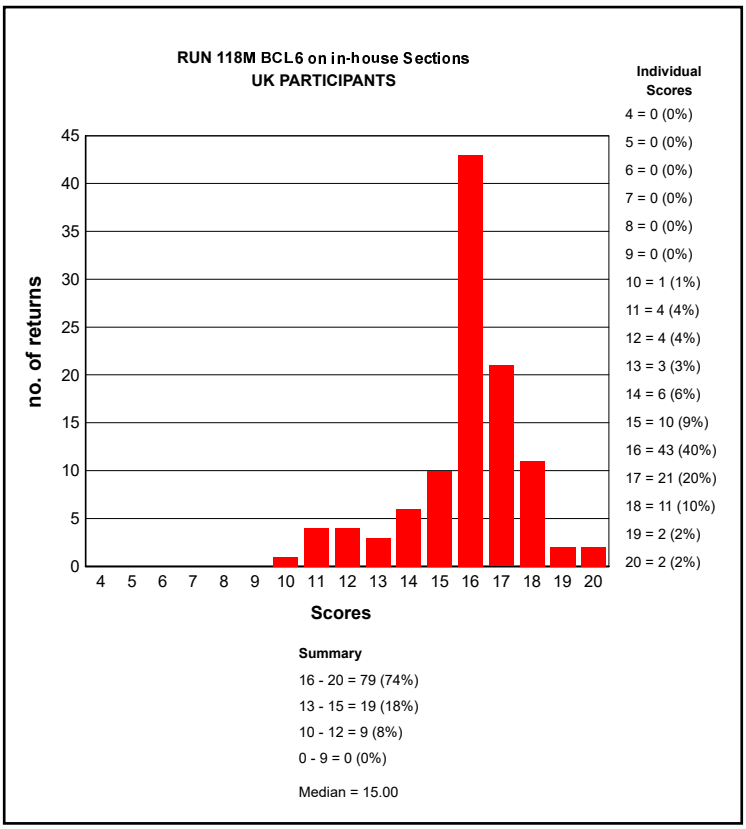
Figure 12: High magnification of a tonsil follicle from an in-house control. The mantle zone is staining stronger and is distinct from the germinal centre B-lymphocytes. The plasma cells with the follicle centre are strong. The membrane staining is crisp and well localised. Method: Dako CD79a (JCB117) (1:200, 20'); PT Link (Ph9.0); Autostainer Link 48 (EnVision FLEX+).

GRAPHICAL REPRESENTATION OF PASS RATES

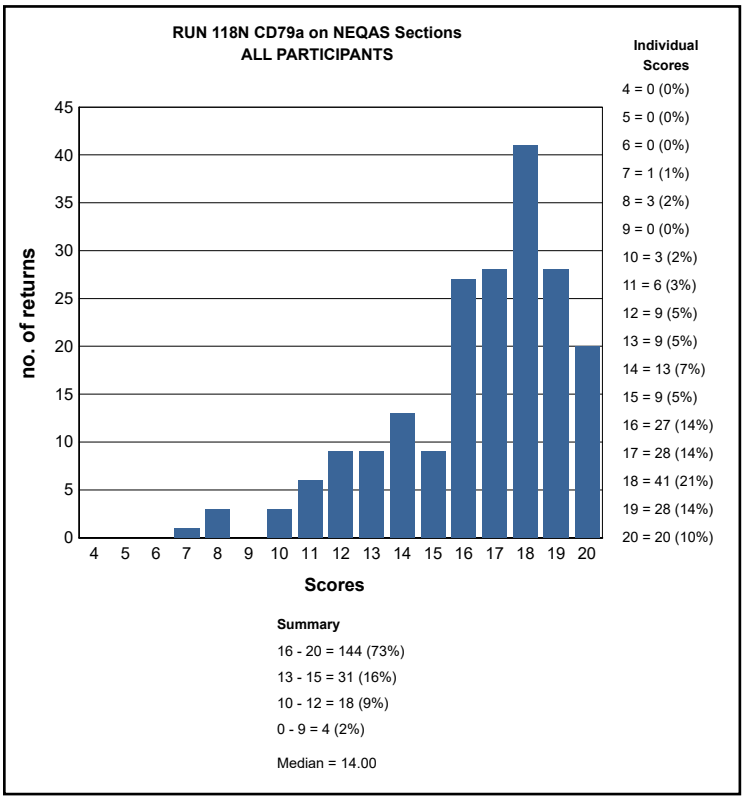


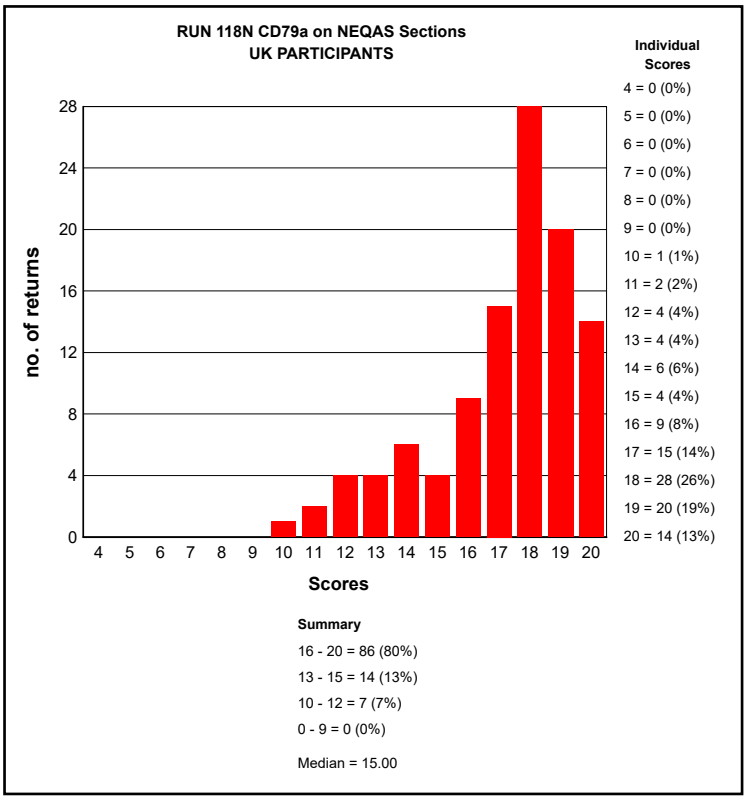


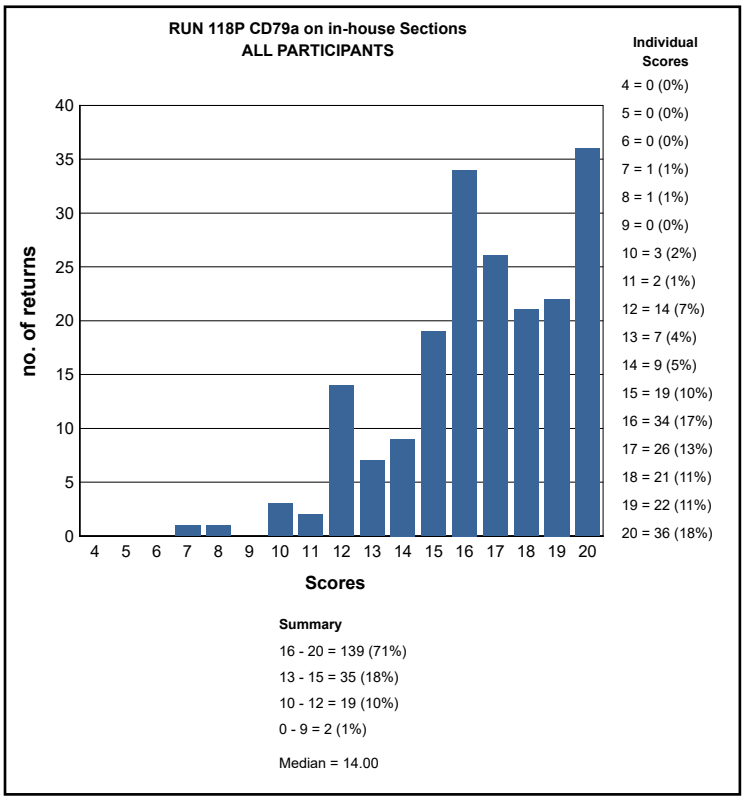


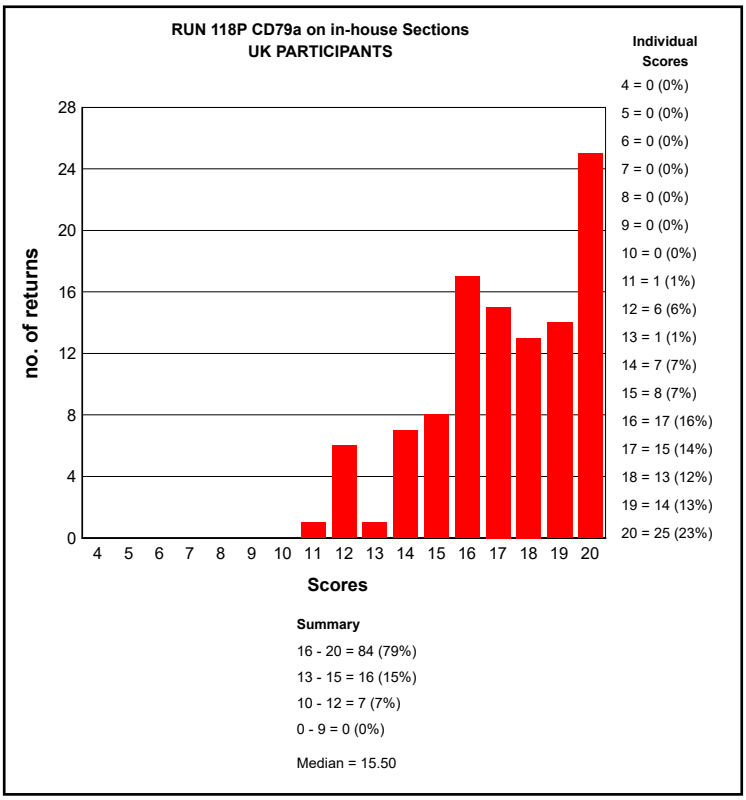


GRAPHICAL REPRESENTATION OF PASS RATES









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: 118

Primary Antibody : BCL6

Antibody Details	N	%
Dako BCL6 M7211 (PG-B6p)	39	82
Labvision Bcl-6 Ab-2 (BL6.02) mm	1	100
Novocastra NCL-BCL-6 (P1F6) mm	2	50
Novocastra NCL-L-BCL-6-564 (LN22) mm	45	98
Other	9	100
Cell marque 227M-94/95/96/97/98	15	60
Dako RTU FLEX Link IR625 (PG-B6p)	5	100
Leica RTU PA0204 (LN22)	17	76
Ventana 760-4241 (1GI191E/A8)	57	91
Dako RTU FLEX Plus IS625 (PG-B6p)	1	100
Dako RTU Omnis (PG-B6p) GA625	6	83

Lymphoma Run: 118

Primary Antibody : CD79a

Antibody Details	N	%
Dako IR621 CD79a (JCB117)	12	100
Dako IS621 CD79a (JCB117)	5	100
Dako M0755 CD20 (L26)	2	100
Dako M7050 CD79a (JCB117)	80	91
Dako M7051 CD79a (HM57)	11	64
Dako N1628 CD79a (JCB117)	5	80
Novocastra NCL-CD79a-192 (11D10)	1	0
Novocastra NCL-CD79a-225 (11E3)	2	50
Novocastra NCL-L-Cd79a-225 (11E3)	4	50
LeicaBond RTU PA0192 (Clone11E3)	13	69
Novocastra PA0906 CD20 (MJ1)	1	100
Novocastra RTU-CD79a-192 (11D10)	3	100
Other	3	100
Thermo RM-9118-x CD79a (SP18)	2	100
Ventana 790 4432 CD79a (SP18)	43	100
Cell Marque 179M-96 CD79a (Clone JCB117)	1	100
Cell Marque 179R-16 CD79a (Clone SP18)	3	100
Cell Marque 179M-98 RTU CD79a (Clone JCB117)	1	0

Lymphoma Run: 118

Heat Mediated Retrieval	BCL6 N	BCL6 %	CD79a N	CD79a %
_Leica BondMax ER1	0	0	1	100
_Leica BondMax ER2	0	0	1	100
_Ventana Benk CC2 (Standard)	0	0	1	100
Dako Omnis	10	90	9	100
Dako PTLink	17	94	16	100
Lab vision PT Module	1	100	1	0
Leica ER1 10 mins	1	0	1	0
Leica ER1 20 mins	0	0	7	86
Leica ER1 30 mins	0	0	12	83
Leica ER1 40 mins	1	100	0	0
Leica ER2 10 mins	0	0	2	0
Leica ER2 20 mins	37	89	31	84
Leica ER2 30 mins	20	80	5	100
Leica ER2 40 mins	6	100	0	0
Microwave	1	100	2	50
Pressure Cooker	1	0	3	67
Ventana CC1 24mins	1	0	2	100
Ventana CC1 32mins	8	88	10	90
Ventana CC1 36mins	1	100	5	100
Ventana CC1 40mins	4	75	9	89
Ventana CC1 48mins	8	88	4	100
Ventana CC1 52mins	2	100	1	100
Ventana CC1 56mins	7	86	3	100
Ventana CC1 64mins	42	88	35	100
Ventana CC1 72mins	3	100	0	0
Ventana CC1 76mins	4	75	1	100
Ventana CC1 80mins	1	100	0	0
Ventana CC1 88mins	1	100	1	100
Ventana CC1 92mins	3	100	0	0
Ventana CC1 extended	5	100	0	0
Ventana CC1 mild	0	0	3	100
Ventana CC1 standard	9	89	11	100
Ventana CC2 64mins	1	100	3	100
Water bath 95-98 OC	2	0	3	0

Lymphoma Run: 118

Enzyme Mediated Retrieval	BCL6 N	BCL6 %	CD79a N	CD79a %
AS PER KIT	3	67	3	67
Dako Protease (S2019)	1	0	0	0
NOT APPLICABLE	96	84	84	87
Other	0	0	1	100
VBS Bond Enzyme 1	0	0	1	100

Lymphoma Run: 118	BCL6		CD79a	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	14	79	16	88
BioGenex HRP (HK 519-06K)	1	100	1	100
BioGenex SSM-link (LP000-UL)	0	0	1	100
Dako EnVision FLEX (K8000/10)	3	100	5	80
Dako EnVision FLEX+ (K8002/12)	14	86	16	100
Dako Envision HRP/DAB (K5007)	1	0	1	100
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	100	0	0
Leica Bond Polymer Refine (DS9800)	57	88	52	81
Other	4	100	6	100
Power Vision DPVB999 HRP	1	100	2	0
Ventana iView system (760-091)	1	0	3	100
Ventana OptiView (760-700) + Amp. (7/860-099)	6	100	2	100
Ventana OptiView Kit (760-700)	47	87	36	94
Ventana UltraView Kit (760-500)	37	95	41	95

Lymphoma Run: 118	BCL6		CD79a	
Automation	**N**	**%**	**N**	**%**
Dako Autostainer Link 48	17	88	14	86
Dako Autostainer plus	0	0	4	75
Dako Autostainer Plus Link	2	100	1	100
Dako Omnis	9	89	9	100
Dako TechMate 500	0	0	1	0
Leica Bond Max	34	82	34	76
Leica Bond-III	34	91	33	85
Menarini - Intellipath FLX	1	0	0	0
None (Manual)	3	67	4	75
Other	1	100	1	0
Ventana Benchmark GX	2	100	3	67
Ventana Benchmark ULTRA	74	88	70	97
Ventana Benchmark XT	21	86	20	95

Lymphoma Run: 118	BCL6		CD79a	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	25	88	33	91
BioGenex liquid DBA (HK-124-7K)	1	100	1	100
DAKO DAB+	1	0	1	100
Dako DAB+ Liquid (K3468)	1	100	3	33
Dako EnVision Plus kits	2	100	4	100
Dako FLEX DAB	21	95	17	94
Dako REAL EnVision K5007 DAB	1	0	1	100
Dako REAL K5001 DAB	1	0	2	50
Leica Bond Polymer Refine kit (DS9800)	60	88	50	78
Other	11	91	8	100
Sigma DAB (D5637)	0	0	1	0
Sigma DAB (D5905)	0	0	1	100
Ventana DAB	32	81	21	90
Ventana iview	2	100	4	100
Ventana Ultraview DAB	38	89	45	96
Vision BioSystems Bond X DAB	0	0	1	100

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

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

BCL6 - Method 2

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

Primary Antibody: Dako BCL6 M7211 (PG-B6p) , 20 Mins, 22 ºC Prediluted
Automation: Dako Omnis
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako Omnis, Buffer: TRS high ph dako
EAR:
Chromogen: Dako FLEX DAB, 22 ºC., Time 1: 5 Mins
Detection: Dako EnVision FLEX+ (K8002/12) , 10 Mins, 22 ºC Prediluted

BCL6 - Method 3

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

Primary Antibody:	Novocastra NCL-L-BCL-6-564 (LN22) mm , 30 Mins Dilution 1: 800
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) , 30 Mins

BCL6 - Method 4

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

Primary Antibody:	Ventana 760-4241 (1GI191E/A8) , 14 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark GX
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 32mins
EAR:	
Chromogen:	AS PER KIT, 37 ºC., Time 1: 8 Mins, Time 2: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 37 ºC Prediluted

BEST METHODS - Secondary Antibody

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

CD79a - Method 1

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

Primary Antibody:	Dako M7050 CD79a (JCB117) , 30 Mins, ambient ºC Dilution 1: 50
Automation:	Dako Autostainer plus
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: DAKO target retrieval high pH, PH: 9
EAR:	
Chromogen:	Dako FLEX DAB, ambie ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako EnVision FLEX (K8000/10) , 20 Mins, ambient ºC Prediluted

CD79a - Method 2

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

Primary Antibody:	Dako M7050 CD79a (JCB117) , 20 Mins, 23 ºC Dilution 1: 50
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590), PH: 7.4
HMAR:	Leica ER1 30 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), Time 1: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 15 Mins, 23 ºC Prediluted

CD79a - Method 3

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

Primary Antibody:	Dako M7050 CD79a (JCB117) , 20 Mins, 22 ºC Dilution 1: 400
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: 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

CD79a - Method 4

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

Primary Antibody:	Ventana 790 4432 CD79a (SP18) , 20 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 48mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700) , 12 Mins, 37 ºC Prediluted

Neil Bilbe

	Gold Standard	Second Antibody
Antigens Assessed:	Ki67/MIB-1	CD68
Tissue Sections circulated:	GBM and Meningioma	Radiation induced necrosis
Number of Registered Participants:	59	
Number of Participants this Run	59 (100%)	

Introduction

Gold Standard: Ki-67

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 MIB1 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: CD68

Cluster of Differentiation 68 (CD68) is a 110kD highly glycosylated lysosomal glycoprotein (LGP), thought to play a role in protecting lysosomal membranes from attack by acidic hydrolases. CD68 is found in cytoplasmic granules and in the cytoplasm of various non-haematopoietic tissues. It is also expressed, to a lesser extent, on the surface of macrophages, monocytes, neutrophils, basophils and large lymphocytes localised in the cytoplasm. Kupffer cells, histiocytes, mast cells and microglia will also stain positive with CD68. The antibody is used to distinguish diseases of similar appearance, such as monocyte/macrophage and lymphoid forms of leukaemia. It is also useful for in the diagnosis of conditions related to proliferation, such as malignant histiocytosis, histocytic lymphoma, and Gaucher's disease. CD68 is a very heterogenous molecule, therefore, different antibodies to CD68 show different cellular relativities (Falini et al., Pulford Et al.). The most widely used clones are KP1 and PG-M1; both show strong specific staining of microglia in the brain.

Features Of Optimal Immunostaining:
- Strong cytoplasmic staining of the microglial cells.
- Minimal background staining with no non-specific staining

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

References:

1. **Falini B, et al.** PG-M1: A new monoclonal antibody directed against a fixative-resistant epitope on the macrophage-restricted form of the CD68 molecule. Am J Pathol 1993; 142:1359-72.
2. **Pulford KAF et al.** KP1: a new monoclonal antibody that detects a monocyte/macrophage associated antigen in routinely processed tissue sections. J Clin Pathol 1989;42:414-21.

Assessment Summary:
All 59 participants submitted slides. One lab did not return any MIB-1 slides (**G**) and another no CD68 (**J**). A total of 232 slides/ sections were assessed: **G=58, H=58, J=58 K=58**

The overall pass rate was 78% (182 slides), borderline 19% (44), and the failed slides rate was 3% (6). The average score for all slides was 15/20. These lower overall results were primarily due to the CD68 outcome (see below).

1) Summary Table - All Slides (MIB-1/Ki67 & CD68)

Slide	Antigen	Pass	Borderline	Fail
G (NEQAS)	MIB-1 (58)	82.8% (48)	17.2% (10)	0% (0)
H (In-House)	MIB-1 (58)	94.8% (55)	5.2% (3)	0% (0)
J (NEQAS)	CD68 (58)	51.7% (30)	37.9% (22)	10.3% (6)
K (In-House)	CD68 (58)	84.5% (49)	15.5% (9)	0% (0)
Total (Average)	232	78%	19%	3%

Ki67/ MIB-1 (**G & H**)
No Gold antigen slides (**G & H**) failed the assessment, although a relatively high number of the NEQAS Ki67/MIB-1 slides were assessed as borderline (17.2%), due primarily to weak staining (8 slides) or non-specific reactions (2 slides) see Image Report Figs 2 & 4*. The two *lowest* scoring slides used: *(1) Biocare (SP6) Ki67, Ventana CC1, on the Benckmark; and (2) Omnis RTU (GA626) MIB-1, on the Omnis platform and Omnis retrieval.

CD68 (**J & K**)
CD68 was last requested in 2013 (Run 103). The Run 118 results were considerably worse than for Run 103 on the NEQAS (**J**) material, with 6 slides failing (two previously), with a pass rate of only 51.7% down from 89% (Run 103). Looking at the data it is apparent that there has been a large number of labs who have moved from a pass score (≥13) to borderline (10 - 12). This is due almost entirely to weak, missing, or uneven staining of microglia (see Image report Figs 7, 9, 12). There was also an increase in failed slides from 2 (3%) to 6 (10.3%). Given these findings, the scheme did a secondary validation of all slides which had initially failed the assessment.

Only three slides achieved a score of >18. All used the PG-M1 clone; two used the Dako M0876 (PG-M1), one on the Omnis and one on the Leica Bond Max; the other the Dako IS613 FLEX RTU Plus (PG-M1) on the Omnis.

Those failing the assessment (6 slides) five employed the KP-1 clone, one lab gave no data. Brief details are as follows:

Dako M0814 (KP1), 1/4000, Leica Bond-III
Dako IR609 FLEX RTU Link (KP1), CC1, Ventana Benchmark XT
Ventana 790-2931 RTU (KP-1), CC1, Ventana Benchmark ULTRA
Dako IR609 FLEX RTU Link (KP1), Water bath 95-98 OC, Autostainer Link 48
No details given.
Dako M0814 (KP1), 1/400, CC1, Ventana Benchmark XT

Selected Images showing Optimal and Sub-optimal Immunostaining

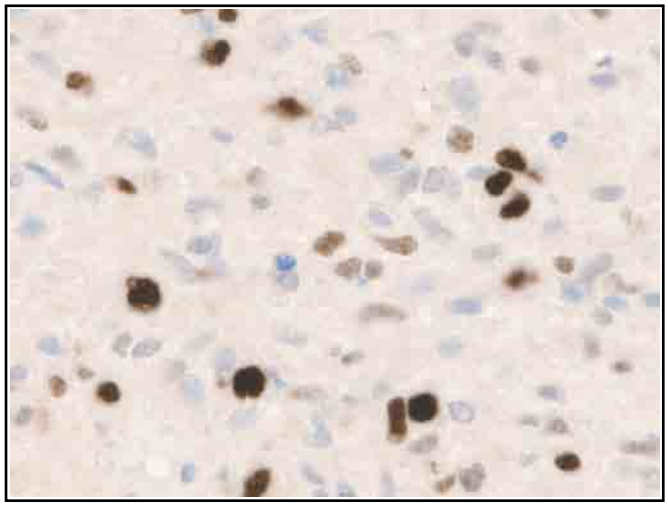


Fig 1. Sub-optimal Ki67 demonstration on the NEQAS GBM sample; some background staining is present, which diminishes the contrast, though still assessed as a pass. Leica/Novocastra RTU (K2) PA0230, 20 mins, with Leica ER2 20 mins, and the RTU Leica Bond Polymer Refine Kit, on a Bond III.

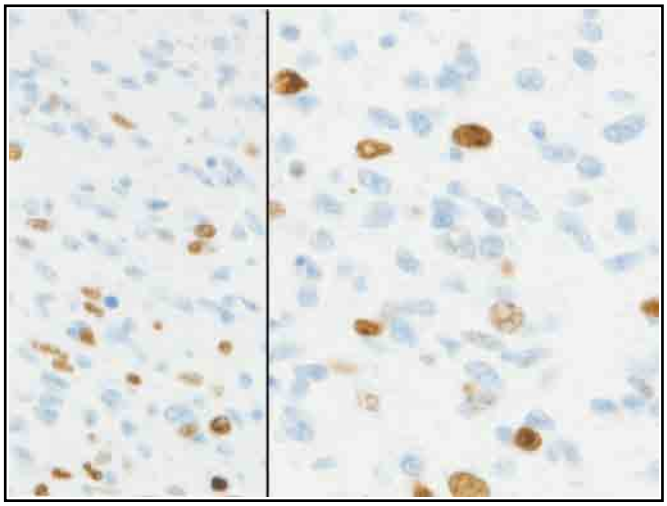


Fig 2. Sub-optimal Ki67 result on NEQAS GBM sample. Staining is weak, and not all of the tumour was adequately demonstrated. Slide was assessed as borderline, with improvement recommended. Dako FLEX RTU Omnis (MIB1) GA626, 20 mins, Dako Omnis PT, on the Omnis platform.

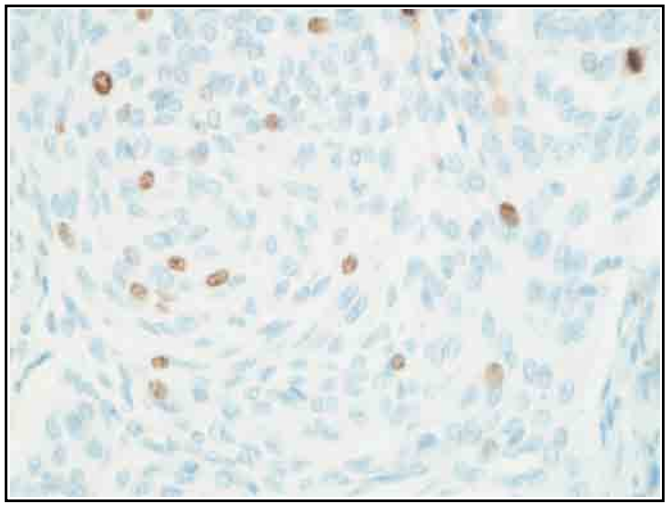


Fig 3. Excellent demonstration of Ki67 on the NEQAS meningioma sample. Staining is crisp, clean and selective. Very few participants obtained this level of staining in the meningioma. Dako M7240 (MIB1), 1:100, 32 mins, using a Ventana CC1 64 mins, Ventana UltraView Kit (760-500), on the Ventana Benchmark ULTRA.

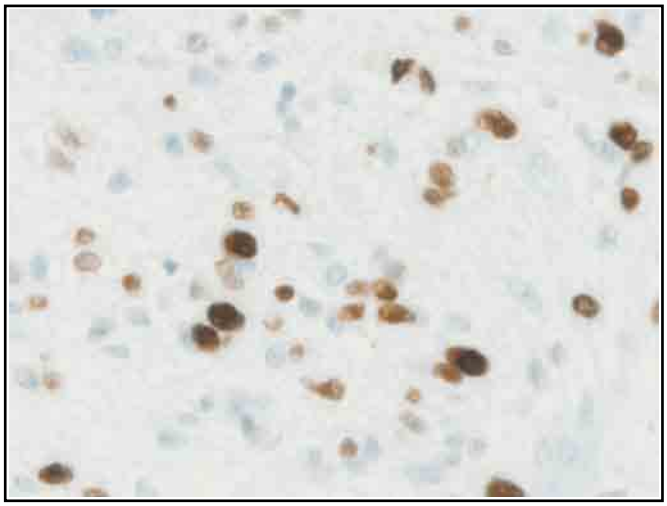


Fig 4. Sub-optimal Ki67 result on NEQAS GBM sample. Non-specific staining is seen, and there is a slight background hue. Borderline assessment outcome. Biocare CRM325 (SP6), 1:30, 24 mins, using Ventana CC1 64 mins, RTU Ventana UltraView Kit (760-500), on the Ventana Benchmark ULTRA.

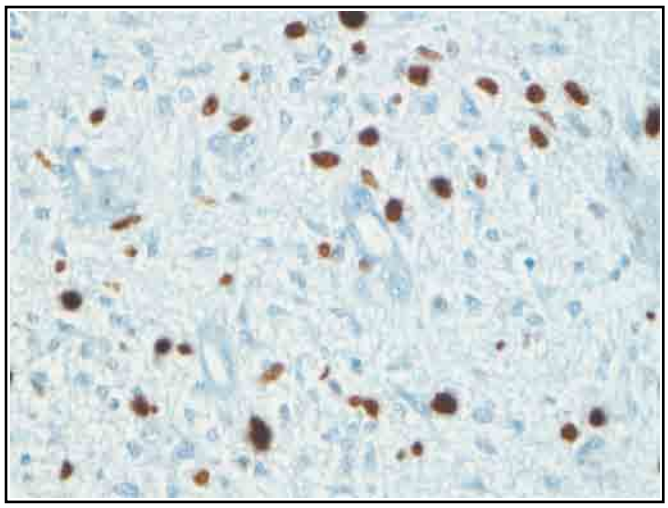


Fig 5. Good primary Ki67 staining on the NEQAS GBM sample, let down by an excessive counterstain intensity, the assessors deducting one mark for this. Dako M7240 (MIB1), 1:100, 32 mins, Ventana CC1 64 mins, using the Ventana OptiView (760-700) + Amp. (7/860-099), on a Ventana Benchmark ULTRA.

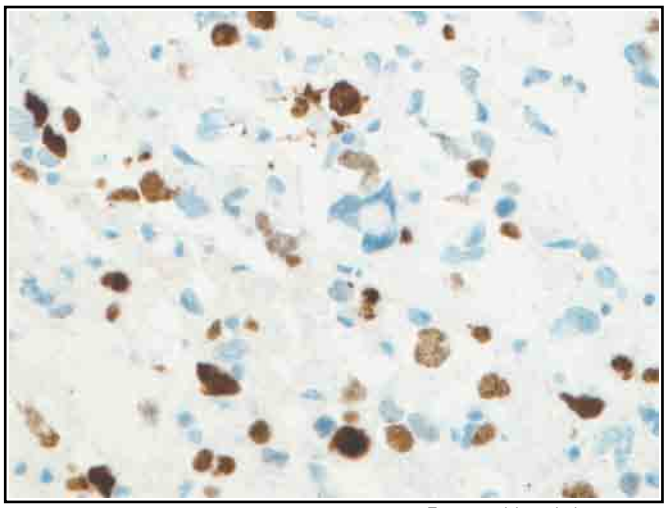


Fig 6. Excellent Ki67 staining on an in-house sample of a glioma. Tumour nuclei are nicely seen against optimal counterstain intensity. Ventana RTU (30-9) 790-4286, 16 mins, using a Ventana CC1 56 mins, a pre-diluted Ventana OptiView Kit (760-700) for 8 mins, on the Ventana Benchmark ULTRA.

Selected Images showing Optimal and Sub-optimal Immunostaining

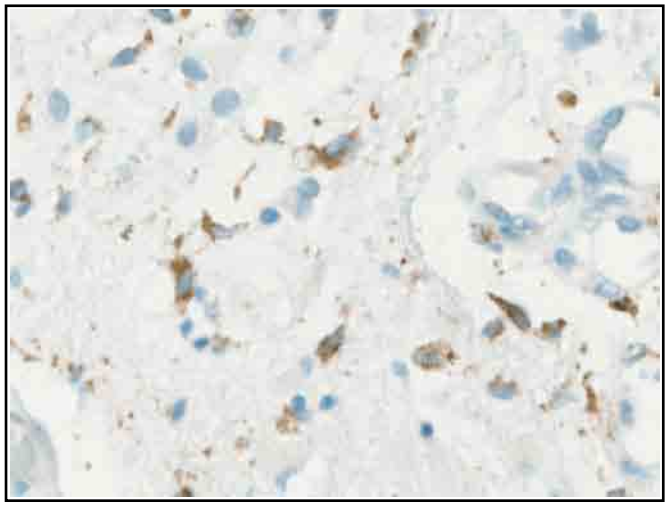


Fig 7. Sub-optimal CD68 demonstration on the NEQAS sample. Microglial staining is weak, and incomplete in some areas (comp Fig 8). Borderline assessment outcome. Dako M0814 (KP1), 1:500, no time given, Leica ER1 10 mins, Leica BondMAx Refine KIT, on the Leica Bond Max.

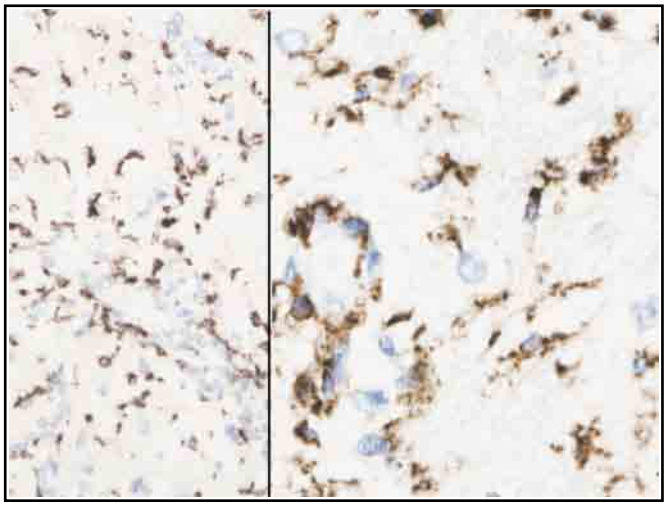


Fig 8. Optimal CD68 demonstration on the NEQAS sample. Microglia are stained in all areas of necrosis. This slide was from the same laboratory as the Golds referenced by the assessors. Dako M0876 (PG-M1), without retrieval, using the Leica Bond Polymer Define (DS9713), on a Leica Bond Max.

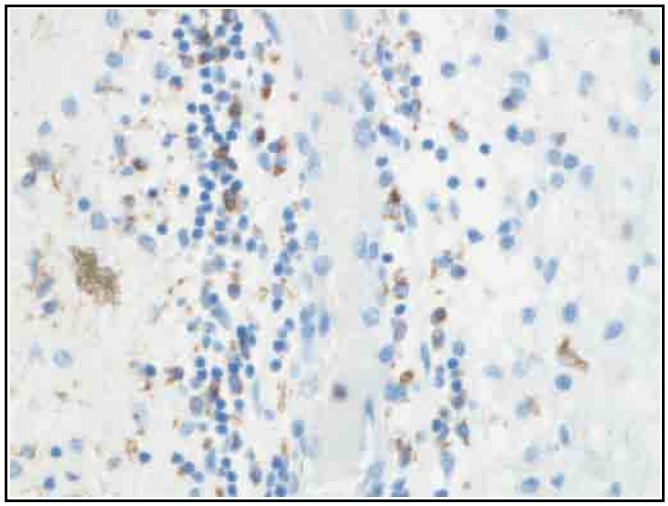


Fig 9. Sub-optimal CD68 demonstration on the NEQAS sample, reaction is weak, some areas of necrosis are unstained, and the haematoxylin is a touch heavy. Slide judged to be a low pass. Dako M0814 (KP1), 1:100, 15 mins, Leica ER1 30 mins, RTU Leica Bond Polymer Refine (DS9800), on a Leica Bond-III.

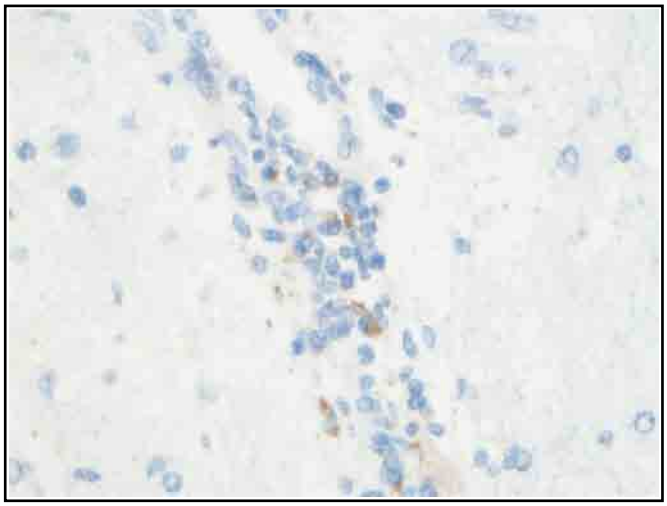


Fig 10. Poor CD68 demonstration on the NEQAS sample, staining is far too weak for reliable interpretation and failed the assessment. Dako M0814 (KP1), 1:4000 (compare dilution with above), no retrieval details given, second layer of Leica Bond Polymer Refine (DS9800), on the Leica Bond-III.

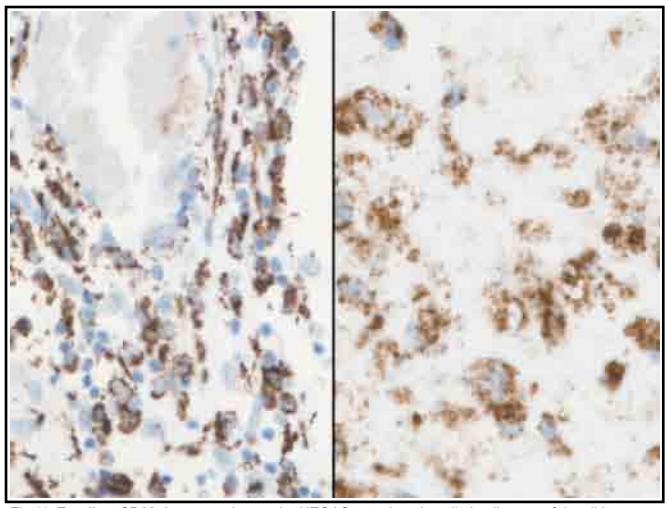


Fig 11. Excellent CD68 demonstration on the NEQAS sample, microglia in all areas of the slide are stained, and the counterstain contrast is optimal. Dako M0876 (PG-M1), 1:50, 15 mins, Dako Omnis HMAR and platform, employing the Dako EnVision FLEX (K8000/10) as a secondary layer.

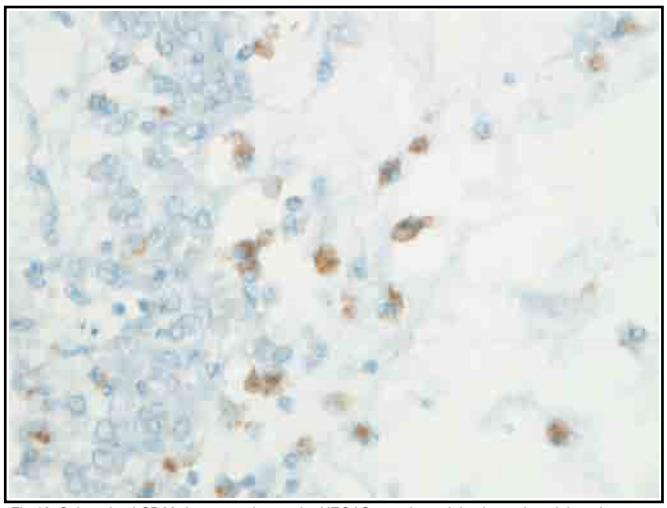
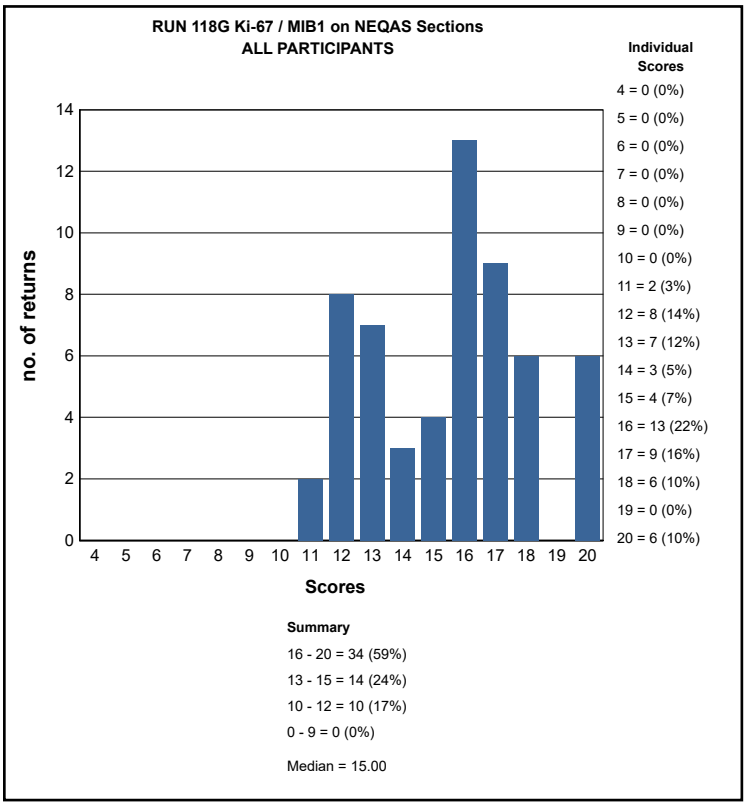
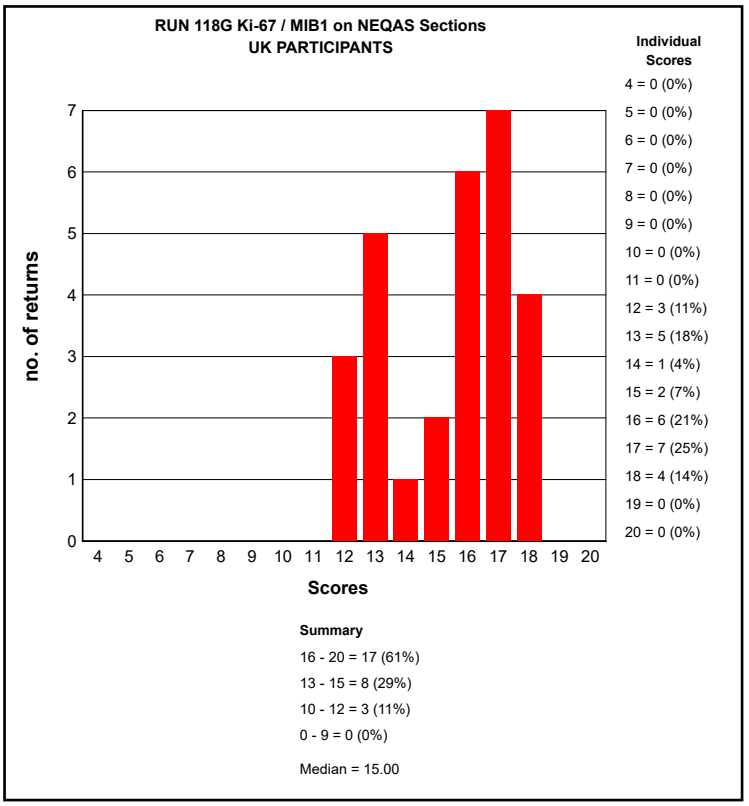
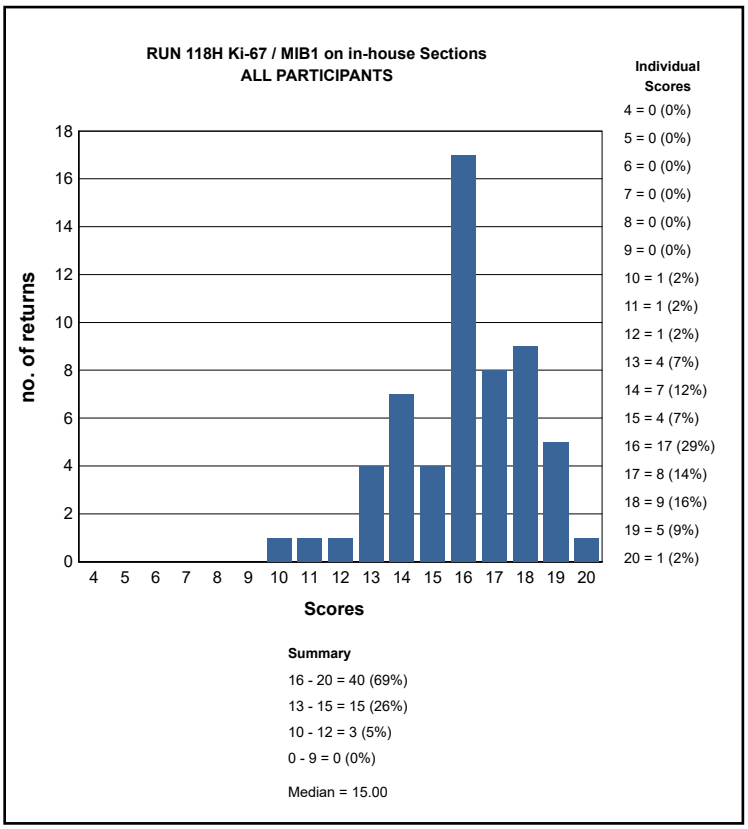


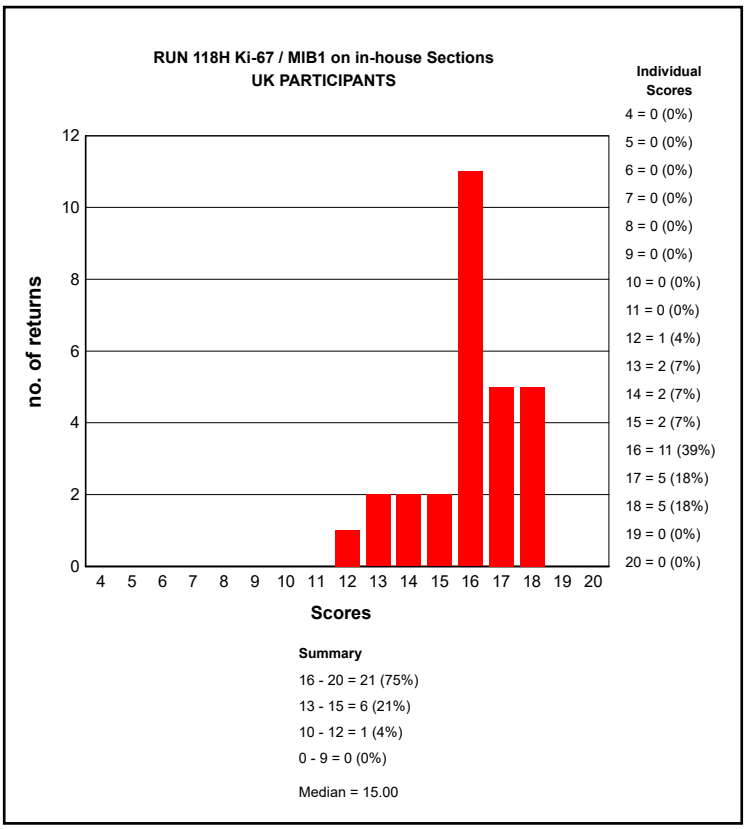
Fig 12. Sub-optimal CD68 demonstration on the NEQAS sample, staining is weak and there is some evidence of morphological damage. The assessors rated this slide as borderline. Dako M0814 (KP1), 1:150, 16 mins, with Ventana CC1 16 mins, the OptiView Kit (760-700), on a Ventana Benchmark ULTRA.

GRAPHICAL REPRESENTATION OF PASS RATES

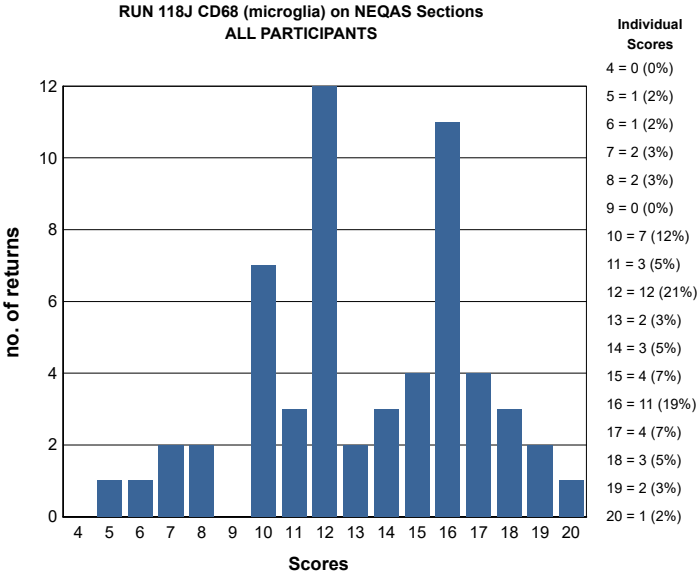


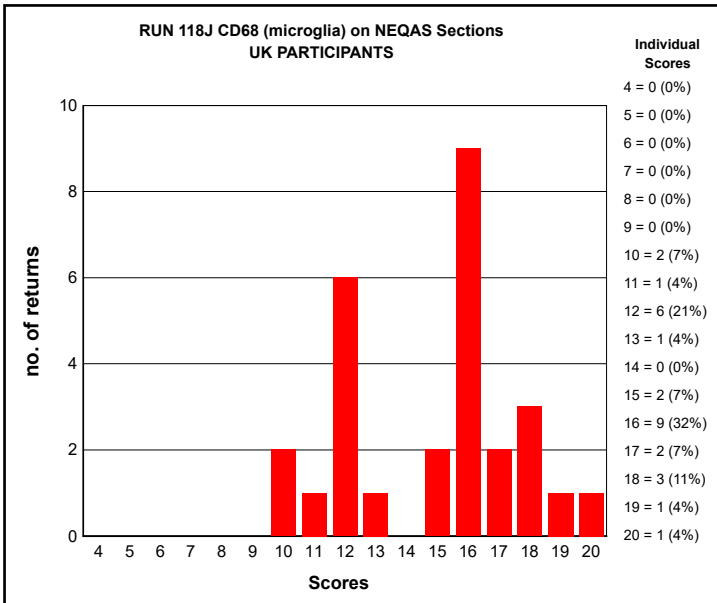


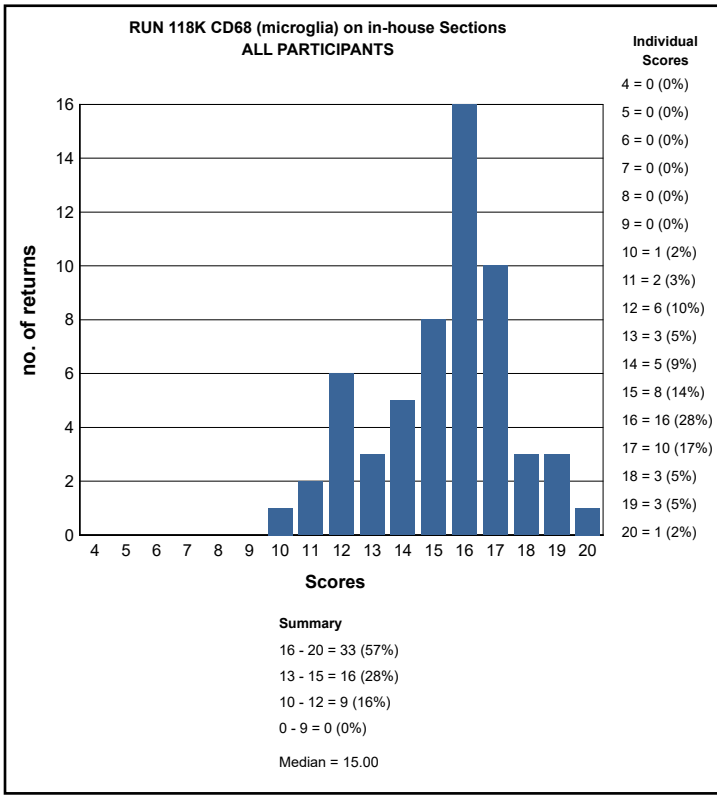


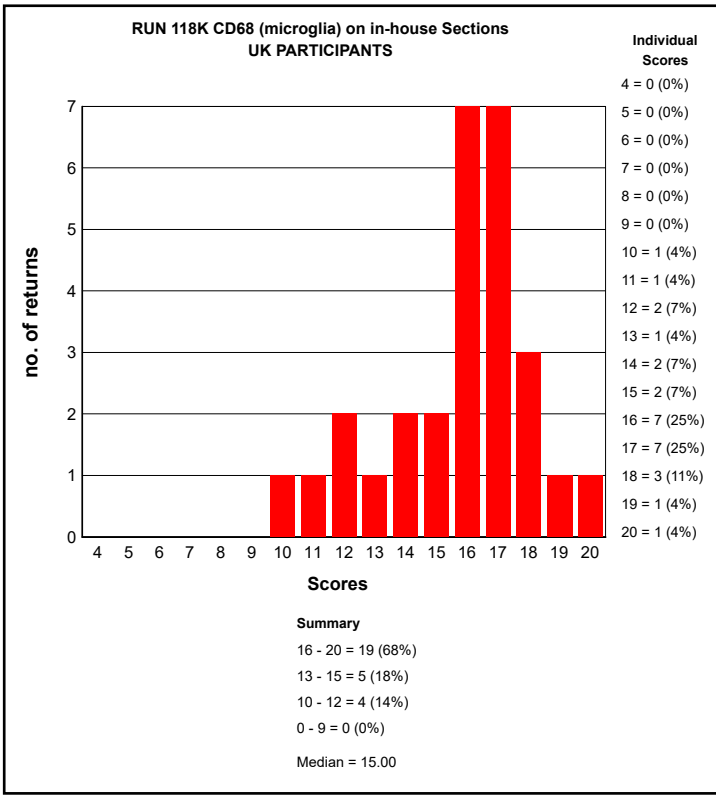


GRAPHICAL REPRESENTATION OF PASS RATES









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: 118

Primary Antibody : Ki-67 / MIB1

Antibody Details	N	%
Dako M7240 (MIB1)	32	84
NeoMarkers/Thermo Sci (SP6) RM 9106	1	100
Leica/Novocastra (MM1) NCL-Ki67-CE	2	50
Other	1	0
Leica/Novocastra RTU (MM1) PA0118	2	100
Leica/Novocastra RTU (K2) PA0230	2	100
Ventana RTU (30-9) 790-4286	9	100
Dako FLEX RTU (MIB1) IR626	2	100
DAKO FLEX RTU Omnis (MIB1) GA626	6	50
Gennova AP10244C (SP6)	1	100

Neuropathology Run: 118

Primary Antibody : CD68 (microglia)

Antibody Details	N	%
Dako M0814 (KP1)	12	42
Dako M0876 (PG-M1)	21	76
Ventana 790-2931 (KP-1)	6	17
Other	3	0
Dako IR613 FLEX RTU Link (PG-M1)	5	80
Dako IS613 FLEX RTU Plus (PG-M1)	1	100
Leica/Novocastra PA0273 RTU (514H12)	3	33
Leica/Novocastra NCL-L-CD68 (514H12)	2	0
Dako IR609 FLEX RTU Link (KP1)	3	33
Dako IS609 FLEX RTU Plus (KP1)	1	100

Neuropathology Run: 118

Heat Mediated Retrieval	CD68 (microglia) N	CD68 (microglia) %	Ki-67 / MIB1 N	Ki-67 / MIB1 %
Dako Omnis	7	86	7	57
Dako PTLink	4	75	4	50
Lab vision PT Module	1	100	1	0
Leica ER1 10 mins	1	0	0	0
Leica ER1 20 mins	2	50	0	0
Leica ER1 30 mins	2	100	0	0
Leica ER2 10 mins	1	0	0	0
Leica ER2 20 mins	8	50	11	100
Leica ER2 30 mins	1	0	3	67
Leica ER2 40 mins	0	0	2	50
Microwave	1	0	1	0
None	2	100	0	0
Other	1	100	1	100
Pressure Cooker	1	100	0	0
Ventana CC1 16mins	2	0	0	0
Ventana CC1 24mins	1	0	1	100
Ventana CC1 32mins	4	50	3	100
Ventana CC1 36mins	3	0	3	100
Ventana CC1 40mins	1	100	0	0
Ventana CC1 48mins	0	0	2	100
Ventana CC1 56mins	0	0	1	100
Ventana CC1 64mins	4	50	9	89
Ventana CC1 8mins	3	0	0	0
Ventana CC1 extended	1	100	1	100
Ventana CC1 mild	2	50	3	100
Ventana CC1 standard	1	100	2	100
Ventana CC2 24mins	1	0	0	0
Ventana CC2 64mins	1	100	2	100
Water bath 95-98 OC	1	0	1	100

Neuropathology Run: 118

Enzyme Mediated Retrieval	CD68 (microglia) N	CD68 (microglia) %	Ki-67 / MIB1 N	Ki-67 / MIB1 %
NOT APPLICABLE	21	62	37	89
VBS Bond Enzyme 1	3	67	0	0

Neuropathology Run: 118	CD68 (microglia)		Ki-67 / MIB1	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	6	17	6	83
Dako EnVision FLEX (K8000/10)	4	100	5	60
Dako EnVision FLEX+ (K8002/12)	4	75	4	75
Dako Envision HRP/DAB (K5007)	2	50	2	50
Leica Bond Polymer Define (DS9713)	1	100	0	0
Leica Bond Polymer Refine (DS9800)	14	50	14	86
Other	2	100	2	50
Power Vision DPVB999 HRP	1	100	1	0
Ventana OptiView (760-700) + Amp. (7/860-099)	1	0	1	100
Ventana OptiView Kit (760-700)	9	33	8	100
Ventana UltraView Kit (760-500)	13	46	15	93

Neuropathology Run: 118	CD68 (microglia)		Ki-67 / MIB1	
Automation	**N**	**%**	**N**	**%**
Dako Autostainer Link 48	4	50	5	60
Dako Autostainer plus	1	100	0	0
Dako Autostainer Plus Link	1	100	0	0
Dako Omnis	7	86	7	57
Leica Bond Max	6	67	4	75
Leica Bond-III	12	42	11	91
None (Manual)	2	50	3	67
Other	1	100	1	0
Ventana Benchmark ULTRA	17	35	19	95
Ventana Benchmark XT	7	43	8	100

Neuropathology Run: 118	CD68 (microglia)		Ki-67 / MIB1	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	7	57	9	100
DAKO DAB+	1	100	1	100
Dako DAB+ Liquid (K3468)	1	100	1	0
Dako DAB+ REAL Detection (K5001)	1	100	1	100
Dako EnVision Plus kits	1	0	1	0
Dako FLEX DAB	8	88	8	50
Dako REAL EnVision K5007 DAB	1	0	1	0
Leica Bond Polymer Refine kit (DS9800)	16	50	14	86
Other	3	33	4	100
Ventana DAB	2	50	2	100
Ventana Ultraview DAB	16	38	16	94

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

Primary Antibody: Leica/Novocastra (MM1) NCL-Ki67-CE , 15 Mins, 25 ºC Dilution 1: 50
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 Prediluted

Ki-67 / MIB1 - Method 2

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

Primary Antibody: Leica/Novocastra RTU (MM1) PA0118
Automation: Leica Bond Max
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 20 mins
EAR: NOT APPLICABLE
Chromogen: Leica Bond Polymer Refine kit (DS9800)
Detection: Leica Bond Polymer Refine (DS9800)

Ki-67 / MIB1 - Method 3

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

Primary Antibody:	Dako M7240 (MIB1) , 32 Mins, 37 ºC Dilution 1: 50
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)

Ki-67 / MIB1 - Method 4

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

Primary Antibody:	Ventana RTU (30-9) 790-4286 , 16 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 56mins
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT, 36 ºC., Time 1: 8 Mins, Time 2: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 36 ºC Prediluted

BEST METHODS - Secondary Antibody

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

CD68 (microglia) - Method 1

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

Primary Antibody:	Dako M0876 (PG-M1)
Automation:	Leica Bond Max
Method:	Indirect
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	None
EAR:	VBS Bond Enzyme 1 Digestion Time In-House: 10 Mins
Chromogen:	AS PER KIT
Detection:	Leica Bond Polymer Define (DS9713)

CD68 (microglia) - Method 2

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

Primary Antibody:	Dako M0876 (PG-M1) , 32 Mins, 37 ºC Dilution 1: 1:200
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	
Chromogen:	Ventana Ultraview DAB, 37 ºC., Time 1: 12 Mins, Time 2: 12 Mins
Detection:	Ventana UltraView Kit (760-500) , 37 Mins, 12 ºC Prediluted

CD68 (microglia) - Method 3

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

Primary Antibody:	Dako M0876 (PG-M1) , 15 Mins Dilution 1: 50
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)

CD68 (microglia) - Method 4

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

Primary Antibody:	Ventana 790-2931 (KP-1) , 16 Mins, 36 ºC
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)

Neil Bilbe and Irena Srebotnik Kirbis

	Gold Standard	Second Antibody
Antigens Assessed:	Ki67	ER
Sample circulated; cytospins and cell block sections:	Cells lines consisting of breast carcinoma, melanoma, and mesothelial cells 65 Cell block (75%), 22 Cytospins (25%)	
Number of Registered Participants:	87	
Number of Participants this Run	85 (98%)	

Introduction

Gold Antigen: Ki 67

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 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

Second Antigen: Oestrogen Receptor

Oestrogen receptor alpha (ER-α) plays a vital role in both the prognosis and predictive response of patients who may be considered for hormone therapy. It is therefore crucial that not only the antibodies are correctly validated prior to patient-tissue use, but also proper control tissues are used to gauge the sensitivity of the test. An incorrect assay can lead to false ER staining which can have a direct impact on patient treatment regime.

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

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

Features of Optimal Immunostaining:

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

Features of Sub-Optimal Immunostaining:

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

References

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

References (cell blocks in cytology)

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

Cytopathology Module 2017- 2018:

87 labs are now registered on the Cytopathology module for Run 118, the first round of the new EQA year. Two participants have withdrawn from the scheme (both non-UK), but four labs have added the cytology module (3 UK, 1 EU) to their repertoire. There are 58 UK & Eire labs and 29 Overseas only two of which are non-EU.

The number of labs requesting cell block sections or cytospins is 75%: 25%. 90% of UK & Eire labs request cell blocks. For the Overseas labs the split is more even: 55% (CS) 45% (CB).

Assessment Summary:

Two participants failed to send in any slides. One lab did not submit any ER slides (**S & T**) and another did not return any in-house samples (**S & U**). This resulted in 336 slides for the assessors. **R = 85, S = 84, T = 84, U = 83.**

Assessment Outcomes:

The overall pass rate was 93% (314 slides), 6% borderline (20), and a 1% failure rate (2).

The average score for all slides was 16/20.

(1) Summary Table - All Slides:

Slide	Antigen	Pass	Borderline	Fail
R (NEQAS)	Ki67 (85)	92% (78)	8% (7)	0% (0)
S (In-House)	Ki67 (84)	94% (79)	5% (4)	1% (1)
T (NEQAS)	ER (84)	96% (81)	4% (3)	0% (0)
U (In-House)	ER (83)	92% (76)	7% (6)	1% (1)
Average (Total)	336	93% (314)	6% (20)	1% (2)

Results were relatively even across all slides and for both antigens, unlike in Run 117, where there was an unusually high level of borderline results for the 2nd antigen.

Ki 67 (R)

All the best performing NEQAS (**R**) slides ($^{20}/_{20}$) were carried out on cytospin samples (See Images Report Fig 3); two used the Dako MIB-1 at 1:100, one on a Leica Bond III and the other the Ventana Benchmark ULTRA. The other two participants both employed the Ventana RTU (30-9), and again both used the Benchmark ULTRA platform.

In terms of the average scores for the NEQAS Ki67 (**R**) samples for cell blocks and cytospins, these are 15.3 (CB) and 16.3 (CS), which again highlights the findings.

N.B. Any retrieval data is taken on trust or with reservations given the nature of how this is recorded (see Important Note Below).

Although no slides failed the assessment, there were seven borderline results on the NEQAS samples; five on cell block sections and two on cytospin preparations. Percentagewise these equate to 7.8% ($^{5}/_{64}$) and 9.5% ($^{2}/_{21}$) respectively.

For the cytospin samples the staining was generally weak (See Images Report Fig 5), whereas for the cell block sections it was more akin to non-specific and background reactions, (See Images Report Fig 2 & 4) due to either antibody dilution and/or pretreatment issues.

ER (T)

The results for the 2nd antigen (Oestrogen Receptor) were slightly better than for the Gold (Ki 67), with a more consistent overall outcome.

The average score of for the NEQAS ER (**T**) slides was $^{16}/_{20}$, compared to $^{15}/_{20}$ for the NEQAS Ki 67 (**R**) slides.

Only three NEQAS ER (**T**) slides scored $^{20}/_{20}$ (four for Ki 67), but only three slides were assessed as borderline (seven for Ki 67), when comparing the NEQAS samples for each antigen (**R & T**).

Of the three best performing NEQAS ER slides (**T**), two were cell block slides and one a cytospin preparation. All three used the same protocol: RTU Ventana 790-4324 (SP1), Ventana CC1 64 mins, using the Ventana Benchmark ULTRA. The participant who submitted a cytospin slide indicated that they had carried out antigen retrieval. It appears looking through the data that many other cytospin labs using this particular marker also did similarly, or more so that for some of the other clones and suppliers; e.g the Leica/ Novocastra NCL-L-ER- 6F11 and Dako (EP1) M3643 clones.

Interestingly, all of the three borderline slides had virtually identical protocols:

- Cell block samples
- Leica/Novocastra 6F11 clone
- Two: NCL-L-ER- 6F11
- One: NCL-ER-6F11 (6F11)
- Two gave no dilution details, the third 1:50 (recommended)
- Leica ER1 between 30 - 40 mins
- Leica Bond Polymer Refine (DS9800)
- Leica Bond Max

Failed Slides

The two failed slides were both in-house samples:

Ki 67 (**S**): Dako MIB-1, Ventana CC1, Benchmark ULTRA.
The lab had scored themselves $^{14}/_{20}$ for which the assessors allocated a combined score of $^{9}/_{20}$.
The participant scored $^{13}/_{20}$ for their NEQAS slide (**R**).

ER (**U**): Ventana SP1, Ventana CC1, Benchmark ULTRA.
The participant assessed themselves as $^{15}/_{20}$ for this slide, NEQAS assessors gave the slide $^{9}/_{20}$.
The participant scored $^{16}/_{20}$ for their NEQAS slide (**T**).

Cell block v Cytospin on NEQAS slides (R & T):

(2) Summary Table - Cell block v Cytospin on NEQAS slides:

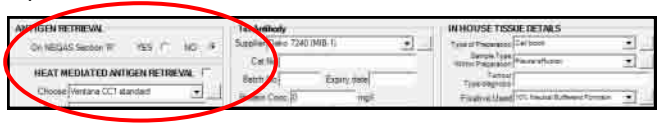
Letter	Antigen	Type	Sample	Average All
R	CK	NEQAS	Cell Block	15
R	CK	NEQAS	Cytospin	16
T	ER	NEQAS	Cell Block	16
T	ER	NEQAS	Cytospin	16

The only significant difference seen was the better results for the NEQAS Ki 67 slides (**R**) by the cytospin labs (16), compared to the cell block participants (15). The rest of the average scores (**S, T, U**) out of 20 were very similar (16).

Important note

It is important to note, that **cell blocks should be treated identically to FFPE blocks** and should therefore receive the same antigen retrieval as a standard FFPE protocol.

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



An example of a ***cell block*** lab that has entered HMAR details: Ventana CC1 Standard, but has checked the Antigen Retrieval on NEQAS sections as **NO**, but has also submitted a cell block section for their in-house control.
When this slide is viewed, and the protocol is requested by the assessors to aid their decision, they have no way of knowing which is the correct set of data for each slide: the NEQAS and the in-house control samples.

Selected Images showing Optimal and Sub-optimal immunostaining

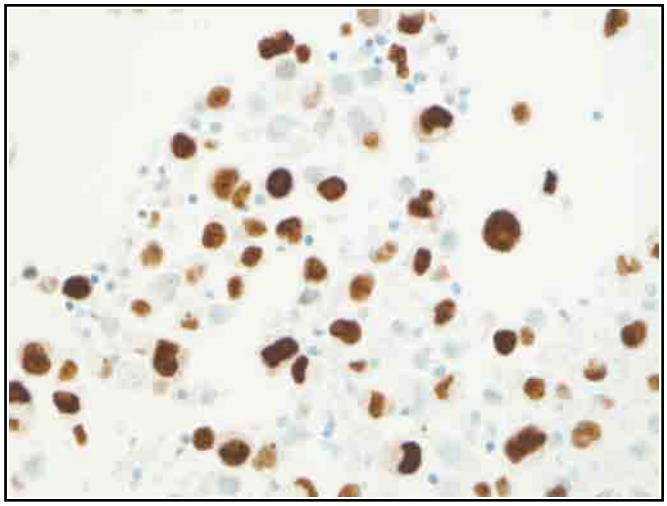


Fig 1. Good Ki67 on a NEQAS cell block section; nuclei are clearly demonstrated but with a slight background hue and a pale counterstain, though still a good pass. Dako 7240 (MIB-1), 1:200, 32 mins; using Ventana CC1 32 mins; a Ventana Optiview kit, on the Ventana Benchmark ULTRA.

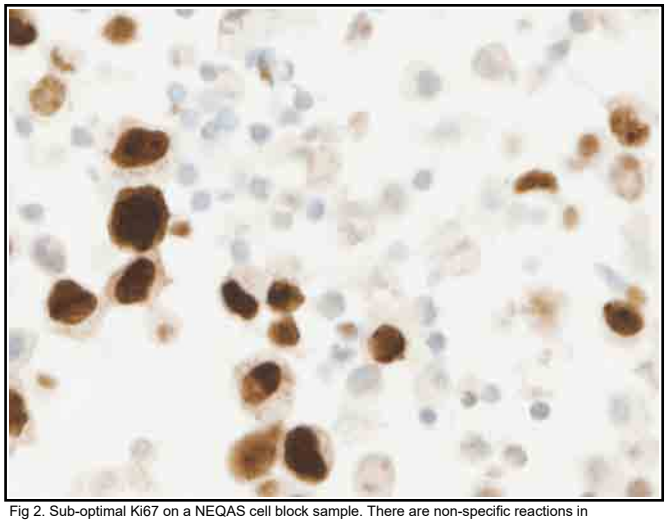


Fig 2. Sub-optimal Ki67 on a NEQAS cell block sample. There are non-specific reactions in non-tumour nuclei. Slide was assessed as borderline. Leica/Novocastra RTU (K2) PA0230, 20 mins; Leica ER2 10 mins; RTU Leica Bond Polymer Refine (DS9800), 8 mins; on a Leica Bond-III.

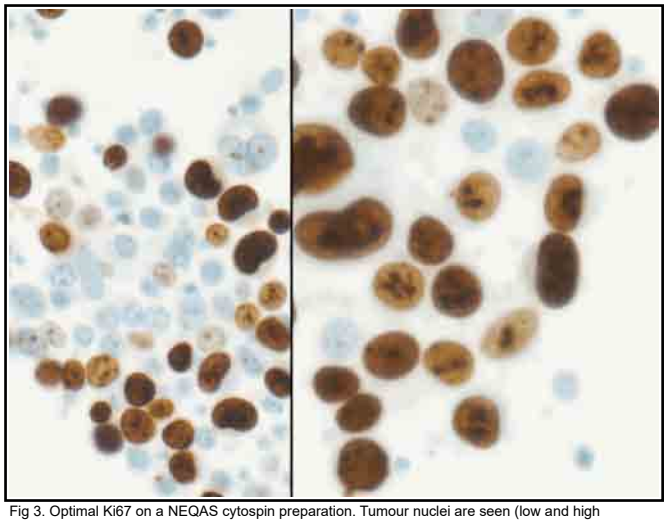


Fig 3. Optimal Ki67 on a NEQAS cytospin preparation. Tumour nuclei are seen (low and high power), background is clean and the counterstain of optimal intensity. Dako 7240 (MIB-1), 1:100, 32 mins; Ventana CC1 64 mins; with a RTU Ventana UltraView Kit (760-500), 12 mins; on Ventana Benchmark ULTRA.

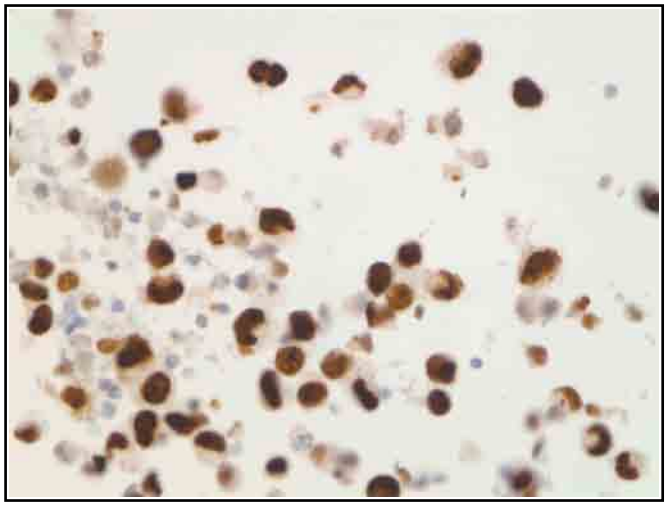


Fig 4. Sub-optimal Ki67 on a NEQAS cell block sample. There is non-specific staining, and a general dirty look to the slide; the haematoxylin colouration is poor. Leica/Novocastra RTU (K2) PA0230, 15 mins; Leica ER2 20 mins; with the Leica Bond Polymer Refine (DS9800) secondary layer, on Leica Bond-III.

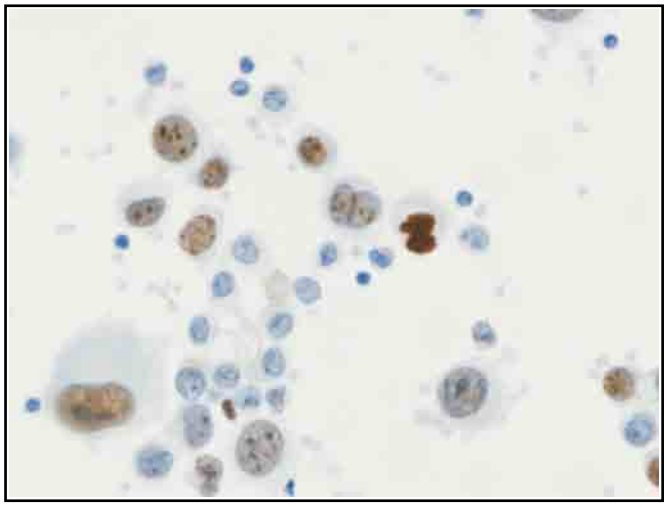


Fig 5. Sub-optimal Ki67 on a NEQAS cytospin preparation. Staining is weak (comp Fig 3), and missing in some areas. Slide was assessed as a low pass. Dako 7240 (MIB-1), no dilution given; without retrieval; with a second layer of Leica Bond Polymer Refine (DS9800); on a Leica Bond-III.

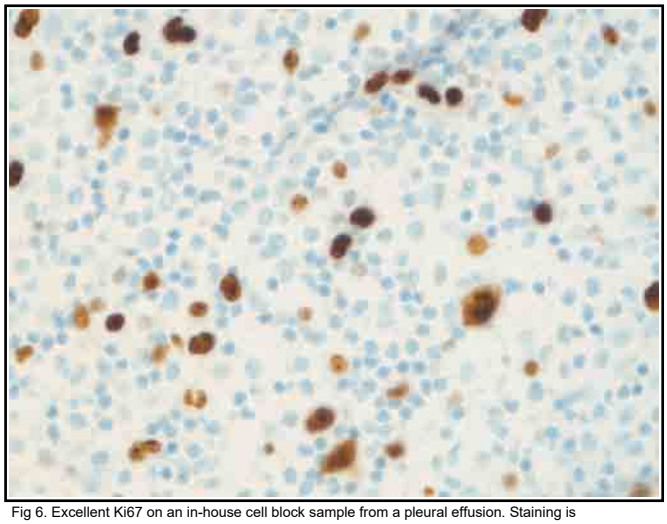


Fig 6. Excellent Ki67 on an in-house cell block sample from a pleural effusion. Staining is clean, precise and selective. Dako 7240 (MIB-1), 1:50, 24 mins; with a Ventana CC1 64 mins; pre-diluted Ventana UltraView Kit (760-500) for 8 mins; on the Ventana Benchmark ULTRA.

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

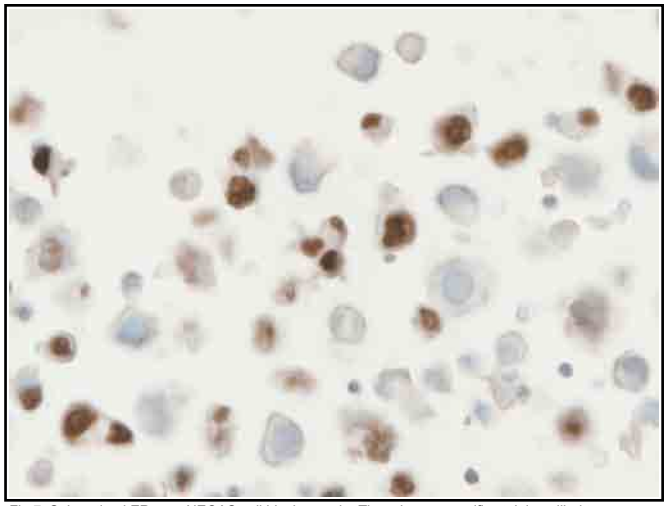


Fig 7. Sub-optimal ER on a NEQAS cell block sample. There is non-specific staining allied to weak/poor haematoxylin. Borderline assessment outcome. Leica/Novocastra NCL-ER-6F11 (6F11), 1:50, 15 mins; Leica ER1 30 mins; using a Leica Bond Polymer Refine (DS9800); on the Leica Bond Max.

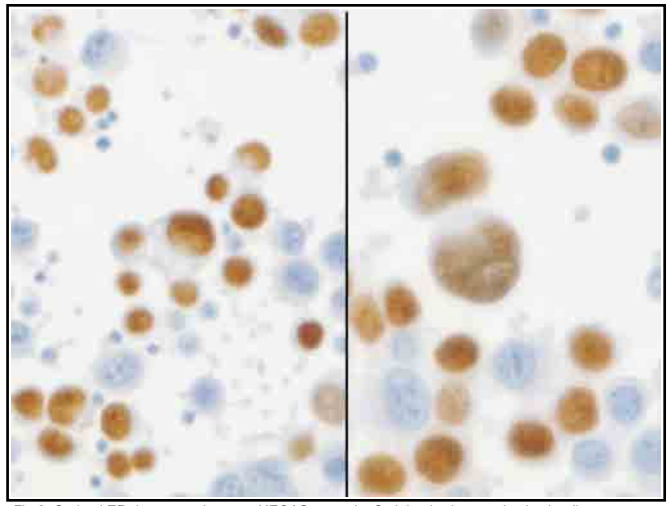


Fig 8. Optimal ER demonstration on a NEQAS cytospin. Staining is clean and selective (low and high power) and the counterstain intensity optimal. RTU Ventana 790-4325 (SP1), 32 mins; Ventana CC1 64 mins; with the Ventana UltraView Kit (760-500) secondary layer; on the Ventana Benchmark ULTRA.

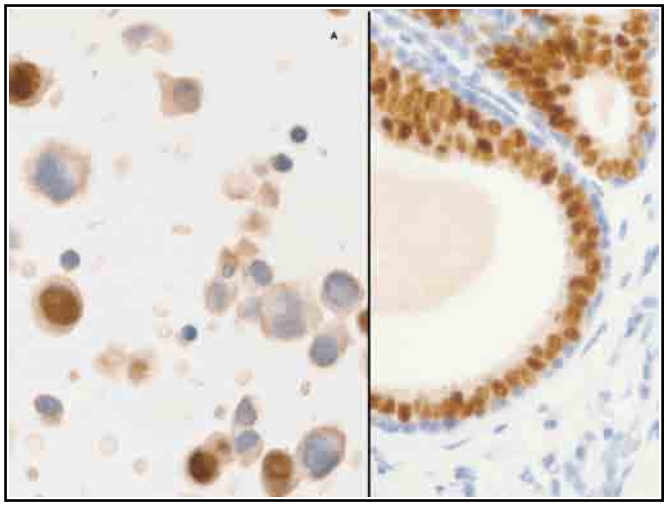


Fig 9. Poor ER staining (borderline) on NEQAS cell block section (A), but with excellent in-house demonstration (pass) in breast FFPE. Leica/Novocastra NCL-ER-6F11 (6F11). Leica ER1 40 mins used, but indicated that this was not used on the NEQAS section. Bond Polymer Refine (DS9800), and the Leica Bond Max.

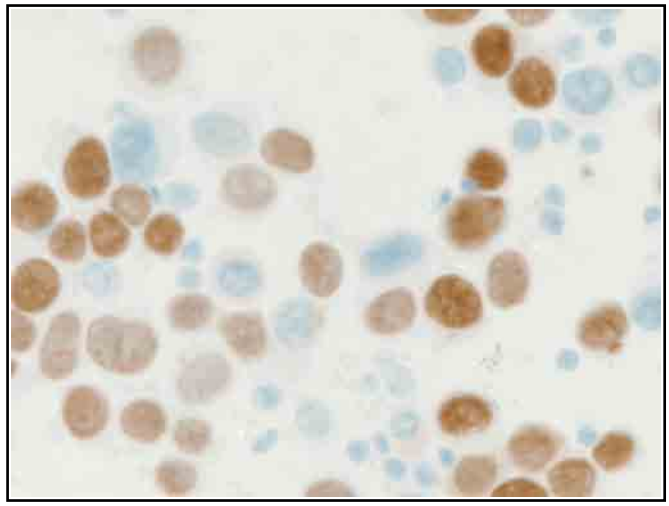


Fig 10. Excellent ER staining on a NEQAS cytospin. There is the merest hint of background, otherwise comparable with Fig 8. RTU Ventana 790-4324 (SP1), 16 mins; Ventana CC1 20 mins (stated not on the NEQAS sample!); RTU Ventana UltraView Kit (760-500), 4 mins; on a Ventana Benchmark ULTRA.

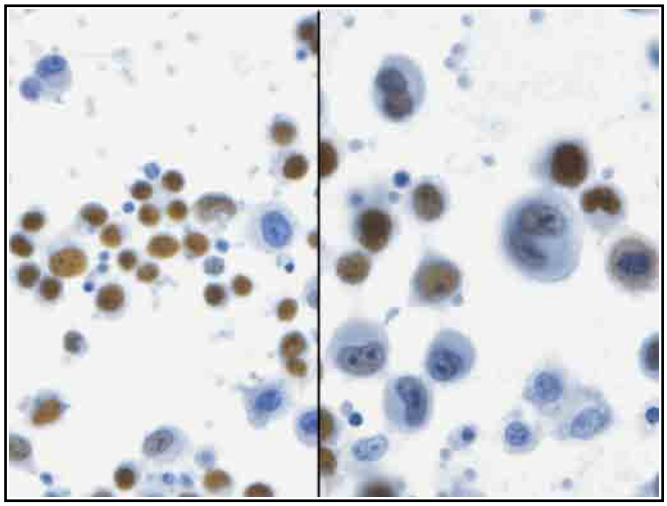


Fig 11. Sub-optimal ER on a NEQAS cytospin sample. Staining is compromised by high counterstain intensity. The assessors deducted one mark for this. Leica/Novocastra NCL-ER-6F11/2; Ventana CC1 20 mins; using a Ventana UltraView Kit (760-500); on the Ventana Benchmark ULTRA.

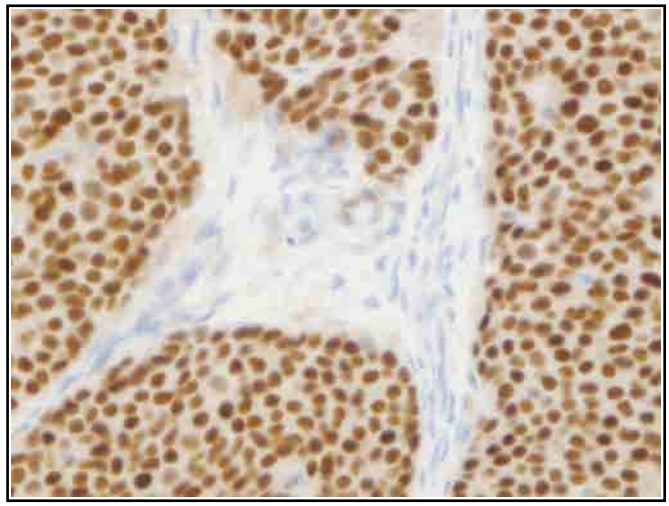
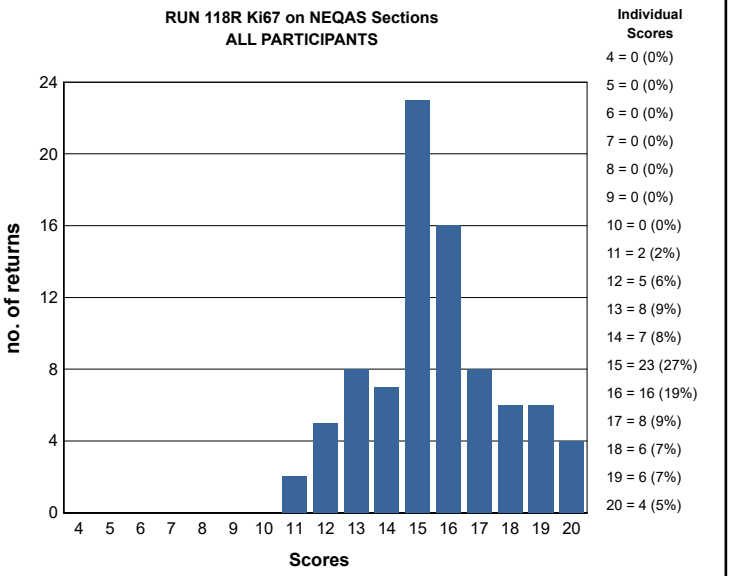
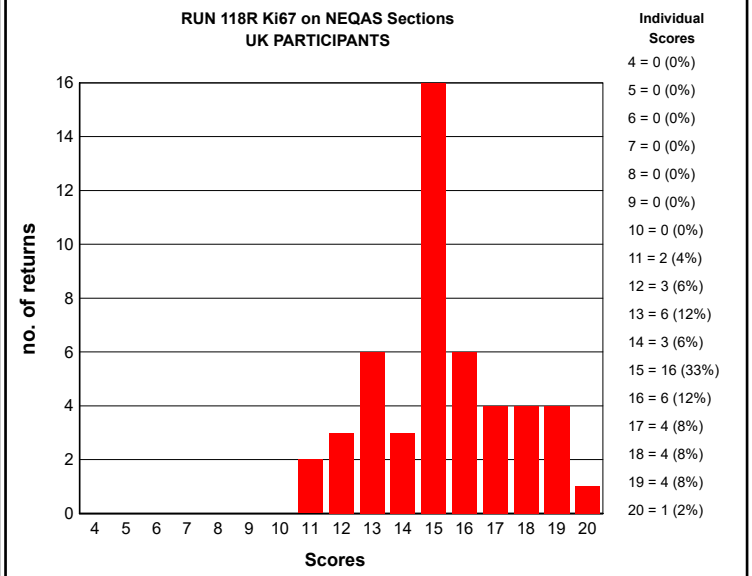
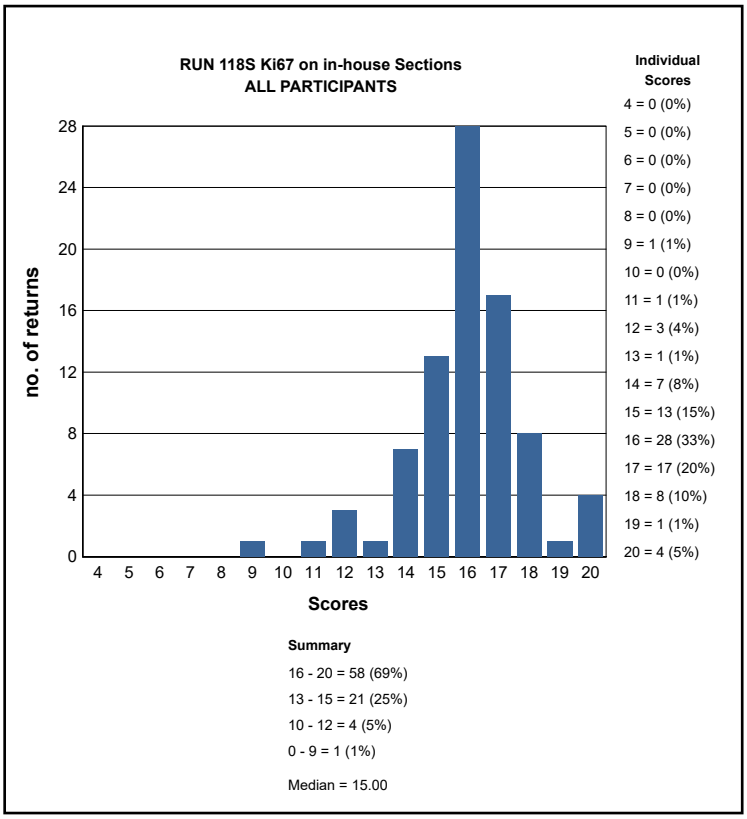


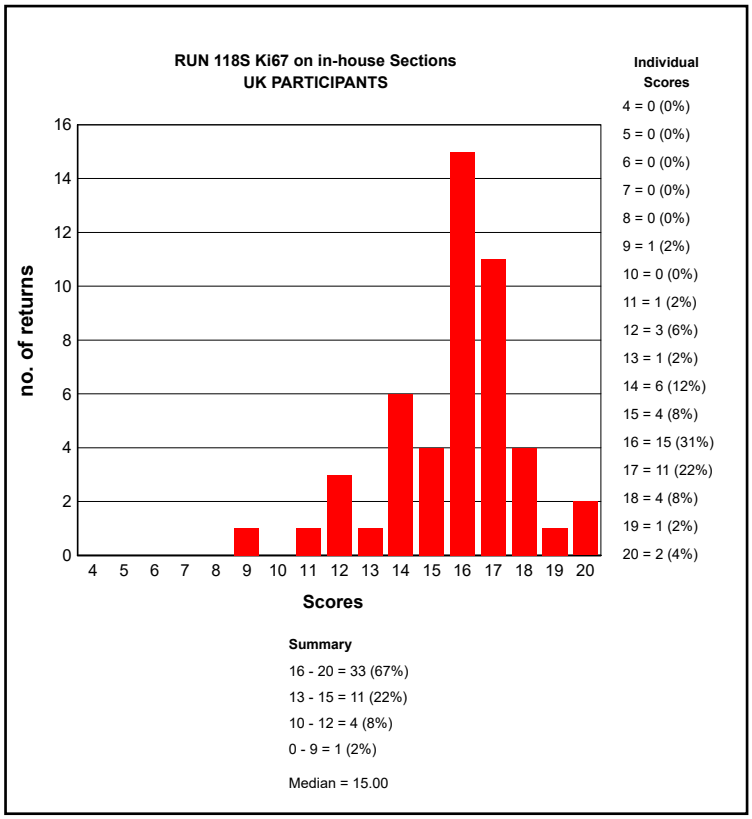
Fig 12. Excellent ER staining on an in-house FFPE section. The breast tumour nuclei are clearly demonstarted against a clean background. RTU Ventana 790-4324 (SP1), 16 mins; Ventana CC1 standard; pre-diluted Ventana UltraView Kit (760-500), 8 mins; on the Ventana Benchmark XT platform.

GRAPHICAL REPRESENTATION OF PASS RATES

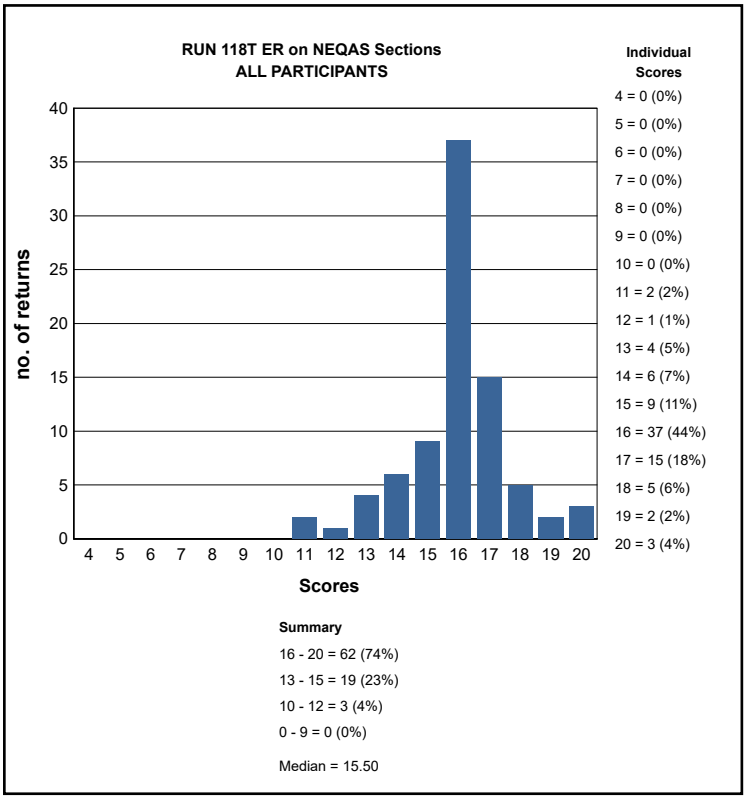


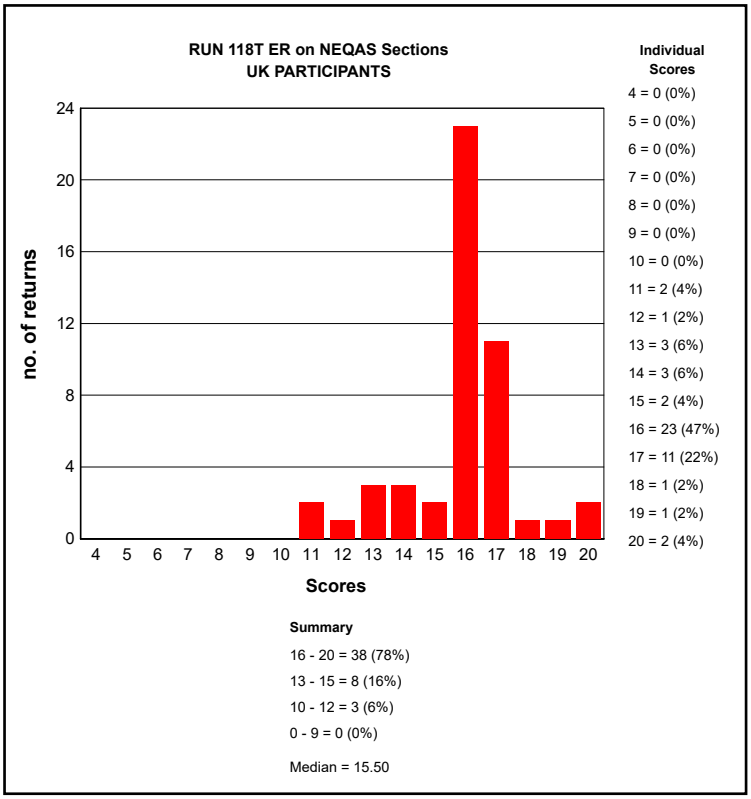


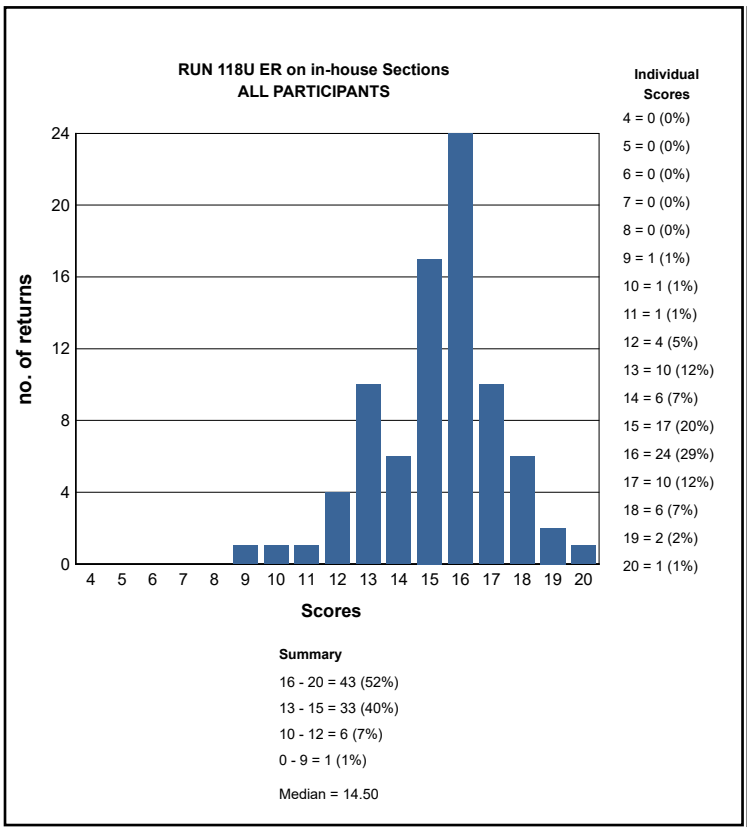


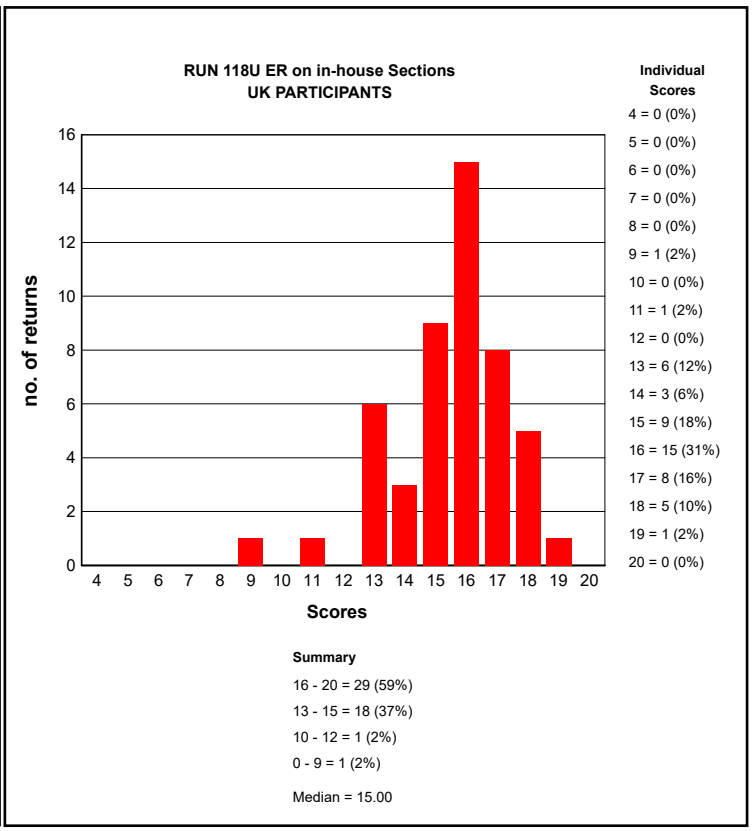


GRAPHICAL REPRESENTATION OF PASS RATES









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: 118

Primary Antibody : Ki67

Antibody Details	N	%
Dako 7240 (MIB-1)	41	95
Dako FLEX RTU IR626 (MIB-1)	5	80
Neomarkers/Thermo Sci (SP6) RM 9106	1	0
Leica/Novocastra RTU (MM1) PA0118	1	100
Leica/Novocastra RTU (K2) PA0230	10	70
Ventana (K2) 760-2910	1	100
Ventana RTU (30-9) 790-4286	21	100
Other	4	100

Cytology Run: 118

Primary Antibody : ER

Antibody Details	N	%
Cell Marque 249-R (SP1)	1	100
Dako M7047 ER (1D5)	1	100
Dako M3634 (SP1)	1	100
Dako (EP1) RTU FLEX IR084	5	100
Dako FLEX (1D5) IR/IS657	1	100
Dako (EP1) M3643	6	100
Leica/Novocastra NCL-ER-6F11 (6F11)	6	67
Leica/Novocastra NCL-L-ER- 6F11	11	91
Leica/Novocastra NCL-ER-6F11/2	4	100
Thermo Fisher/ Neomarkers RM 9101-S (SP1)	4	100
Ventana 250- 2596 ER (6F11)	1	100
Ventana 790-4324 (SP1)	26	100
Ventana 790-4325 (SP1)	14	100
Other	2	100

Cytology Run: 118

Primary Antibody : Ki67

Antigen Retrieval	N	%
YES	35	41
NO	50	59
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	35	
Not Specified	0	

Cytology Run: 118

Primary Antibody : ER

Antigen Retrieval	N	%
YES	32	38
NO	53	62
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	32	
Not Specified	0	

Cytology Run: 118

Heat Mediated Retrieval

Cytology Run: 118

Heat Mediated Retrieval

Cytology Run: 118

Enzyme Mediated Retrieval

Cytology Run: 118

Enzyme Mediated Retrieval

Cytology Run: 118

Detection

	ER		Ki67	
	N	%	N	%
AS PER KIT	9	100	7	100
Dako EnVision FLEX (K8000/10)	1	100	3	100
Dako EnVision FLEX+ (K8002/12)	5	100	5	100
Dako Envision+ HRP mouse K4004/5/6/7	1	0	1	100
Leica Bond Polymer Define (DS9713)	1	100	0	0
Leica Bond Polymer Refine (DS9800)	14	79	18	72
Other	3	100	3	100
Power Vision DPVB999 HRP	1	100	1	100
Ventana iView system (760-091)	1	100	2	100
Ventana OptiView (760-700) + Amp. (7/860-099)	0	0	1	100
Ventana OptiView Kit (760-700)	7	100	18	94
Ventana UltraView Kit (760-500)	37	100	23	100

Cytology Run: 118

Automation

	ER		Ki67	
	N	%	N	%
BioGenex GenoMX 6000i	0	0	1	100
Dako Autostainer Link 48	8	88	8	100
Dako Autostainer Plus Link	1	100	1	100
Dako Omnis	2	100	3	100
Leica Bond Max	8	63	7	100
Leica Bond-III	10	100	14	57
Other	1	100	1	100
Ventana Benchmark GX	3	100	3	67
Ventana Benchmark ULTRA	37	100	36	100
Ventana Benchmark XT	14	100	10	100

Cytology Run: 118

Chromogen

	ER		Ki67	
	N	%	N	%
AS PER KIT	12	92	15	93
Dako DAB K3468	1	100	0	0
DAKO DAB+	2	50	1	100
Dako DAB+ Liquid (K3468)	0	0	1	100
Dako FLEX DAB	6	100	8	100
Leica Bond Polymer Refine kit (DS9800)	15	87	17	71
Other	3	100	6	100
Ventana DAB	5	100	11	91
Ventana iview	3	100	2	100
Ventana Ultraview DAB	37	100	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 19/20 (UK NEQAS Slide) and 16/20 (In House slide) using this method.

Primary Antibody:	Dako FLEX RTU IR626 (MIB-1)
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	AS PER KIT

Ki67 - Method 2

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

Primary Antibody:	Dako 7240 (MIB-1) , 15 Mins, 24 ºC Dilution 1: 100
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590), PH: 7.6
HMAR:	None
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), 24 ºC., Time 1: 10 Mins, Time 2: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, 24 ºC Prediluted

Ki67 - Method 3

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

Primary Antibody:	Dako FLEX RTU IR626 (MIB-1) , 25 Mins, RT ºC Prediluted
Automation:	Dako Omnis
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis, Buffer: 0
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, RT ºC., Time 1: 5 Mins
Detection:	Dako EnVision FLEX (K8000/10) , 20 Mins, RT ºC Prediluted

Ki67 - Method 4

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

Primary Antibody:	Ventana RTU (30-9) 790-4286 , 16 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 8mins, PH: 9
EAR:	
Chromogen:	Ventana Ultraview DAB, Time 1: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC Prediluted

BEST METHODS - Secondary Antibody

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

ER - Method 1

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

Primary Antibody:	Ventana 790-4324 (SP1) , 16 Mins, RT ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	
Chromogen:	Ventana Ultraview DAB, RT ºC., Time 1: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, RT ºC Prediluted

ER - Method 2

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

Primary Antibody:	Thermo Fisher/ Neomarkers RM 9101-S (SP1) , 32 Mins, 37 ºC Dilution 1: 1/100
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 36mins
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	AS PER KIT

ER - Method 3

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

Primary Antibody:	Dako (EP1) M3643 , 30 Mins, 21 ºC Dilution 1: 40
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: FLEX HIGH PH
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 21 ºC., Time 1: 10 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 30 Mins, 21 ºC Prediluted

ER - Method 4

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

Primary Antibody:	Leica/Novocastra NCL-L-ER- 6F11 , 32 Mins, 37 ºC Dilution 1: 50
Automation:	Ventana Benchmark GX
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	
EAR:	
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700)

The Alimentary Tract Module: GIST Run 118

Amy Newman

	First Antibody	Second Antibody
Antigens Assessed:	CD117	DOG-1
Tissue Sections circulated:	Normal Appendix, GIST and Desmoid tumours	
Number of Registered Participants:	119	
Number of Participants this Run	118 (99%)	

Introduction

Gold Standard: CD117

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

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

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

Features of Sub-optimal Immunostaining: **(See Figs 2, 3b, 5 & 6)**

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

Second Antibody: 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 (see Figs 7, 10 & 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 (see Figs 8 &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 (Note: Mast cells are not expected to stain with DOG-1)

Tissue Distribution and Assessment Procedure

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

Assessment was carried out around a multi-header microscope with each slide being assessed by 4 independent assessors and a microscope lead. All 3 samples on the slide were viewed, and then a final overall score out of 5 was given by each individual assessor. These four scores were then combined to give an overall score out of 20.

Assessment Summary:

CD117

Results from the **CD117** assessment showed an overall acceptable pass rate of 84%. This is an improvement from the previous Run 117, where the pass rate was 75%. The predominant reason for sub-optimal marks was due to very weak staining of the GIST. Non-specific and inappropriate false-positive staining was often observed in the desmoid tumour which should be negative for CD117 expression. Inappropriate antibody dilution factor and antigen retrieval were the main causes of these issues. The staining in the appendix TMA control showed to be the least variable in terms of expression.

The most popular CD117 antibody of choice still remains the Dako polyclonal, used by 84 participants and showed an acceptable pass rate of 86% in this assessment run. The number of laboratories submitting an in-house control containing both a normal CD117 expression in the mast cells in GI epithelium and demonstration of tumour cells of a GIST.

NEQAS Pass Rates Run 117 v 118 CD117		
Run no	117	118
Acceptable	75% (N=85)	84% (N=98)
Borderline	18% (N=20)	13% (N=15)
Unacceptable	7% (N=8)	3% (N=3)

DOG-1

Pass rates were slightly lower for DOG-1 than CD117 at 78% compared to 84% respectively on the NEQAS submitted tissue. Pass rates for DOG-1 in-house control material was 81%. The main assessors comments on borderline and failing submissions focused on weak to very weak demonstration of the DOG-1 antigen.

The Leica (K9) antibody in both RTU and concentrate forms is most commonly used by 68% of participants who completed the on-line methods. The other antibody commonly employed was the Ventana (SP31) used by 21% of the cohort.

References

1. Cordless et al., Biology of Gastrointestinal Stromal Tumours. J Clin Oncol 2004, 22(18): 3813-3825.
2. Ostrowski J et al, Functional features of gene expression profiles differentiating gastrointestinal stromal tumours according to KIT mutations and expression, BMC Cancer, 2009; 9:413.
3. Lamba G et al, Current Management and Prognostic Features for Gastrointestinal Stromal Tumour (GIST), Exp Hematol Oncol 2012; 1:14.
4. Blay et al., Consensus meeting for the management of gastrointestinal stromal tumors. Report of the GIST Consensus Conference of 20-21 March 2004, under the auspices of ESMO. Ann Oncol 2005 6: 566-578.
5. Pisters PW and Patel SR., Gastrointestinal stromal tumours: current management, J Surg Oncol., 2010 Oct 1: 102(5):530-8.
6. West RB, Corless CL, Chen X et al. The novel marker, DOG1, is expressed ubiquitously in gastrointestinal stromal tumors irrespective of KIT or PDGFRA mutation status. Am. J. Pathol. 2004; 65; 107–113.
7. Espinosa I, Lee CH, Kim MK et al. A novel monoclonal antibody against DOG1 is a sensitive and specific marker for gastrointestinal stromal tumors. Am. J. Surg. Pathol. 2008; 32; 210– 218.
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.

Selected Images showing Optimal and Sub-optimal Immunostaining

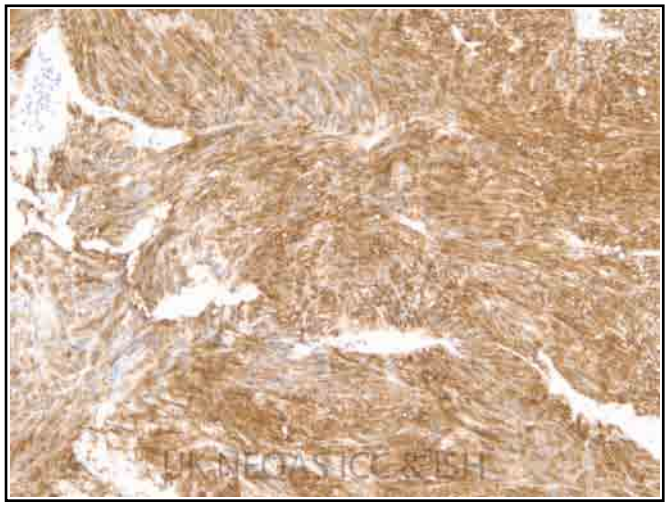


Figure 1: Excellent example of the NEQAS GIST using CD117. The staining is strong and specific to the tumour. Method: CD117 clone: Leica RTU (EP10); Leica Bond III (ER2, 30'; Polymer Refine kit). Score 20/20.

Figure 2: Weak staining of the NEQAS GIST sample with CD117. The tumour is positive giving the correct diagnosis, however, the staining could be stronger. Method: Dako A4502 (1:40); Leica BondmaX (ER1, 30'; Polymer Refine kit). Score 11/20.

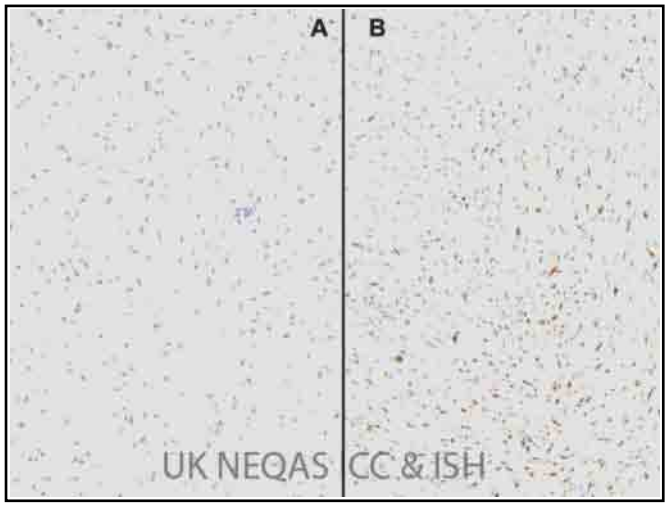


Figure 3A: Good example of NEQAS Desmoid tumour which should be negative for CD117 expression. Figure 3B: False-positive CD117 staining in the desmoid tumour which could lead to incorrect diagnosis. Method 3B: Dako A4502; PT Link; Autostainer Link 48 (FLEX+ kit). Score 9/20

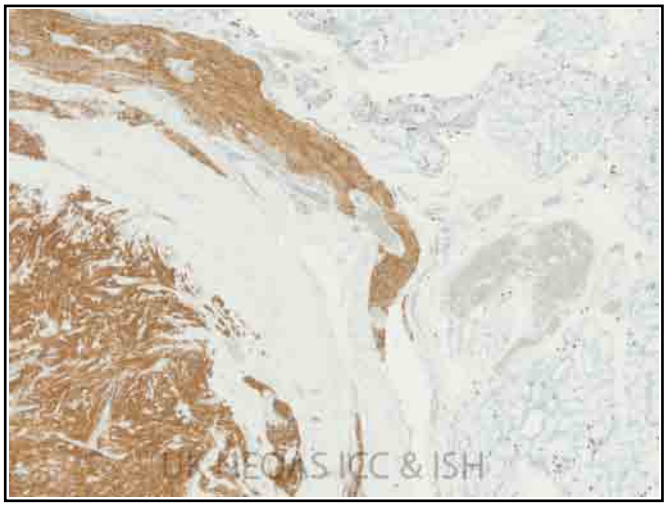


Figure 4: Excellent demonstration of an in-house control for CD117. There is excellent contrast between the positive GIST tumour and negative normal duodenal mucosa containing positive Cells of Cajal. Method: Dako A4502 (1:200); PT Link (pH9.0); Autostainer Link 48 (Flex+). Score 19/20

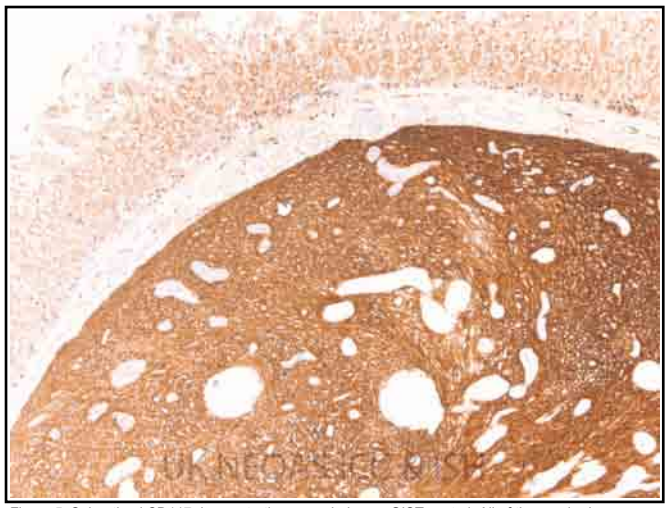


Figure 5: Suboptimal CD117 demonstration on an in-house GIST control. All of the required components are present, however, there is an unacceptable level of non-specific background staining within the normal mucosa which should be completely negative apart from the interstitial cells of Cajal. Method: As in Figure 3B. Antibody dilution and antigen retrieval pH not

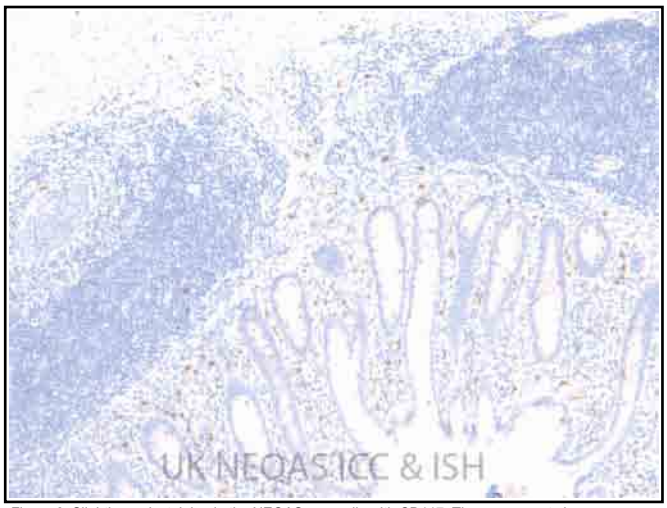


Figure 6: Slightly weak staining in the NEQAS appendix with CD117. The components have been demonstrated, however the staining could be stronger. Method: Dako A4502; Ventana Ultra (CC1 36'; UltraView Kit). Score 13/20.

Selected Images showing Optimal and Sub-optimal Immunostaining

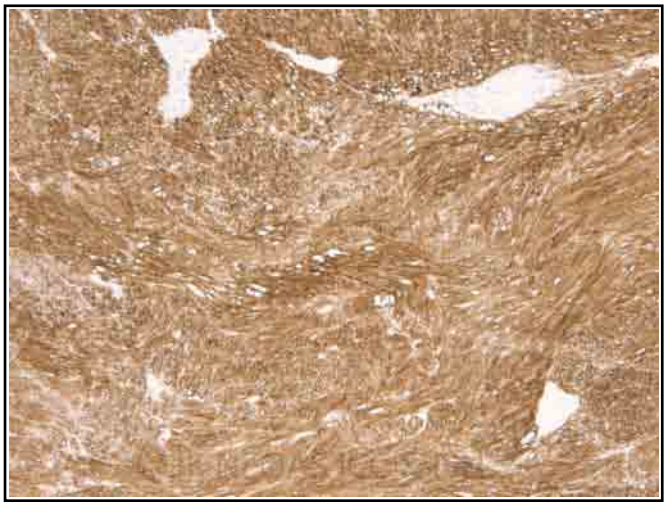


Figure 7: Good example of DOG-1 on the NEQAS GIST sample. The staining is strong and specific to the GIST tumour cells. Method: Leica PA0219 (K9); Leica Bond III (ER2, 20'; Polymer Refine).

Figure 8: Weak demonstration of DOG-1 on the NEQAS GIST sample. The correct components are expressing DOG-1, however, the staining could be stronger. Method: Leica NCL-L-DOG1 (K9) (1:60); Ventana ULTRA (CC1 64'; UltraView Kit). Score: 9/20

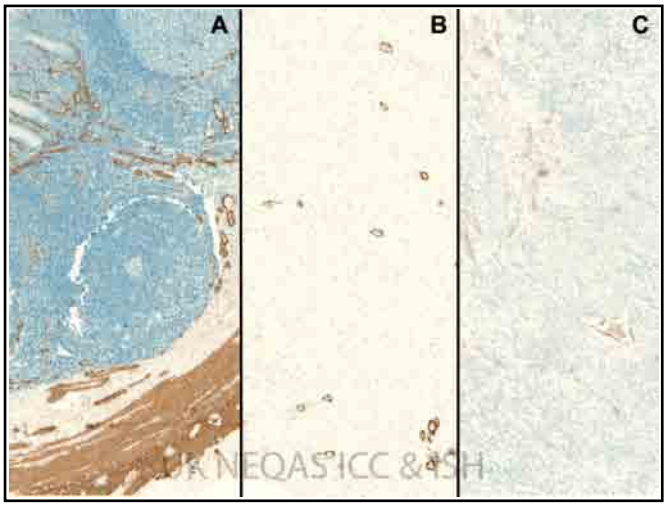


Figure 9: Alternative antibody SMA on the NEQAS distributed samples. (A) Appendix with staining in the smooth muscle fibres of the muscle wall, capillaries and vessels within the lymphoid and mucosal elements. (B & C) SMA expression in the blood vessel walls and negative in the desmoid and GIST tumour respectively.

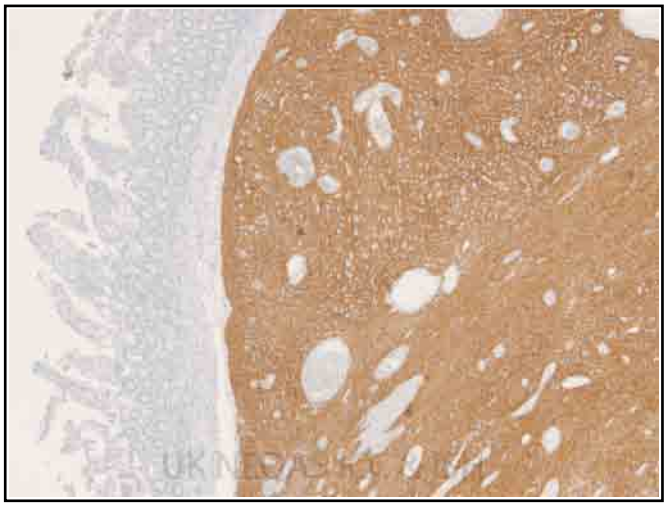
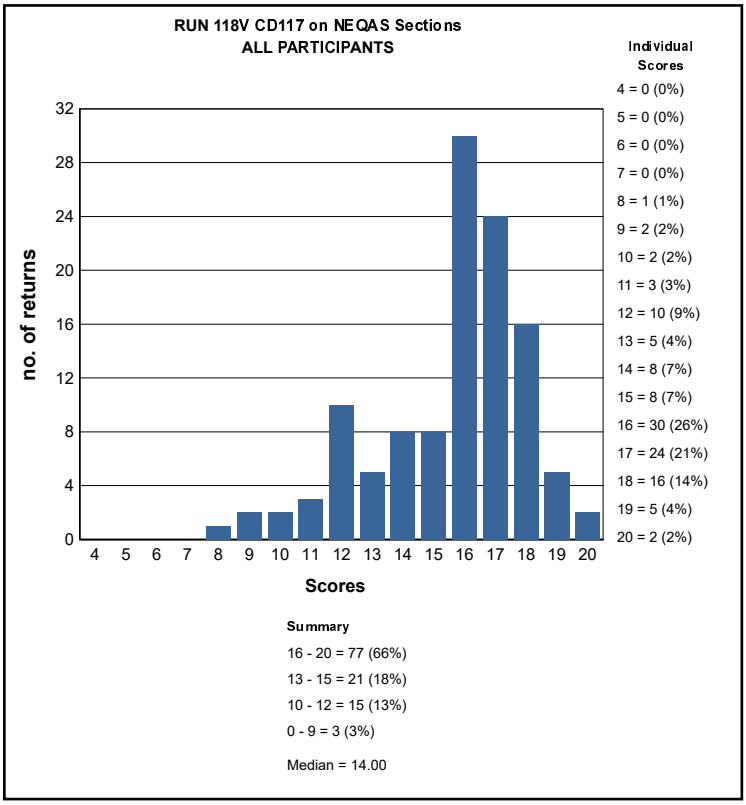


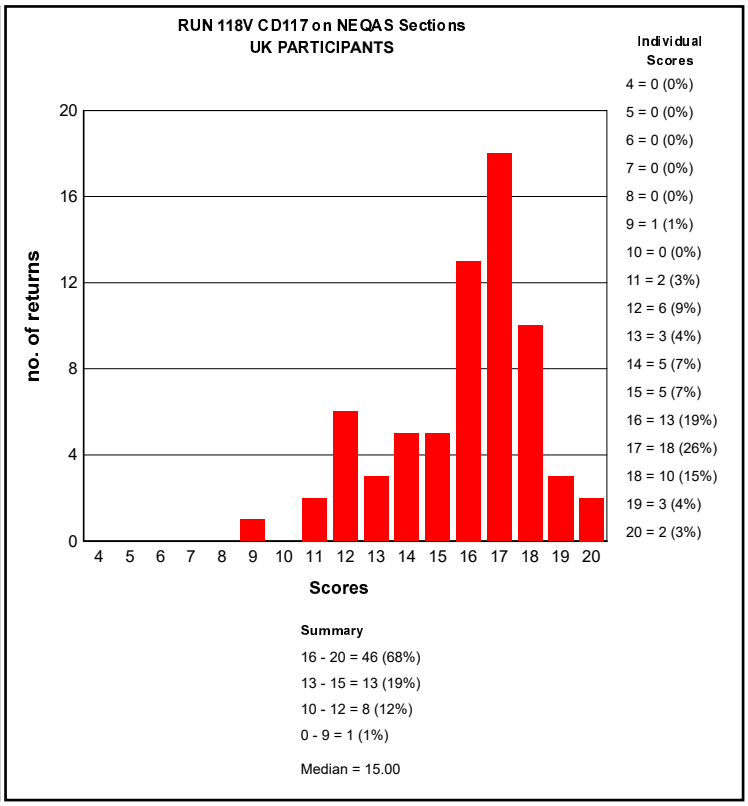
Figure 10: Excellent example of an in-house control for DOG-1. The GIST is positive and the normal mucosa is completely negative. Method: Abcam (ab53212); PT Link; Autostainer Link 48 (FLEX+ kit).

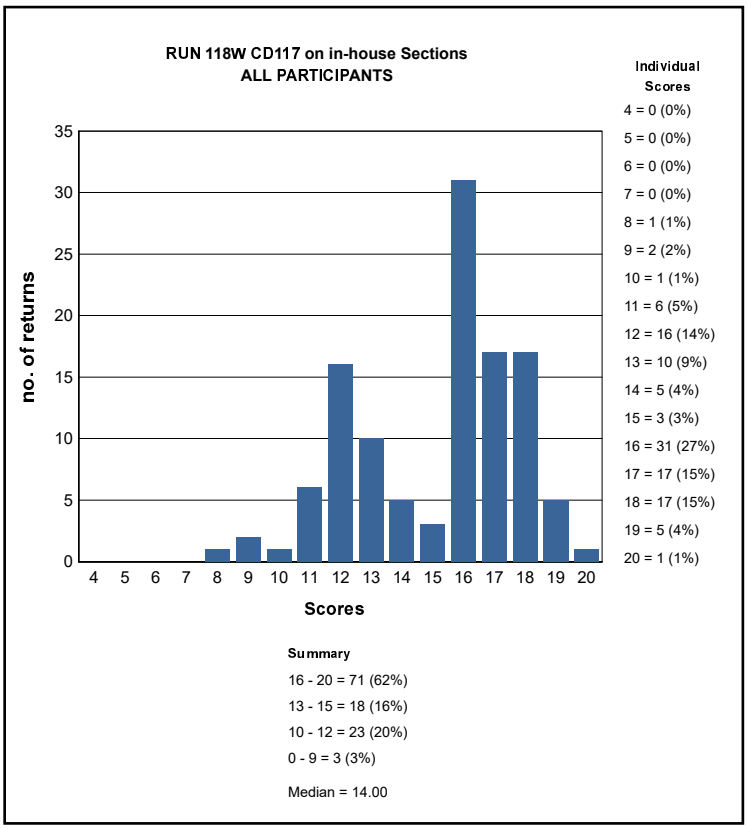
Figure 11: Good example of negative appendix on the NEQAS sample. DOG-1 should not demonstrate any components within the appendix as it is specific to GIST tumours.

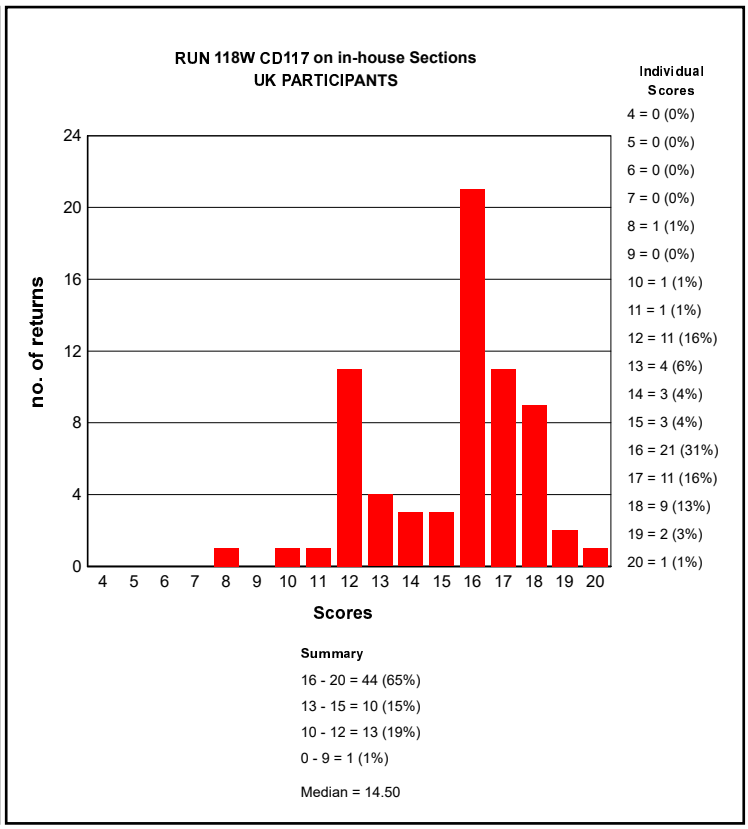
Figure 12: False-positive staining present within the lymphoid element on the NEQAS appendix sample. The expression presents as membrane staining in the lymphoid areas. This was a common feature noted with a specific DOG-1 clone. Method: Ventana (SP31) 760-4590; Ventana Ultra (CC1, 32'; OptiView). Score 12/20.

GRAPHICAL REPRESENTATION OF PASS RATES

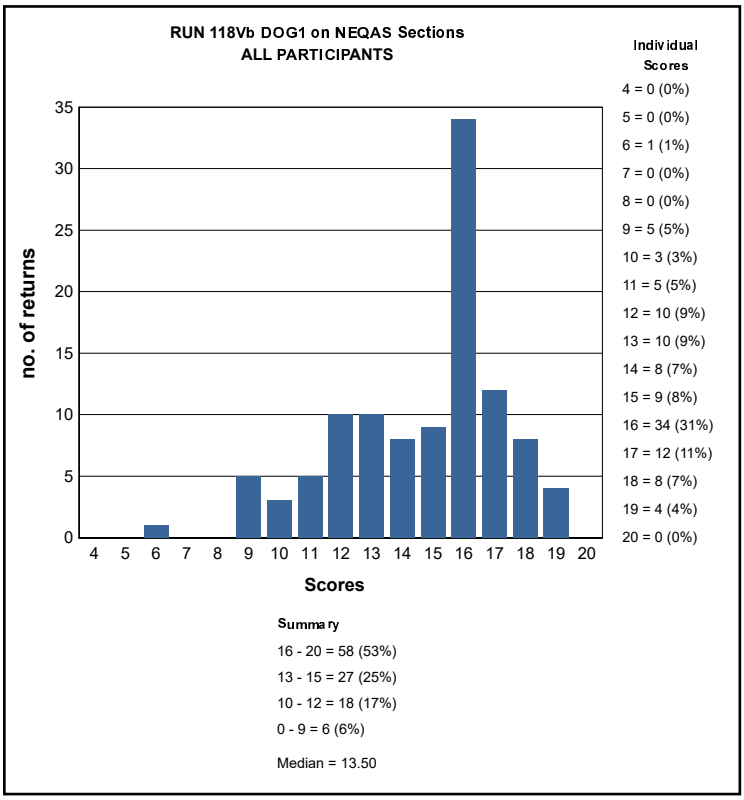


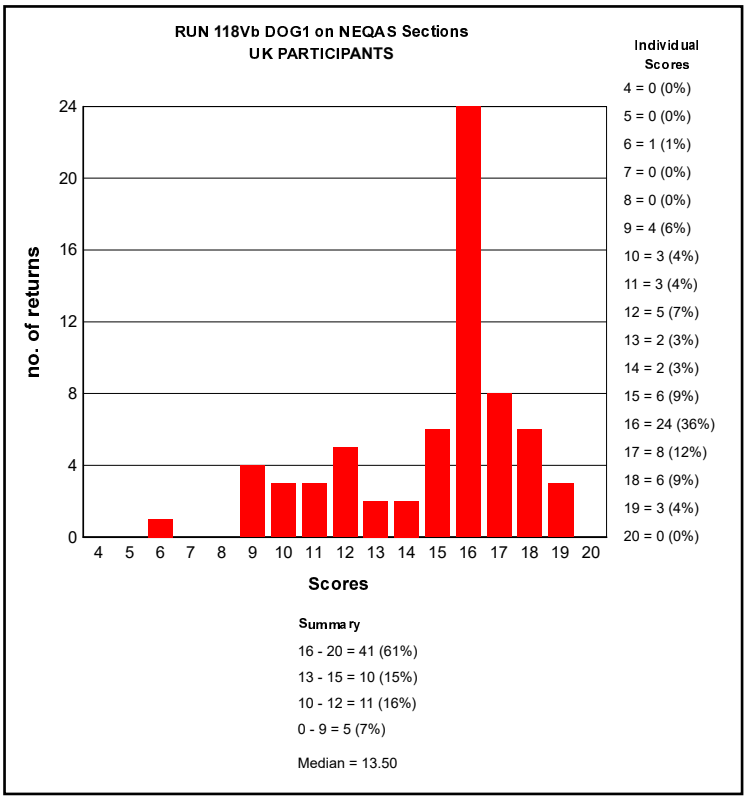


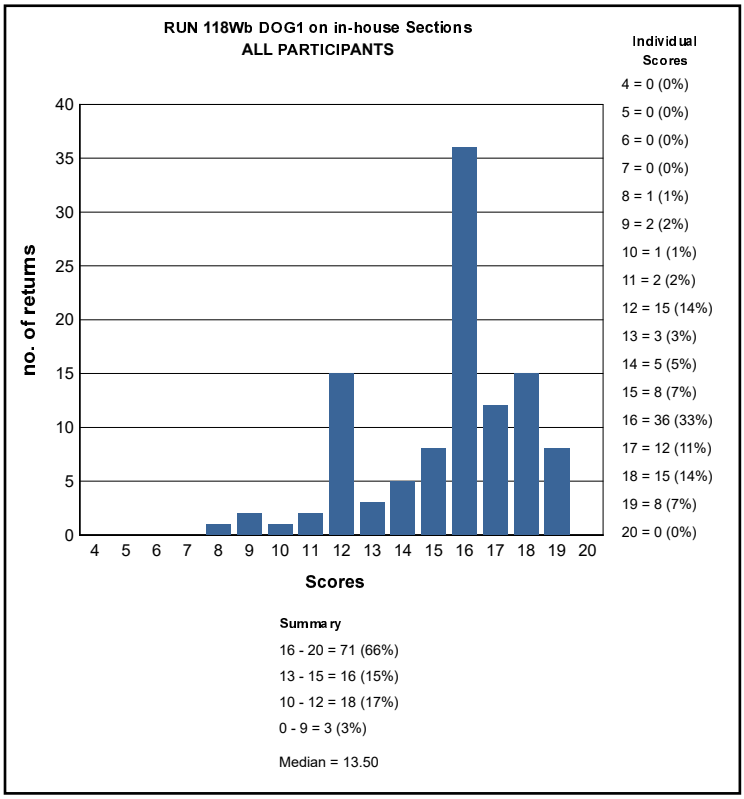


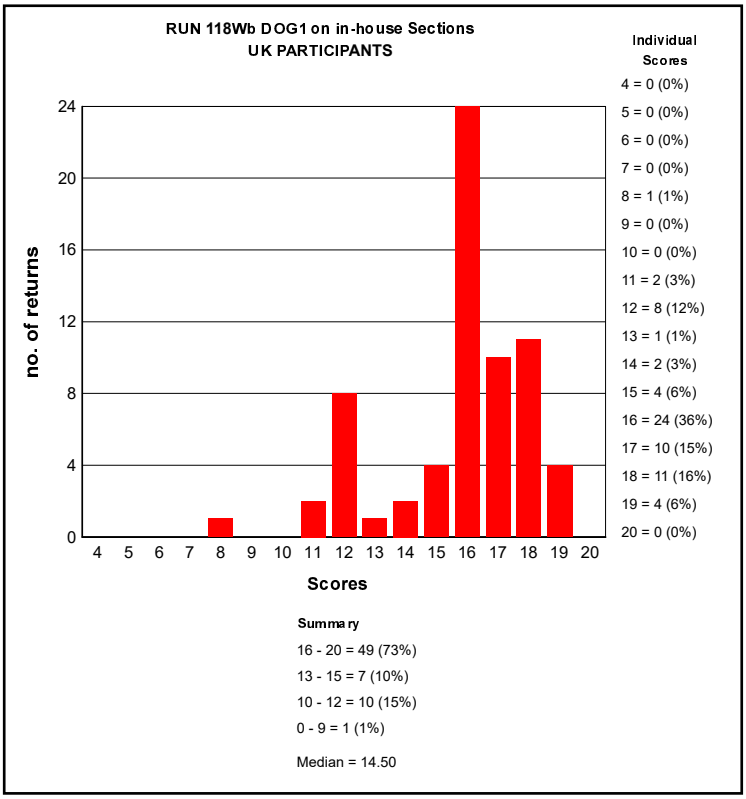


GRAPHICAL REPRESENTATION OF PASS RATES









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: 118

Primary Antibody : CD117

Antibody Details	N	%
Dako A4502 (rb poly)	84	86
Ventana 790-2939 (rb poly)	4	25
Cell Marque 117R/S-xx (YR145)	10	100
Leica/Novocastra NCL-L-CD117 (T595)	1	0
Ventana 790-2951 (9.7)	9	78
Other	1	100
Leica RTU (EP10) PA0007	7	100

Alimentary Tract Pathology Run: 118

Primary Antibody : DOG1

Antibody Details	N	%
Biocare CM 385 (1.1)	1	100
Cell Marque 244R-14/15/16 (SP31)	1	0
Leica NCL-L-DOG-1 (K9)	50	74
Leica PA0219 (K9)	23	96
Thermo RM-9132-R7 (SP31)	1	0
Other	5	100
Spring Biosciences M3311 (SP31)	1	0
Abcam TMEM16A (ab53212)	1	100
Ventana (SP31) 760-4590	23	78
Menarini MP-385-CM01/1	1	0
Diagnostic Biosystems Mob466 (DOG1.1)	1	100

Alimentary Tract Pathology Run: 118

Heat Mediated Retrieval	CD117 N	CD117 %	DOG1 N	DOG1 %
_Ventana Benk CC1 (Standard)	1	0	0	0
Dako Omnis	4	50	3	100
Dako PTLink	10	70	11	64
Leica ER1 10 mins	0	0	1	0
Leica ER1 20 mins	5	100	5	20
Leica ER1 30 mins	5	80	1	100
Leica ER2 10 mins	1	100	1	100
Leica ER2 20 mins	25	100	24	88
Leica ER2 30 mins	4	75	3	100
Leica ER2 40 mins	0	0	2	100
None	2	50	2	100
Pressure Cooker	0	0	1	0
Ventana CC1 16mins	1	100	0	0
Ventana CC1 24mins	0	0	2	100
Ventana CC1 32mins	9	56	9	67
Ventana CC1 36mins	6	67	2	50
Ventana CC1 40mins	1	100	0	0
Ventana CC1 48mins	2	100	3	100
Ventana CC1 52mins	1	100	0	0
Ventana CC1 56mins	4	100	1	100
Ventana CC1 64mins	18	94	20	80
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	4	75
Ventana CC1 92mins	1	100	0	0
Ventana CC1 mild	3	100	2	100
Ventana CC1 standard	8	75	10	90
Ventana CC2 mild	1	100	0	0

Alimentary Tract Pathology Run: 118

Enzyme Mediated Retrieval	CD117 N	CD117 %	DOG1 N	DOG1 %
AS PER KIT	1	100	1	100
NOT APPLICABLE	81	89	69	75

Alimentary Tract Pathology Run: 118	CD117		DOG1	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	7	86	8	75
Dako EnVision FLEX (K8000/10)	2	50	2	100
Dako EnVision FLEX+ (K8002/12)	7	71	6	67
Dako Envision+ HRP mouse K4004/5/6/7	0	0	1	0
Dako Envision+ HRP rabbit K4008/9/10/11	1	0	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)	38	95	34	79
None	1	100	0	0
Other	2	100	2	50
Ventana OptiView (760-700) + Amp. (7/860-099)	1	100	1	100
Ventana OptiView Kit (760-700)	27	81	23	78
Ventana UltraView Kit (760-500)	28	82	28	79

Alimentary Tract Pathology Run: 118	CD117		DOG1	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	0	0	1	0
Dako Autostainer Link 48	9	67	9	56
Dako Autostainer Plus Link	2	100	2	100
Dako Omnis	4	50	3	100
Leica Bond Max	14	86	13	85
Leica Bond-III	26	100	25	76
Ventana Benchmark GX	1	100	1	100
Ventana Benchmark ULTRA	45	82	41	80
Ventana Benchmark XT	14	79	12	83

Alimentary Tract Pathology Run: 118	CD117		DOG1	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	14	93	12	67
DAKO DAB+	2	0	0	0
Dako EnVision Plus kits	1	100	1	100
Dako FLEX DAB	10	70	9	67
Leica Bond Polymer Refine kit (DS9800)	38	95	36	81
Other	5	60	5	100
Ventana DAB	14	86	16	75
Ventana Ultraview DAB	31	84	29	79

BEST METHODS - Gold Standard Antibody

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

CD117 - Method 1

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

Primary Antibody: Dako A4502 (rb poly) , 15 Mins Dilution 1: 1/200
Automation: Leica Bond Max
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 20 mins
EAR:
Chromogen: Leica Bond Polymer Refine kit (DS9800)
Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins, rt ºC Prediluted

CD117 - Method 2

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

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

CD117 - Method 3

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

Primary Antibody:	Dako A4502 (rb poly) , 30 Mins, 20 ºC Dilution 1: 200
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer, PH: 7.6
HMAR:	Dako PTLink, Buffer: High pH TRS, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 20 ºC., Time 1: 10 Mins, Time 2: 10 Mins
Detection:	Dako EnVision FLEX+ (K8002/12)

CD117 - Method 4

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

Primary Antibody:	Cell Marque 117R/S-xx (YR145) , 60 Mins, 37 ºC Dilution 1: 200
Automation:	Ventana Benchmark XT
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 88mins, Buffer: CC1
EAR:	
Chromogen:	AS PER KIT, 37 ºC., Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 37 ºC Prediluted

BEST METHODS - Secondary Antibody

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

DOG1 - Method 1

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

Primary Antibody:	Leica NCL-L-DOG-1 (K9) , 15 Mins, 23 ºC
Automation:	Leica Bond-III
Method:	Other
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 30 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800), 23 ºC., Time 1: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, 23 ºC

DOG1 - Method 2

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

Primary Antibody:	Leica PA0219 (K9) , 30 Mins, RT ºC Prediluted
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800), RT ºC., Time 1: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, RT ºC Prediluted

DOG1 - Method 3

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

Primary Antibody:	Leica NCL-L-DOG-1 (K9) , 15 Mins Dilution 1: 25
Automation:	Dako Omnis
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB
Detection:	Dako EnVision FLEX (K8000/10)

DOG1 - Method 4

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

Primary Antibody:	Leica NCL-L-DOG-1 (K9) , 32 Mins, 36 ºC Dilution 1: 50
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	
HMAR:	Ventana CC1 64mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB, 36 ºC., Time 1: 8 Mins, Time 2: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 36 ºC Prediluted

Lynch Syndrome/HNPCC Run 118

Suzanne Parry and Amy Newman

	Gold Standard	Second Antibody
Antigens Assessed:	MLH1	PMS2
Tissue Sections circulated:	Positive and negative colonic tumours and normal appendix	
Number of Registered Participants:	93	
Number of Participants This Run:	MLH1: 91(98%), PMS2: 92 (99%)	

General Introduction

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

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

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

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

Mismatch Repair Markers

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

Clinical Interpretation & Reporting Guidelines

We recommend reporting MMR IHC findings in tumours as either:

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

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

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

Clinical Reporting

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

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

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

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

Further Discussion on MMR proteins

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

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

Assessment Procedure:

Composite slides were distributed to all participants for them to stain with **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:
Appendix: (Figs 1, 9)
Strong nuclear staining of the epithelium at the base and lower third-half of the crypts with fading of nuclear staining intensity in the middle and upper thirds of the crypts, to negative or weak staining of epithelial nuclei at the luminal surface.

- Strong staining of lymphoid follicles.

Tumour without loss of MMR protein: (Figs 3, 8)

. Strong nuclear staining in the tumour cells.

- Strong nuclear staining in the lymphocytes and stromal cells.

Tumour with loss of MMR protein: (Figs 2, 7)

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

Features of Suboptimal Immunostaining:
Appendix: (Fig 4)

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

Tumour without loss of MMR protein: (Figs 5, 12)

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

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

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

Assessment Summary:

The NEQAS section pass rates for the **MLH1** assessment were similar to the previous time (Run 116) this antibody was assessed, with 73% of labs achieving an acceptable pass, and a further 16% of participants receiving a borderline score (10-12/20), and therefore an overall pass of 89%. There was a fail rate of 11%. The predominant reasons for a borderline or failed result was due to either weak staining or background staining. The in-house MLH1 had a slightly better result with only 4% (4 laboratories) receiving a fail result, again due to very weak demonstration of antigen and/or excessive background. The Ventana (M1) clone was the most popular choice of antibody (used by 45% of participants), and showed an acceptable pass rate of 76%. The Dako (ES05) clone was also popular , used by 26% of laboratories and showed an acceptable pass rate of 62%.

The **PMS2** assessment showed a lower pass rate to that of the previous run (Run 116). 71% of participants received an acceptable pass, and a further 14% received a borderline pass. The failure rate was higher than previous runs at 15% (n=14) Similarly to the MLH1 assessment, weak staining and excessive background were the main reasons for failure or borderline scores (depending on the severity). The Ventana (EPR3947) clone was the most popular choice of PMS2 antibody used in this assessment by 45% of participants, and showed an acceptable pass rate of 78%. The Dako (EP51) clone was also commonly used, and showed an overall acceptable pass rate of 70%.

In-House Control Tissue Recommendations

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

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1. Vasen HF, Möslein G, Alonso A et al.,(2007) Guidelines for the clinical management of Lynch syndrome (hereditary non-polyposis cancer). J Med Genet. 44 6):353-62. Free download from: http://www. jmg.bmj.com/cgi/content/full/44/6/353.
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4. Frayling IM, Happerfield L, Mattocks C, Oakhill K, Arends MJ. Application of Molecular Diagnostics to Hereditary Nonpolyposis Colorectal Cancer (chapter 32). In: "Molecular Diagnostics, For the Clinical Laboratorian" (2nd Edition). Eds: Coleman WB & Tsongalis GJ. Humana Press Inc., NJ. 2005. ISBN: 1-59259-928-1, ISBN13: 978-1-58829-356-5; ISBN10: 1-58829-356-4. pp 375 – 392.
5. Poulogiannis G, Frayling IM, Arends MJ. DNA Mismatch Repair Deficiency in Sporadic Colorectal Cancer and Lynch Syndrome. *Histopathology* 2010; 56: 167 - 179.a.
6. Vasen F, et al. Revised guidelines for the clinical management of Lynch syndrome (HNPCC): recommendations by a group of European experts. Gut 2013;62: 812-823.

Selected Images showing Optimal and Sub-optimal Immunostaining

Figure 1: Good example of NEQAS appendix with MLH1. The staining is strongest towards the lower part of the crypts and decreases towards to the top. There is strong nuclear staining in the lymphoid follicles. Method: Ventana (M1) RTU; Ventana Ultra (CC1, 64'; Optiview + amp).

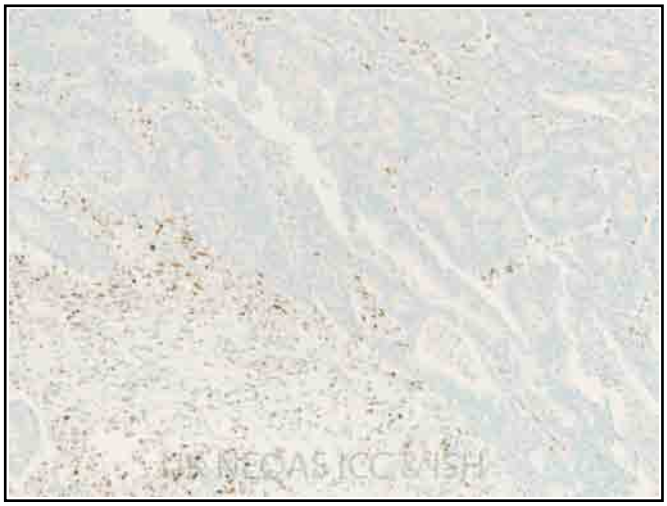


Figure 2: Excellent staining in the NEQAS negative control. There is loss of MLH1 expression within the tumour and positive internal controls of lymphocytes and stromal cells. Method as in Figure 1.

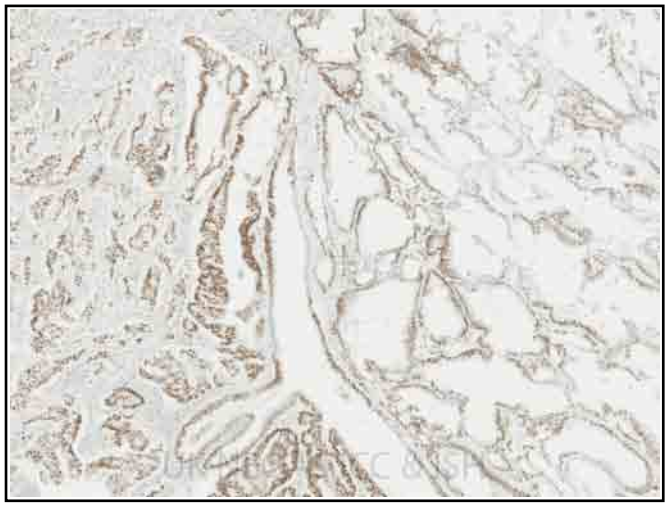


Figure 3: Good example of an in-house control with positive tumour expression for MLH1. The nuclear staining is strong and specific and there is also normal lymphoid and stromal cells staining. Method as in Figure 1.

Figure 4. Shows sub-optimal staining of the NEQAS appendix. The MLH1 staining is weak and there is minimal staining within the crypts accompanied by a slight non-specific background staining. Method: Leica RTU (ES05); Leica Bond III (ER1 30'; Polymer Refine). Score 9/20.

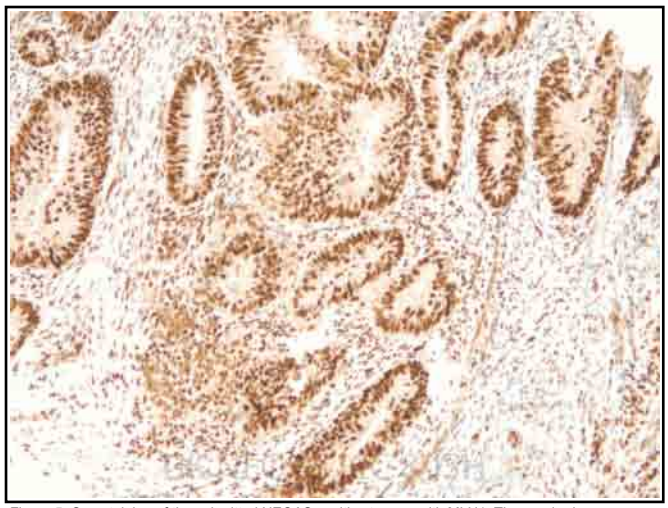


Figure 5: Overstaining of the submitted NEQAS positive tumour with MLH1. The required components are staining, however, there is also non-specific background staining. Method: Dako (ES05) (1:50); Ventana XT (CC1, 88'; Optiview + amp). Score 11/20.

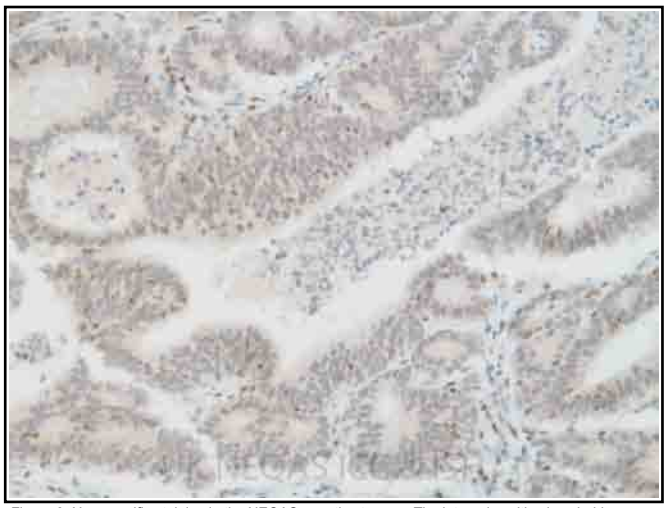


Figure 6: Non-specific staining in the NEQAS negative tumour. The internal positive lymphoid and stromal cells are staining, however there is a background hue giving the appearance of weak false-positivity in some of the negative tumour cells. Method: Dako (ES05) (1:50, 60'); Biocare Decloaker (Tris EDTA); Manual staining + Dako EnVision REAL detection. Score 9/20.

Selected Images showing Optimal and Sub-optimal Immunostaining

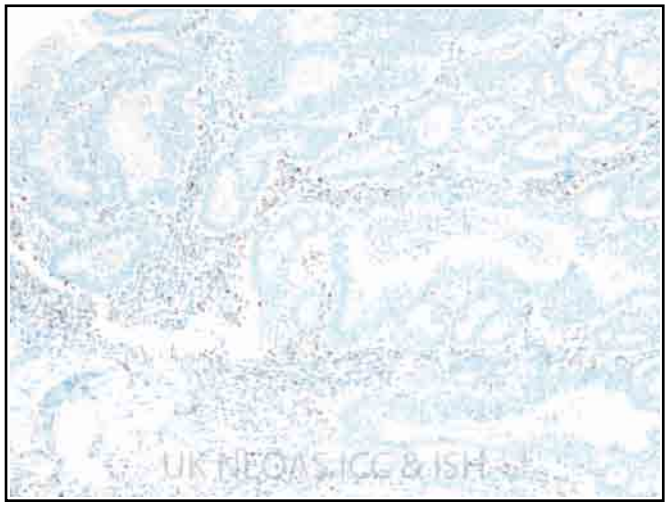


Figure 7: Good demonstration of loss of PMS2 expression in NEQAS negative tumour. The surrounding lymphocytes and stromal cells have good nuclear staining and act as an internal positive control. Method: Ventana (EPR3947); Ventana ULTRA (CC1 92'; Optiview + amp).

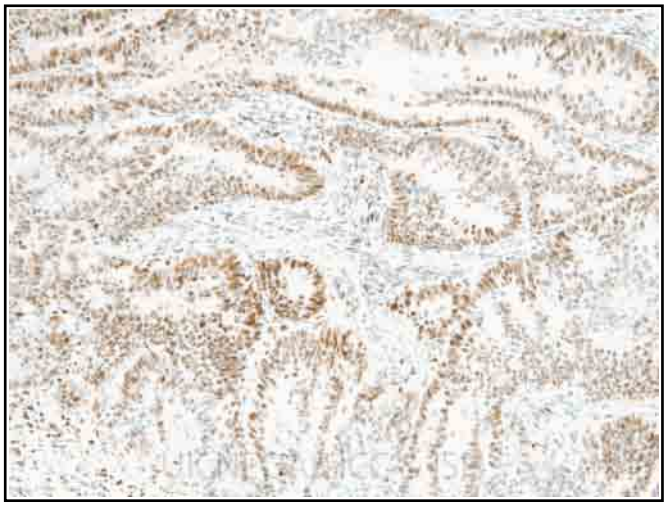


Figure 8: This method as described in Figure 7 also produced excellent staining in the NEQAS positive tumour. There is strong specific nuclear staining within the tumour cells and surrounding normal tissue. Score: 18/20.

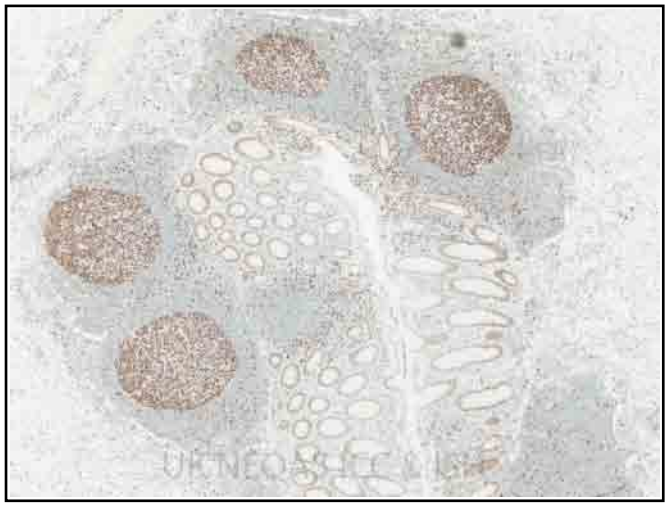


Figure 9: Good demonstration of PMS2 staining on an in-house appendix. The staining in the lower crypts could be slightly stronger, however the lymphoid tissue is staining adequately. Method: Ventana (EPR3947) RTU; Ventana ULTRA (CC2 92'; Optiview + amp).

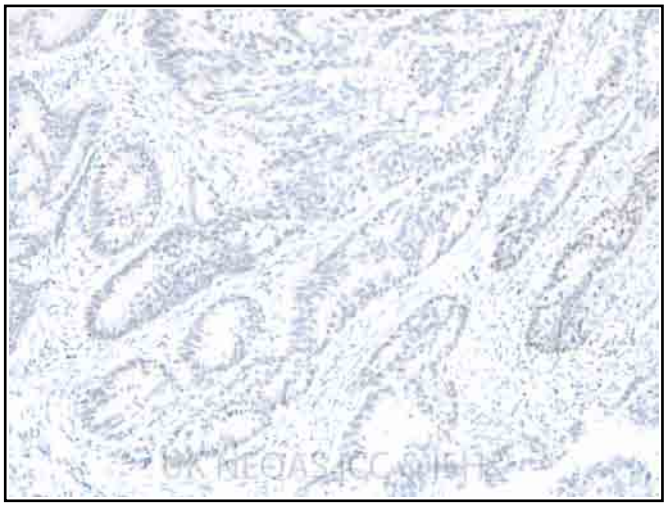


Figure 10: Weak demonstration of PMS2 in the NEQAS positive tumour. The staining is very weak and in many nuclei it is negative. Method: Bio SB (EP51) (1:20; 52') (Cat: BSB2124); Ventana ULTRA (CC1 48'; Optiview + amp).

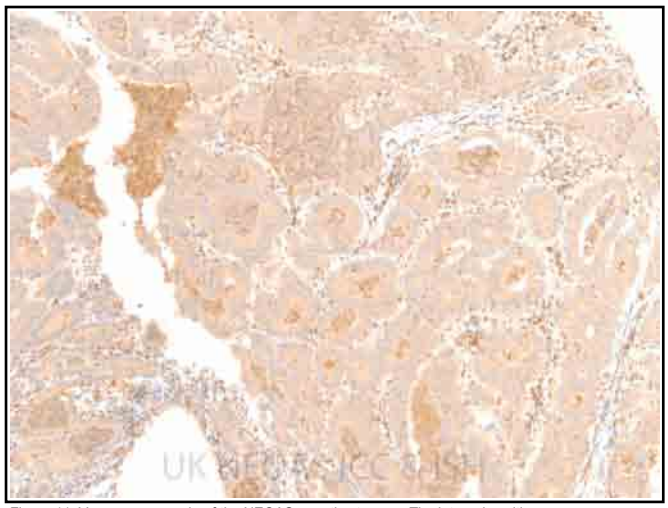


Figure 11: Very poor example of the NEQAS negative tumour. The internal positive lymphocytes show diffuse staining and there is excessive non-specific background staining in the tumour. Method: Dako (EP51) (1:30; 15'); Leica Bond III (ER2 40'; Polymer Refine 8', 8').

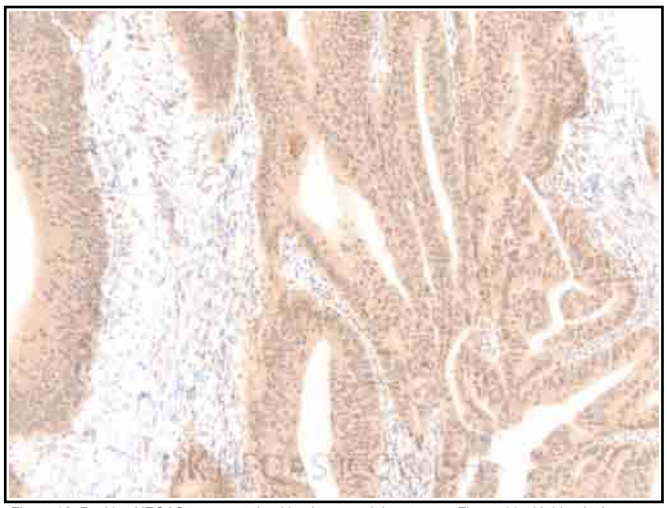
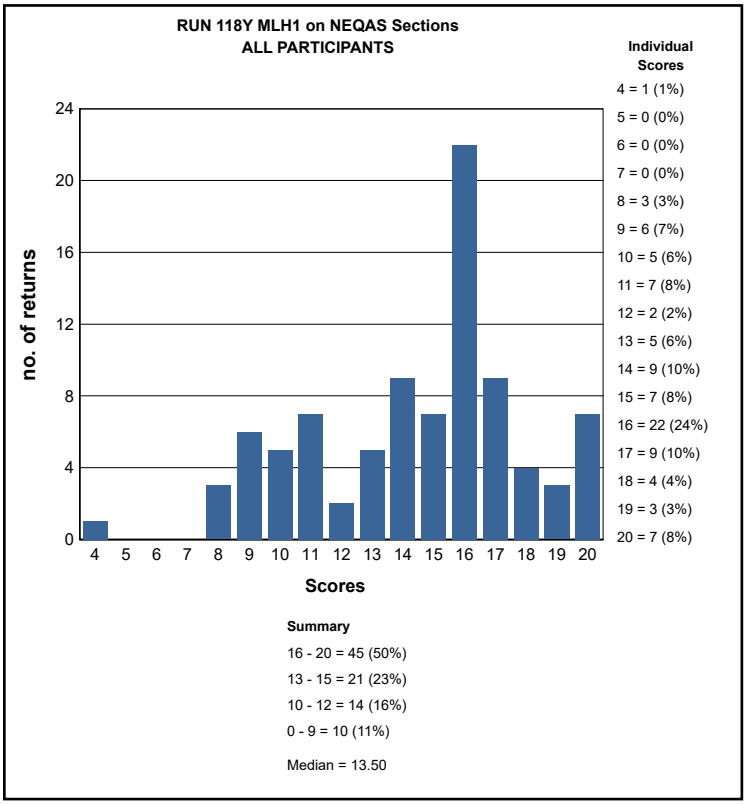
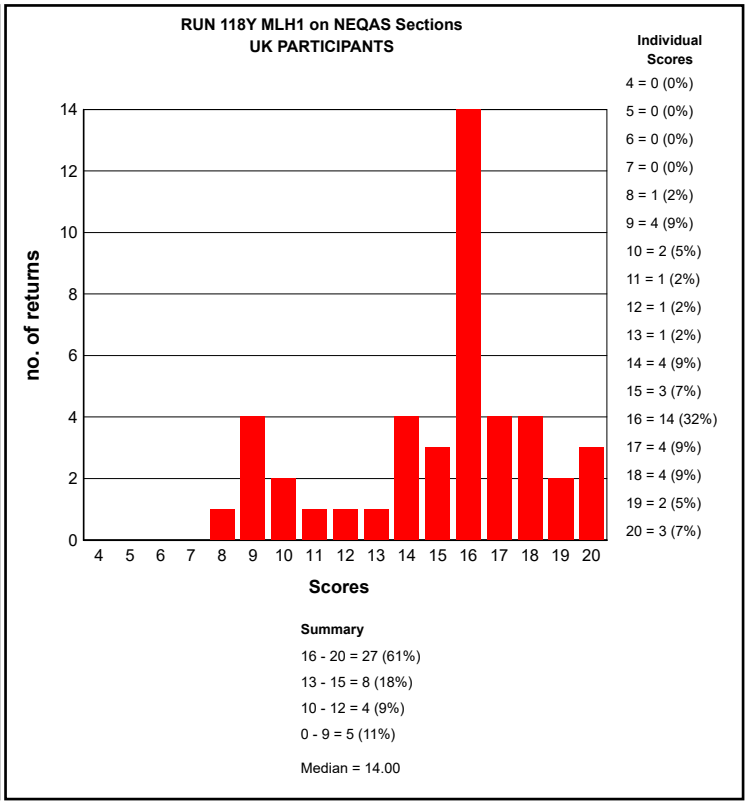
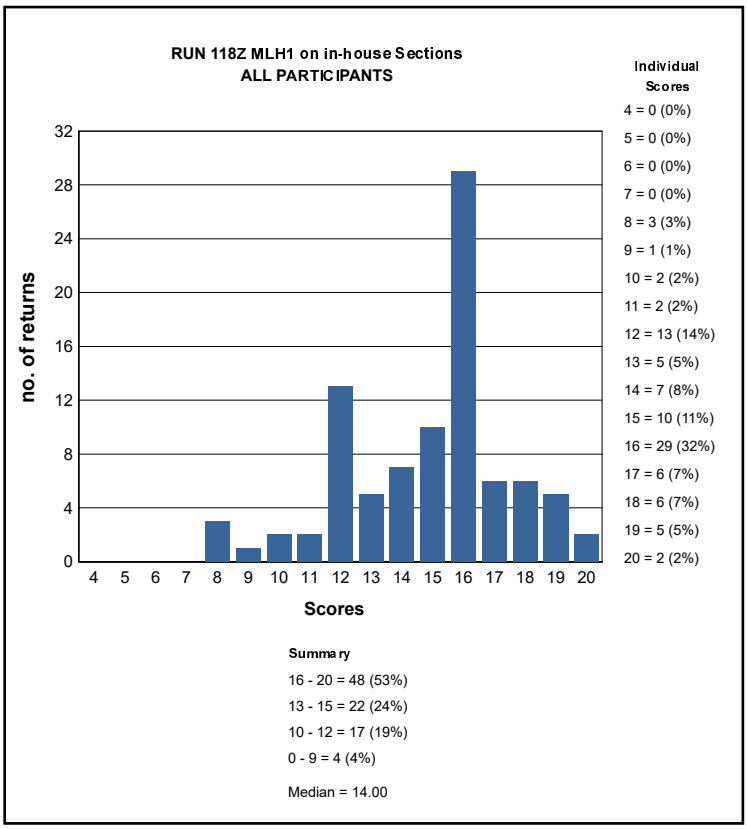


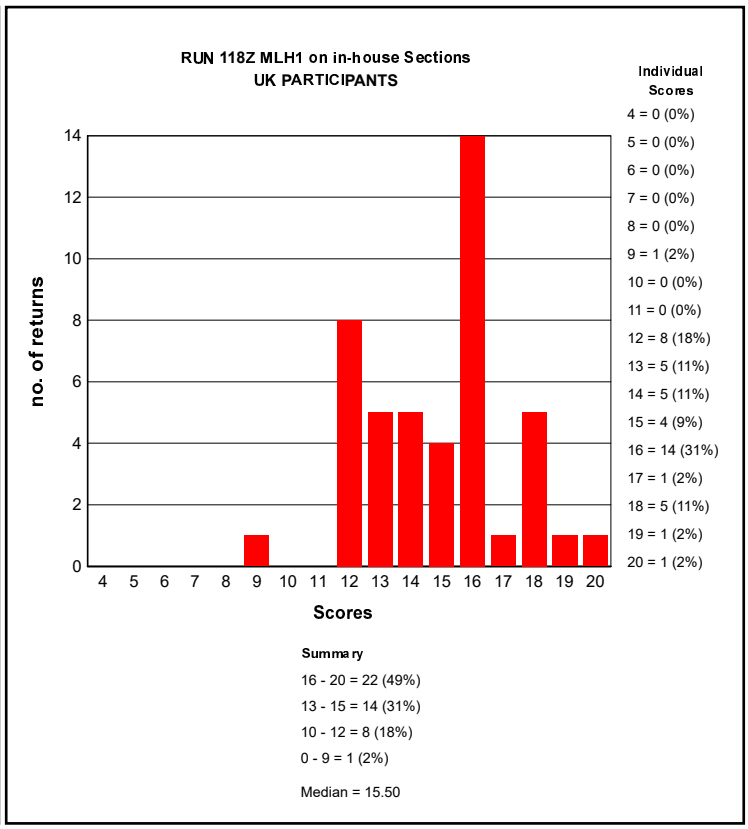
Figure 12: Positive NEQAS tumour stained by the same laboratory as Figure 11 with identical protocol. The staining is weak and not localised to the tumour nuclei. From the methods described in Figure 11, the incubation times are shorter than recommended for mismatch repair proteins. Refer to Best Methods section for optimal protocols. Score 9/20.

GRAPHICAL REPRESENTATION OF PASS RATES

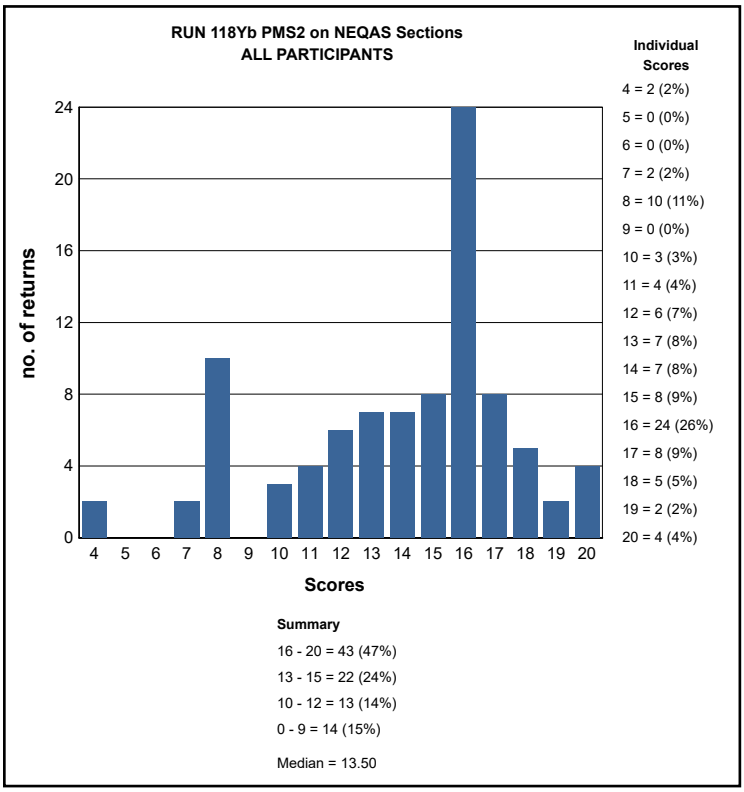


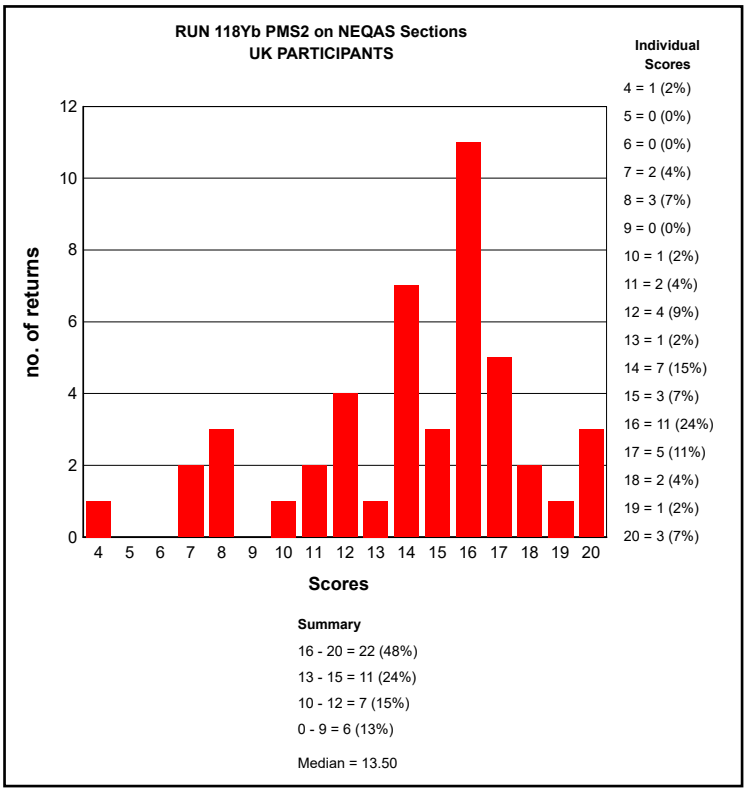


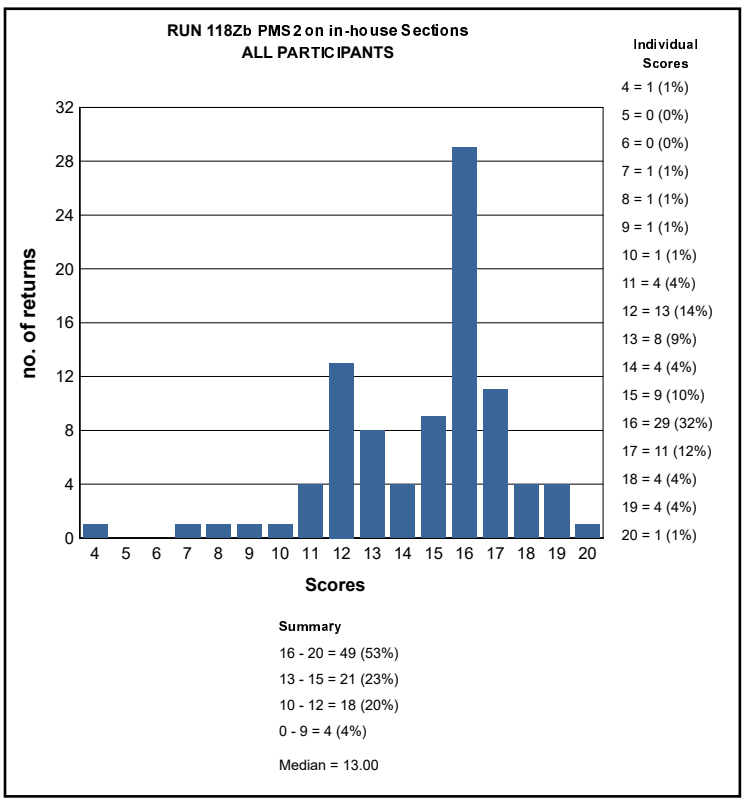


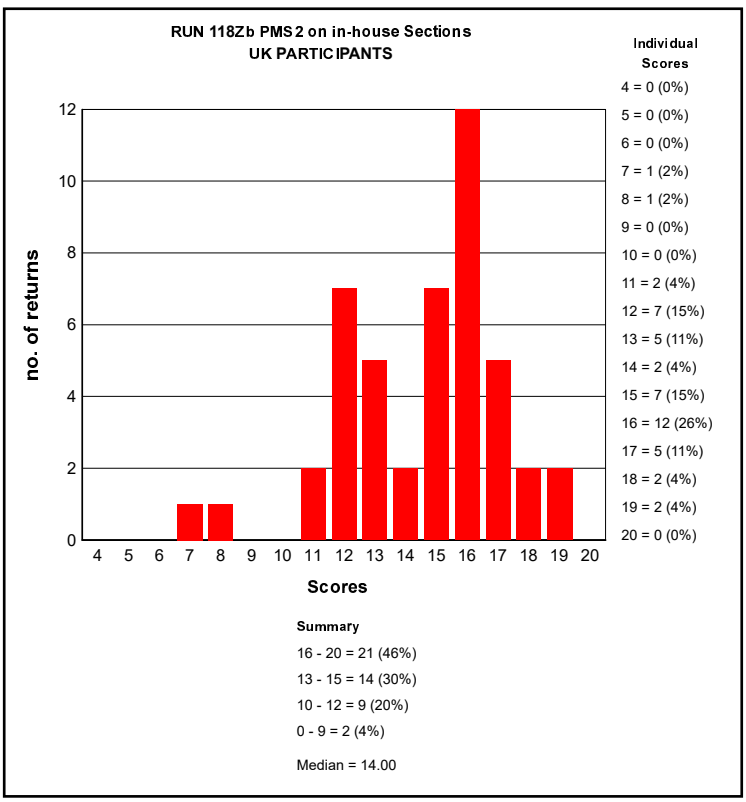


GRAPHICAL REPRESENTATION OF PASS RATES









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: 118

Primary Antibody : MLH1

Antibody Details	N	%
Other	1	0
BD Pharmingen (G168-15)	5	80
Biocare medical CM/PM 220 (G168-15)	2	100
Ventana 760-4264 (G168-728)	1	100
Novocastra NCL-L-MLH1 (ES05)	13	85
Dako M3640 (ES05)	14	64
Leica Bond RTU PA0610 (ES05)	3	67
Dako Flex RTU IR079/IS079 (ES05)	10	60
Ventana 790-4535 (M1)	41	76

Primary Antibody : PMS2

Antibody Details	N	%
BD Bio/Pharmingen 556415 (A16-4)	9	67
Cell Marque 288R -17/18 (EPR3947)	4	75
Leica/Novoca NCL-L-PMS2 (MOR4G)	3	0
Other	1	0
Ventana 760-4531 (EPR3947)	41	78
Cell Marque 288M -16 (MRQ28)	1	100
Dako M3647 (EP51)	19	68
Dako RTU FLEX IR087 (EP51)	12	75
Epitomics AC-0049 (EP51)	2	50

HNPCC Run: 118

Heat Mediated Retrieval	MLH1 N	MLH1 %	PMS2 N	PMS2 %
Biocare Decloaking Chamber	1	0	1	100
Dako Omnis	2	50	3	33
Dako PTLink	9	89	9	100
Lab vision PT Module	1	0	1	0
Leica ER1 30 mins	2	0	1	0
Leica ER1 40 mins	2	50	1	0
Leica ER2 20 mins	5	80	5	60
Leica ER2 30 mins	9	78	7	71
Leica ER2 40 mins	8	88	11	64
Other	1	100	1	100
Ventana CC1 24mins	1	0	0	0
Ventana CC1 32mins	4	50	1	100
Ventana CC1 36mins	1	0	1	100
Ventana CC1 40mins	5	80	2	50
Ventana CC1 48mins	4	25	2	50
Ventana CC1 56mins	3	67	4	25
Ventana CC1 64mins	22	91	10	60
Ventana CC1 72mins	1	100	1	100
Ventana CC1 80mins	2	100	4	75
Ventana CC1 88mins	2	50	2	100
Ventana CC1 92mins	1	0	19	95
Ventana CC1 standard	5	80	3	67
Ventana CC2 64mins	0	0	1	0
Ventana CC2 92mins	0	0	2	50

HNPCC Run: 118

Enzyme Mediated Retrieval	MLH1 N	MLH1 %	PMS2 N	PMS2 %
AS PER KIT	3	100	2	100
NOT APPLICABLE	47	74	53	70

HNPCC Run: 118	MLH1		PMS2	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	4	75	3	33
Dako EnVision FLEX (K8000/10)	2	50	2	50
Dako EnVision FLEX+ (K8002/12)	7	86	8	88
Dako Envision+ HRP mouse K4004/5/6/7	1	100	1	100
Leica Bond Polymer Define (DS9713)	0	0	1	100
Leica Bond Polymer Refine (DS9800)	24	75	23	65
Other	3	67	3	33
Ventana OptiView (760-700) + Amp. (7/860-099)	15	87	21	81
Ventana OptiView Kit (760-700)	29	66	24	75
Ventana UltraView Kit (760-500)	5	60	3	33

HNPCC Run: 118	MLH1		PMS2	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	0	0	1	100
Dako Autostainer	1	0	1	0
Dako Autostainer Link 48	7	86	7	100
Dako Autostainer plus	1	100	1	100
Dako Autostainer Plus Link	1	100	1	100
Dako Omnis	2	50	3	33
Leica Bond Max	10	80	8	75
Leica Bond-III	17	71	18	56
None (Manual)	1	0	1	100
Ventana Benchmark GX	1	0	1	0
Ventana Benchmark ULTRA	40	75	41	76
Ventana Benchmark XT	10	70	9	67

HNPCC Run: 118	MLH1		PMS2	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	17	65	17	59
Dako EnVision Plus kits	1	100	1	100
Dako FLEX DAB	9	78	10	80
Dako REAL EnVision K5007 DAB	1	0	1	100
Leica Bond Polymer Refine kit (DS9800)	24	75	23	70
Other	8	88	7	57
Ventana DAB	22	82	26	77
Ventana Enhanced Alk. Phos. Red Detection Kit	0	0	1	0
Ventana Ultraview DAB	9	44	6	83

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) , 32 Mins, 37 ºC Prediluted
Automation: Ventana Benchmark ULTRA
Method: Ventana Optiview
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 64mins
EAR:
Chromogen: AS PER KIT
Detection: Ventana OptiView (760-700) + Amp. (7/860-099)

MLH1 - Method 2

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

Primary Antibody: Biocare medical CM/PM 220 (G168-15) Dilution 1: 100
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 40 mins
EAR: NOT APPLICABLE
Chromogen: Leica Bond Polymer Refine kit (DS9800)
Detection: Leica Bond Polymer Refine (DS9800)

MLH1 - Method 3

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

Primary Antibody:	Dako M3640 (ES05) , 40 Mins, 21 ºC Dilution 1: 50
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: HIGH PH ANTIGEN RETRIEVAL, PH: 9
EAR:	
Chromogen:	Dako FLEX DAB, 21 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 20 Mins, 21 ºC Prediluted

MLH1 - Method 4

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

Primary Antibody:	Leica Bond RTU PA0610 (ES05) , 25 Mins, 20 ºC Prediluted
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

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 18/20 (In House slide) using this method.

Primary Antibody:	Dako M3647 (EP51) , 40 Mins, 21 ºC Dilution 1: 50
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako Wash Buffer (S3006)
HMAR:	Dako PTLink, Buffer: HIGH PH ANTIGEN RETRIEVAL
EAR:	
Chromogen:	Dako FLEX DAB, 21 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 20 Mins, 21 ºC Prediluted

PMS2 - Method 2

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

Primary Antibody:	BD Bio/Pharmingen 556415 (A16-4) , 30 Mins, 20 ºC Dilution 1: 100
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer, PH: 7.6
HMAR:	Dako PTLink, Buffer: High pH TRS, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 20 ºC., Time 1: 10 Mins, Time 2: 10 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 15 Mins, 20 ºC Prediluted

PMS2 - Method 3

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

Primary Antibody:	Dako M3647 (EP51) , 60 Mins, 37 ºC Dilution 1: 50
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 88mins, Buffer: CC1
EAR:	
Chromogen:	AS PER KIT, 37 ºC., Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 37 ºC Prediluted

PMS2 - Method 4

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

Primary Antibody:	Cell Marque 288M -16 (MRQ28)
Automation:	Dako Autostainer plus
Method:	Dako FLEX+ kit
Main Buffer:	AS PER KIT, PH: 7.4
HMAR:	Dako PTLink, PH: 9
EAR:	AS PER KIT
Chromogen:	AS PER KIT
Detection:	Dako Envision+ HRP mouse K4004/5/6/7

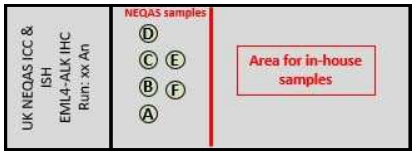
ALK for NSCLC Module Run 118

Suzanne Parry

Gene Assessed:	ALK
Sections Circulated:	Composite slide consisting of ell lines and Non-small cell lung carcinoma (NSCLC) tissue samples with different levels of ALK expression, and normal appendix tissue
Number of Registered Participants:	63
Number of Participants This Run	61 (97%)

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

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



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. A more recent publication by Savic and colleagues[12] indicates that certain IHC antibodies perform equally well but still recommend a combined use with FISH testing.

Assessment Criteria

Same slide NEQAS & In-house controls

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

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

Interpretation criteria incorporating staining intensity

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

Assessment scoring guidelines

- A team of four assessors independently but simultaneously scored all of the returned slides, and provided interpretation and feedback on technical issues.
- Each of the 4 assessors also provided a possible score out of 5, with marks summed together to give a final score out of 20.

Table 2: Assessment interpretation

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

Features of Optimal Staining. (Figs 1, 3, 5, 6, 7 & 8)

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

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Features of Sub-optimal Staining (Figs 2 & 4)

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

Results & Discussion

Distributed NEQAS Sample Results

There was a decrease in the number of participants receiving an acceptable pass, compared to the previous assessment run, with 65% (N=46) receiving scores of more than 12/20, compared to 80% in the Run 117 assessment. In the current run a further 15% of labs (N=9) received borderline passes (scores of 10-12/20), and 6 labs (10%) failed the assessment. The borderline scores were mostly due to weak staining or inappropriate background or non-specific staining. Those laboratories that failed the assessment showed a significantly low level of staining, and therefore a cause for concern of a clinically incorrect result. One particular laboratory showed no staining of the appendix ganglion cells. This control is a very good control to help gauge then sensitivity of the assay. The appendix should show strong staining of the ganglion and axons, and if this is not staining, then this suggests that the assay is not working optimally.

Most laboratories are now using the recommended Ventana/ Roche (D5F3) assay. In the current run this was used by 49 participants (80%) and showed an acceptable pass rate of 82%. One lab using this assay failed the assessment as they did not include the amplification step in the method which is required for this assay, therefore the staining was seen to be very weak: In a clinical setting this method would not detect some ALK positive cases. The Cell Signalling Technology D5F3 antibody was used by 4 participants, and this showed an acceptable pass rate of 75%.

In-house Control Results

All participating laboratories taking part in this assessment also submitted their in-house controls. The acceptable pass rates on the in-house material was only slightly higher than that on the NEQAS material; with 68% (N=42) receiving an acceptable pass. A further 28% (N=17) participants obtained a borderline pass result and 2 (3%) participants failed with the in-house sections. Many of the borderline passes lost marks due to not providing the required in-house material as outlined below (ALK IHC control recommendation). Laboratories not submitting the required in-house control received a maximum borderline score of 12/20.

Lymphoma control

Lymphoma is not recommended as a control in the lung setting. A lymphoma control can lead to potential false-negative result in the lung setting. Participating labs are provided with instructions on the recommendations set out by UK NEQAS ICC for the choice of in-house controls. (See recommendations below). Any labs that do not provide this composite control tissue is scored a maximum borderline mark of 12/20.

ALK IHC Control Recommendation

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

a. NSCLC ALK IHC positive tumour: Gauges sensitivity
b. NSCLC ALK IHC negative tumour: Gauges specificity

Appendix may also be used alongside the lung tumour controls to gauge both the sensitivity & specificity, but should not be used 'alone' as a single control.

- Commercially available control material, such as cell lines, showing at least positive and negative ALK IHC expression are also acceptable when used alongside a participants own in=house material.
- In all cases the control material should initially be validated using FISH.
- It is also recommended and good practice to cut and place control material alongside the clinical sample being tested on every slide.

References:

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14. Georg Hutarew, Cornelia Hauser-Kronberger, Felix Strasser et al., (2014) Immunohistochemistry as a screening tool for ALK rearrangement in NSCLC: evaluation of five different ALK antibody clones and ALK FISH. histopahology 65:398-407.

Selected Images showing Optimal and Sub-optimal Immunostaining

Fig 1. Good staining of the UK NEQAS distributed positive tumour (sample A). The section shows moderate to strong membranous and cytoplasmic staining in the neoplastic cells. Stained with the Ventana D5F3 assay on the Benchmark XT, CC1 extended, Optiview detection with amplification.

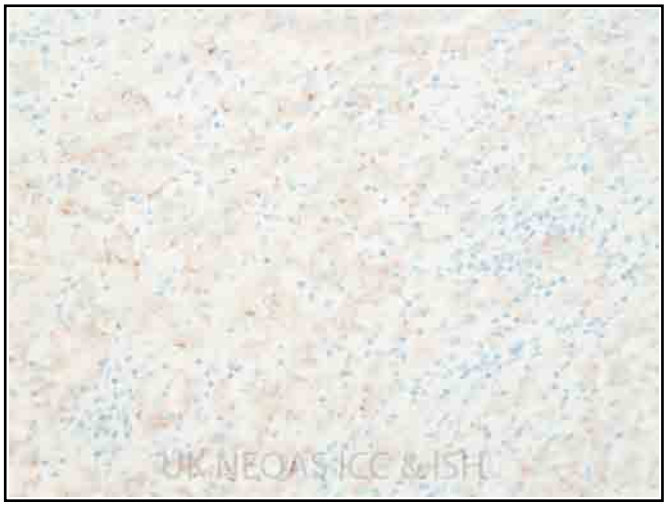


Fig 2. Sub-optimal ALK staining of the UK NEQAS distributed positive tumour (sample A) (compare with Fig 1). The staining is much weaker than expected. Section stained with the Ventana D5F3 assay on the Ventana Benchmark XT. (Same protocol as Fig 1).

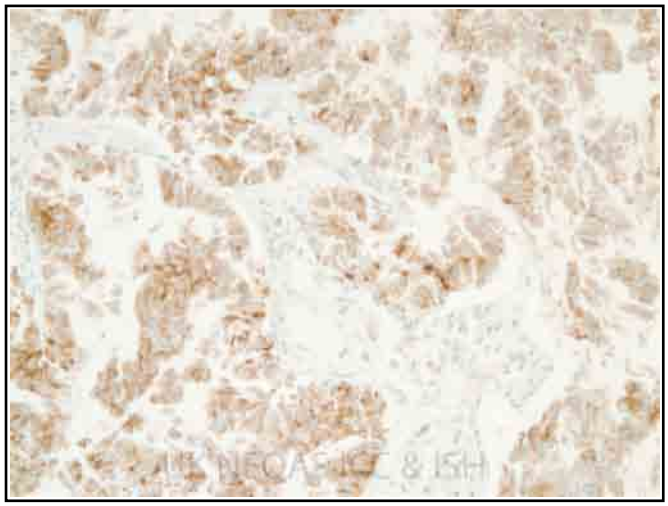


Fig 3. Good staining on the UK NEQAS distributed positive tumour (sample B). This tumour was slightly weaker than tumour A, and showed moderate to strong staining. The example was stained using a laboratory-devised technique with the Cell Signalling D5F3 clone on the Ventana Benchmark XT platform, Optivew detection with amplification.

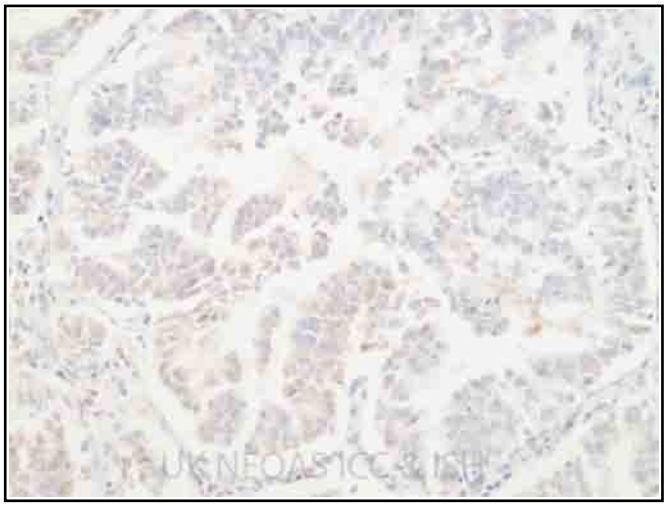


Fig 4. Sub-optimal staining of the UK NEQAS distributed positive tumour (sample B) stained with the Ventana D5F3 clone (compare to Fig 3). The staining is much weaker than expected for this clone; most likely due to the omission of the amplification step required for this assay.

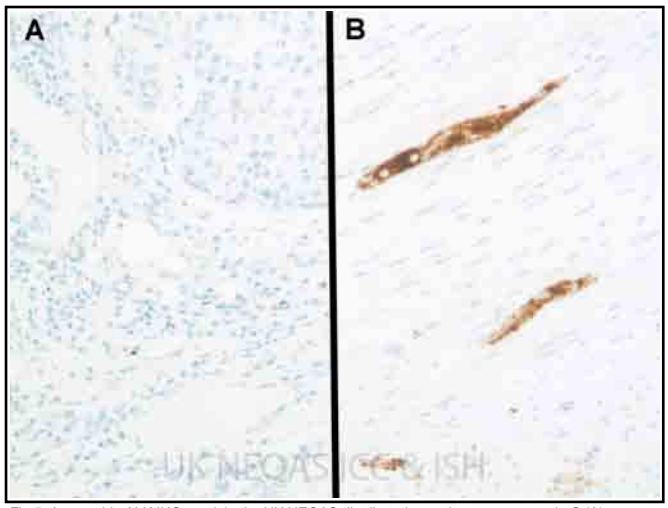


Fig 5. Acceptable ALK IHC result in the UK NEQAS distributed negative tumour sample C (A), and the appendix sample D (B). The appendix shows the expected positive staining of ganglion cells and axons. Both sections stained using the Ventana D5F3 assay with the recommended protocol.

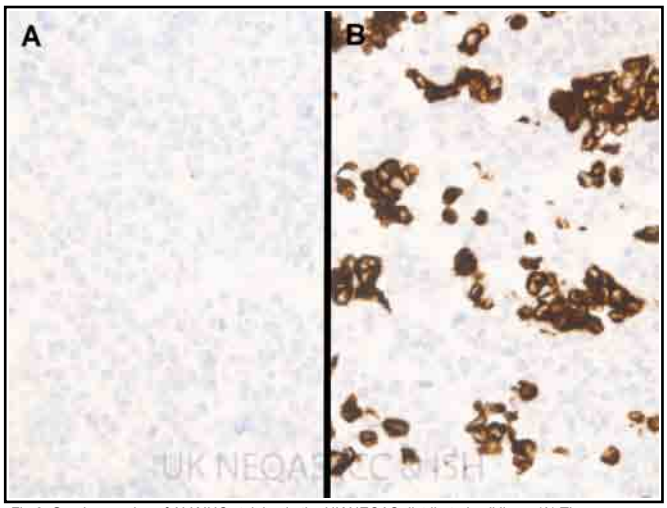


Fig 6. Good examples of ALK IHC staining in the UK NEQAS distributed cell lines: (A) The negative cell line, (sample E) shows the expected result and is therefore negative for ALK. Whereas the image in (B) (sample F) is the positive cell, showing strong membranous and cytoplasmic staining of the expected proportion of neoplastic cells. Stained with the Ventana

Selected Images showing Optimal and Sub-optimal Immunostaining

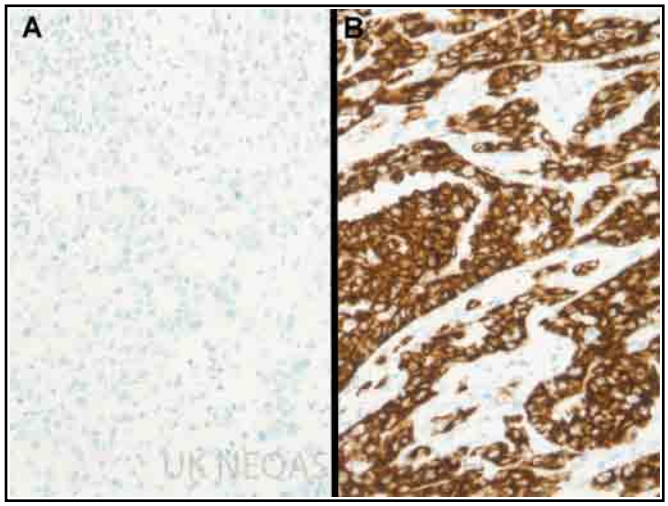
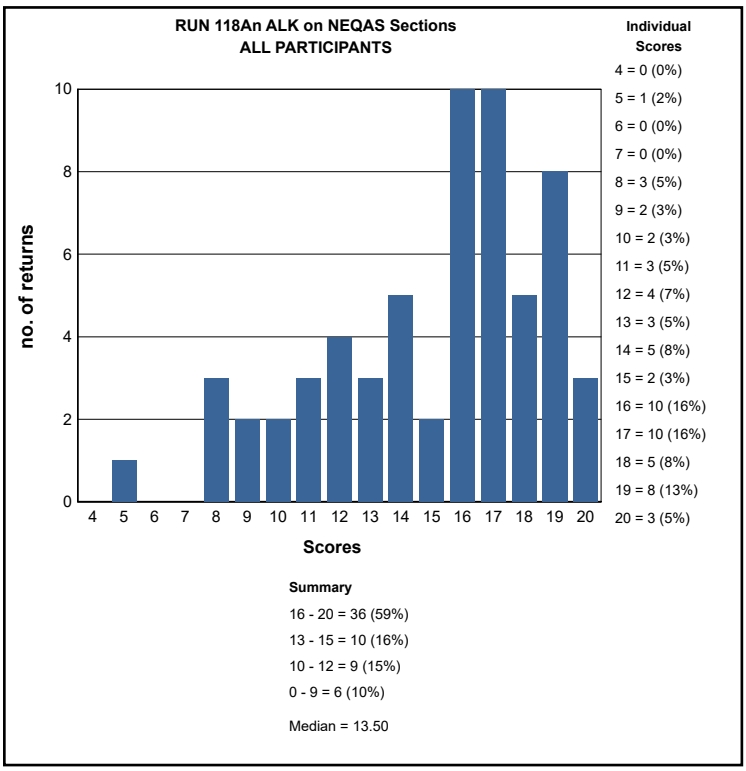
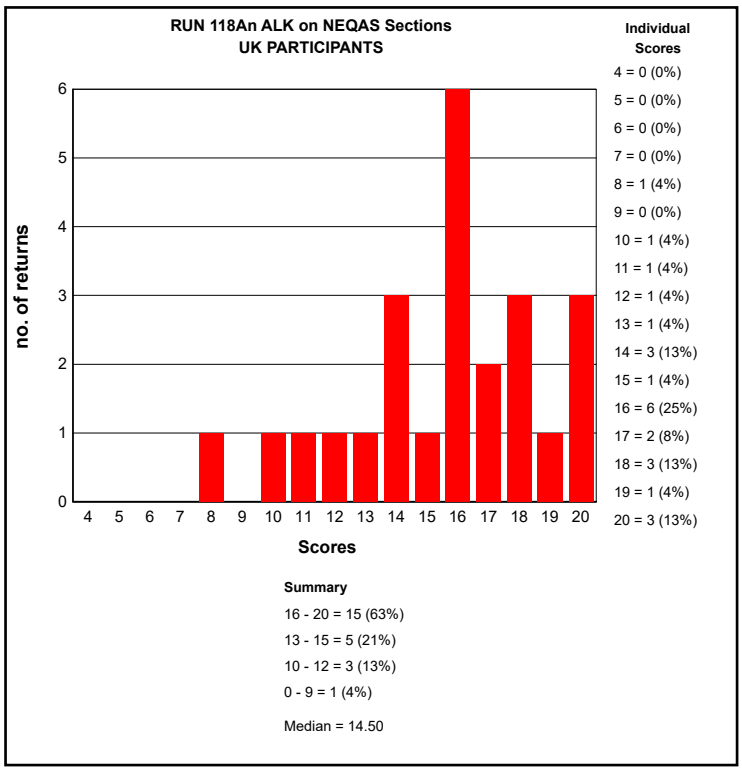


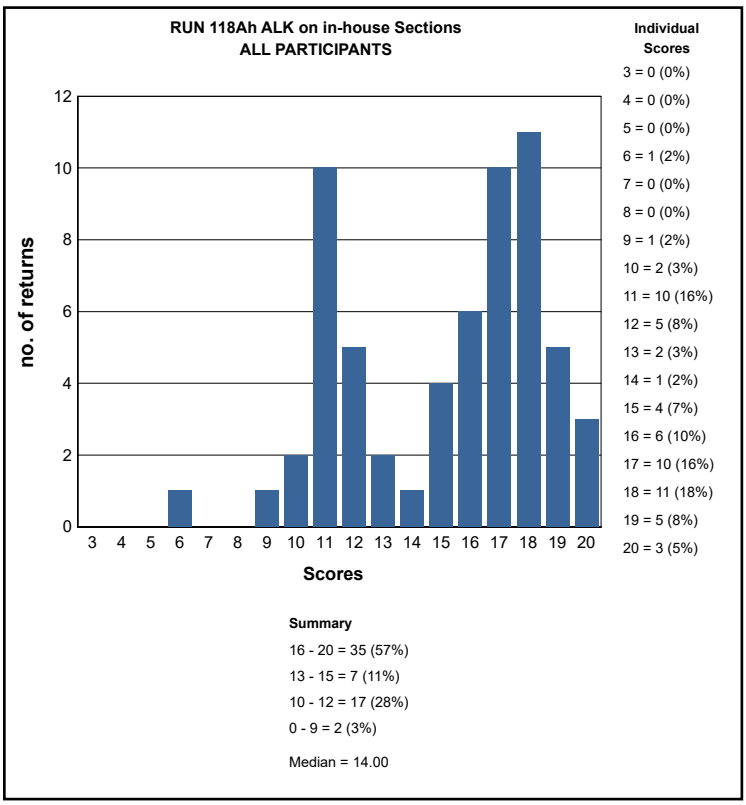
Fig 7. Good in-house NSCLC ALK negative and positive controls (see also Fig 8 for the accompanying appendix control submitted by the participant). Stained using the Ventana D5F3 assay on a Benchmark XT with the recommended protocols.

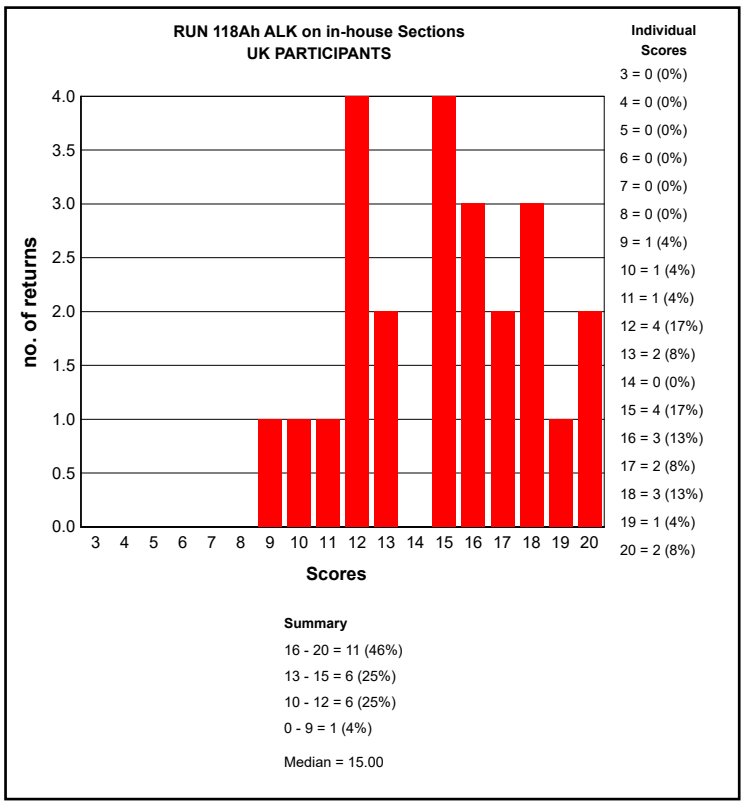
Fig 8. Good in-house appendix control stained with the Ventana D5F3 ALK IHC assay (see also Fig 7 for the accompanying ALK negative and positive tumours submitted by the participant). The image shows the expected strong positive staining of ganglion cells and axons.

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: 118

Primary Antibody	N	%
Cell Sign. Tech. (D5F3)	4	75
Dako M7195 (ALK1)	1	0
Novocastra NCL-ALK (5A4)	4	50
Novocastra PA0306 (5A4)	1	100
Other	1	0
Ventana/Roche (D5F3)	49	82

ALK NSCLC Run: 118

Automation	N	%
Dako Autostainer Plus Link	1	0
Dako Omnis	1	100
Leica Bond Max	2	50
Leica Bond-III	2	100
Ventana Benchmark GX	3	67
Ventana Benchmark ULTRA	29	79
Ventana Benchmark XT	22	77

ALK NSCLC Run: 118

Heat Mediated Retrieval	N	%
Dako Omnis	1	100
Dako PTLink	1	0
Leica ER2 20 mins	3	67
Other	4	100
Ventana CC1 36mins	1	100
Ventana CC1 64mins	2	50
Ventana CC1 88mins	1	100
Ventana CC1 92mins	39	77
Ventana CC1 extended	5	80
Ventana CC1 standard	3	67

ALK NSCLC Run: 118

Detection	N	%
AS PER KIT	2	50
Dako EnVision FLEX+ (K8002/12)	1	100
Leica Bond Polymer Refine (DS9800)	2	100
Ventana OptiView (760-700) + Amp. (7/860-099)	34	79
Ventana OptiView Kit (760-700)	19	79
Ventana UltraView Kit (760-500)	1	0

ALK NSCLC Run: 118

Enzyme Retrieval	N	%
AS PER KIT	2	100
NOT APPLICABLE	30	77

ALK NSCLC Run: 118

Chromogen	N	%
AS PER KIT	18	72
DAKO DAB+	1	100
Dako FLEX DAB	1	0
Leica Bond Polymer Refine kit (DS9800)	4	75
Ventana DAB	32	84
Ventana Ultraview DAB	4	50

BEST METHODS

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

ALK - Method 1

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

Primary Antibody:	Ventana/Roche (D5F3) , 16 Mins Prediluted
Automation:	Ventana Benchmark XT
Method:	
Main Buffer:	
HMAR:	Ventana CC1 extended
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099) , 12 Mins

ALK - Method 2

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

Primary Antibody:	Cell Sign. Tech. (D5F3) , 60 Mins Dilution 1: 100
Automation:	Ventana Benchmark XT
Method:	
Main Buffer:	
HMAR:	Ventana CC1 88mins
EAR:	
Chromogen:	Ventana DAB
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099) , 8 Mins

ALK - Method 3

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

Primary Antibody:	Ventana/Roche (D5F3) , 16 Mins Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	
Main Buffer:	
HMAR:	Ventana CC1 92mins
EAR:	
Chromogen:	Ventana DAB
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099)

VENTANA PD-L1 (SP263) Assay

Guiding immunotherapy for NSCLC

Now available for use on the **Benchmark systems** with **KEYTRUDA®** (pembrolizumab) and **OPDIVO®** (nivolumab) in **NSCLC**

VENTANA PD-L1 (SP263) Assay is intended for the qualitative detection of the Programmed Death Ligand 1 (PD-L1) in formalin-fixed, paraffin-embedded (FFPE) non-small cell lung cancer (NSCLC) and other tumour tissues stained with OptiView DAB IHC Detection Kit on a BenchMark IHC/ISH instrument.

PD-L1 expression in tumour cell (TC) membrane as detected by VENTANA PD-L1 (SP263) Assay in NSCLC is indicated as an aid in identifying patients for treatment with KEYTRUDA (pembrolizumab). PD-L1 expression in tumour cell (TC) membrane as detected by VENTANA PD-L1 (SP263) Assay in NSCLC may be associated with enhanced survival from OPDIVO (nivolumab).

Therapy and associated indication

Indication for use	Therapy	PD-L1 Expression-Therapeutic Line
NSCLC	KEYTRUDA	≥50% TC – First Line
		≥1% TC – Second Line
	OPDIVO	≥1%, ≥5% and ≥10% TC – Second Line

Roche Diagnostics Ltd.
Charles Avenue, Burgess Hill, West Sussex, RH15 9RY, United Kingdom. Registration number: 571546

The HER2 ISH Module - Interpretive Run 47

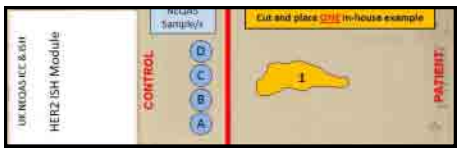
Suzanne Parry

Gene Assessed:	HER2
Sections Circulated:	Composite slide consisting of 4 breast carcinoma sections (see table below)
Number of Registered Participants:	156
Number of Participants This Run	121 (78%)

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

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

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



Introduction

Breast cancer HER2 status should be determined in all newly diagnosed and recurrent and metastatic breast cancers. In-situ hybridization (ISH), using either fluorescent (FISH) or brightfield chromogenic methods (CISH) 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), 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. 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

Several laboratories did not provide their interpretive results, although they sent their slides in for the All laboratories employed a dual probe and a ratio scoring algorithm. 74% of participants achieved excellent or acceptable results. 21% received a borderline pass and 7 laboratories (6%) had an unacceptable interpretation result. There was one unacceptable results from a UK laboratory. The most common brightfield method was the Ventana DDISH with 42 (34%) laboratories using this technique. The most popular FISH method was the Pathvysion Vysis Kit with 35 (29%) laboratories using this technique. The pass rates of these two assays were 64% and 86% respectively.

Overall there was a slight increase in the pass rates compared to the previous assessment (run 46). In parallel there was a drop in the unacceptable result which has fallen from 11 labs (8%) in run 46 to 7 labs (6%) in the current run 47.

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.

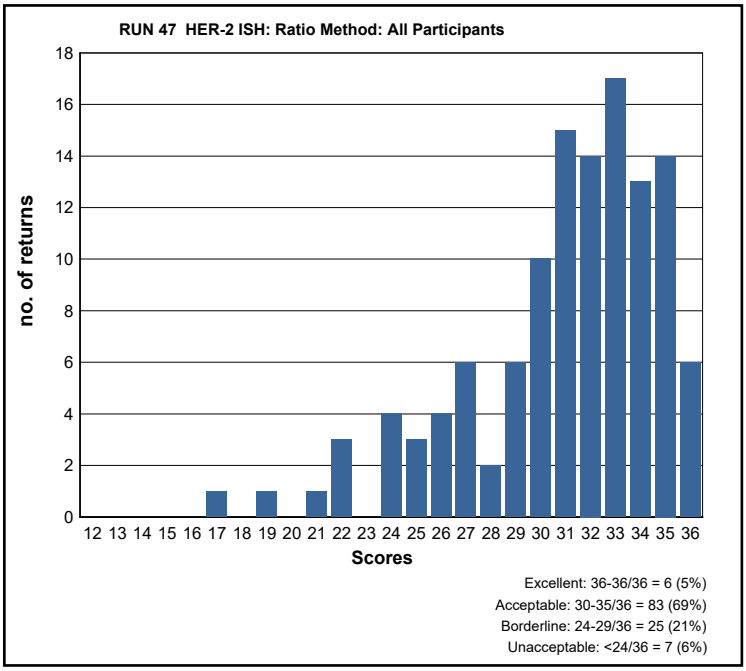
HER2 ISH Method and Probe Enumeration

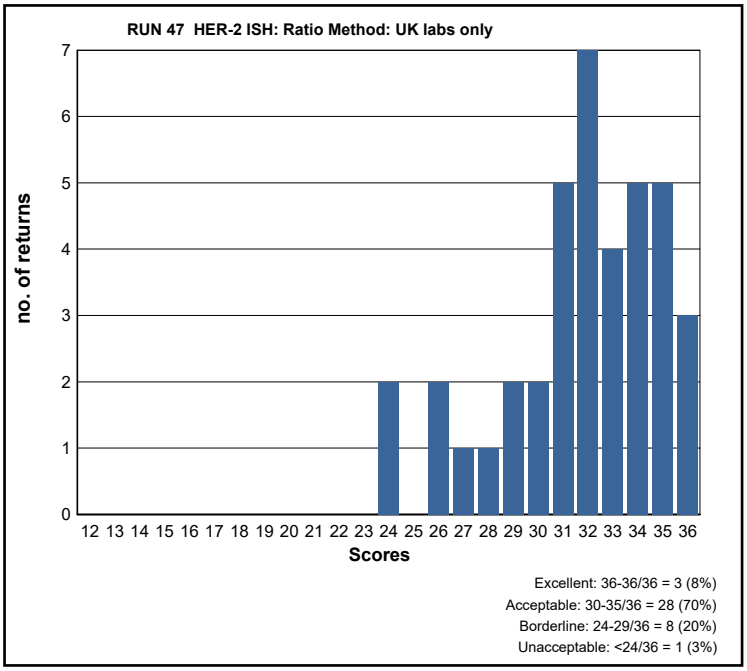
Table 1 shows the various ISH methods employed along with mean HER2, Cen17 counts and ratio calculations.

Bibliography

1. Rakha, E., Pinder, S., Bartlett, J., Ibrahim, M., Starczynski, J., Carder, P., Provenzano, E., Hanby, A., Hales, S., Lee, A. and Ellis, I. (2014). Updated UK Recommendations for HER2 assessment in breast cancer. *Journal of Clinical Pathology*, 68(2), pp.93-99.
2. Bartlett, J., Starczynski, J., Atkey, N., Kay, E., O'Grady, A., Gandy, M., Ibrahim, M., Jasani, B., Ellis, I., Pinder, S. and Walker, R. (2011). HER2 testing in the UK: recommendations for breast and gastric in-situ hybridisation methods. *Journal of Clinical Pathology*, 64(8), pp.649-653.
3. Moore, D., McCabe, G. and Craig, B. (2011). *Introduction to the practice of statistics*. 7th ed. New York, NY: Freeman.

GRAPHICAL REPRESENTATION OF PASS RATES (ratio methods only)





METHODS USED and PASS RATES

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

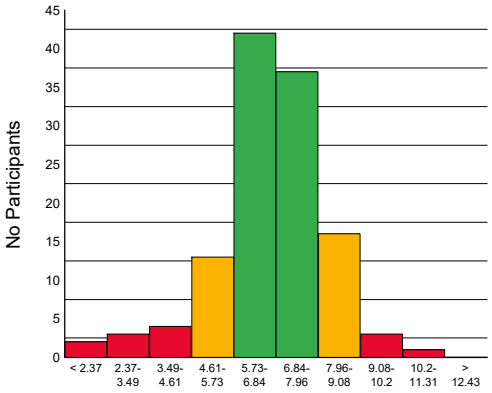
Ratio Method	N	% Pass (score ≥ 30/36)
Ratio: Dako DuoCISH	1	100%
Ratio: Dako IQFISH pharmDX	13	77%
Ratio: Dako Pharm Dx	1	0%
Ratio: In house FISH	1	100%
Ratio: Kreatech Probes	3	67%
Ratio: Leica HER2 FISH TA9217	5	100%
Ratio: Other - CISH	1	100%
Ratio: Other - FISH	2	50%
Ratio: Pathvysion Vysis Kit	35	86%
Ratio: Ventana BDISH 800-098/505	6	50%
Ratio: Ventana DDISH (780/800-4422)	42	64%
Ratio: Ventana Inform Silver ISH	2	50%
Ratio: Zytovision ZytoDot 2C	1	0%
Ratio: Zytovision ZytoLight	7	100%

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

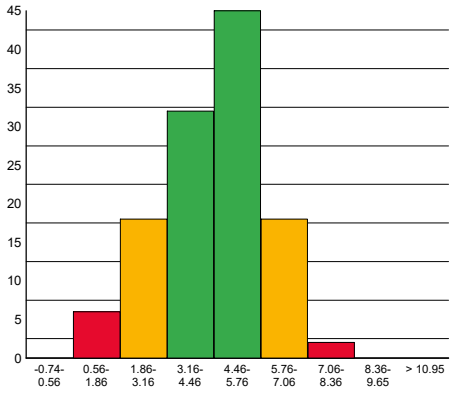
FREQUENCY HISTOGRAMS

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

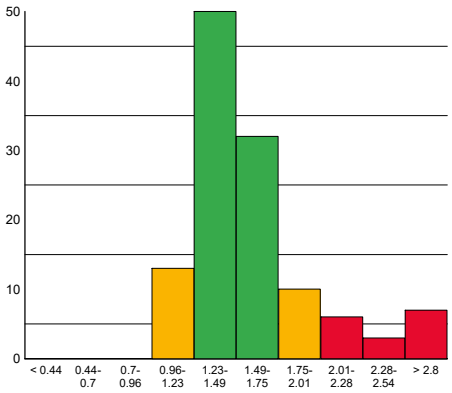
Green = mean +/- 1 Stdev Amber = mean +/- 2 Stdev Red = mean ≥ +/- 3 stdev



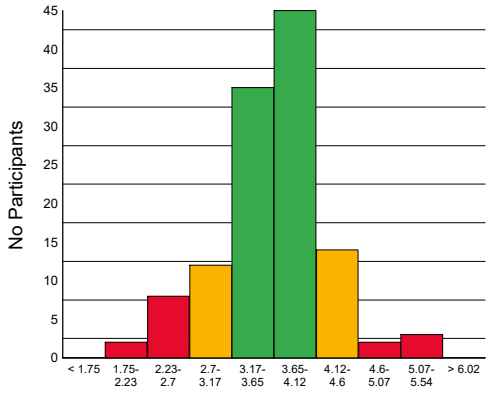
Sample A: Av. HER2 copy



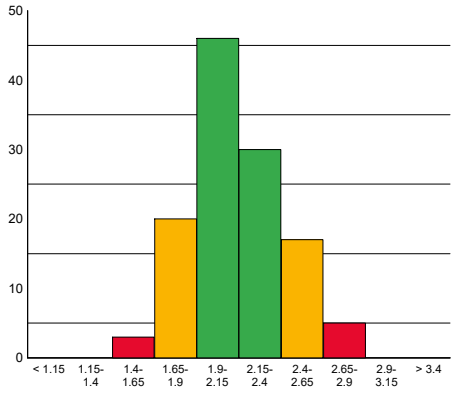
Sample A: Av. Chr17



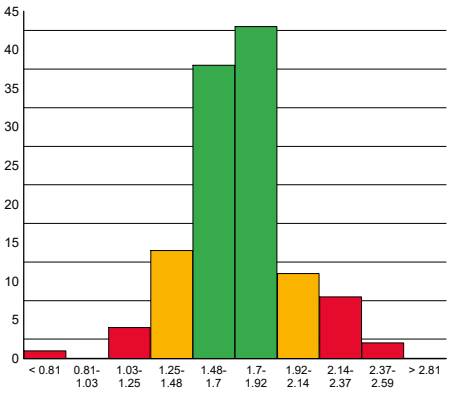
Sample A: Ratio



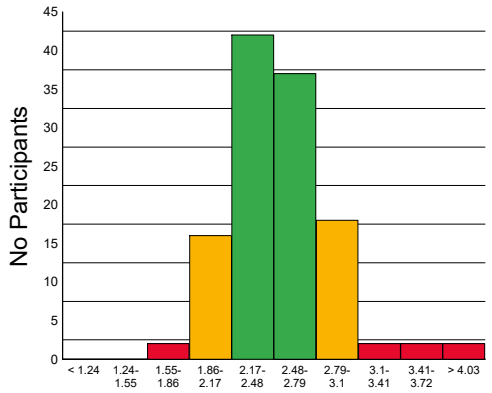
Sample B: Av. HER2 copy



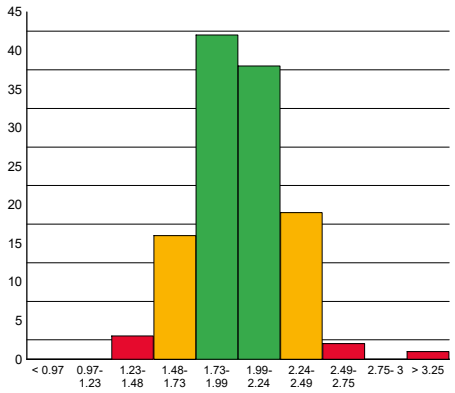
Sample B: Av. Chr17



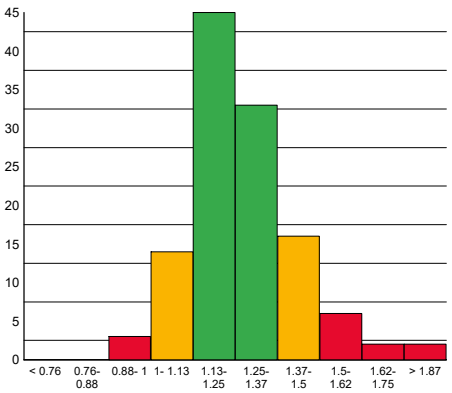
Sample B: Ratio



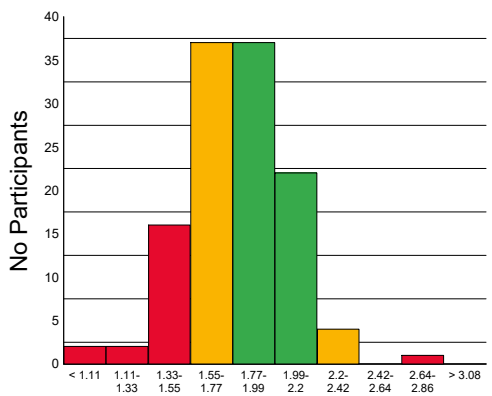
Sample C: Av. HER2 copy



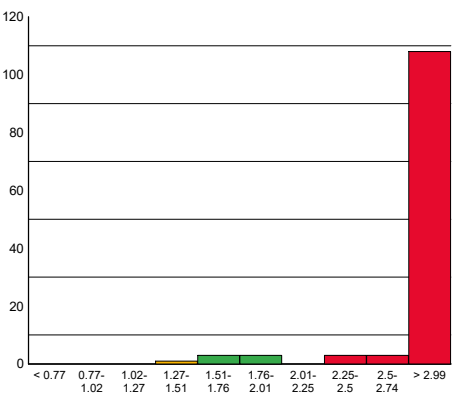
Sample C: Av. Chr17



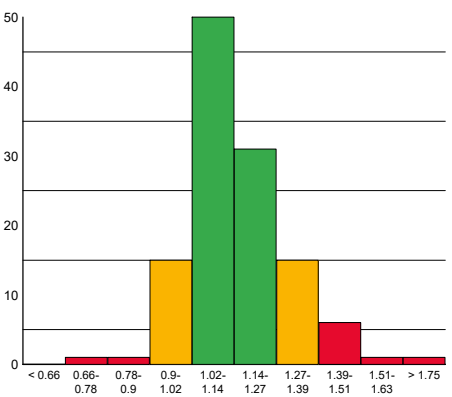
Sample C: Ratio



Sample D: Av. HER2 copy



Sample D: Av. Chr17



Sample D: Ratio

The HER2 ISH Module - Technical Run 47

Suzanne Parry and Amy Newman

Gene Assessed:	HER2
Sections circulated:	Composite slide consisting of 4 breast carcinoma sections (see table below)
Number of Registered Participants:	156
Number of Participants Taking Part this Run	120 (77%) (66 Fluorescent and 54 Chromogenic)

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

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

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

Assessment Procedure

Brightfield ISH (CISH / SISH / BDISH / DDISH etc.) was assessed around a multi-header microscope. Each slide was reviewed by 4 independent assessors, each providing scores from 1-5 for the slide as a whole (not the individual tissue sections). The scores are added together to give an overall score out of 20 along with any assessor feedback comments.

Fluorescent ISH (FISH) was assessed by a team of assessors who viewed the slides together on a large HD screen which was linked to the fluorescent microscope. Each assessor provided their opinion and feedback, and then an overall score was given out of 20.

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

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

In-house 'example/s' are now requested for this module and participants will be required to place them on the distributed NEQAS slides.

Results Summary

CISH Results

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

The overall results showed similar pass rates compared to the previous run (Run 46). 32 (59%) of laboratories achieved an acceptable pass rate on this Run (47), which was the same as that for Run 46. A further 28% (N=15) received a borderline pass, 6 laboratories (13%) failed the assessment.
Again, the failures were predominantly due to weak or no Cen17 signals, as seen in previous assessment runs over the last 12-18 months. The borderline passes were given for weak, but diagnostically readable signals; again, this was mostly due to weak Cen17 signals. Some slides showed dust-like silver chromogen signals, but unless obscuring the specific HER2 signals and the slide was deemed still readable, the section did not fail, but was given a borderline pass. These observational results refer to the Ventana BDISH, Inform or DDISH methods, which 89% of laboratories have adopted who submitted brightfield ISH slides for this technical assessment.

The other CISH methods were used by only a small number of laboratories, such as the Dako DuoCISH and the ZytoDot 2C assays. Both of these users failed the assessment. Another laboratory used the Ventana single silver probe method which received a borderline pass at assessment.

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

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

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

FISH Results

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

There was a marked improvement in the current Run 47 compared to the previous Run (46) where there was a high level of FISH submissions with weak or no HER2 and/or Cen17 signals. This may have been attributed to technical, storage or transport errors, as it did not appear to be assay specific. The failure rate dropped from 34% (Run 46) to 17% (Current Run 47).
This is reflected in the overall pass rate with an increase of acceptable pass rates from 56% (Run 46) to 71% (Run 47).
The Pathvysion Vysis Kit is most commonly used by laboratories for FISH (53%). Pass rates using the Vysis Kit for this Run have risen compared to Run 46 (86% vs 72% respectively). The Dako IQFISH and Zytovision FISH assays were used by 20% and 10% of participants respectively. They achieved a 77% and 100% acceptable pass rate respectively. Other FISH assays used include the Dako Pharm Dx, Kreatech Probes and the Leica FISH probes, all of which performed well, 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**	**Overall Feedback**
Appropriate or Acceptable	Acceptable	**Acceptable** The NEQAS samples show a good standard of staining **and** has been interpreted correctly
Unacceptable	Acceptable	**Unacceptable** The NEQAS samples show a good standard of staining **BUT** there appears to be issues with interpretation i.e. HER2 copy number and/or Cen17 overestimated/underestimated. **Recommend that scoring/counting criteria is reviewed**
Appropriate or Acceptable	Borderline	**Borderline Acceptable** The NEQAS samples are of borderline acceptability for staining quality. **Recommend that technical method (kit/assay) is further optimised.**
Unacceptable	Borderline	**Unacceptable** The technical staining can be improved as this may be effecting interpretation. **Recommend that technical method (kit/assay) is optimised (or where relevant a standardised kit is used as per instructions) as interpretation may be affected by the borderline quality of staining.**
Appropriate or Acceptable	Unacceptable	**Unacceptable** The NEQAS samples are unacceptable for interpretation and caution should be taken when interpreting from these slides. **Recommend that technical method (kit/assay) is optimised (or where relevant a standardised kit is used as per instructions) as interpretation, although appears correct, may lead to incorrect interpretation of clinical cases.**
Unacceptable	Unacceptable	**Unacceptable** The NEQAS samples are unacceptable for technical staining and interpretation. **Reporting from such cases is very likely to lead to incorrect interpretation of clinical cases.** **If there is persistent underperformance:** • **seek assistance from kit/assay manufacturer** • **seek assistance from UK NEQAS or colleagues** • **re-validate protocol (retrospectively and prospectively)** • **review scoring criteria** • **send clinical cases to a reference centre to confirm your results**

Selected Images showing Optimal and Sub-optimal Immunostaining

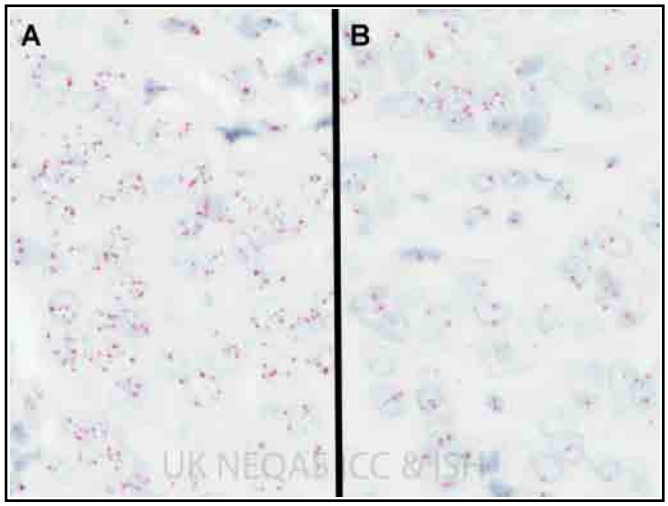


Fig 1. Acceptable Ventana DDISH in the UK NEQAS distributed samples 'A' (amplified case) and 'B' (non-amplified). Both examples show distinct HER2 signals (black) and Chr17 signals (red).

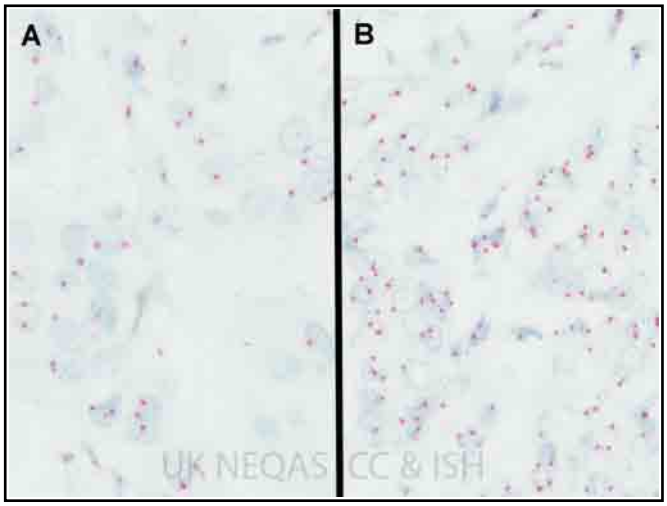


Fig 2. Acceptable Ventana DDISH in the UK NEQAS distributed non-amplified samples 'C' and 'D'. The sections show strong HER2 signals (black) and Chr17 signals (red) and the expected copy numbers per cell.

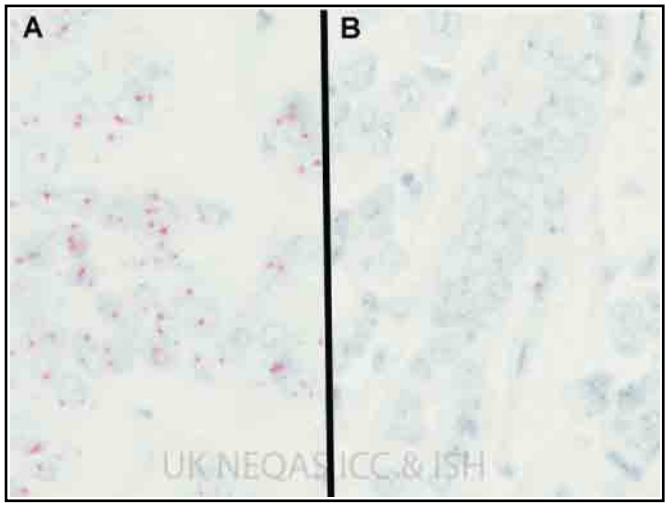


Fig 3. Two examples showing unacceptable DDISH staining in the UK NEQAS non-amplified sample 'B'. In example A the HER2 signals are very weak and unreadable. While in example B there are virtually no HER2 signals and the Chr17 signals are very weak and patchy.

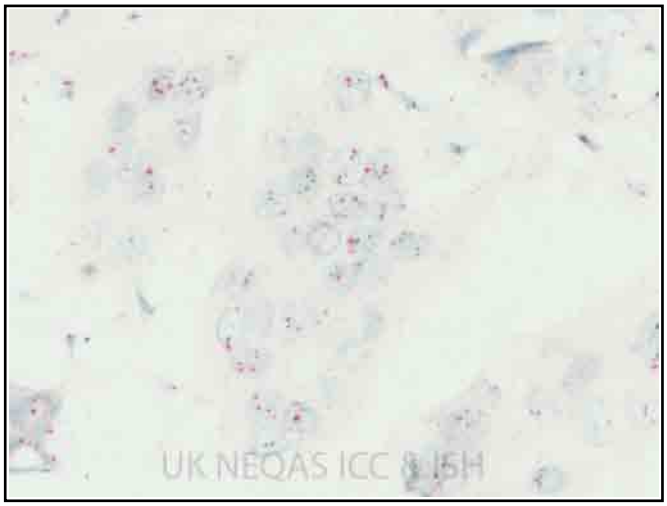


Fig 4. Borderline pass demonstration of Ventana DDISH in the UK NEQAS non-amplified sample 'C'. Although the signals are visible, there is also excessive silver deposit and signals outside of the nuclei.

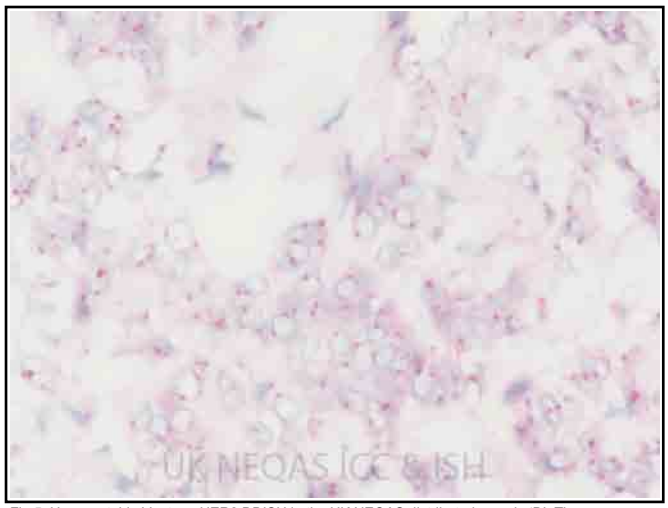


Fig 5. Unacceptable Ventana HER2 DDISH in the UK NEQAS distributed sample 'D'. The section shows excessive leaching of the Chr17 probe, and no HER2 signals.

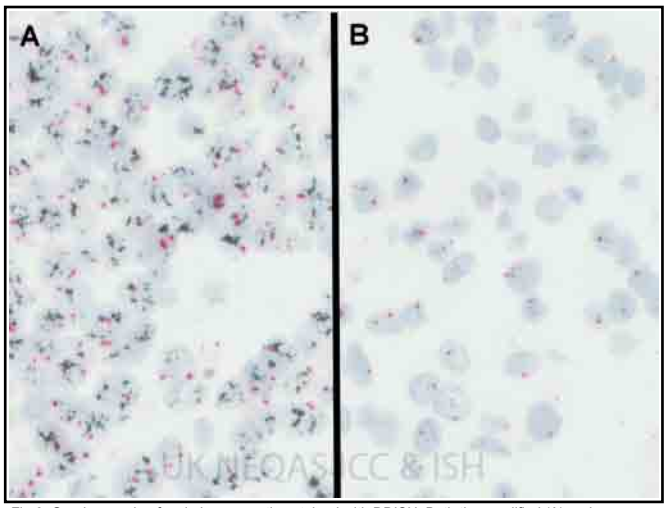


Fig 6. Good example of an in house section stained with DDISH. Both the amplified (A) and non-amplified (B) samples show strong and distinct HER2 and Chr17 signals.

Selected Images showing Optimal and Sub-optimal Immunostaining

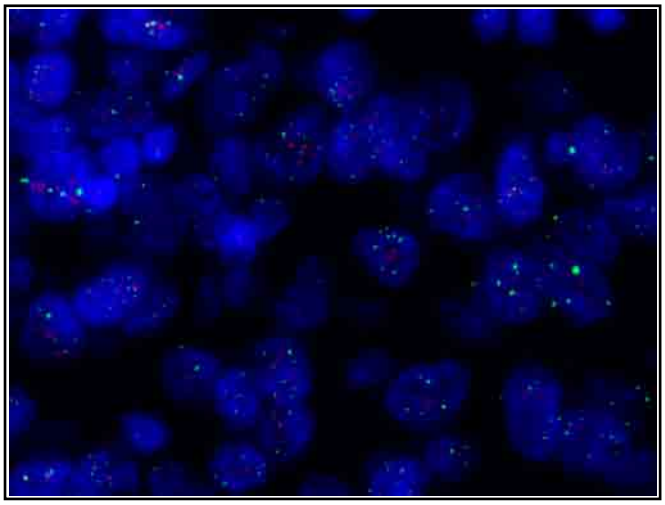


Figure 7: Excellent demonstration of Core A on the NEQAS ISH section. The HER2 (orange) and CEP17 (green) signals are clear displaying co-amplification. Method: PathVysion Kit.

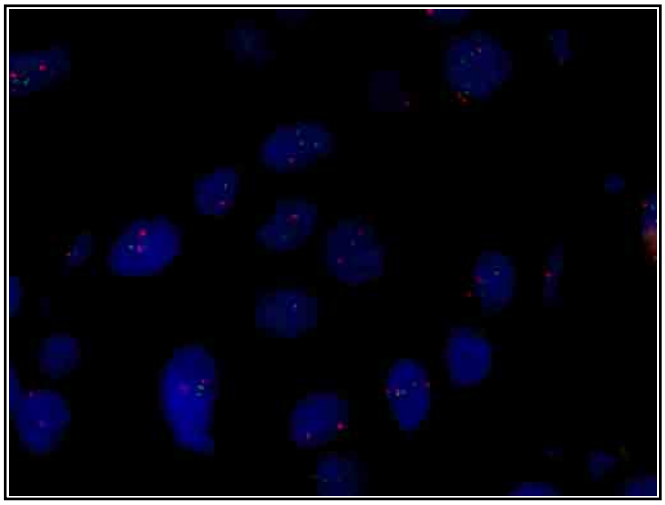


Figure 8: Good demonstration of Core C on the NEQAS ISH sample. Method: FISH probe - Zytovision ZytoLight; Automation - Leica Bond III (Pepsin + ER2).

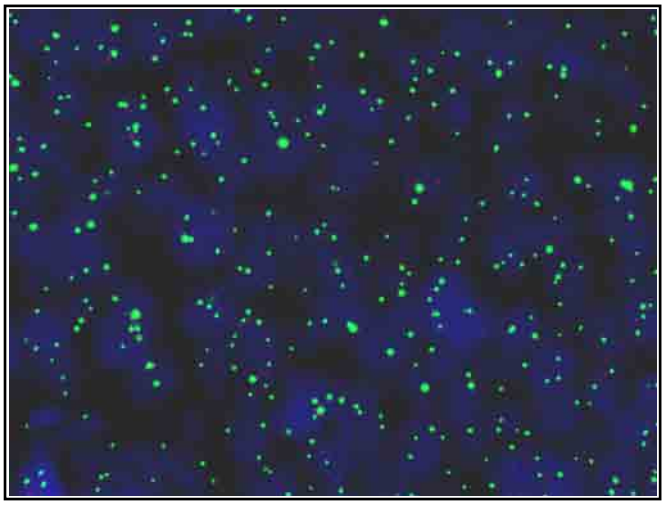


Figure 9: Excessive, non-specific CEP17 (green) signals on Core C on the NEQAS ISH sample. This can obscure the HER2 signals and may lead to an incorrect HER2:CEP17 ratio. Method: Pathvysion Vysis Kit (VP2000; Protease I).

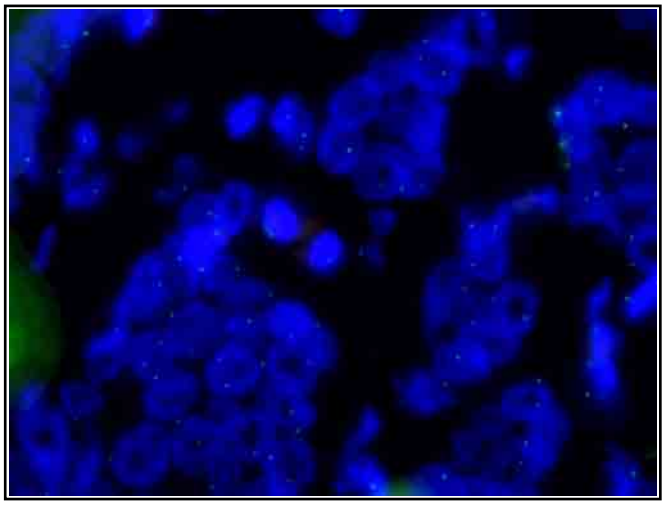


Figure 10: Very weak HER2 (orange) signals in core D. Damaged nuclei suggest possible over-digestion with pepsin. Method: HER2 probe (GC, Italia); (Manual; Vysis pretreatment kit + pepsin, 10').

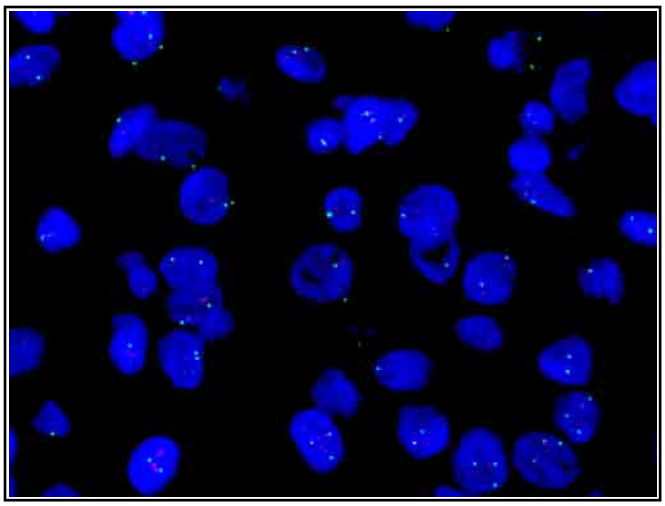


Figure 11: Excellent FISH signals on Core B on the NEQAS ISH sample. Method: Pathvysion Vysis kit + Pepsin.

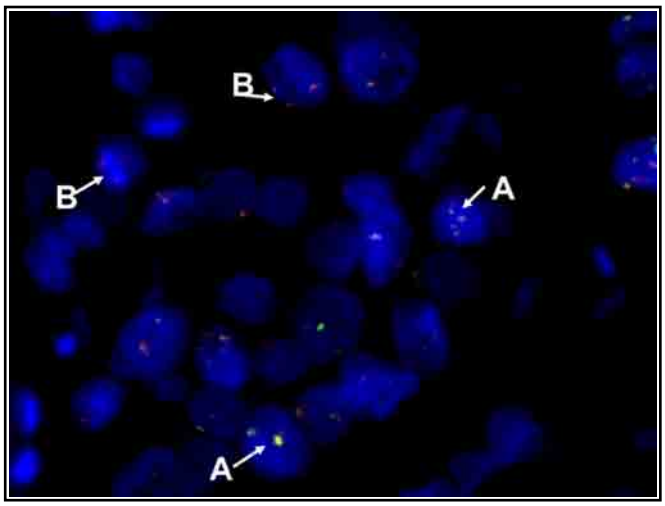
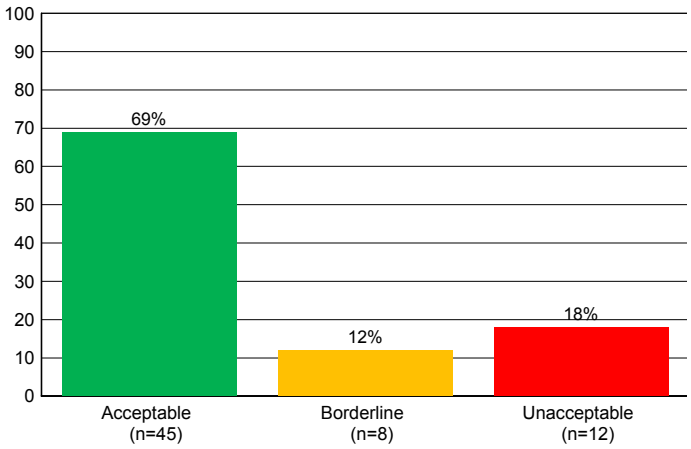


Figure 12: In-house FISH control demonstrating clear HER2 and CEP17 signals. This particular case shows a good contrast of HER2/CEP17 co-amplification in the tumour cells (A) adjacent to normal stromal cells (B). Methods not indicated.

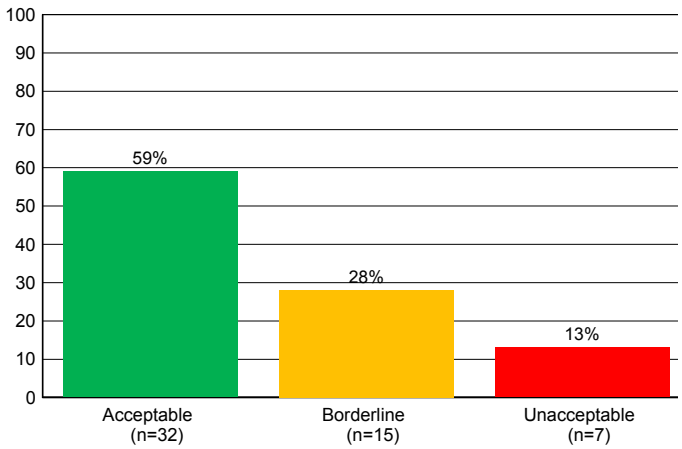
Technical ISH: Pass Rates and Methods

Overall Pass Rates

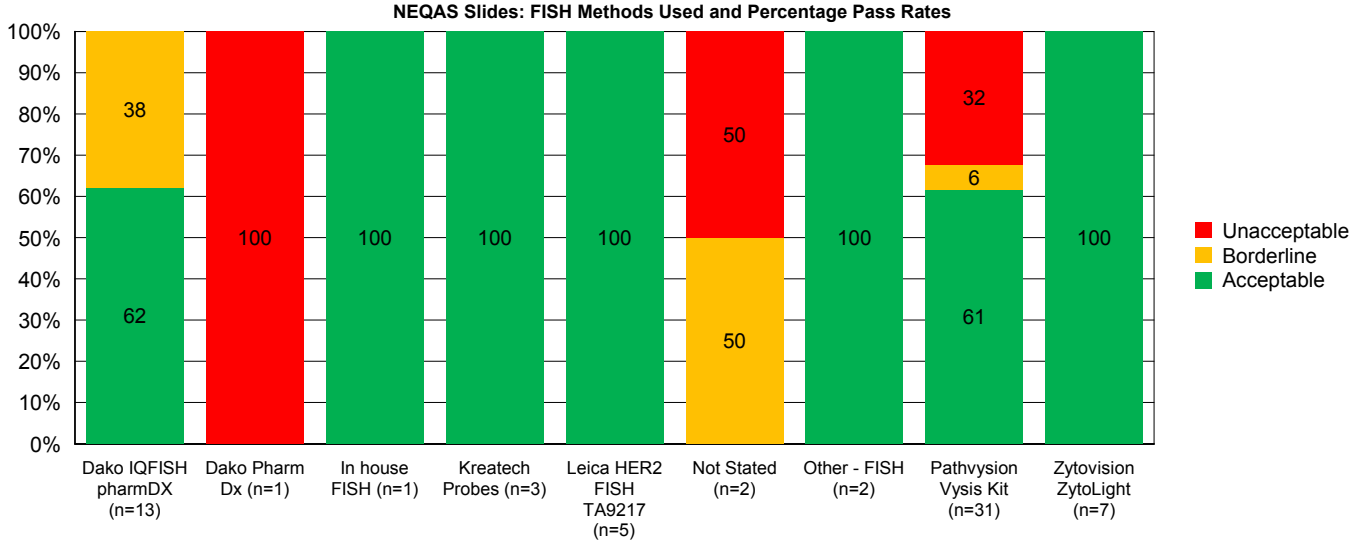
FISH NEQAS slide (n=65)



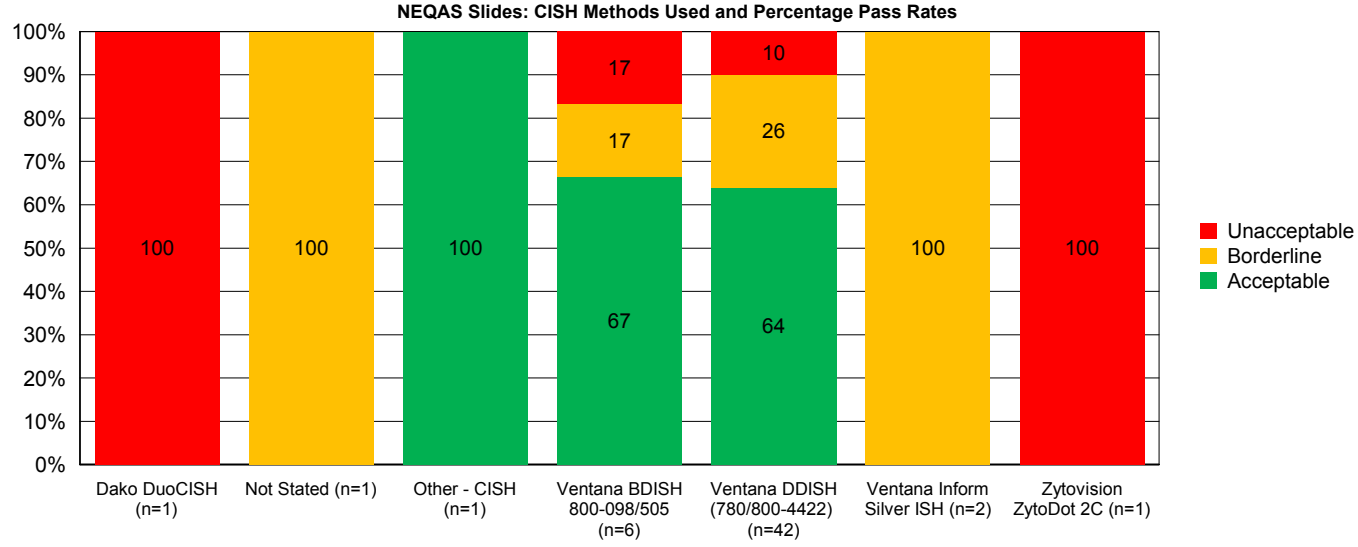
CISH NEQAS Slide (n=54)



NEQAS Slides: FISH Methods Used and Percentage Pass Rates



NEQAS Slides: CISH Methods Used and Percentage Pass Rates



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