UK NEQAS
International Quality Expertise

Immunocytochemistry

Improving Immunocytochemistry for Over 25 Years

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

Assessment Dates: 2nd — 17th October 2017

Immunocytochemistry Modules

In situ Hybridisation Modules

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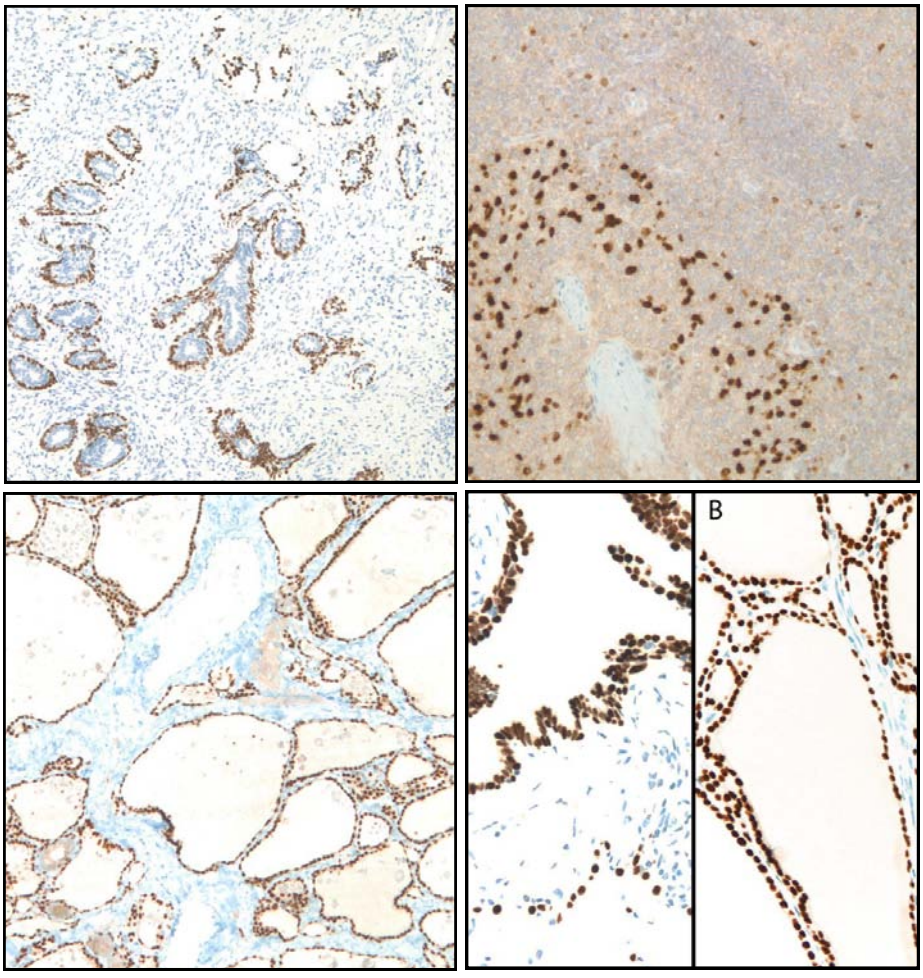

Cover Photo: Taken from the General module

Top Left: Optimal p63 staining on the NEQAS sample of prostate
Top Right: Sub-optimal p63 demonstration in the NEQAS sample
Bottom Left: Optimal TTF-1 demonstration in the NEQAS thyroid sample
Bottom Right: Excellent TTF-1 staining on an in-house control slide

General Information

UK NEQAS
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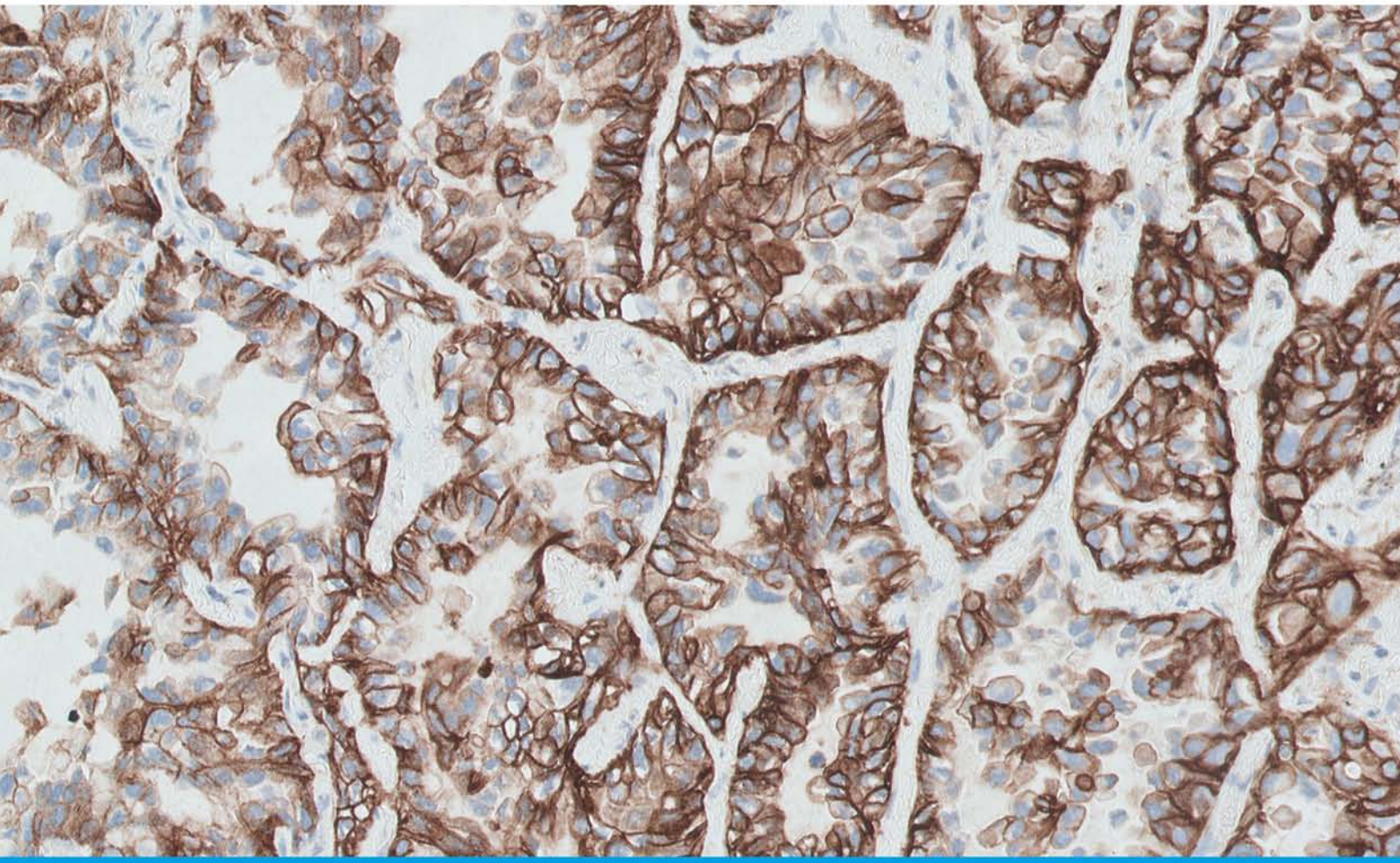


50249 2016JUN09

Agilent Technologies

The General Pathology Module Run 119

Gavin Rock and Jamie Hughes

	Gold Standard	Second Antibody
Antigens Assessed:	TTF-1	p63
Tissue Sections circulated:	Normal thyroid and adenocarcinoma of lung	Hyperplastic prostate and normal tonsil
Number of Registered Participants:	312	
Number of Participants this Run	296 (95%)	

Introduction

Gold Standard: TTF-1

Thyroid transcription factor (TTF1) is a member of the family of homeodomain (protein structural domain that binds DNA or RNA) transcription factors and is a gene regulatory factor expressed in the follicular epithelial and para-follicular c-cells of the thyroid, epithelial cells of the lung, brain and pituitary gland. TTF1 is essential for the normal development of both the thyroid and lung, and increased TTF1 immunohistochemical expression is seen in both pulmonary adenocarcinomas and thyroid neoplasms of follicular origin (follicular adenoma and follicular and papillary carcinoma). Not all pulmonary adenocarcinomas have been shown to be positive for TTF1, with a positivity range of between 65-80 (Compérat et al., 2005), depending on the antibody clone used. TTF1 in small lung carcinomas have been shown in about 96% of cases (Ordóñez, 2000) and in 75% of non-small cell pulmonary carcinomas, but generally it is not thought to be expressed in typical pulmonary carcinoids, although the literature does have some contrasting views. TTF1 can also be used to differentiate between tumours of primary and metastatic origin e.g. primary lung adenocarcinoma (TTF1+ve/ CDX2-ve) vs. metastatic gastrointestinal adenocarcinomas (TTF1+ve/CDX2-ve). Furthermore, TTF1 alongside other antibodies such as CK20 can help to further identify tumour types including Merkel cell carcinoma (TTF1-ve/CK20+ve) and metastatic small cell lung carcinoma (TTF1+ve/CK20-ve).

Features of Optimal Immunostaining:
Normal Thyroid: (Fig 3 & 5B)
- Strong, nuclear staining of the follicular cells.
- Clean background.

Lung Adenocarcinoma: (Fig 1 & 5A)
- Strong, crisp, nuclear staining of neoplastic cells.
- Strong, nuclear staining of the basal epithelial cells lining the bronchial ducts.
- Weak to moderate nuclear staining of bronchial luminal epithelial cells.
- Clean background.

Features of Suboptimal Immunostaining: (Figs 6 & 2)
- Weak staining of the basal epithelial cells lining the bronchial ducts.
- Weak staining of neoplastic cells of the tumour.
- Non-specific nuclear staining.
- Excessive background staining.

References

1. Comperat E., Zhang, F., Perrotin, C., Molina, T., Magdeleinat, P., Marmey, B., Régnard, J., Audouin, J. and Camilleri-Broët, S. (2005); Variable sensitivity and specificity of TTF1 antibodies in lung metastatic adenocarcinoma of colorectal origin. Modern Pathology. 18(10). pp. 1371-1376.
2. Ordóñez N. (2000). Value of thyroid transcription factor-1 immunostaining in distinguishing small cell lung carcinoma from other Small Cell Carcinomas. *The American journal of Surgical Pathology,* 24(9),pp.1217-1223.

Second Antigen: p63

p63 is a 63KDa nuclear homologue protein of the tumour suppressor p53 family of transcription factors, which is found in the basal and progenitor cells of many epithelial cells, including those of the breast, prostate, bladder and oesophagus (Signoretti et al., 2000) p63 has been identified as a particularly useful aide in the differentiation between benign and malignant lesions of the prostate (Humphrey, 2007), and has also shown to be a sensitive marker for squamous cell carcinomas of the lung. In breast tissue, p63 is expressed in the myoepithelial cells of normal ducts and is therefore useful within a panel of IHC markers for the assessment of breast lesions due to the differential expression of luminal versus basal and myoepithelial markers (Reis-Filho et al., 2006)

Features of Optimal Immunostaining:
Tonsil (Fig 7)
- Strong nuclear staining in almost all of the epithelial cells, with higher intensity in the basal elements.
- Minimal background staining.
-

Prostate - Benign prostatic hyperplasia: (Fig 9)
- Strong nuclear staining in the basal cells lining the gland.
- Minimal background staining.

Suboptimal Immunostaining: (Figs 10 8, & 12)
- Weak, uneven or no staining.
- Diffuse staining.
- Non-specific staining of cell types not expected to stain.
- Excessive background staining.

References:

1. Humphrey, P. (2007). Diagnosis of adenocarcinoma in prostate needle biopsy tissue. *Journal of Clinical Pathology*, 60(1), pp.35-42.
2. Reis-Filho, J., Milanezi, F., Steele, D., Savage, K., Simpson, P., Nesland, J., Pereira, E., Lakhani, S. and Schmitt, F. (2006). Metaplastic breast carcinomas are basal-like tumours. *Histopathology*, 49(1), pp.10-21.
3. Signoretti, S., Waltregny, D., Dilks, J., Isaac, B., Lin, D., Garraway, L., Yang, A., Montironi, R., McKeon, F. and Loda, M. (2000). p63 Is a Prostate Basal Cell Marker and Is Required for Prostate Development. *The American Journal of Pathology*, 157(6), pp.1769-1775.

Assessment Summary

TTF-1

294 laboratories submitted slides for the TTF-1 assessment, and all but 2 laboratories submitted their in-house control sections for this run. The results show an increase of 4% in acceptable results from Run 107, as shown in the table below:

TTF-1 Pass Rates : NEQAS section		
	Run 107	Run 119
Acceptable	84% (N=263)	88% (N=260)
Borderline	13% (N=42)	10% (N=30)
Unacceptable	3% (N=10)	1% (N=4)

The most common reason for failure/ loss of marks in run 119 was due to weak/very weak demonstration of the antigen, and less so due to background staining being observed.

The most popular antibody used in this run was Ventana TTF-1 (SP141) used by 64 participants with a pass rate of 95%. The second most popular antibody was Leica NCL-L-TTF1 (SPT24) used by 62 participants with a pass rate of 98%. Another popular choice was Dako M3575 (8G7G3/1) used by 55 participants with a 82% acceptable pass rate. The acceptable pass rate of the in-house tissue was 96% compared to the NEQAS distributed tissue of 88.44%.

The most common automated platform for TTF-1 was the Ventana Benchmark Ultra used by 103 participants with a pass rate of 90%. The second most common was the Leica Bond III used by 56 participants with a 95% pass rate.

p63

296 laboratories submitted slides for the assessment, and all but 2 laboratories also submitted their in-house material. The results showed an increase of 1% in acceptable results from run 110 to run 119, as shown in the table below:

p63 Pass Rates: NEQAS section		
	Run 110	Run 119
Acceptable	90% (N=280)	91% (N=270)
Borderline	7% (N=22)	7% (N=21)
Unacceptable	3% (N=9)	2% (N=5)

The most common reasons for failure/ loss of marks in run 119 was due to weak demonstration of antigen and background staining being observed.

The most popular antibody used in this run was Ventana 790-4509 (454) used by 94 participants with a pass rate of 97%. The second most popular used was Dako M7317 (DAK-p63) used by 47 participants with a pass rate of 91%. Another popular antibody was Leica NCL-L-p63 used by 44 participants with a pass rate of 77%. The acceptable pass rate for the in-house tissue was 94% compared to the NEQAS distributed tissue of 91%.

The most common automated platform for p63 was the Ventana Benchmark Ultra used by 101 participants with a pass rate of 96%. The second most common was the Leica Bond III used by 54 participants with a 85% pass rate.

Selected Images showing Optimal and Sub-optimal immunostaining

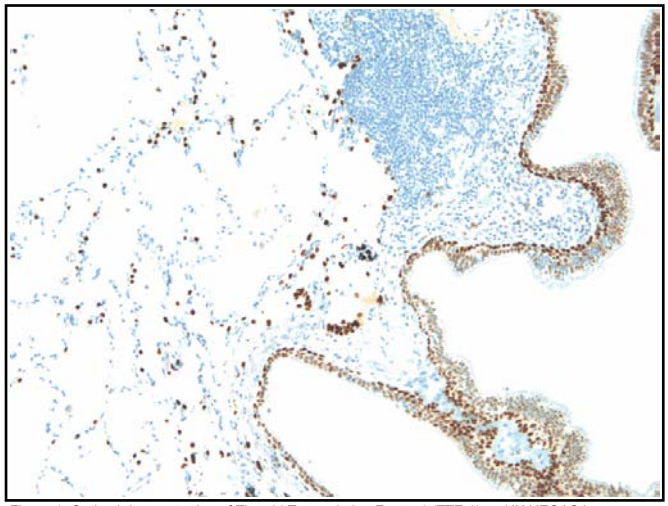


Figure 1: Optimal demonstration of Thyroid Transcription Factor 1 (TTF-1) on UK NEQAS lung tumour sample. The example shows strong staining of the basal epithelial cells lining the bronchial duct, while the staining of the luminal epithelial cells are of moderate intensity. Ventana (SP141) 12 mins 37°C; Ventana Ultra; CC1 40mins: OptiView Kit.

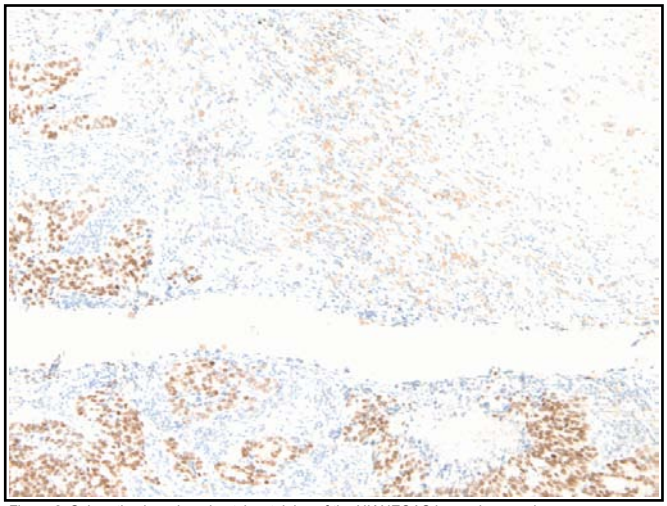


Figure 2: Sub-optimal weak and patchy staining of the UK NEQAS lung adenocarcinoma sample. Dako (8G7G3/1) RTU 20mins 32°C; Dako Omnis; Dako Envision+ HRP mouse.

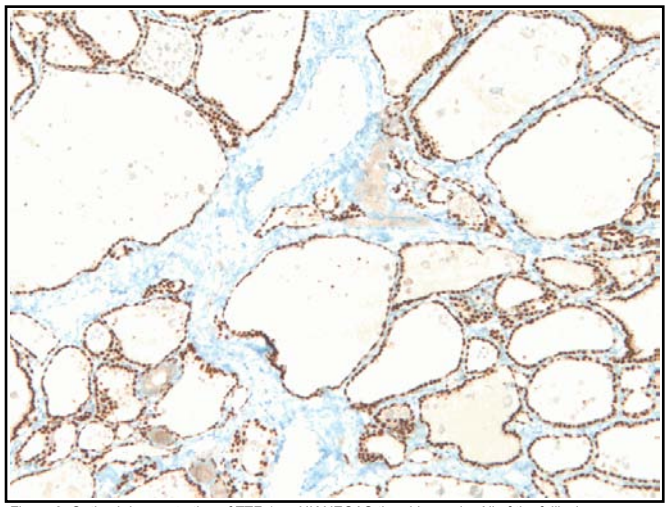


Figure 3: Optimal demonstration of TTF-1 on UK NEQAS thyroid sample. All of the follicular epithelial cells show strong nuclear staining, while the background remains clean. Protocol as per fig. 1.

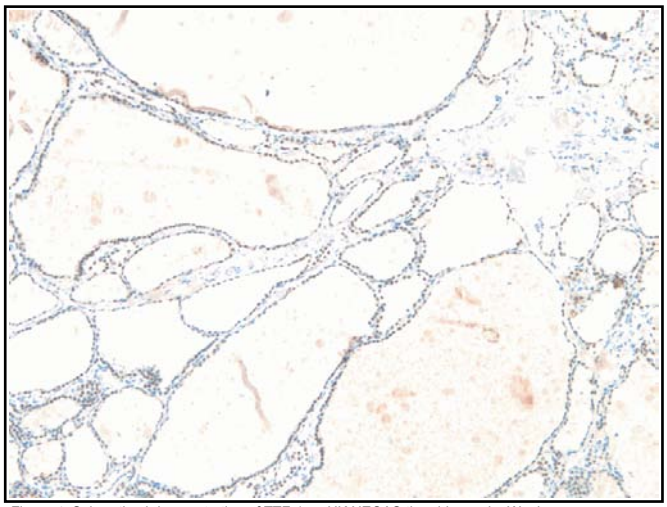


Figure 4: Sub-optimal demonstration of TTF-1 on UK NEQAS thyroid sample. Weak demonstration of antigen and slight background staining. Dako (M3575) 1:800 15mins 20°C; Leica Bond Max; ER2 20 mins; Leica Bond Polymer Refine.

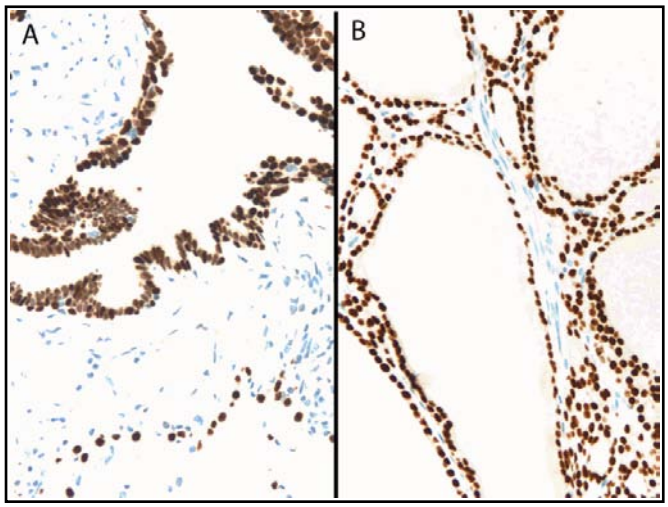


Figure 5: Excellent demonstration of TTF-1 on in-house sample with normal lung (A) and normal thyroid (B). Both images show strong and distinct staining while the background remains clear. A) Leica NCL-L-TTF 1:200 32 mins 37°C; Ventana XT; CC1 standard; UltraView Kit. B) Ventana (SP141) 24mins 36°C; Ventana Ultra; CC1 64mins; Ventana OptiView Kit.

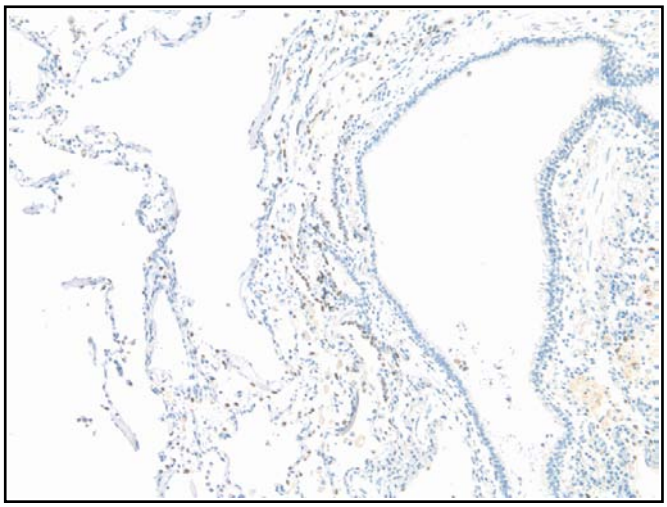


Figure 6: Poor example of TTF-1 staining of in-house lung sample. Weak to no demonstration of antigen. Dako (8G7G3/1) 1:800 15mins 20°C; Leica Bond Max; ER2 20 mins; Leica Bond Polymer Refine.

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

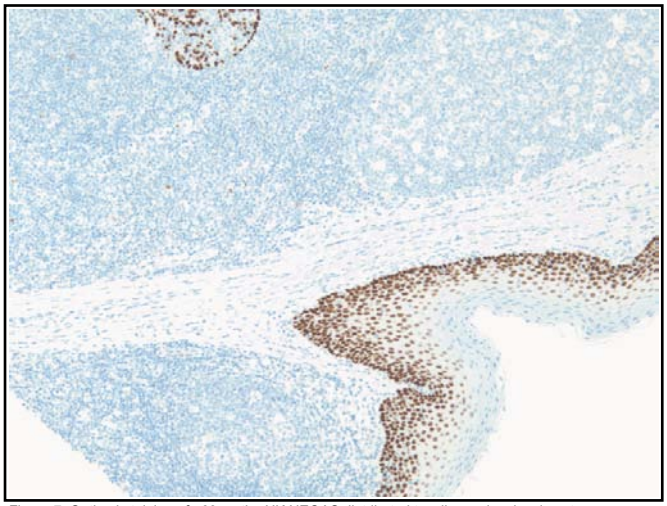


Figure 7: Optimal staining of p63 on the UK NEQAS distributed tonsil sample, showing strong nuclear epithelial staining. Ventana (454) 40 mins at RT; Ventana Ultra; CC1 64mins; OptiView Kit.

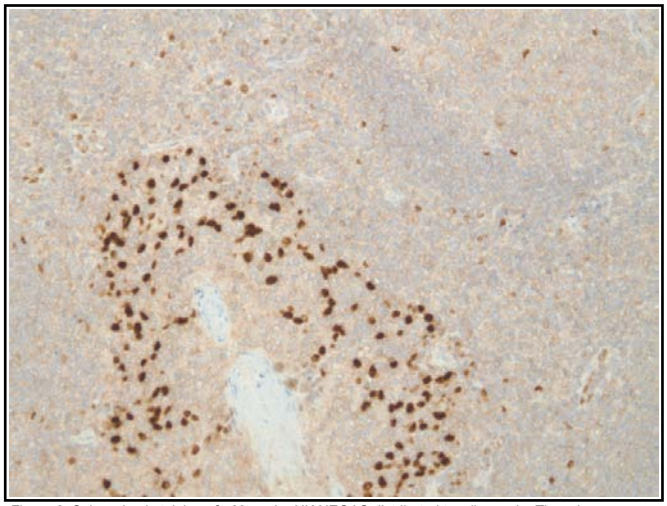


Figure 8: Sub-optimal staining of p63 on the UK NEQAS distributed tonsil sample. There is excessive background staining and inadequate demonstration of the antigen. Dako (DAK-p63) 1:25; Dako Autostainer Link 48; Dako PTLink; As per kit.

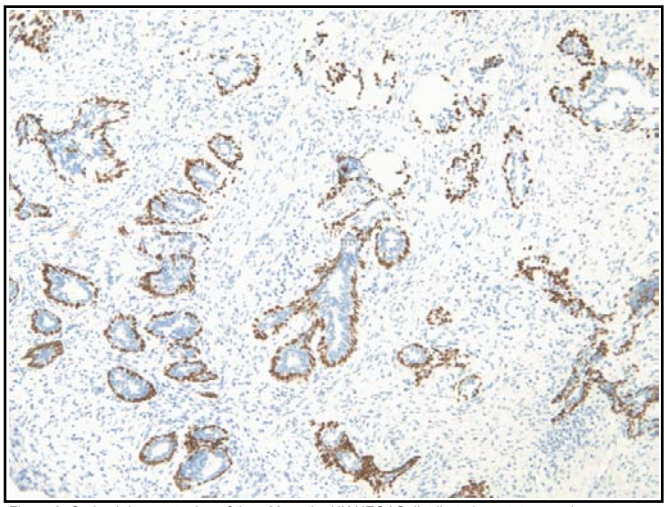


Figure 9: Optimal demonstration of the p63 on the UK NEQAS distributed prostate sample. There is strong nuclear staining of the basal cells with a clean background. Dako (DAK-p63) RTU 20mins 32°C; Dako Omnis; EnVision FLEX

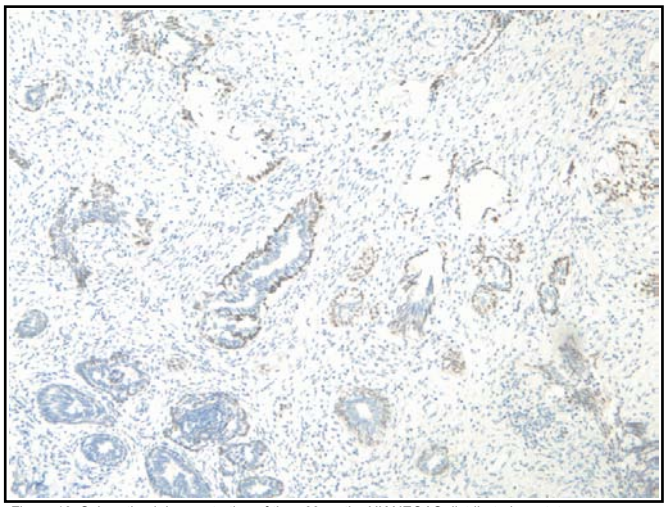


Figure 10: Sub-optimal demonstration of the p63 on the UK NEQAS distributed prostate sample. There is very weak demonstration of the antigen. Leica (NCL-L-p63) 1:25 32mins; Ventana XT; CC1 64mins; UltraView Kit.

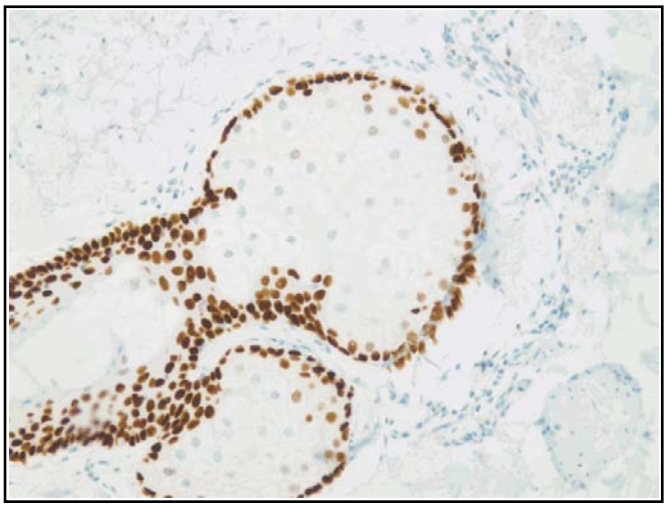


Figure 11: Excellent example of an in-house skin control stained with p63. The staining is strong and distinct while the background remains clean. Dako M7317 (DAK-p63) 1:50 32mins RT; Ventana Ultra; CC1 standard; OptiView Kit.

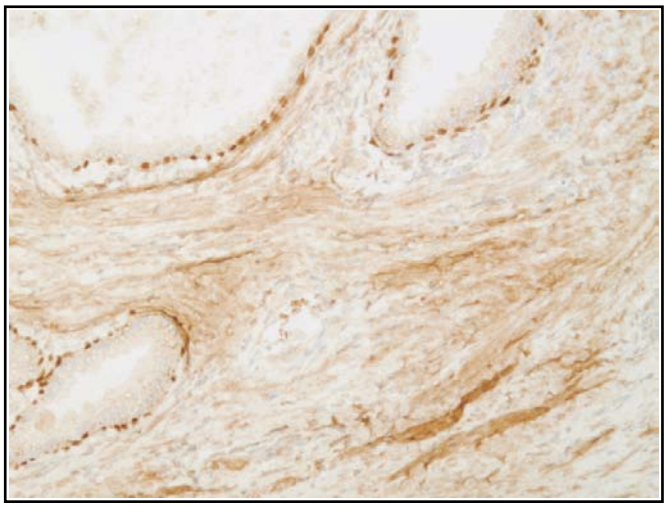
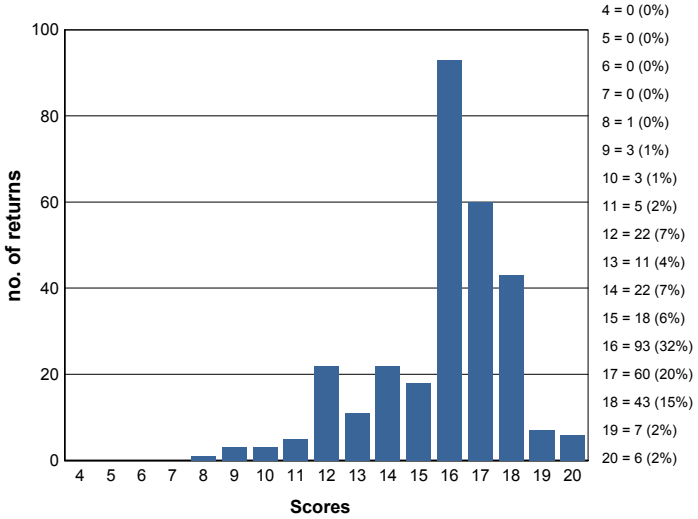


Figure 12: Poor example of an in-house control stained with p63. There is excessive background with little to no counterstain. Protocol as per fig 8.

GRAPHICAL REPRESENTATION OF PASS RATES

RUN 119A Thyroid Transcription Factor 1 (TTF1) on NEQAS Sections
ALL PARTICIPANTS

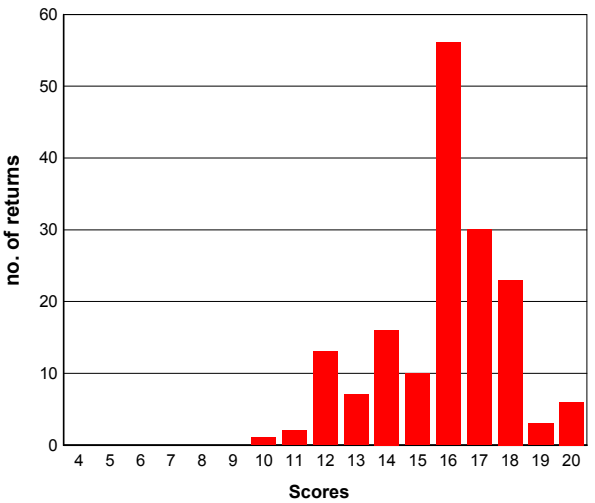


Individual Scores
4 = 0 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 0 (0%)
8 = 1 (0%)
9 = 3 (1%)
10 = 3 (1%)
11 = 5 (2%)
12 = 22 (7%)
13 = 11 (4%)
14 = 22 (7%)
15 = 18 (6%)
16 = 93 (32%)
17 = 60 (20%)
18 = 43 (15%)
19 = 7 (2%)
20 = 6 (2%)

Summary
16 - 20 = 209 (71%)
13 - 15 = 51 (17%)
10 - 12 = 30 (10%)
0 - 9 = 4 (1%)

Median = 14.00

RUN 119A Thyroid Transcription Factor 1 (TTF1) on NEQAS Sections
UK PARTICIPANTS

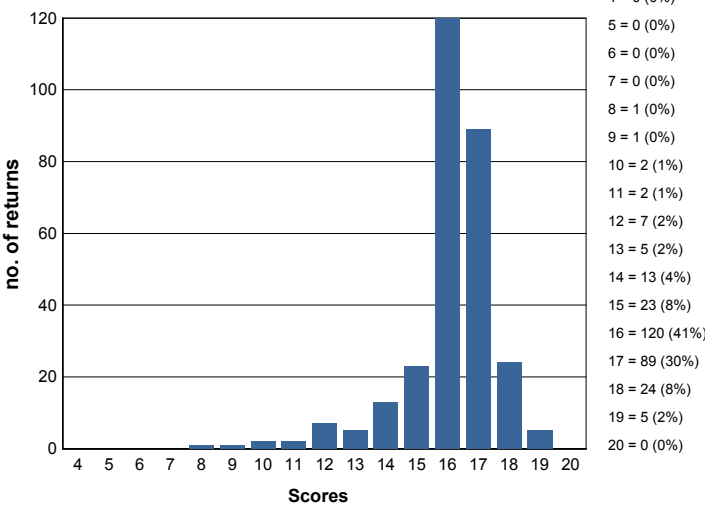


Individual Scores
4 = 0 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 0 (0%)
8 = 0 (0%)
9 = 0 (0%)
10 = 1 (1%)
11 = 2 (1%)
12 = 13 (8%)
13 = 7 (4%)
14 = 16 (10%)
15 = 10 (6%)
16 = 56 (34%)
17 = 30 (18%)
18 = 23 (14%)
19 = 3 (2%)
20 = 6 (4%)

Summary
16 - 20 = 118 (71%)
13 - 15 = 33 (20%)
10 - 12 = 16 (10%)
0 - 9 = 0 (0%)

Median = 15.00

RUN 119B Thyroid Transcription Factor 1 (TTF1) on in-house Sections
ALL PARTICIPANTS

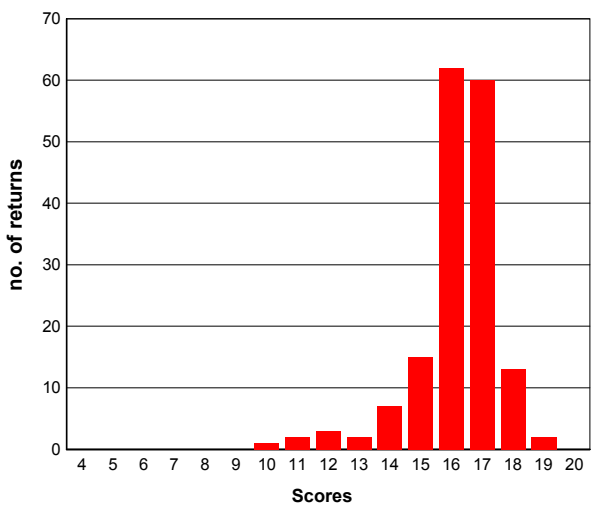


Individual Scores
4 = 0 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 0 (0%)
8 = 1 (0%)
9 = 1 (0%)
10 = 2 (1%)
11 = 2 (1%)
12 = 7 (2%)
13 = 5 (2%)
14 = 13 (4%)
15 = 23 (8%)
16 = 120 (41%)
17 = 89 (30%)
18 = 24 (8%)
19 = 5 (2%)
20 = 0 (0%)

Summary
16 - 20 = 238 (82%)
13 - 15 = 41 (14%)
10 - 12 = 11 (4%)
0 - 9 = 2 (1%)

Median = 13.50

RUN 119B Thyroid Transcription Factor 1 (TTF1) on in-house Sections
UK PARTICIPANTS

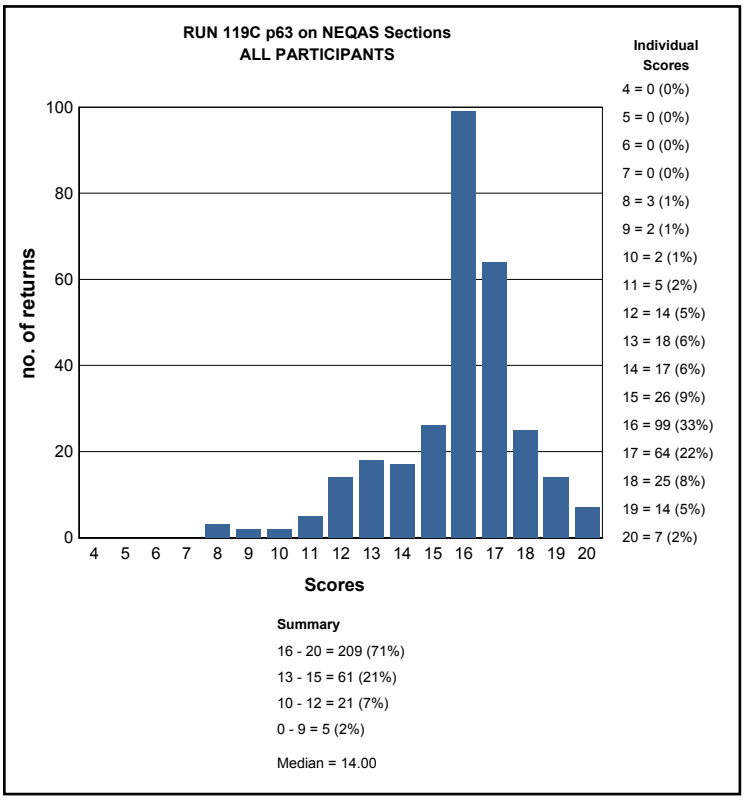


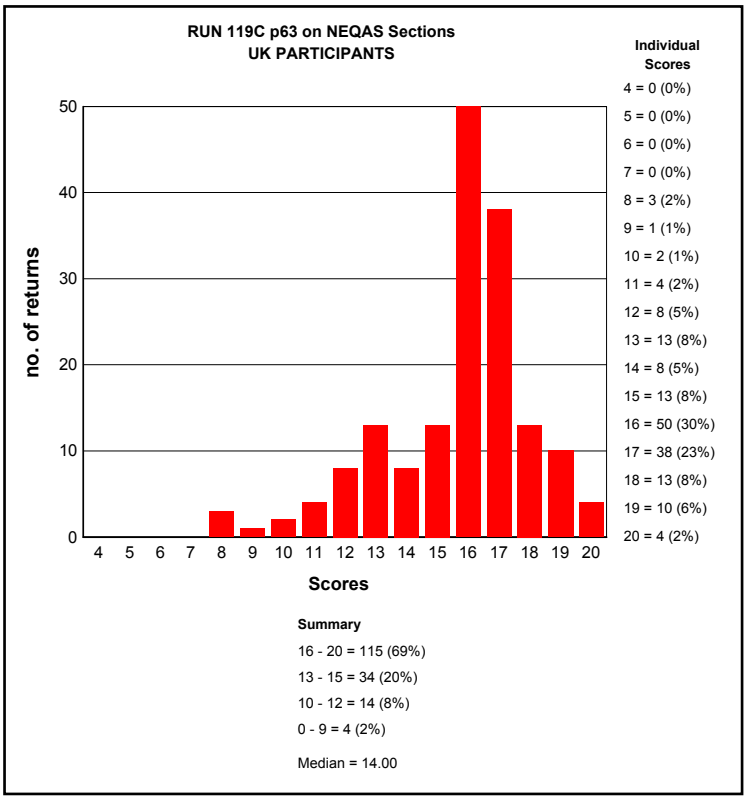
Individual Scores
4 = 0 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 0 (0%)
8 = 0 (0%)
9 = 0 (0%)
10 = 1 (1%)
11 = 2 (1%)
12 = 3 (2%)
13 = 2 (1%)
14 = 7 (4%)
15 = 15 (9%)
16 = 62 (37%)
17 = 60 (36%)
18 = 13 (8%)
19 = 2 (1%)
20 = 0 (0%)

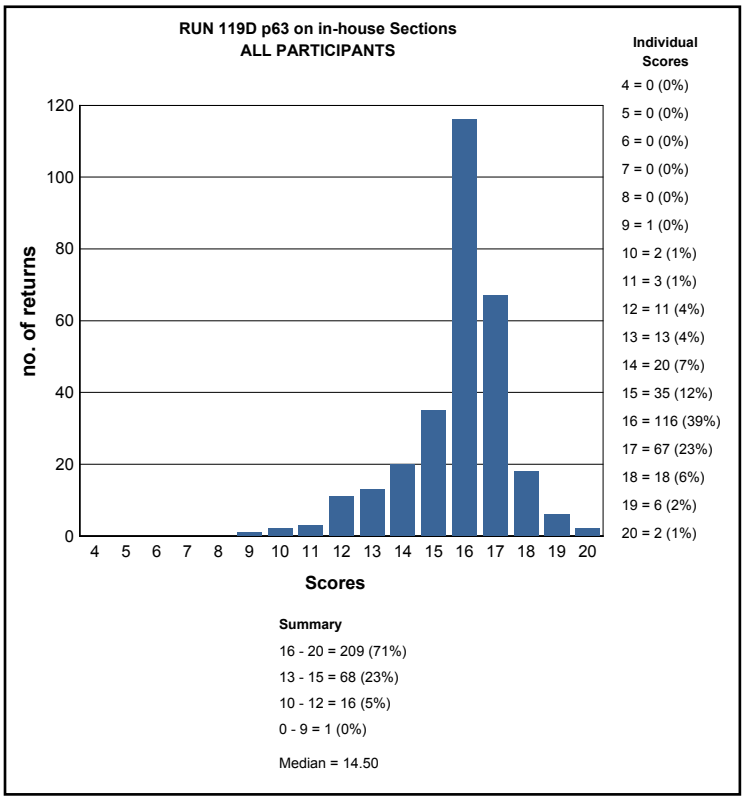
Summary
16 - 20 = 137 (82%)
13 - 15 = 24 (14%)
10 - 12 = 6 (4%)
0 - 9 = 0 (0%)

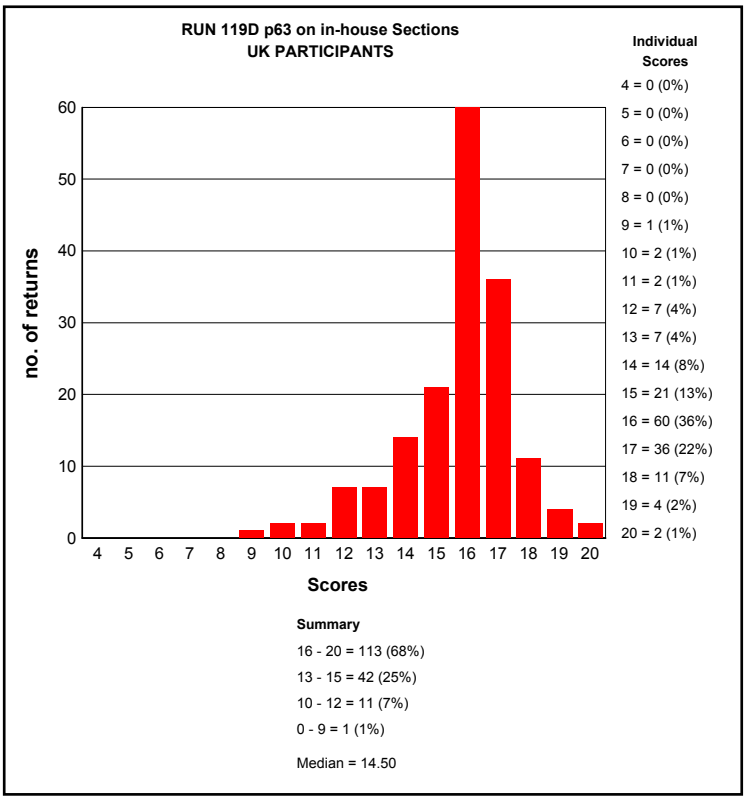
Median = 14.50

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: 119

Primary Antibody : Thyroid Transcription Factor 1 (TTF1)

Antibody Details	N	%
Dako IR056 (8G7G3/1)	12	67
Dako IS056 (8G7G3/1)	2	50
Dako M3575 (8G7G3/1)	55	82
Invitrogen 081221 (8G7G3/1)	1	0
Invitrogen 180221 (8G7G3/1)	2	50
Neomarkers MS-69-XX (8G7G3/1)	1	0
Novocastra NCL-L-TTF-1 (SPT24)	50	98
Novocastra NCL-TTF-1 (SPT24)	12	100
Novocastra PA0364 (SPT24)	6	100
Ventana 760-2829 (8G7G3/1)	9	78
Other	14	79
Zymed 08-1221 (8G7G3/1)	1	100
Biogenex MU397-UC	1	100
BOND RTU TTF-1 (SPT24) PA0364	5	100
Cell Marque 343M-95/96/97 (8G7G3/1)	5	60
Labvision MS-699	1	0
Leica Bond TTF1 SPT24 (RTU) PA0364	20	90
Leica NCL-L-TTF L136446	21	95
THermo-Scientific MS/699P1	1	0
Ventana TTF-1 (SP141) 790-4756	51	98
Cell Path MOB 285 (8G7G3/1)	2	100
Ventana TTF-1 (SP141) 790-4398	13	85

General Pathology Run: 119

Primary Antibody : p63

Antibody Details	N	%
Biocare Medical PM163 (4A4)	8	88
Dako M7317 (DAK-p63)	47	91
Dako IR662 RTU FLEX Link (DAK-p63)	27	96
Dako M7247 (4A4)	1	100
Leica/Novocastra PA0103 RTU (7JUL)	25	92
Leica/Novocastra NCL-L-p63 (7JUL)	44	77
Minarini MP-163-CM (4A4)	17	88
Santa Cruz sc8431 (4A4)	2	100
Ventana 790-4509 (454)	94	97
Other	20	85

General Pathology Run: 119

Heat Mediated Retrieval	p63 N	p63 %	Thyroid Transcription Factor 1 (TTF1) N	Thyroid Transcription Factor 1 (TTF1) %
_Ventana Benk CC1 (Extended)	0	0	1	100
_Ventana Benk CC1 (Standard)	0	0	1	100
Dako Omnis	15	87	12	75
Dako PTLink	24	92	30	90
Lab vision PT Module	2	50	1	100
Leica ER1 10 mins	2	50	0	0
Leica ER1 20 mins	3	67	24	88
Leica ER1 30 mins	1	100	12	92
Leica ER1 40 mins	0	0	1	0
Leica ER2 10 mins	5	100	2	100
Leica ER2 20 mins	50	88	34	88
Leica ER2 30 mins	15	80	8	100
Leica ER2 40 mins	6	83	1	100
Microwave	4	75	4	75
None	0	0	2	100
Other	1	100	1	100
Pressure Cooker	3	100	0	0
Pressure Cooker in Microwave Oven	1	100	1	100
Steamer	2	50	2	100
Ventana CC1 16mins	1	100	0	0
Ventana CC1 24mins	1	100	3	100
Ventana CC1 32mins	15	100	8	100
Ventana CC1 36mins	4	100	7	71
Ventana CC1 40mins	10	100	10	90
Ventana CC1 48mins	7	100	7	100
Ventana CC1 52mins	0	0	1	100
Ventana CC1 56mins	8	100	9	100
Ventana CC1 64mins	54	96	60	87
Ventana CC1 72mins	0	0	2	100
Ventana CC1 76mins	1	100	2	100
Ventana CC1 80mins	1	100	0	0
Ventana CC1 88mins	1	100	0	0
Ventana CC1 92mins	1	100	0	0
Ventana CC1 extended	2	100	3	100
Ventana CC1 mild	9	89	5	80
Ventana CC1 standard	32	91	27	85
Ventana CC2 48mins	1	100	0	0
Ventana CC2 64mins	0	0	1	100
Water bath 68 OC	1	100	1	0
Water bath 95-98 OC	1	0	1	0

General Pathology Run: 119

Enzyme Mediated Retrieval	p63 N	p63 %	Thyroid Transcription Factor 1 N	Thyroid Transcription Factor 1 %
AS PER KIT	6	67	7	57
NOT APPLICABLE	124	90	153	90
Other	0	0	1	100
Ventana Protease 1 (760-2018)	1	0	1	100

General Pathology Run: 119 Detection	p63 N	p63 %	Thyroid Transcription Factor 1 N	Thyroid Transcription Factor 1 %
AS PER KIT	22	73	17	71
Dako EnVision FLEX (K8000/10)	9	78	9	67
Dako EnVision FLEX+ (K8002/12)	21	100	25	96
Dako Envision HRP/DAB (K5007)	4	100	2	50
Dako Envision+ HRP mouse K4004/5/6/7	1	0	4	75
LabVision UltraVision LP HRP (TL 125 HLJ)	1	0	1	100
Leica Bond Polymer Define (DS9713)	2	50	0	0
Leica Bond Polymer Refine (DS9800)	72	89	76	91
None	2	100	0	0
NOT APPLICABLE	0	0	1	100
Novocastra Novolink PDS (RE7-140/150/280/290-K)	2	50	2	100
Other	7	86	7	57
Ventana iView system (760-091)	3	33	3	100
Ventana OptiView (760-700) + Amp. (7/860-099)	3	100	4	100
Ventana OptiView Kit (760-700)	62	100	63	94
Ventana UltraView Kit (760-500)	67	96	66	86

General Pathology Run: 119 Automation	p63 N	p63 %	Thyroid Transcription Factor 1 (TTF1) N	Thyroid Transcription Factor 1 (TTF1) %
BioGenex GenoMX 6000i	0	0	1	100
Dako Autostainer	1	0	2	100
Dako Autostainer Link 48	23	91	24	92
Dako Autostainer plus	0	0	1	100
Dako Autostainer Plus Link	3	100	4	75
Dako Omnis	14	86	12	75
LabVision Autostainer	2	50	2	100
Leica Bond Max	28	86	32	78
Leica Bond-III	54	85	56	95
None (Manual)	7	71	4	50
Other	1	100	1	100
Shandon Sequenza	1	100	1	0
Ventana Benchmark GX	5	100	4	100
Ventana Benchmark ULTRA	101	96	103	90
Ventana Benchmark XT	45	96	38	87
Ventana NexES	0	0	1	100

General Pathology Run: 119 Chromogen	p63 N	p63 %	Thyroid Transcription Factor 1 (TTF1) N	Thyroid Transcription Factor 1 (TTF1) %
AS PER KIT	40	88	41	90
BioGenex liquid DBA (HK-124-7K)	0	0	1	100
Dako DAB K3468	1	0	0	0
Dako DAB+ Liquid (K3468)	3	67	3	100
Dako EnVision Plus kits	3	100	3	67
Dako FLEX DAB	32	91	33	85
Dako REAL EnVision K5007 DAB	3	67	4	50
Leica Bond Polymer Refine kit (DS9800)	71	87	73	90
Other	12	100	13	85
Sigma DAB (D5637)	1	0	1	100
Sigma DAB (D5905)	1	100	0	0
Ventana DAB	40	100	38	95
Ventana iview	5	60	6	83
Ventana Ultraview DAB	72	96	70	86

BEST METHODS - Gold Standard Antibody

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

Thyroid Transcription Factor 1 (TTF1) - Method 1

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

Primary Antibody: Ventana TTF-1 (SP141) 790-4398
Automation: Ventana Benchmark ULTRA
Method: Ventana Optiview
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 36mins
EAR:
Chromogen: Ventana DAB
Detection: Ventana OptiView Kit (760-700)

Thyroid Transcription Factor 1 (TTF1) - Method 2

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

Primary Antibody: Novocastra NCL-L-TTF-1 (SPT24) , 15 Mins, 37 ºC Dilution 1: 1:200
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 30 mins
EAR:
Chromogen: Leica Bond Polymer Refine kit (DS9800), Time 1: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins, RT ºC Prediluted

Thyroid Transcription Factor 1 (TTF1) - Method 3

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

Primary Antibody:	Leica NCL-L-TTF L136446 , 60 Mins, 20 ºC Dilution 1: 50
Automation:	Dako Autostainer
Method:	Envision
Main Buffer:	Tris Buffered Saline (TBS)
HMAR:	Microwave, Buffer: Tris/EDTA, PH: 8.5
EAR:	
Chromogen:	Dako DAB+ Liquid (K3468), 20 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Other , 30 Mins Prediluted

Thyroid Transcription Factor 1 (TTF1) - Method 4

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

Primary Antibody:	Novocastra NCL-L-TTF-1 (SPT24) , 32 Mins, 36 ºC Dilution 1: 1/200
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700) Prediluted

BEST METHODS - Secondary Antibody

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

p63 - Method 1

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

Primary Antibody:	Ventana 790-4509 (454) , 40 Mins, RT ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB, RT ºC., Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 16 Mins, RT ºC Prediluted

p63 - Method 2

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

Primary Antibody:	Dako IR662 RTU FLEX Link (DAK-p63) , 20 Mins, 32 ºC Prediluted
Automation:	Dako Omnis
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis, PH: 9
EAR:	
Chromogen:	Dako FLEX DAB, 32 ºC., Time 1: 5 Mins
Detection:	Dako EnVision FLEX (K8000/10) , 20 Mins, 32 ºC

p63 - Method 3

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

Primary Antibody:	Dako M7317 (DAK-p63) , 32 Mins, 36 ºC Dilution 1: 100
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 32mins, Buffer: Cell Conditioner 1 (CC1), PH: 8
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT, 36 ºC., Time 1: 8 Mins
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099) , 8 Mins, 36 ºC Prediluted

p63 - Method 4

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

Primary Antibody:	Leica/Novocastra NCL-L-p63 (7JUL) Dilution 1: 50
Automation:	Ventana Benchmark XT
Method:	Ventana Optiview
Main Buffer:	AS PER KIT
HMAR:	Ventana CC1 standard
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	AS PER KIT

The Breast Hormone Receptor Module Run 119

Suzanne Parry and Jamie Hughes

Antigen Assessed:	Progesterone Receptor (PR)
Tissue Sections circulated:	Composite slide consisting of 3 breast carcinomas with different levels of receptor expression and normal tonsil.
Number of Registered Participants:	263
Number of Participants This Run	255 (97%)

Circulated Tissue
The table below shows the staining characteristics of the tissue sections circulated during Run 119. 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 PgR (clone 16) and Dako PgR 1294.

Sections	% Positivity	Expected Staining Intensity	Allred / Quick Score
A. IDC	**>95%**	**High**	**8**
B. IDC	**11-33%**	**Mid**	**4 (the expression level varied depending on the serial section received)**
C. IDC	**0%**	**Negative**	**0**
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/inadequate choice of in-house control tissue (poor/inadequate fixation, damaged cell morphology, over retrieval etc.)

In-House Tissue Recommendations:

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

The required composite control should consist of the following samples:

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

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

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, PR can be useful in specific clinical situations to predict response to ER-antagonists (Ciocca and Elledge, 2000). Therefore, accurate staining protocols and validated staining protocols are 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 PR 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 1A6. clone. This made sure that any heterogeneity in the tissue samples did not result in a participant being penalised.

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.

Features of Optimal Immunostaining (Figures 1-6 & 9)

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

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

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

NEQAS Section Assessment Results

255 laboratories submitted their slides for the PR assessment. The pass rates are slightly lower than the previous PR assessment (Run 114), but in keeping with the pass rates seen over previous runs.

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

PR NEQAS Pass Rates :			
	Run 112	Run 114	Run 119
Acceptable	88%(N=242)	85%(N=221)	81%(N=207)
Borderline	8%(N=21)	10%(N=25)	13%(N=34)
Unacceptable	4%(N=10)	5%(N=14)	6%(N=14)

The borderline and failed marks for this assessment were mostly due to weak staining, particularly in the mid-expressing tumour. A small number of laboratories 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 excessive, 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=178) compared to the NEQAS sections. The in-house samples showed a higher percentage of borderline passes, 28% (N=71) compared to the NEQAS borderline pass rate. Only 4 laboratories (2%) 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 PR tumour (as outlined in the in-house recommendations section of this article).

Methodologies

The most popular antibody was the Ventana 1E2 clone, used by 39% (N=99) of participants and showed an acceptable pass rate of 80%. The second most popular clone was Leica PgR Clone 16, used by 17% (N=45), and showed an acceptable pass rate of 97%. The third most popular clone was the Dako PgR 636 clone, used by 16% (N=41) and showed an acceptable pass rates of 78%.

The Ventana 1E2 clone is a pre-diluted antibody, and was only seen at the Run 119 assessment to be used on the Ventana automated platforms, for which the antibody was validated. While both the Dako PgR 636 and Leica 16 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. 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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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 'Gold standard' slides with their respective assays.

Selected Images showing Optimal and Sub-optimal immunostaining

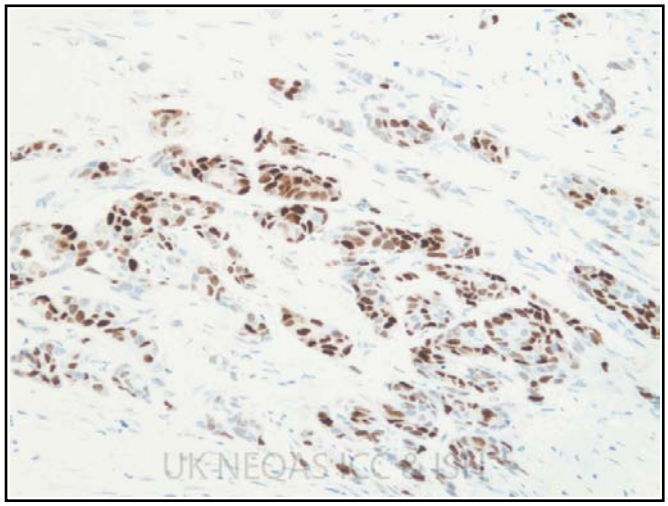


Fig 1. Optimal demonstration of PR in the UK NEQAS high expressing tumour. Over 95% of neoplastic cells are staining, with mostly moderate to strong intensity. Stained using the Leica/NCL-L-PGR-312 (16) (A) antibody on the Bond III with ER1 retrieval for 30 minutes.

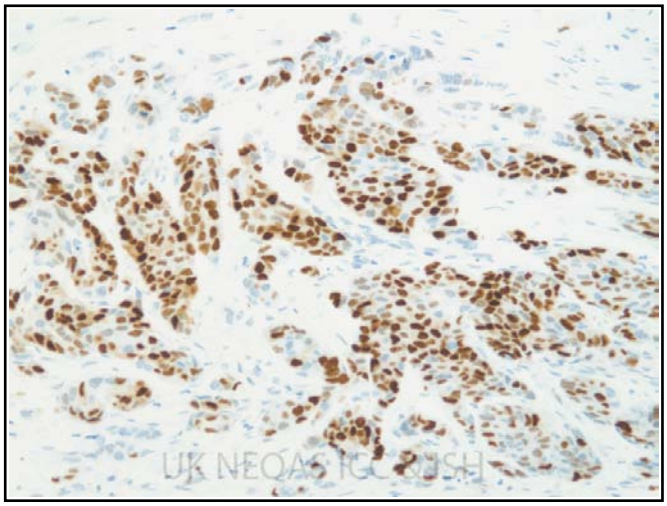


Fig 2. Expected level of PR staining in the UK NEQAS high expressing tumour. Similarly to Figure 1, the staining is seen in over 95% of neoplastic cells with varying intensity; many showing moderate to strong expression. Stained with the Dako RTU PR 1294 on the Omnis platform.

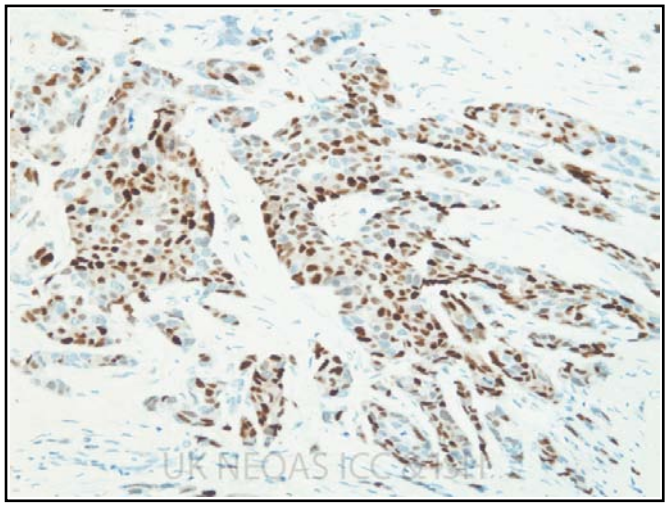


Fig 3. Optimal demonstration of PR in the UK NEQAS higher expressing tumour, showing the expected level of staining. Stained with the 1E2 (A&B) pre-diluted antibody on the Benchmark XT, CC1 retrieval for 20 minutes and Ultraview detection.

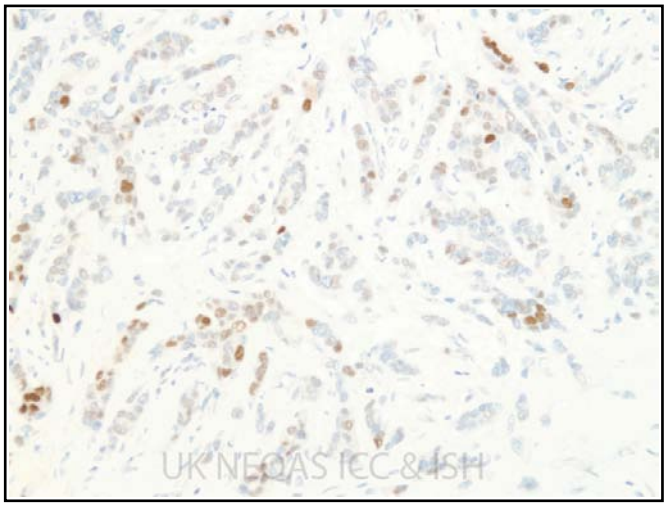


Fig 4. Unacceptable PR staining of the UK NEQAS high expressing tumour. The staining is weak with fewer positive tumour cells staining than expected. The same method was used as the image in Fig 1, except shorter antigen retrieval time (compare to Figs 1-3).

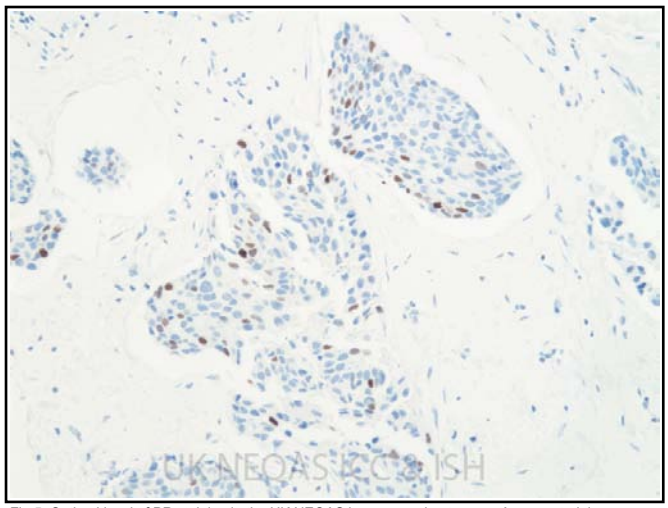


Fig 5. Optimal level of PR staining in the UK NEQAS low expressing tumour. As expected, less than 10% of tumour cells are staining with moderate to high intensity. (Same protocol as Fig 1).

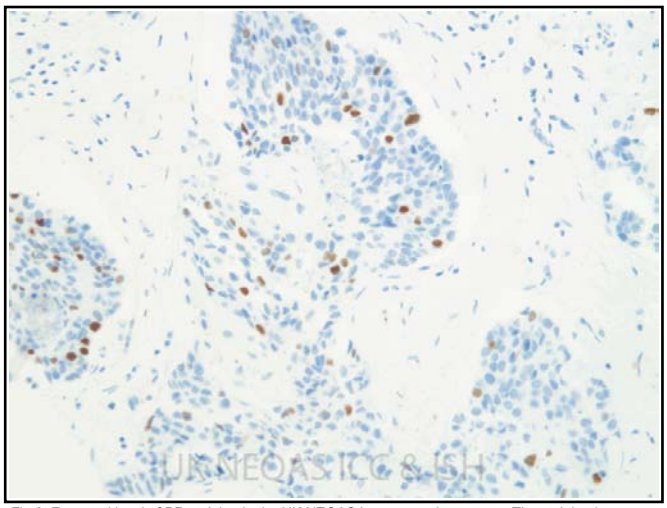


Fig 6. Expected level of PR staining in the UK NEQAS low expressing tumour. The staining is strong in less than 10% of neoplastic cells. (Same protocol as Fig 2).

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

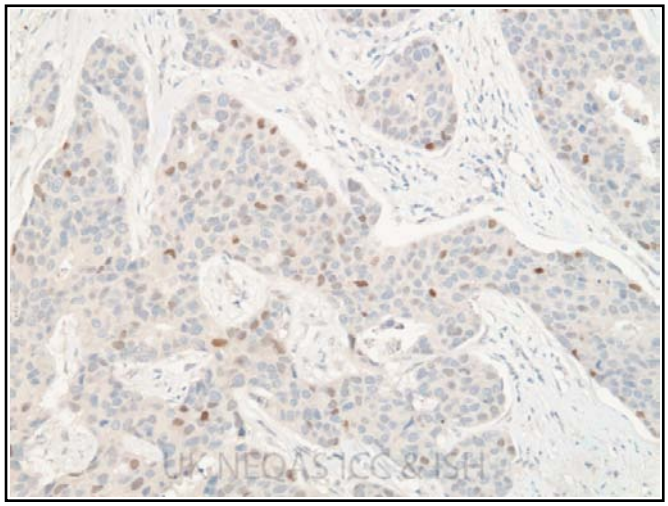


Fig 7. Sub-optimal demonstration of PR the UK NEQAS low expressing tumour. The level of tumour cells staining is as expected, but there is also non-specific background staining, which is most likely caused by excessive antigen retrieval. Staining was carried out on the Ventana XT with extended retrieval.

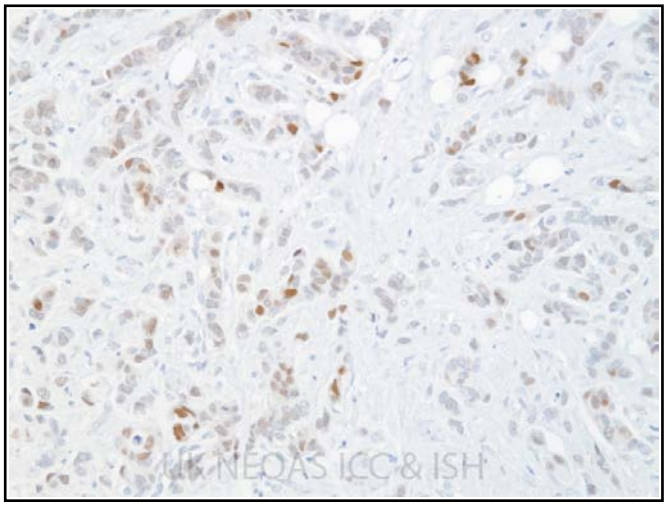


Fig 8. Unacceptable over-staining of the UK NEQAS low expressing tumour. The percentage of tumour cells staining is much higher than expected, resulting in an incorrect clinical outcome. Stained with the Ventana pre-diluted 1E2 (A&B) clone on the ULTRA CC1 36 minutes retrieval.

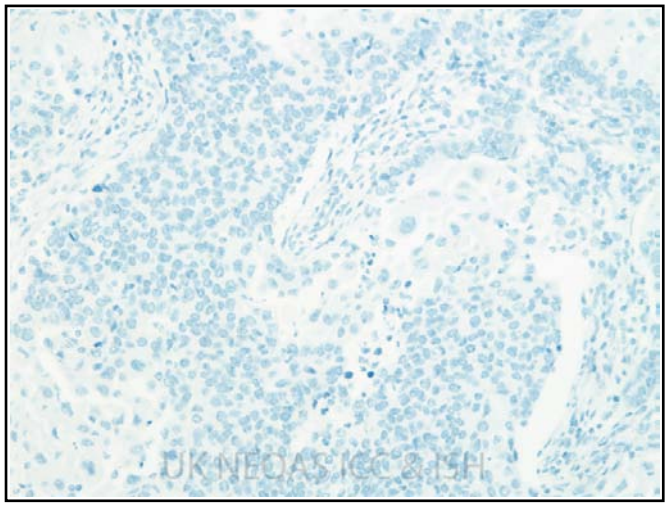


Fig 9. Expected staining result on the PR negative tumour. None of the tumour cells show positivity of PR. (Same protocol as Fig 3).

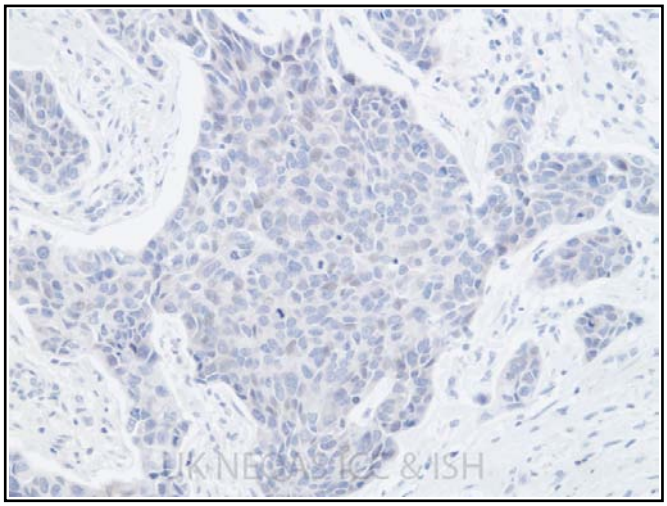


Fig 10. Unacceptable inappropriate staining seen in the UK NEQAS negative tumour sample. The staining is therefore showing a false-positive reaction. (Same protocol as Fig 8).

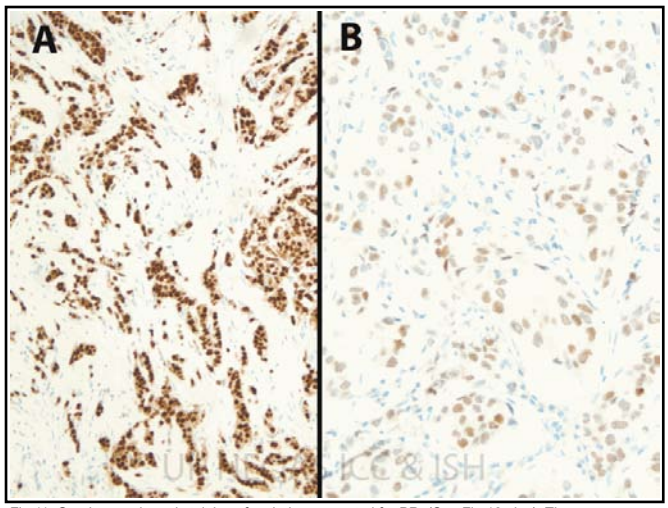


Fig 11. Good example and staining of an in-house control for PR. (See Fig 12 also). The multi-block section contained high- and negative-expressing tumours shown in this image (A & B respectively). Stained using the Ventana 1E2 (A&B) clone on the ULTRA, CC1 for 64 minutes retrieval.

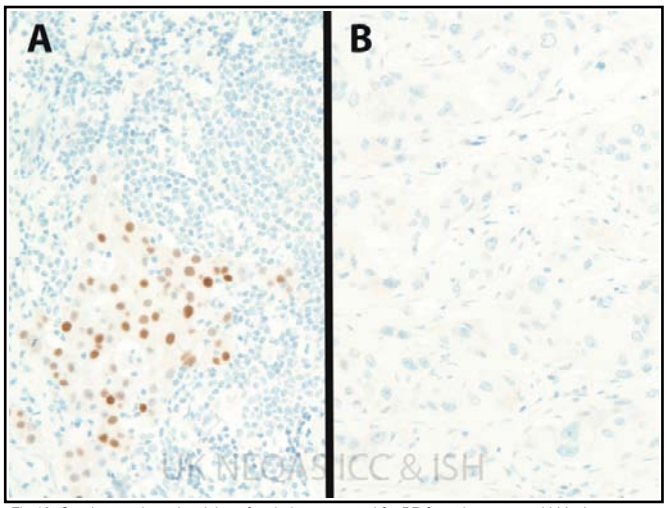
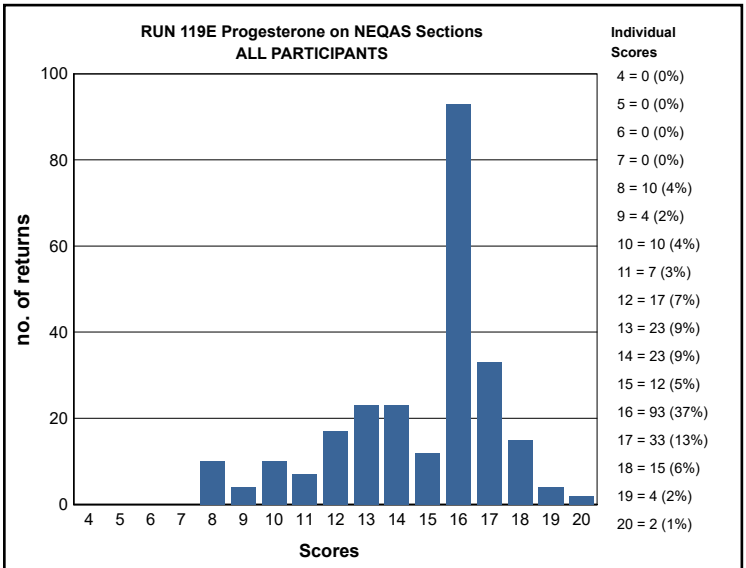
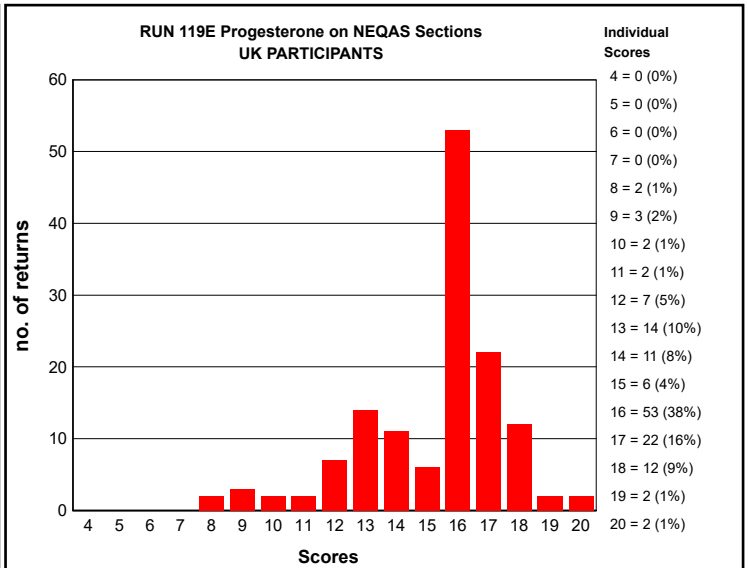
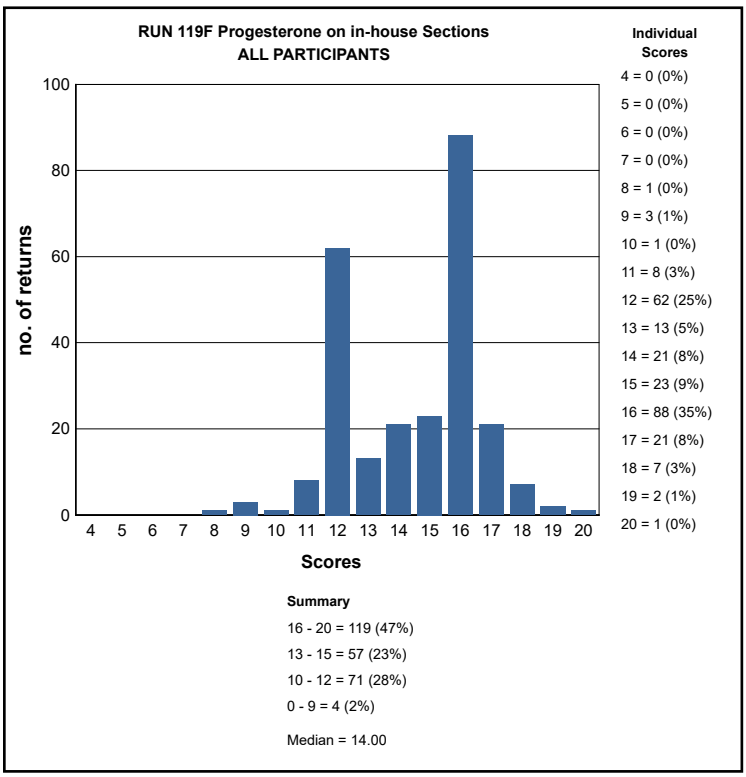


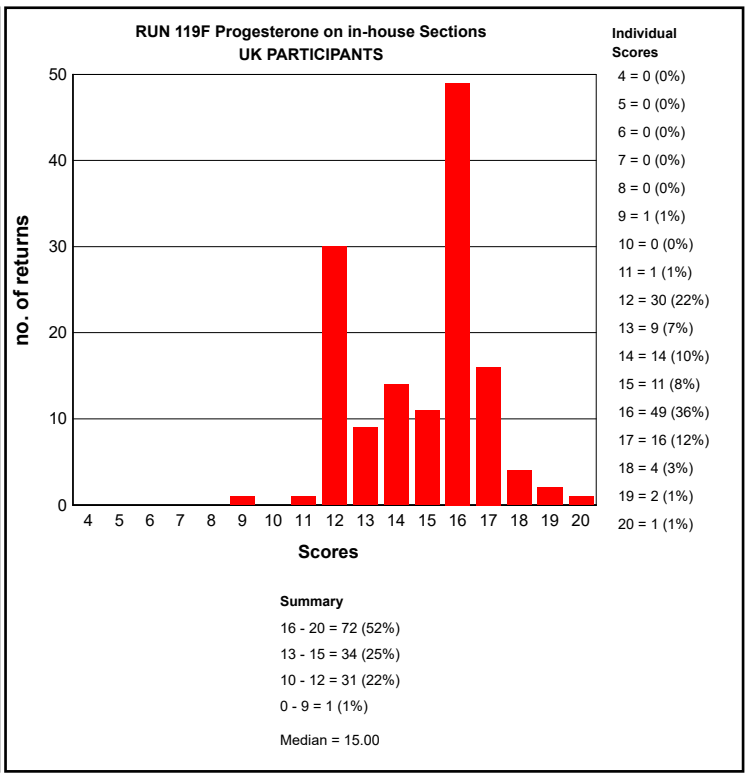
Fig 12. Good example and staining of an in-house control for PR from the same multi-block control shown in Fig 11. (A) low expressing PR tumour, and (B) is negative for PR.

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: 119

Primary Antibody : Progesterone

Antibody Details	N	%
Dako M3569 (PgR 636) (A&B)	28	68
Dako K1904 (PgR 1294 (b))	1	0
Novocastra NCL-PGR (1A6) (A&B)	2	100
Novocastra NCL-PGR-312 (16) (A)	11	100
Novocastra NCL-L-PGR-312 (16) (A)	33	94
Ventana 760 2547 PgR (1A6) (A&B)	1	0
Ventana 790-4296 (1E2) (A&B)	34	82
Novocastra NCL-L-PGR-AB (16+SAN27) (A&B)	7	100
Novocastra RTU-PGR-312 (16) (A)	2	100
Novocastra PA0312 (16) (A)	12	83
Novocastra NCL-L-PGR/2 (1A6) (A&B)	2	50
Novocastra NCL-PGR-AB (16+SAN27)	4	100
Ventana 790-2223 (1E2) (A&B)	65	77
Dako N1630 RTU (PgR 636) (A&B)	1	100
Dako IR068 (PgR 636) (A&B)	9	78
Dako IS068 (PgR 636) (A&B)	3	67
Ventana 790-4324 (SP2) (A&B)	3	67
Cell Marque 323R-16 (A)	1	0
Other	19	84
Dako M3568 (PgR 1294)	2	100

Breast Steroid Hormone Receptor Run: 119

Automation — Progesterone

	N	%
Dako Autostainer	2	100
Dako Autostainer Link 48	18	89
Dako Autostainer Plus Link	4	50
Dako Omnis	10	90
LabVision Autostainer	3	67
Leica Bond Max	17	100
Leica Bond-III	48	92
None (Manual)	3	67
Ventana Benchmark GX	8	63
Ventana Benchmark ULTRA	102	78
Ventana Benchmark XT	34	68

Breast Steroid Hormone Receptor Run: 119

Heat Mediated Retrieval — Progesterone

	N	%
Biocare Decloaking Chamber	1	100
Dako Omnis	9	100
Dako PTLink	22	77
Lab vision PT Module	2	50
Leica ER1 20 mins	12	83
Leica ER1 30 mins	15	100
Leica ER1 40 mins	3	67
Leica ER2 10 mins	2	100
Leica ER2 20 mins	31	97
Leica ER2 30 mins	3	100
Microwave	2	100
Pressure Cooker	2	100
Pressure Cooker in Microwave Oven	1	100
Ventana CC1 16mins	2	0
Ventana CC1 20mins	1	100
Ventana CC1 24mins	1	100
Ventana CC1 32mins	6	67
Ventana CC1 36mins	18	67
Ventana CC1 40mins	3	100
Ventana CC1 44mins	1	100
Ventana CC1 48mins	3	67
Ventana CC1 52mins	8	88
Ventana CC1 56mins	1	100
Ventana CC1 64mins	49	78
Ventana CC1 72mins	1	0
Ventana CC1 76mins	3	67
Ventana CC1 8mins	1	0
Ventana CC1 92mins	1	100
Ventana CC1 extended	3	33
Ventana CC1 mild	13	69
Ventana CC1 standard	27	85
Water bath 95-98 OC	1	0

Breast Steroid Hormone Receptor Run: 119

Enzyme Mediated Retrieval — Progesterone

	N	%
AS PER KIT	8	88
NOT APPLICABLE	166	82
Ventana Protease 1 (760-2018)	1	100

Breast Steroid Hormone Receptor Run: 119

Detection	Progesterone N	Progesterone %
AS PER KIT	12	67
BioGenex SS Polymer (QD 430-XAKE)	1	100
Dako EnVision FLEX (K8000/10)	7	100
Dako EnVision FLEX+ (K8002/12)	16	75
Dako Envision HRP/DAB (K5007)	2	100
Dako Envision+ HRP mouse K4004/5/6/7	2	100
Dako REAL HRP/DAB (K5001)	1	100
LabVision UltraVision LP HRP (TL 125 HLJ)	1	0
Leica Bond Polymer Refine (DS9800)	60	93
NOT APPLICABLE	2	100
Other	3	100
Ventana iView system (760-091)	2	100
Ventana OptiView (760-700) + Amp. (7/860-099)	1	100
Ventana OptiView Kit (760-700)	17	71
Ventana UltraView Kit (760-500)	117	76

Breast Steroid Hormone Receptor Run: 119

Chromogen	Progesterone N	Progesterone %
AS PER KIT	18	83
BioGenex liquid DBA (HK-124-7K)	1	100
Dako DAB K3468	1	0
DAKO DAB+	1	100
Dako EnVision Plus kits	3	33
Dako FLEX DAB	23	91
Dako REAL EnVision K5007 DAB	4	75
Dako REAL K5001 DAB	1	100
Leica Bond Polymer Refine kit (DS9800)	60	93
Other	2	100
Ventana DAB	14	71
Ventana Enhanced Alk. Phos. Red Detection Kit	1	0
Ventana iview	3	67
Ventana Ultraview DAB	117	76

BEST METHODS

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

Progesterone - Method 1

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

Primary Antibody: Novocastra NCL-L-PGR-312 (16) (A) , 15 Mins, ambient ºC Dilution 1: 200
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER1 30 mins
EAR: NOT APPLICABLE
Chromogen: Leica Bond Polymer Refine kit (DS9800)
Detection: Leica Bond Polymer Refine (DS9800)

Progesterone - Method 2

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

Primary Antibody: Ventana 790-2223 (1E2) (A&B)
Automation: Ventana Benchmark XT
Method: Ventana UltraView DAB
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 20mins
EAR:
Chromogen: Ventana Ultraview DAB
Detection: Ventana UltraView Kit (760-500)

Progesterone - Method 3

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

Primary Antibody: Novocastra NCL-L-PGR-312 (16) (A) , 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 standard
EAR: NOT APPLICABLE
Chromogen: Ventana Ultraview DAB
Detection: Ventana UltraView Kit (760-500)

Progesterone - Method 4

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

Primary Antibody:	Dako M3569 (PgR 636) (A&B) , 30 Mins, room ºC Dilution 1: 200
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), room ºC., Time 1: 5 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, room ºC

The Breast HER2 ICC Module Run 119

Amy Newman and Keith Miller

Antigen Assessed:	HER2
Sections Circulated:	4 breast cancer cell lines of varying levels of HER2 expression.
Number of Registered Participants:	249
Number of Participants this Run	232 (93%)

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: Negative	No staining in the '0' control cell line	No staining in the '0' control cell line
B: 1+	Faint barely perceptible incomplete staining	Faint barely perceptible incomplete membrane staining in >10% of cells
C: 2+	Weak to moderate complete cell membrane staining	Weak to moderate complete cell membrane staining in >10% of tumour cells
D: 3+	Strong complete membrane staining	Strong complete cell membrane staining in >30% of tumour cells

Validation of Distributed UK NEQAS ICC & ISH Cell Lines

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

Updated Assessment and Scoring Procedure

UK NEQAS Specific Membrane Scoring Algorithm:

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

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

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

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

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

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

Introduction

HER2 immunohistochemistry has been routinely used as a predictive marker in breast cancer for more than 10 years. Patients with HER2 positive tumours generally have a poor overall prognosis. However, patients with HER2 positve metastatic disease showed an improved rate of survival when the humanized monoclonal antibody Trastuzumab (Herceptin) was given alone or added with chemotherapy treatment (Slamon et al., 1998). Herceptin, in the metastatic setting was made available in the UK in 2000 by the UK National Institute for Clinical Excellence (NICE). In 2005, Herceptin in the adjuvant setting was also shown to be effective in primary breast cancers by reducing the risk of recurrence and mortality (see articles by Wolff et al., (2006) and Walker et al., (2008), and in 2006 NICE approved primary breast screening for HER2. In the UK alone this has resulted in approximately 40,000 breast cancers per year being tested for HER2.
With so many more cases now being tested worldwide, it is imperative that correct protocols and methodologies are followed and adhered to. The UK NEQAS HER2 IHC module assesses the technical quality of staining achieved by laboratories and provides feedback to laboratories to help improve the analytical phase of patient diagnosis.
Recommended HER2 testing guidelines are: The ASCO/CAP guidelines by Wolff et al.(2007), the UK guidelines by Walker et al., (2008), the ASCO/CAP guidelines by Wolff et al., 2013. , and the updated UK guidelines by Rakha et al., (2015). These publications provide invaluable guidelines covering interpretation, tissue fixation and antibody validation. The articles by Walker et. al. and Rhaka et al. also provide guidelines on the minimum number of tests per year that labs should be carrying out in order to provide a robust HER2 IHC service. The recommendations are 250 tests for HER2 IHC and 100 tests for HER2 ISH.
Participants who continue to have difficulty in producing acceptable staining results should be aware that the members of staff at UK NEQAS ICC are always willing to assist any laboratory struggling to meet the required standard of HER2 immunostaining, and would be happy to receive requests for assistance via e-mail in the first instance (see front of journal or the UK NEQAS ICC & ISH website for contact details).

In-House Control Tissue Recommendations

Correct choice of in-house control tissue and good morphological preservation is paramount to gauge the sensitivity of the HER2 test, which can have an impact on clinical interpretation. UK NEQAS ICC & ISH therefore recommend the following:
In-house control tissue (or cell lines) must include 3+, 2+ and 1+/0 invasive breast cancer cases. However, it has become quite apparent that as patient tumour size and respective biopsies become smaller laboratories may be having difficulty finding appropriate invasive control material. It is therefore acceptable, if laboratories are having problems in finding appropriate invasive control material, to submit DCIS tissue showing differing levels of membrane staining as an acceptable alternative. Laboratories must indicate on their datasheet which component they have scored otherwise the invasive component (if present) will be assessed.

Assessment Summary:

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

	Pass Rates Run 119:	
	NEQAS	**In-House**
Acceptable	**72% (N=157)**	**62% (N=133)**
Borderline	**20% (N=43)**	**31% (N=66)**
Unacceptable	**8% (N=18)**	**7% (N=16)**

The predominant issue observed with this run, was weak staining in the participants 1+ NEQAS cell line compared with the Gold Standard. Assessors agreed that if any membrane staining (>1 membrane) showed HER2 staining, then participants would not be marked down for this and their 1+ core was assessed as a 0/1+. However, if weaker staining was also noted in the 2+ cell line core, participants were marked down accordingly.
As with previous runs the most popular antibody was the Ventana 4B5, used by 70% (n=163) of participants and showed an overall acceptable pass rate of 80%. 10 laboratories employed the Dako HercepTest, with 60% of participants achieving an acceptable pass rate. 14 laboratories are using the Leica Oracle assay kit with an acceptable pass rate of 71%. 24 laboratories used laboratory devised tests (LDTs), incorporating a variety of antibodies (most commonly Dako A0485), pre-treatment methods and staining platforms. These laboratories showed an acceptable pass rate of 30%. All data is based on the methodology information submitted by participants, but unfortunately not all methodology was provided by all participants.

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

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

Selected Images showing Optimal and Sub-optimal immunostaining

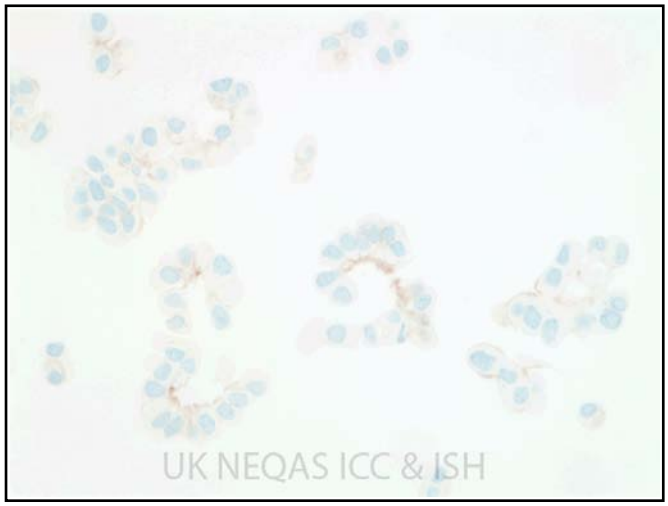


Figure 1: Optimal staining in the 1+ NEQAS cell line. There is weak and delicate incomplete membrane staining in some cells. Note: the stronger brush border (indicated by the arrow) is NOT included in the scoring assessment. Method: Ventana 4B5 Pathway (16 mins); (Benchmark ULTRA, CC1 36 mins; UltraView).

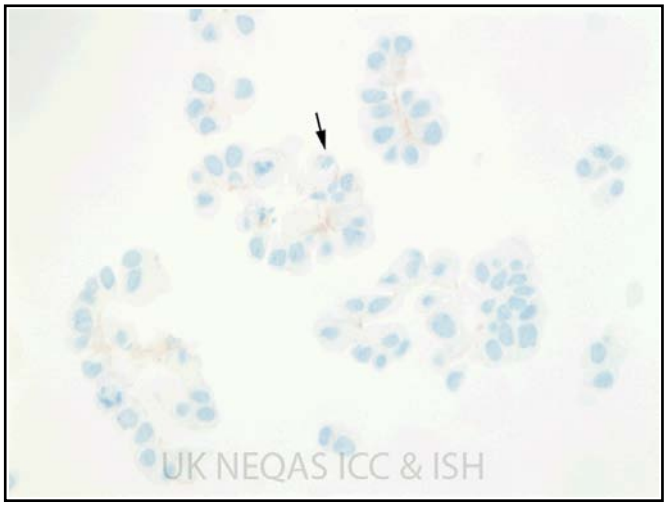


Figure 2: Weak staining in Core B (1+ cell line) on the NEQAS sample. Scored by assessors as 0 or 0/1+ due to barely present membrane staining as indicated by arrow. Method: Ventana 4B5 Pathway (16 mins, 37C); (Benchmark XT, CC1 32 mins, Ultraview).

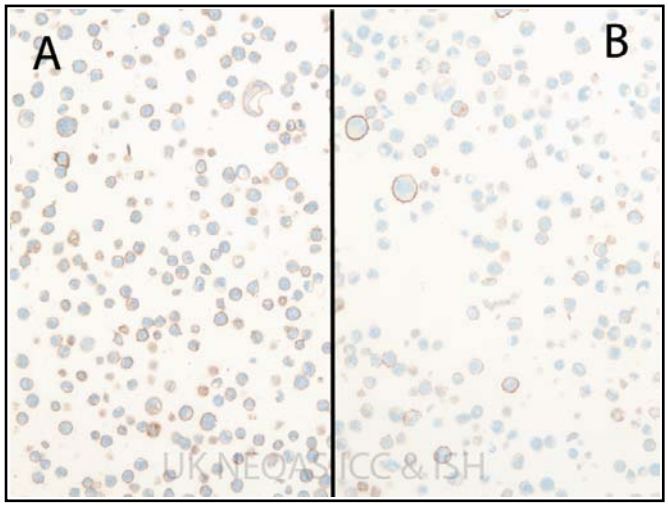


Figure 3: (A) Good example of the 2+ NEQAS distributed cell line. The staining is of moderate intensity with complete membrane staining. Method: Dako HercepTest (PT Link + Autostainer). (B) staining in the 2+ cell line is less intense but still acceptable. Method: As is Figure 2.

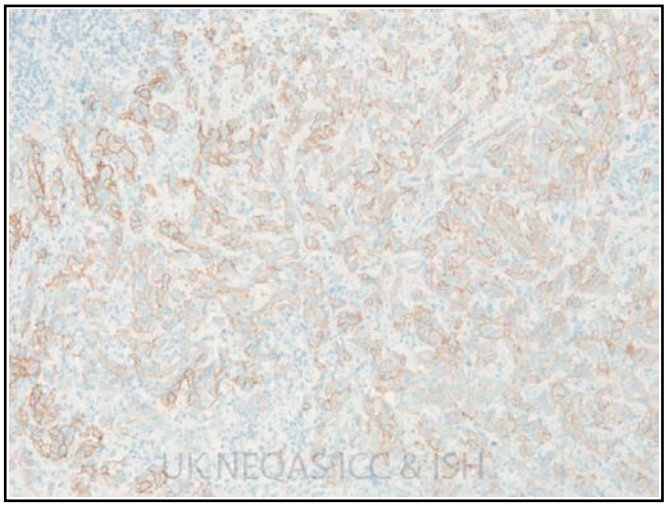


Figure 4: Excellent representation of a 2+ in-house HER2 breast control. There is moderate intensity staining in >10% of tumour cells. Method: Ventana 4B5 Confirm (Benchmark ULTRA, CC1 Mild, UltraView).

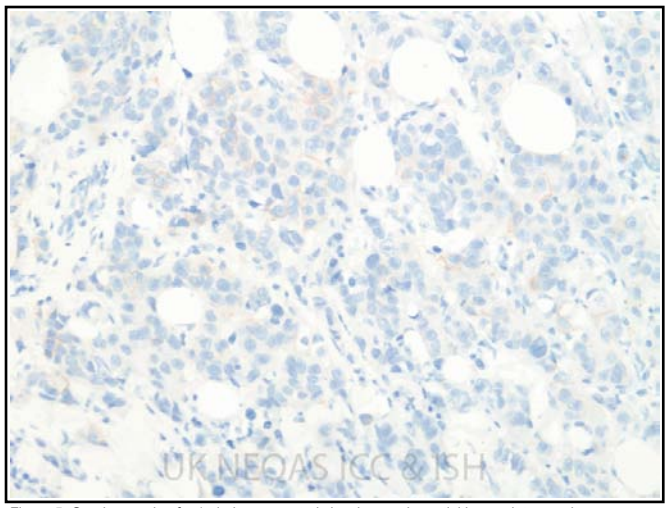


Figure 5: Good example of a 1+ in-house control showing weak, partial incomplete membrane staining in tumour cells. Method: Biogenex (EP1045Y) (1:25 for 8 mins @ 37C); (BOND III; ER1, 40'; Polymer Refine).

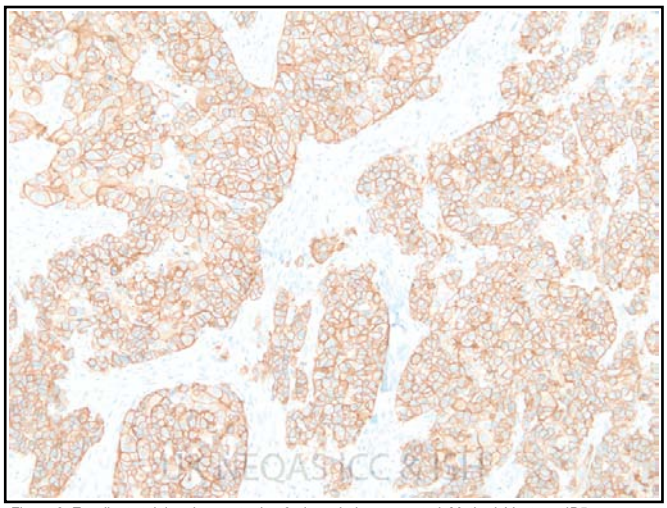
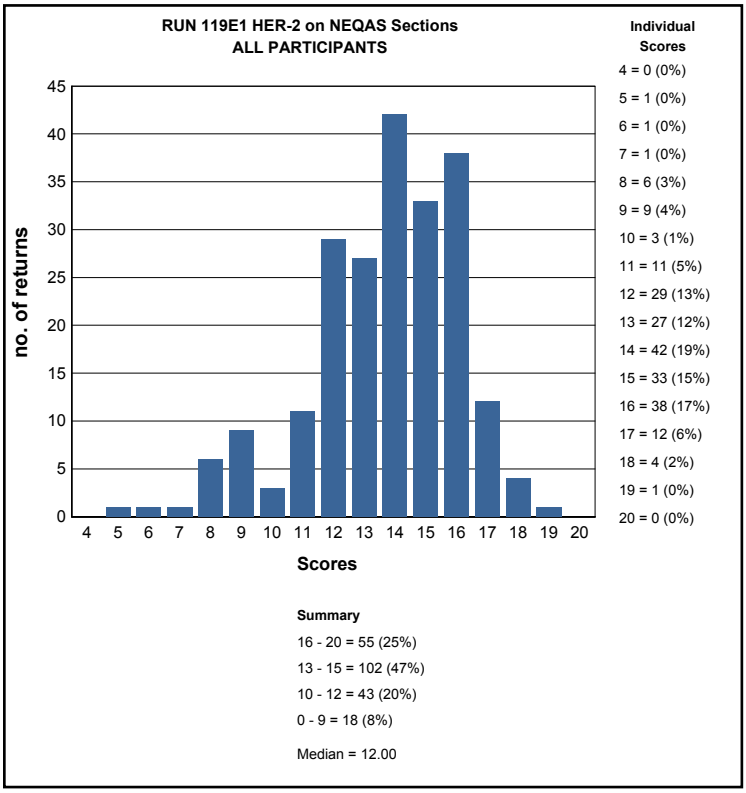
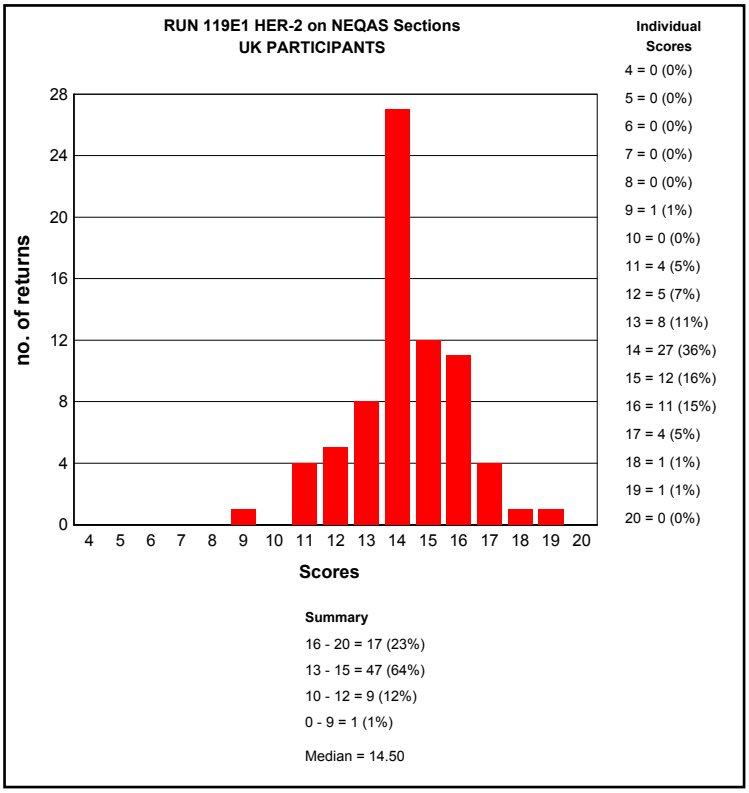
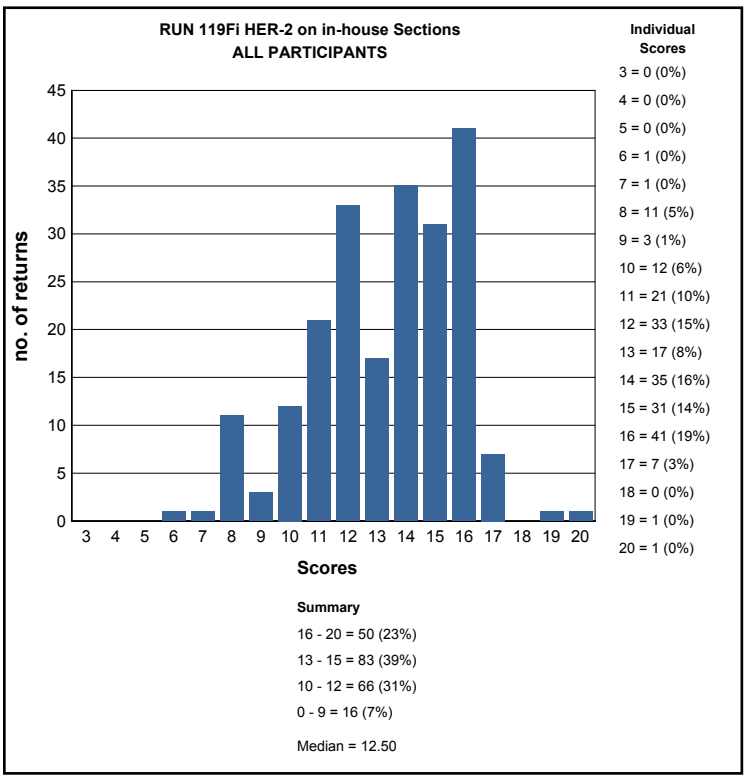


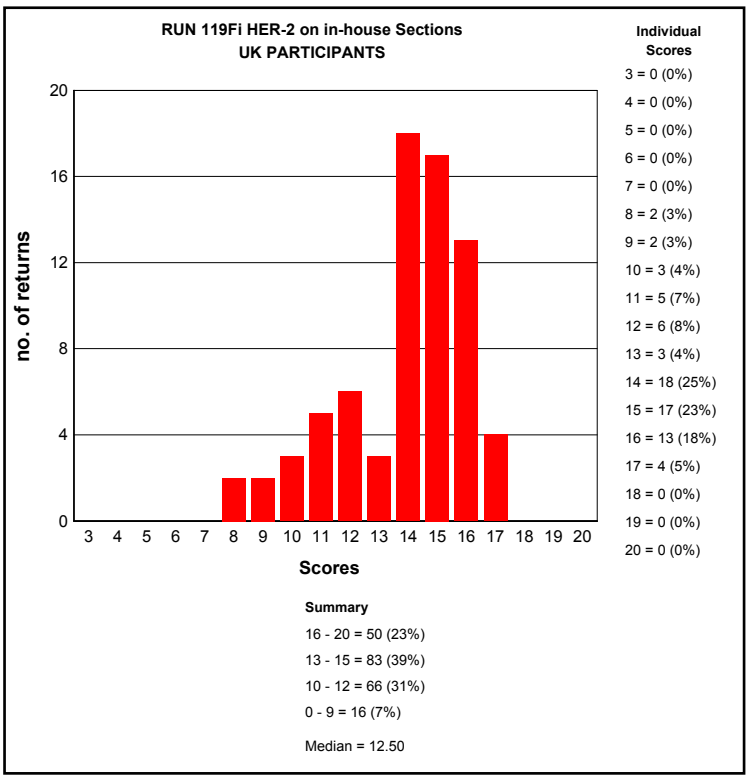
Figure 6: Excellent staining demonstrating 3+ in an in-house control. Method: Ventana 4B5 Confirm (12mins); (Benchmark ULTRA; CC1 36 mins; UltraView).

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: 119

Primary Antibody	N	%
Dako HercepTest K5204 (poly)	2	0
Dako HercepTest K5205 (poly)	1	0
Dako HercepTest K5207 (poly)	1	0
Dako A0485 C-erB-2 (poly)	17	35
Cell Marque CMA 601 (CB11)	1	0
Ventana Pathway 790-100 (4B5)	6	83
Leica Oracle HER2 Bond IHC (CB11)	14	71
Dako Link HercepTest SK001 (poly)	6	100
BioGenex (EP1045Y) rb mono	1	0
Ventana Confirm 790-4493 (4B5)	40	73
Ventana Pathway 790-2991 (4B5)	117	82
Novocastra NCL-L-CB11 (CB11)	3	33
Other	2	0

Breast HER2 ICC Run: 119

Automation	N	%
Dako Autostainer	1	0
Dako Autostainer Link 48	10	60
Dako Autostainer Plus Link	3	67
Dako Omnis	1	100
LabVision Autostainer	1	0
Leica Bond Max	9	22
Leica Bond-III	16	69
None (Manual)	5	0
Ventana Benchmark GX	8	88
Ventana Benchmark ULTRA	105	84
Ventana Benchmark XT	51	69

Breast HER2 ICC Run: 119

Heat Mediated Retrieval	N	%
Biocare Decloaking Chamber	1	0
Dako Omnis	1	100
Dako PTLink	12	67
Lab vision PT Module	2	0
Leica ER1 10 mins	1	0
Leica ER1 20 mins	7	71
Leica ER1 25 mins	11	64
Leica ER1 30 mins	1	0
Leica ER1 40 mins	1	0
Leica ER2 10 mins	1	0
Leica ER2 30 mins	1	0
Microwave	2	0
None	2	50
Other	2	50
Ventana CC1 16mins	2	100
Ventana CC1 20mins	1	100
Ventana CC1 24mins	2	100
Ventana CC1 32mins	14	71
Ventana CC1 36mins	56	86
Ventana CC1 40mins	1	0
Ventana CC1 48mins	1	0
Ventana CC1 52mins	3	100
Ventana CC1 56mins	4	50
Ventana CC1 64mins	9	89
Ventana CC1 76mins	1	0
Ventana CC1 8mins	1	0
Ventana CC1 mild	57	77
Ventana CC1 standard	10	80
Water bath 95-98 OC	2	0

Breast HER2 ICC Run: 119

Detection	N	%
AS PER KIT	18	61
BioGenex SS Polymer (QD 420-YIKE)	1	0
Dako HerCep Test (K5204)	1	0
Dako EnVision FLEX (K8000/10)	2	50
Dako EnVision FLEX+ (K8002/12)	5	40
Dako Envision HRP/DAB (K5007)	1	0
Dako HerCep Test Autor (SK001)	5	100
LabVision UltraVision LP HRP (TL 125 HLJ)	1	0
Leica Bond Polymer Refine (DS9800)	13	38
Other	1	0
Ventana iView system (760-091)	5	100
Ventana OptiView Kit (760-700)	11	73
Ventana UltraView Kit (760-500)	141	80

Breast HER2 ICC Run: 119

Enzyme Retrieval	N	%
AS PER KIT	11	73
NOT APPLICABLE	122	73
Ventana Protease 1 (760-2018)	1	100

Breast HER2 ICC Run: 119

Chromogen	N	%
AS PER KIT	30	70
BioGenex liquid DBA (HK-124-7K)	1	0
DAKO DAB+	1	100
Dako DAB+ Liquid (K3468)	1	0
Dako FLEX DAB	10	60
Dako REAL EnVision K5007 DAB	4	0
Leica Bond Polymer Refine kit (DS9800)	12	42
Other	4	50
Ventana DAB	7	71
Ventana iview	3	100
Ventana Ultraview DAB	138	80

BEST METHODS

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

HER-2 - Method 1

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

Primary Antibody:	Ventana Pathway 790-2991 (4B5) , 20 Mins, 36 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:	AS PER KIT
Detection:	Ventana UltraView Kit (760-500)

HER-2 - Method 2

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

Primary Antibody:	Dako Link HercepTest SK001 (poly) , 30 Mins, 21 ºC Prediluted
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: HERCEPTEST EPITOPE RETRIEVAL
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 21 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako HerCep Test Autor (SK001) , 20 Mins, 21 ºC Prediluted

HER-2 - Method 3

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

Primary Antibody:	Ventana Confirm 790-4493 (4B5) , 16 Mins, 36 ºC
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 32mins
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 36 ºC

Suzanne Parry

Antigen Assessed:	**HER2 for IHC**
The NEQAS distributed sections consisted of a composite slide containing 4 surgical /resections of intestinal gastric carcinoma:	The expected expression levels of each tissue sample were: **A.** 3+ **B**. 2+, 3+ or 1+ depending on the block or serial section received **C**. 0, 1+ or 2+ depending on the block or serial section received **D**. 0
Number of Registered Participants	66
Number of Participants this Run	60 (91%)

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

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

Validation of Distributed Samples

IHC Validation of Distributed Samples

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

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

Table 2: HER2 IHC staining and & ISH results

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

Assessment Procedure

Assessment is carried out around a multi-header microscope with each slide being assessed by 4 independent assessors and a microscope lead. Each of the NEQAS samples (A-D) (as well as in-house samples 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: (Figures 1, 2, 3a, 4, 5 & 6)

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

Features Of Suboptimal or Unacceptable Staining: (Figure 3b)

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

Additional Comment:

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

Pass Rates

The pass rates for the NEQAS distributed samples are summarised in the table below:

NEQAS Pass Rates Run 119:	
Acceptable	87% (N=52)
Borderline	3% (N=2)
Unacceptable	10% (N=6)

The pass rate for this module has remained at a pleasingly high level for the past number of Runs. Only 6 laboratories (10%) obtained an unacceptable result due to weak staining in cores B and/or C. In the clinical setting, false negative results may lead to eligible patients not being put forward for Herceptin therapy, and equally concerning is that many patients may be over-treated: For instance, a HER2 IHC result of 3+ will automatically mean that the patient is put forward for Herceptin therapy. Also, as IHC 2+ cases are also reflex tested, incorrect over-staining could mean that more samples than necessary are being put forward for ISH reflex testing.
Most labs are using the recommended standardised protocols for their particular automated systems, such as CC1 Mild antigen retrieval on the Ventana XT with the 4B5 pre-diluted antibody clone. However, a few labs are not using the recommended protocols, and this may have the potential to induce weaker or stronger membrane staining than expected.

All laboratories submitted in-house control material for assessment and the results for these are summarised below:

In-House Pass Rates Run 119:	
Acceptable	69% (N=41)
Borderline	30% (N=18)
Unacceptable	2% (N=1)

The overall pass rates on the in-house controls was lower than on the Neqas samples. More laboratories received a borderline pass on the in-house samples. The majority of these borderline passes were given because the laboratories did not submit ideal composite control material, consisting of a 3+, 2+ and 1+/0 expressing gastric/breast control tumour sample. These labs were therefore given a maximum score of 12/20. Other labs were marked down for poor tissue quality or fixation. Another consideration for the 2+ controls sent by some laboratories were not truly 2+. Recommendations for control tissue are described below.

Methodologies

The majority of labs are using the Ventana 4B5 standardised kit for their gastric HER2 testing: In this particular assessment run the Ventana assay was used by 88% of participants with an average acceptable pass rate of 85%. 3 laboratories used the Dako Hercept test with 67% acceptable pass rate.

Control Tissue and Recommendations

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

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

References:

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

Acknowledgments

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

Selected Images showing Optimal and Sub-optimal immunostaining

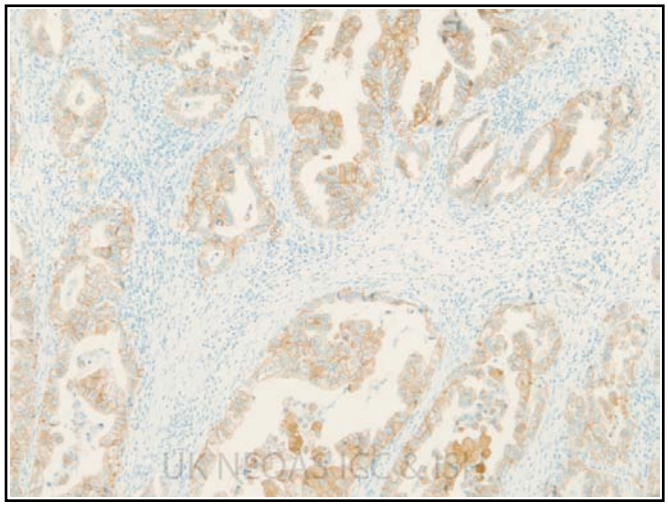


Figure 1. Good example of expected level of 3+ HER2 expression for Core A on the NEQAS distributed sample. Stained using the Ventana 4B5 Pathway on the Ventana Benchmark XT. CC1 32mins.

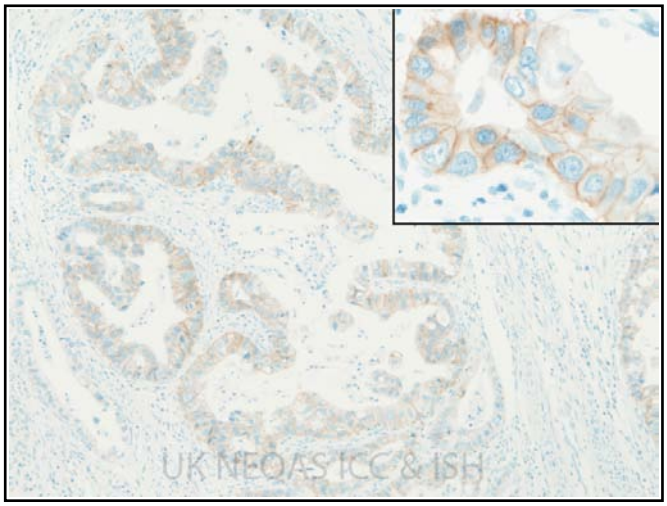


Figure 2. Expected level of staining for the 2+ Core B on the NEQAS sample. The expression level varied depending on which block and serial section was distributed (See Fig. 3). Staining method as in Figure 1.

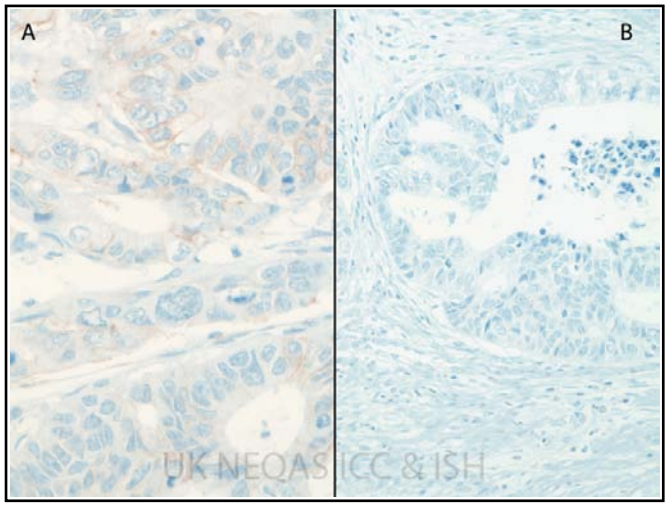


Figure 3. Figure 3A demonstrates acceptable 1+ staining in Core B on the NEQAS sample. Method as described in Figure 1. Figure 3B shows an unacceptable absence of staining in the 1+ Core B. Method: Ventana 4B5 on the Ventana Benchmark Ultra. CC1 36 mins.

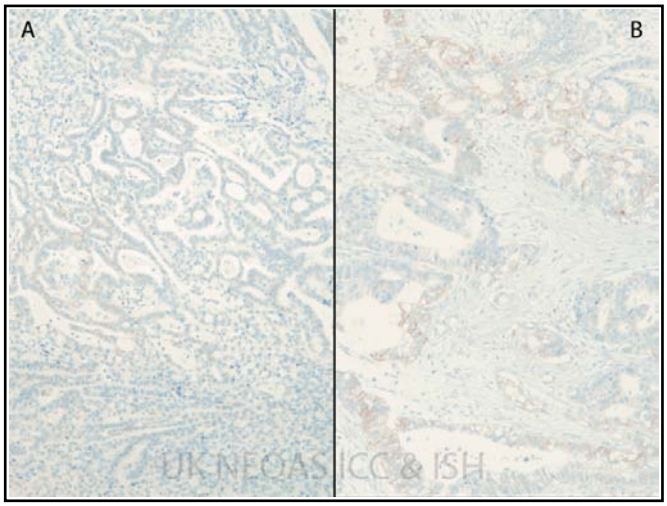


Figure 4. The expression in Core C varied from 1+ to 2+ depending on the block and serial section. Figure 4A shows excellent example of 1+ staining in Core C. Figure 4B demonstrates stronger staining of 2+. Both A&B stained using the method described in Figure 1.

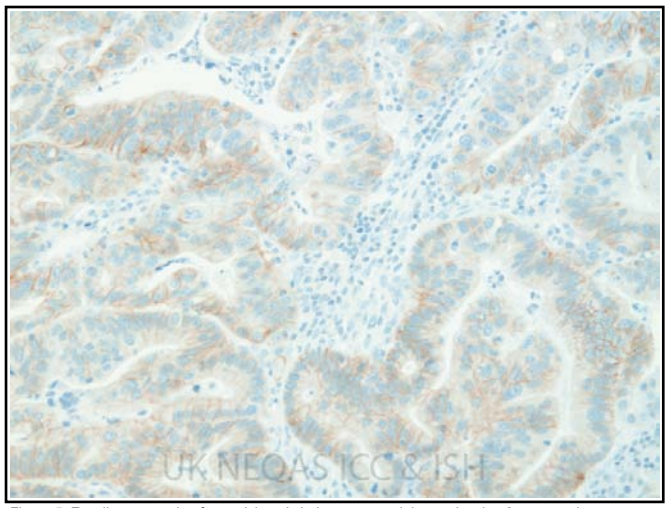


Figure 5. Excellent example of a participant's in-house control tissue showing 2+ expression. Stained using Ventana 4B4 Confirm on the Ventana Benchmark ULTRA. CC1 36 mins.

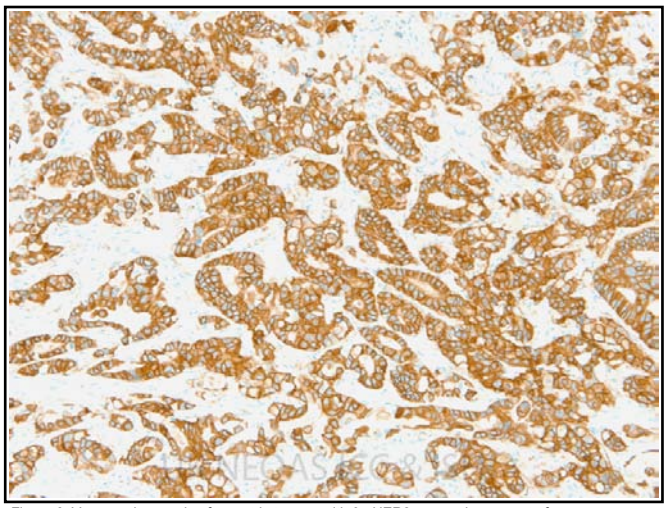
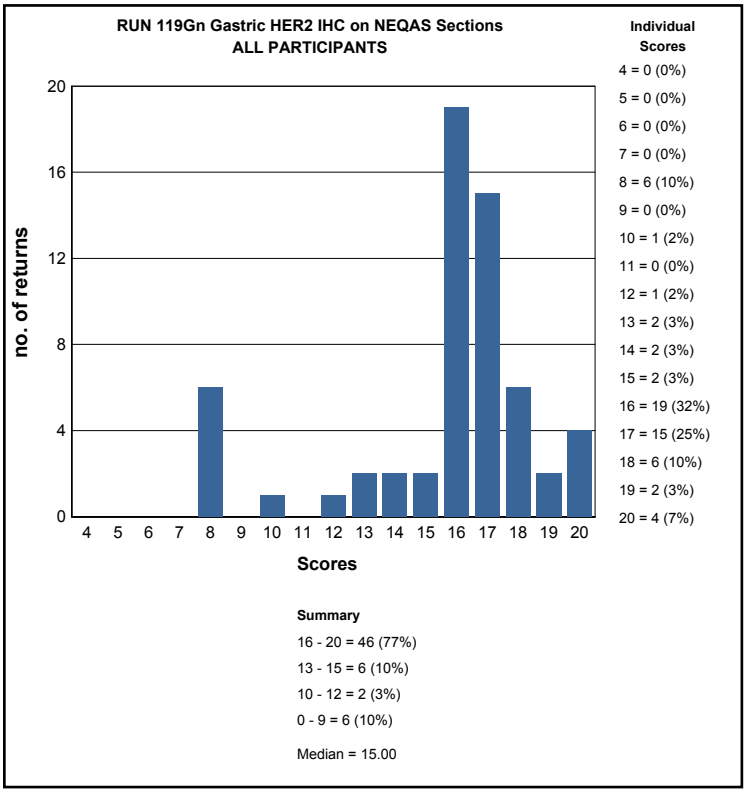
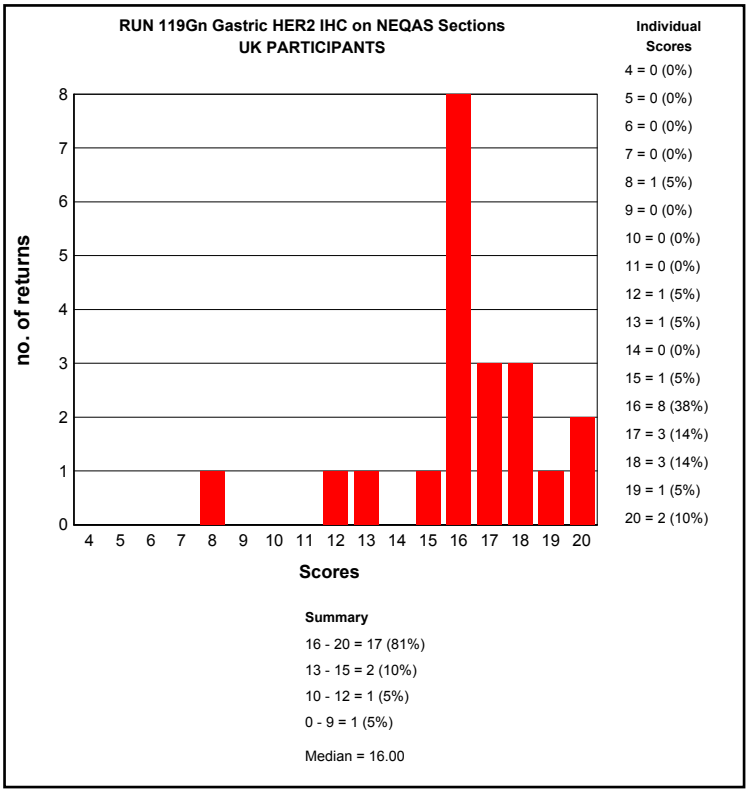
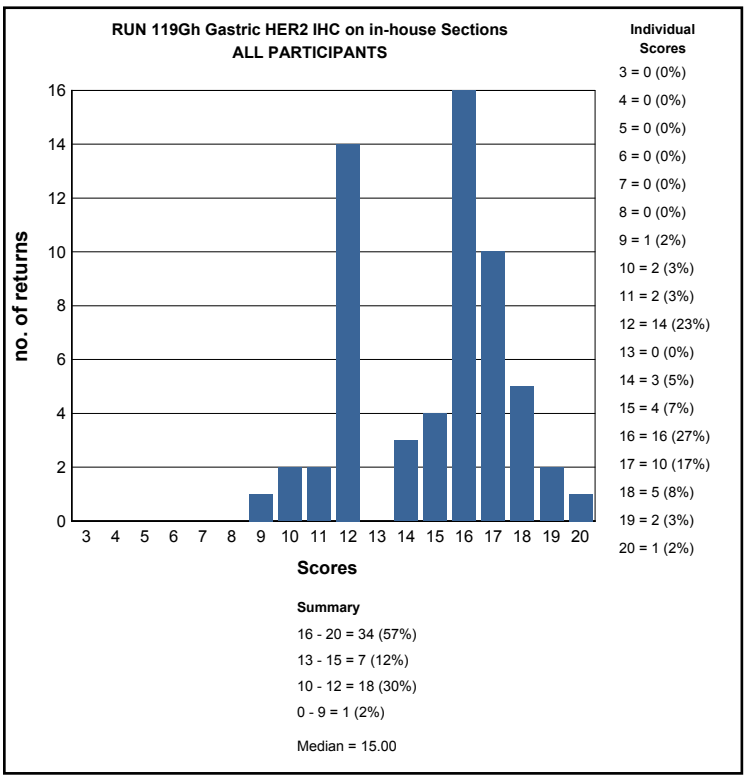


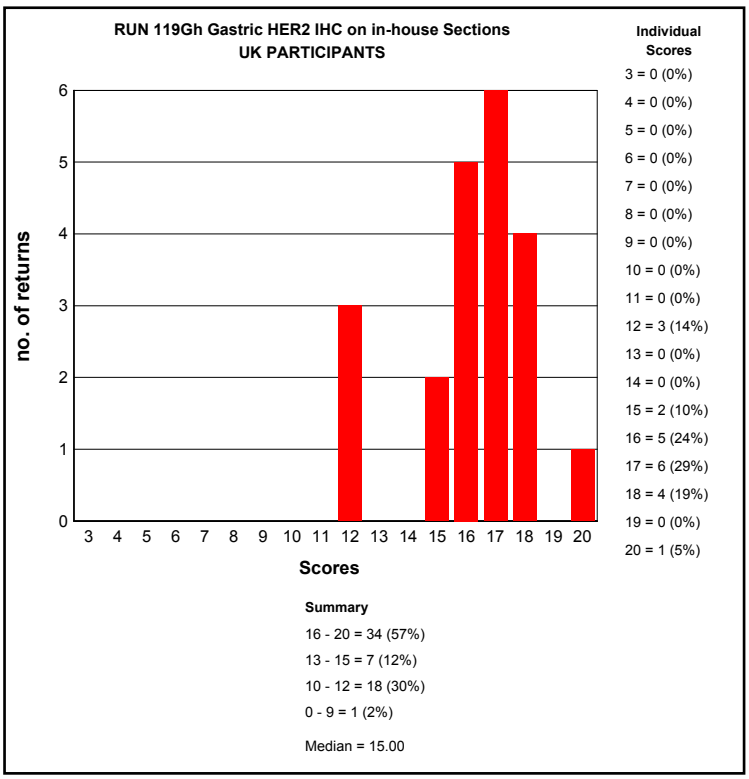
Figure 6. Very good example of a gastric tumour with 3+ HER2 expression as part of a participant's composite control block. Method as described in Figure 5.

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: 119

Primary Antibody	N	%
Dako A0485 C-erB-2 (poly)	2	100
Dako Link HercepTest SK001 (poly)	3	67
Ventana Pathway 790-100 (4B5)	2	100
Ventana Pathway 790-2991 (4B5)	37	92
Other	1	0
Ventana Confirm 790-4493 (4B5)	14	79

Gastric HER2 ICC Run: 119

Automation	N	%
Dako Autostainer Link 48	1	0
Dako Autostainer Plus Link	2	100
Dako Omnis	2	100
Other	1	0
Ventana Benchmark GX	2	100
Ventana Benchmark ULTRA	33	85
Ventana Benchmark XT	18	94

Gastric HER2 ICC Run: 119

Heat Mediated Retrieval	N	%
Dako Omnis	2	100
Dako PTLink	3	67
Lab vision PT Module	1	0
Ventana CC1 16mins	2	50
Ventana CC1 24mins	1	100
Ventana CC1 32mins	6	100
Ventana CC1 36mins	15	80
Ventana CC1 52mins	1	100
Ventana CC1 56mins	2	50
Ventana CC1 64mins	6	100
Ventana CC1 mild	15	93
Ventana CC1 standard	5	100

Gastric HER2 ICC Run: 119

Detection	N	%
AS PER KIT	5	60
Dako EnVision FLEX (K8000/10)	1	100
Dako EnVision FLEX+ (K8002/12)	1	100
Dako HerCep Test Autor (SK001)	2	100
Power Vision DPVB999 HRP	1	0
Ventana iView system (760-091)	1	100
Ventana OptiView Kit (760-700)	5	60
Ventana UltraView Kit (760-500)	42	93

Gastric HER2 ICC Run: 119

Enzyme Retrieval	N	%
AS PER KIT	3	100
NOT APPLICABLE	28	93
Ventana Protease 1 (760-2018)	2	100

Gastric HER2 ICC Run: 119

Chromogen	N	%
AS PER KIT	10	80
Dako DAB+ Liquid (K3468)	1	0
Dako FLEX DAB	4	100
Other	1	100
Ventana DAB	3	67
Ventana iview	1	100
Ventana Ultraview DAB	39	90

BEST METHODS

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

Gastric HER2 IHC - Method 1

Participant scored 20/20 (UK NEQAS Slide) and 18/20 (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), PH: 7.6
HMAR:	Ventana CC1 16mins, PH: 8.4
EAR:	
Chromogen:	Ventana Ultraview DAB, Time 1: 37 Mins, Time 2: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC Prediluted

Gastric HER2 IHC - Method 2

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

Primary Antibody:	Ventana Pathway 790-100 (4B5) , 16 Mins
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.54
HMAR:	Ventana CC1 mild
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB, Time 1: 8 Mins
Detection:	Ventana UltraView Kit (760-500)

David Blythe and Suzanne Parry

	Gold Standard	Second Antibody
Antigens Assessed:	BCL-6	CD15
Tissue Sections circulated:	Diffuse large B-cell lymphoma (LN) and reactive tonsil	Hodgkin's disease lymph node.
Number of Registered Participants:	210	
Number of Participants this Run	201 (96%)	

Introduction

Gold Standard: BCL-6

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

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

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

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

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

References:

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

Second Antigen: CD15

CD15 is a complex cluster of differentiation antigen, expressed on glycoproteins, glycolipids and proteoglycans, and is also referred to as Lewis X (LeX) antigen[1,]. CD15 is expressed in normal mature myeloid cells, but it has been predominantly used as an immunohistochemical marker to identify Reed-Sternberg Cells (RSC) in Classical Hodgkin Lymphoma (CHL), used to distinguish CHL from reactive lymphadenitis, lymphocyte-predominance Hodgkin lymphoma, non-Hodgkin lymphomas and HD-like neoplasms[2,3]. CD15 is also useful within a panel to aid in the differential diagnosis and classification of other neoplasms, such as the differentiation of mesothelioma from adenocarcinoma, or the differentiation between malignant gliomas and non-neoplastic glial cells. CD15 is also used to indicate a poorer prognosis in certain tumours, such as gastric and colonic carcinoma, and thyroid medullary carcinoma.

Features of Optimal Immunostaining (Figures 7, 8, 9, 11 & 12):

• Moderate to strong predominantly membranous staining of most of the Hodgkin and Reed-Sternberg cells.
- Clean background with no non-specific staining.

Features of Sub-optimal Immunostaining (Figure 10):

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

References

1.Kerr MA, Stocks SC The role of CD15-(Le(X))-related carbohydrates in neutrophil adhesion. Histochem. J. 1992; 24 (11): 811–26.
2. Dorfman RF, et al. An evaluation of the utility of anti-granulocyte and anti-leucocyte monoclonal antibodies in the diagnosis of Hodgkin's disease. Am J Pathol 1986. 123:508–19.
3. Vassallo J. et al. Mimicking nodular sclerosis Hodgkin's lymphoma: report of 10 cases. Am J Surg Pathol 2006. 30:223–9.
4. Barry TS, et al. Peripheral T-cell lymphomas expressing CD30 and CD15. Am J Surg Pathol 2003; 27:1513–22.

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 88% of participants achieving an acceptable result for their NEQAS submission, 11% received a borderline score, and only 3% received unacceptable results. 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 33% of participants respectively. The acceptable pass rate for the participants in-house tissue was 92%, therefore, slightly higher than that achieved on the NEQAS material. A further 8% received a borderline pass, and no laboratories failed on their in-house tissue. Many laboratories are only using an appendix as an in-house positive control. While this is an appropriate tissue which stains positively with BCL-6, NEQAS recommends for best practice to use a multi-tissue composite control to help measure the sensitivity of the assay, such as a tonsil and a positive tumour control (i.e. DLBCL). Laboratories were not penalised for only using a single control.

NEQAS and In-house Pass Rates Run 119 BCL-6		
	NEQAS	In-house
Acceptable	88% (N=174)	92% (N= 183)
Borderline	11% (N=21)	8% (N=16)
Unacceptable	3% (N=5)	0% (N=0)

CD15

201 laboratories submitted their stained NEQAS material for the CD15 assessment. Only 1 of these laboratories did not

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submit their in-house material. It was the first time that NEQAS has requested CD15 as the marker to be assessed for a while, and the results were generally good. 82% (N=164) achieved an acceptable pass, and a further 9% (N=19) received a borderline pass. However, 18 laboratories (9%) failed the assessment on the NEQAS material.

Pass rates on the in-house material were lower than that on the NEQAS material, with less participants receiving an acceptable pass, and more laboratories receiving borderline passes. 11 laboratories (9%) failed on their in-house material. These pass rates are summarised in the table below:

NEQAS and In-house Pass Rates Run 119 CD15		
	NEQAS	In-house
Acceptable	82% (N=164)	66% (N= 130)
Borderline	9% (N=19)	30% (N=59)
Unacceptable	9% (N=18)	6% (N=11)

The reason for failure on both the NEQAS and in-house material was mostly due to no or little staining of the expected Hodgkin and Reed-Sternberg cells. It was noted during the assessment and after reviewing the data, that the Leica Carb-1 antibody was showing particularly poor staining. Even though most laboratories using this clone are carrying out the recommended antigen retrieval with acceptable dilution ranges, the results highlighted this antibody to be underperforming. Of the 16 participants using this clone, no laboratories achieved an acceptable pass, and only 2 laboratories received a borderline. The results with this clone were similar on the NEQAS and in-house material. The MMA clone from Leica was used by 3 participants, and this antibody performed well in the assessment, with all 3 users achieving acceptable pass scores. Other popular antibodies used in the assessment include the Ventana MMA antibody, used by 67 participants, and showed an acceptable pass rate of 94%. The Dako Carb-3 antibody was used by 42 participants, either as a ready-to-use for the autostainers or the Omnis staining machines, or diluted out from the concentrate. Results with this antibody were excellent, and all laboratories achieved acceptable passes. Borderline passes were given mainly for weaker staining, where the expression was present, but the staining could be improved for better technical quality.

Selected Images showing Optimal and Sub-optimal immunostaining

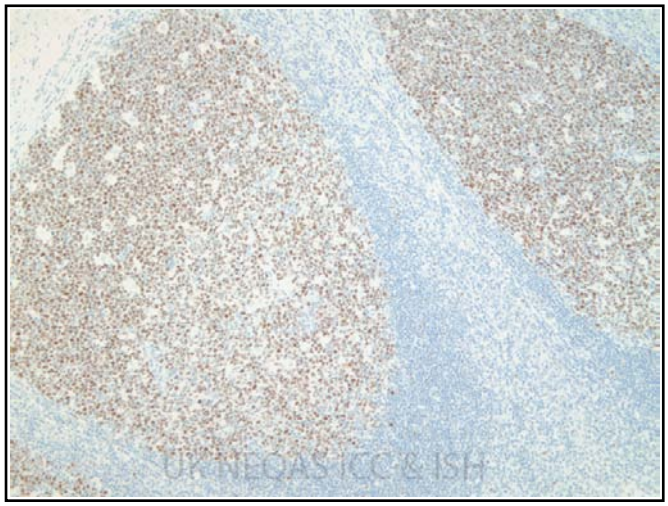


Figure 1: Acceptable demonstration of BCL-6 on the UK NEQAS reactive tonsil. The germinal centre B-cells show moderate nuclear staining while the background remains clean. Stained with the Dako RTU PG-B6p antibody clone on the Omnis autostainer, antigen retrieval for 30 minutes.

Figure 2: Weak demonstration of BCL-6 on NEQAS reactive tonsil. This was most likely due to insufficient antigen retrieval. The section was stained using the Cell Marque antibody on the Leica BondMax with ER1 antigen retrieval for 10 minutes.

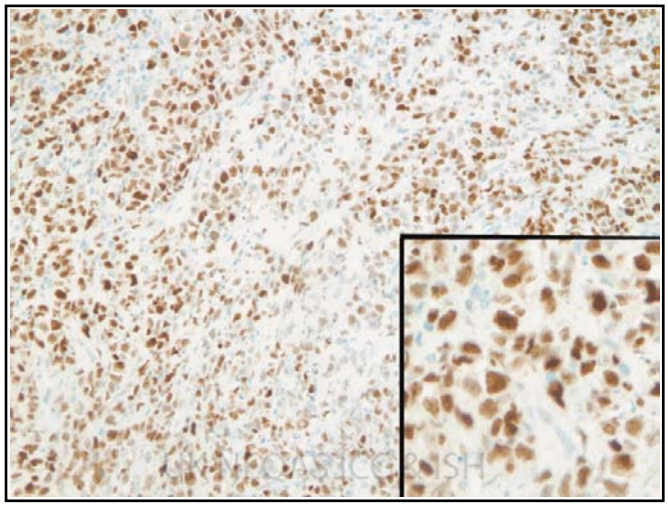


Figure 3. Good demonstration of BCL-6 on the UK NEQAS diffuse large B-cell lymphoma (DLBCL) sample. Virtually all of the neoplastic cells show moderate to strong, distinct nuclear staining. Section stained with the Cell Marque GI191E/A8 clone on the Ventana XT with CC1 extended antigen retrieval.

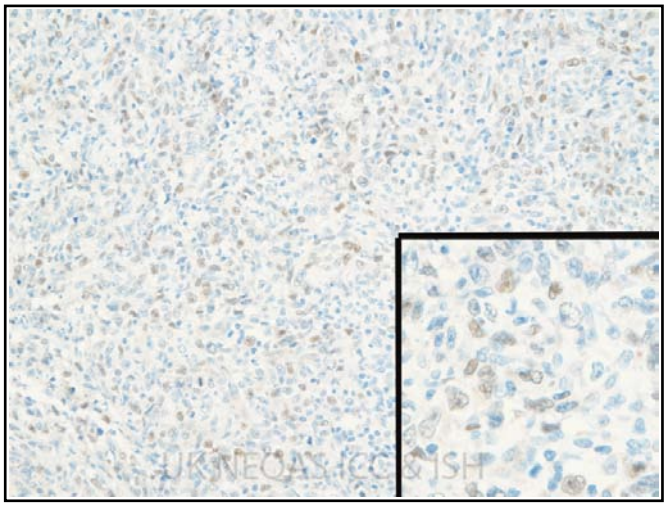


Figure 4. Sub-optimal demonstration of BCL-6 on the UK NEQAS DLBCL sample (compare to Fig 4). The staining pattern is weak and the antigen is poorly localised. (Same protocol as Fig 2).

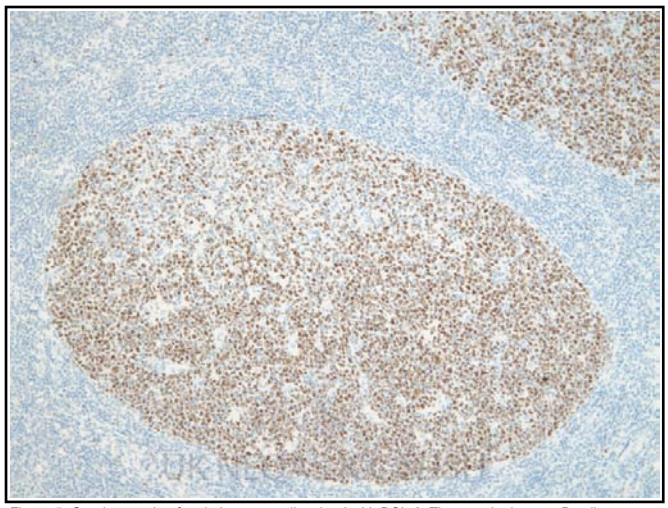


Figure 5. Good example of an in house tonsil stained with BCL-6. The germinal centre B cells show distinct nuclear staining and there is no background staining. (Same protocol as Fig 1).

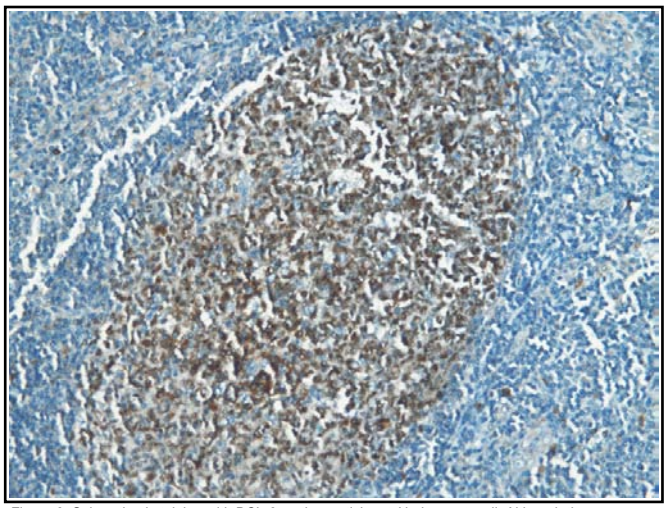


Figure 6. Sub-optimal staining with BCL-6 on the participants' in-house tonsil. Although the B-cells are demonstrated, the tissue is damaged, most likely due to excessive antigen retrieval . The counterstain is also excessive. Stained with a manual method with antigen retrieval in the water bath.

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

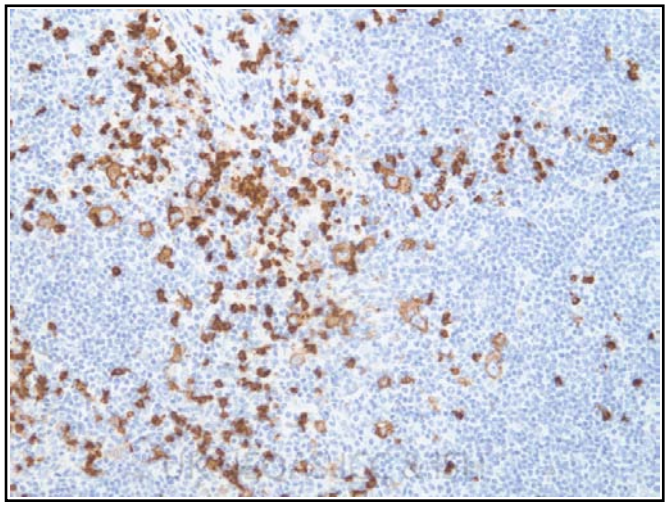


Figure 7. Good demonstration of CD15 on the UK NEQAS Hodgkin's lymphoma sample, showing the expected level of staining. The example was stained using the Becton Dickinson LEU M1 clone, 1:50, on the Dako Omnis autostainer with FLEX detection kit.

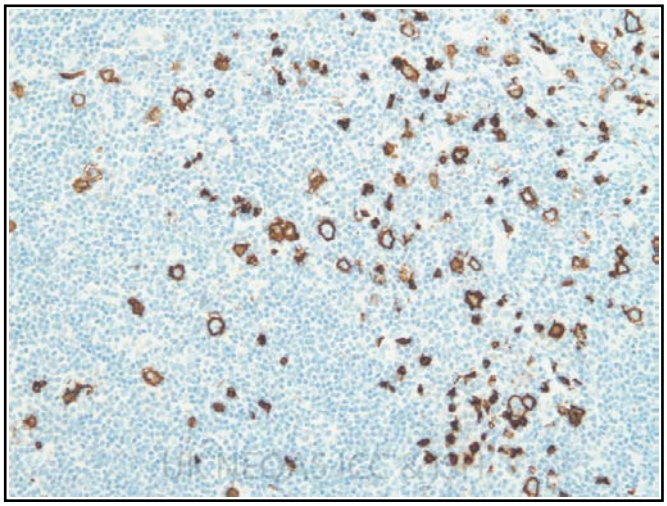


Figure 8. Optimal demonstration of CD15 on the UK NEQAS distributed Hodgkin's lymphoma sample, showing strong well-localised membrane staining of the Hodgkin's tumour cells. Stained using the Dako Carb-3 antibody, 1:40, on the Ventana ULTRA with CC1 standard retrieval.

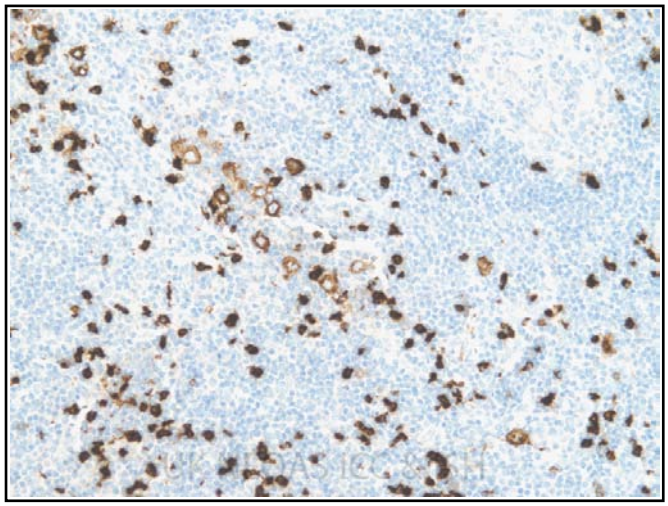


Figure 9. Good demonstration of CD15 on the UK NEQAS distributed Hodgkin's lymphoma. Even at low power the Hodgkin cells show strong membranous staining with dot-like positivity. Stained with the Ventana pre-diluted MMA antibody on the Benchmark ULTRA ,64 minutes retrieval in CC1 buffer.

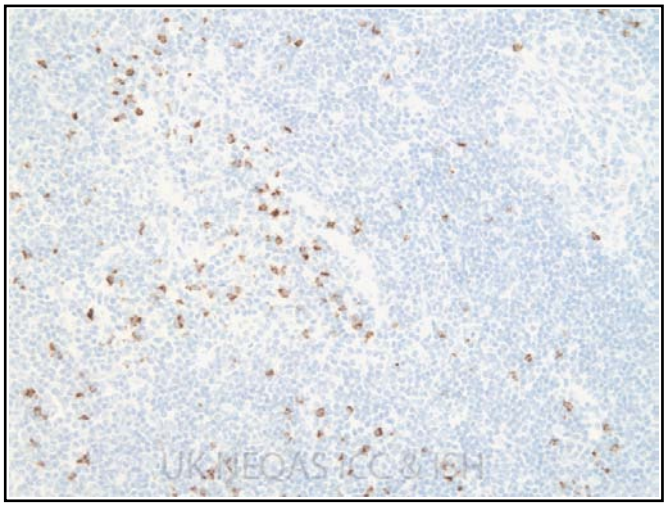


Figure 10. Poor demonstration of CD15 in the UK NEQAS Hodgkin's lymphoma sample (compare to Figs7-9&11):The example shows a false negative reaction, with only scattered neoplastic cells staining weakly. Stained with the Leica Carb-1 RTU antibody on the Bond III platform.

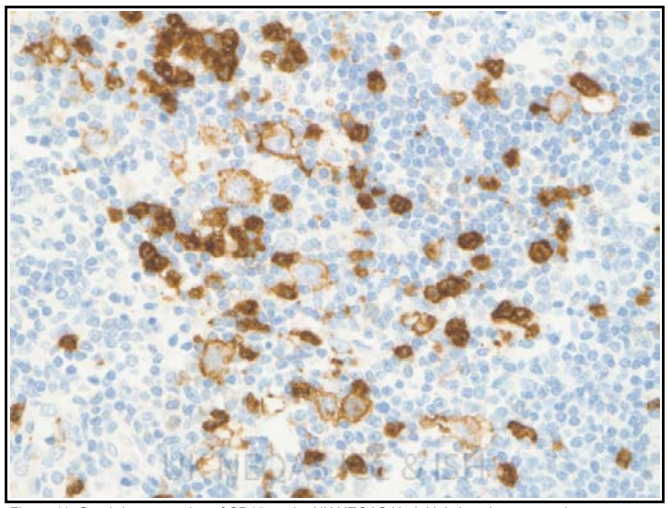


Figure 11. Good demonstration of CD15 on the UK NEQAS Hodgkin's lymphoma sample. Staining was carried out using the BD Biosciences LEU M1 antibody, 1:50 on the Leica Bond III autostainer with Refine detection.

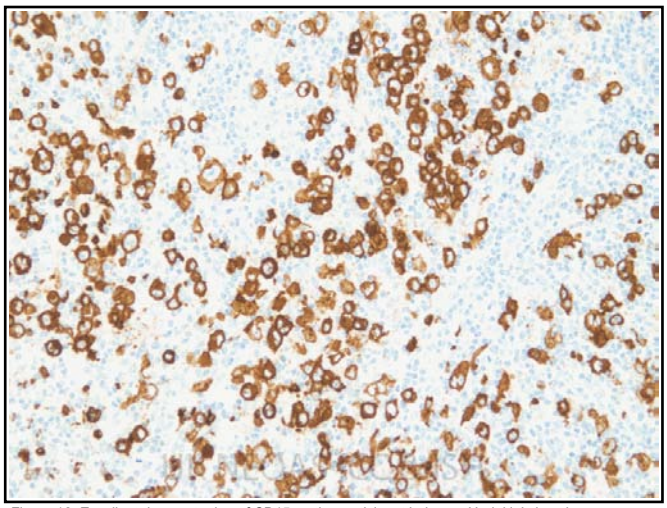
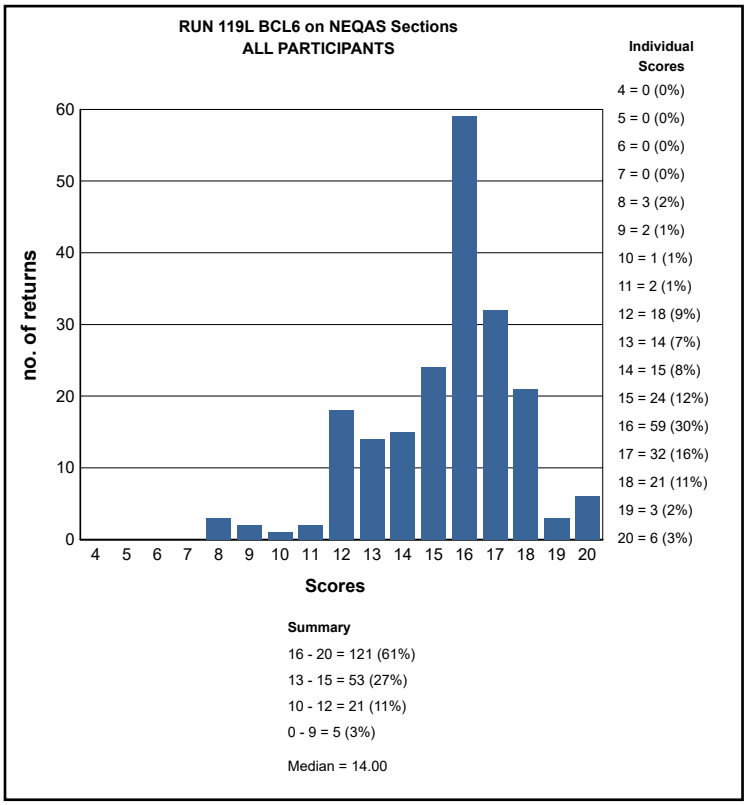
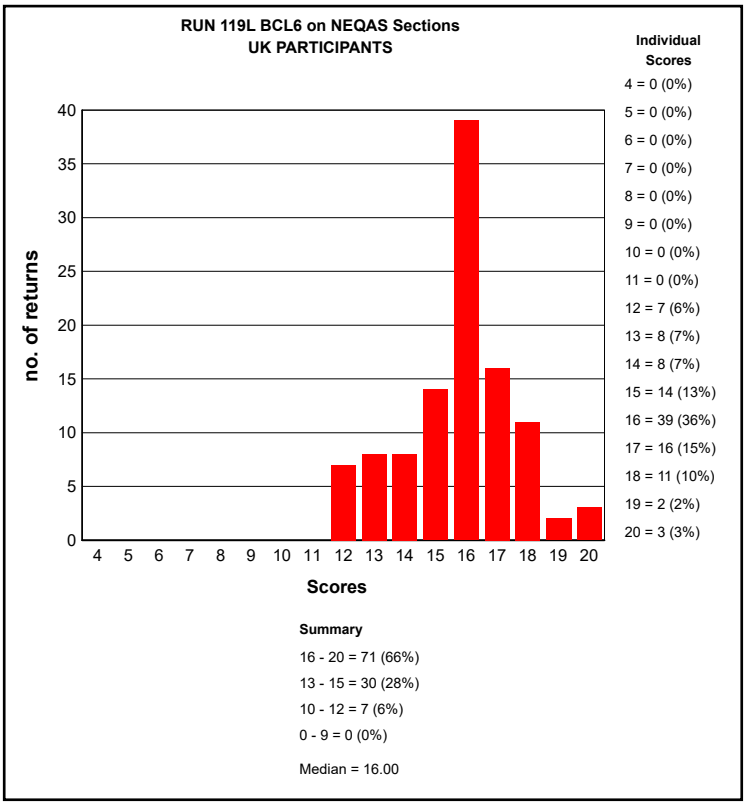
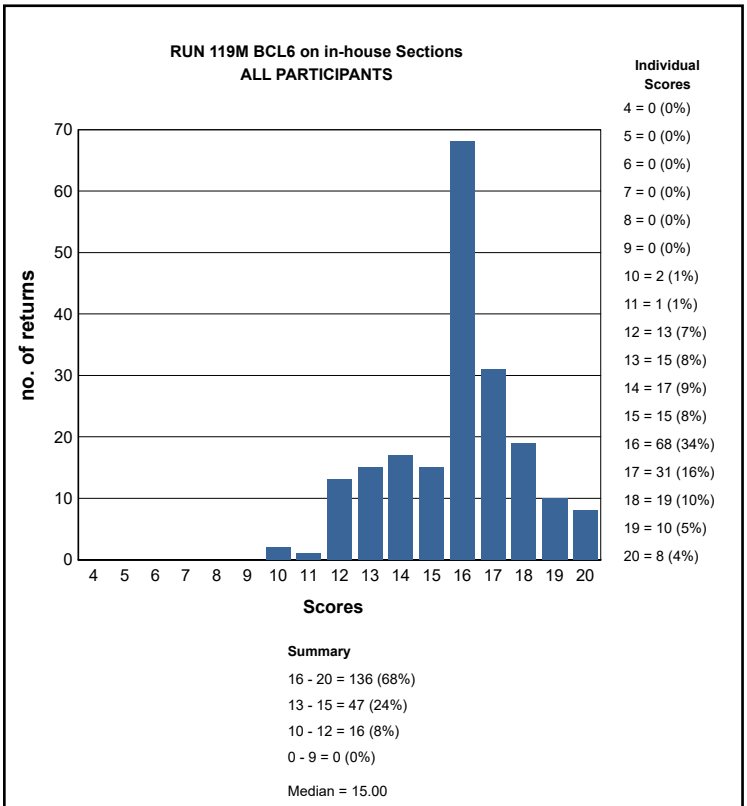


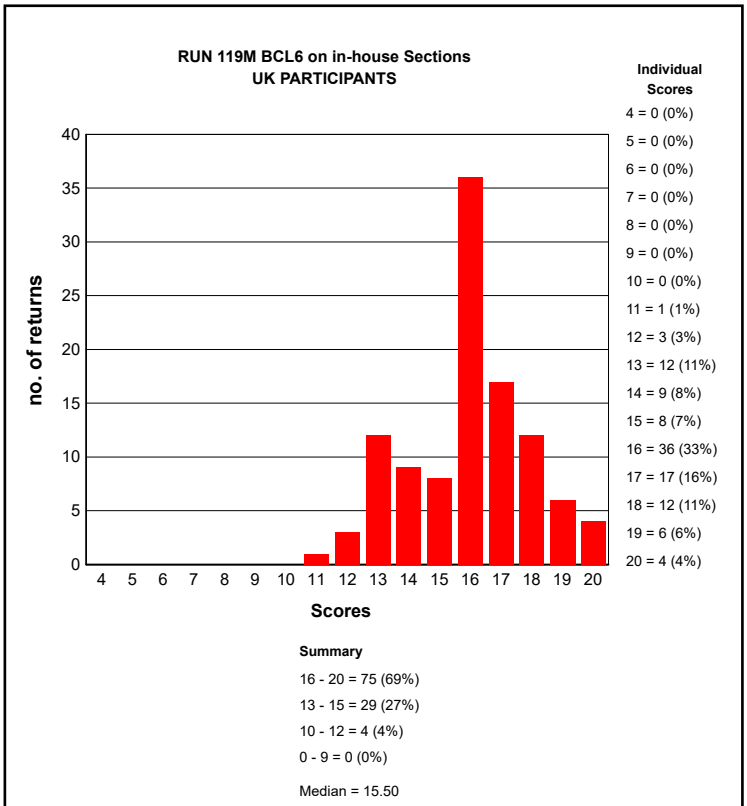
Figure 12: Excellent demonstration of CD15 on the participant in-house Hodgkin's lymphoma control. The example shows strong, well localised membrane staining in the Reed-Sternberg and Hodgkin's tumour cells. Stained using the Dako RTU Carb-3 antibody on the Dako Omnis with FLEX detection.

GRAPHICAL REPRESENTATION OF PASS RATES

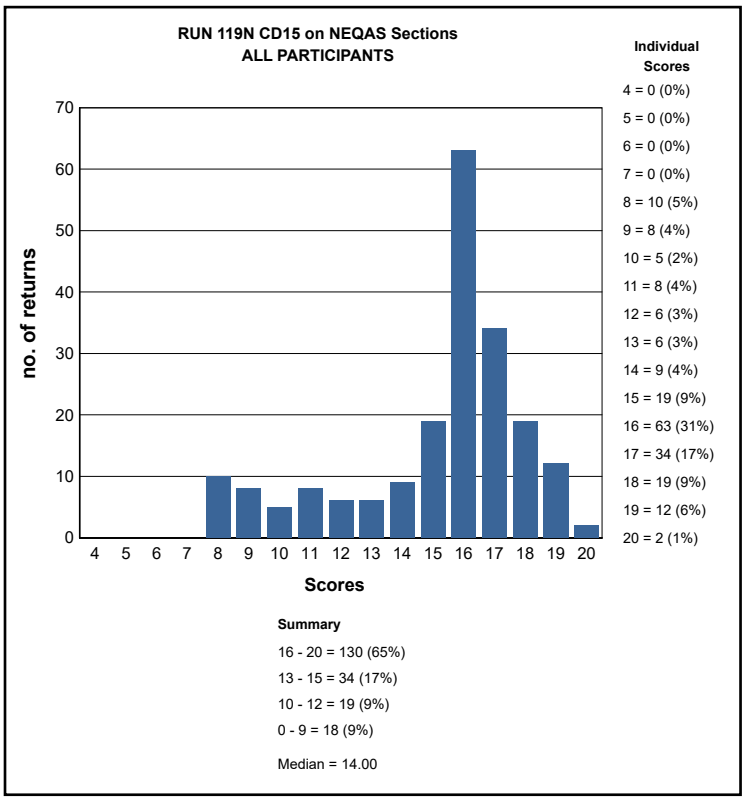


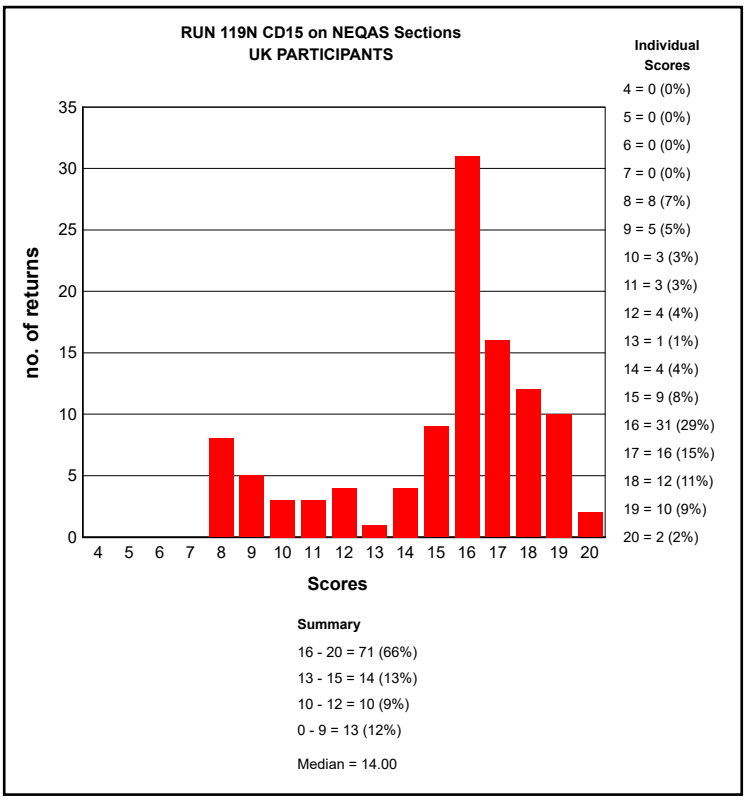


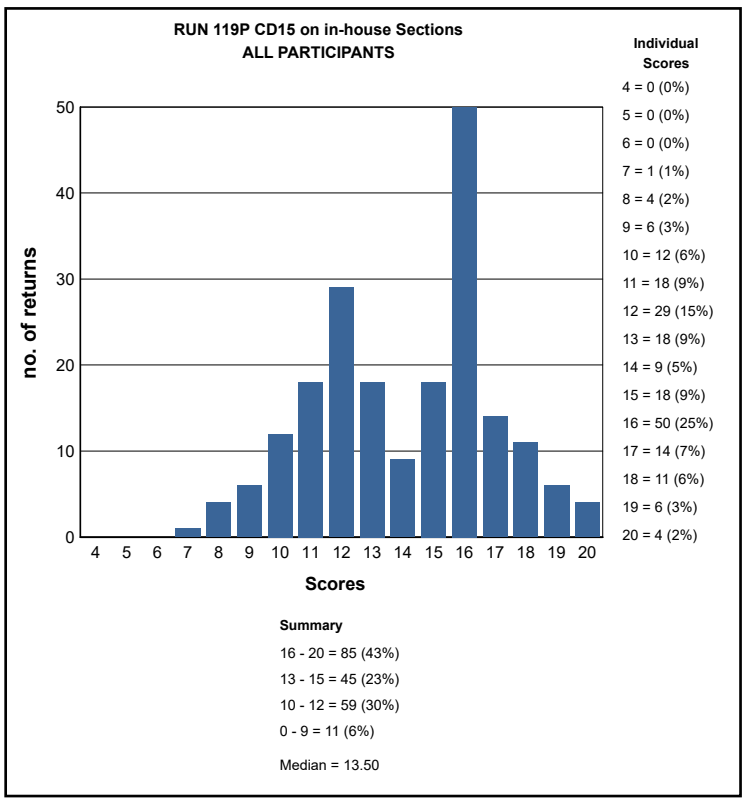


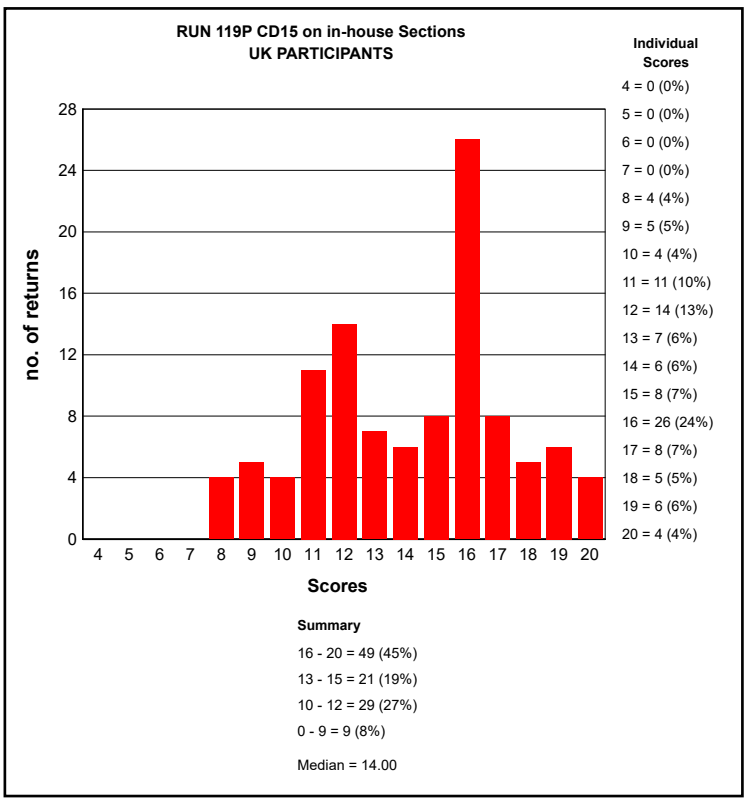


GRAPHICAL REPRESENTATION OF PASS RATES









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: 119

Primary Antibody : BCL6

Antibody Details	N	%
Dako BCL6 M7211 (PG-B6p)	36	86
Labvision Bcl-6 Ab-2 (BL6.02) mm	1	0
Novocastra NCL-BCL-6 (P1F6) mm	2	50
Novocastra NCL-L-BCL-6-564 (LN22) mm	48	96
Other	10	90
Cell marque 227M-94/95/96/97/98	12	75
Dako RTU FLEX Link IR625 (PG-B6p)	7	57
Leica RTU PA0204 (LN22)	18	78
Ventana 760-4241 (1GI191E/A8)	57	93
Dako RTU Omnis (PG-B6p) GA625	6	83

Lymphoma Run: 119

Primary Antibody : CD15

Antibody Details	N	%
Becton Dickinson 347420 (clone LEU M1)	25	88
Biocare CM073A	1	0
Cell Marque CMA 316 MMA	2	100
Dako M0733 (clone C3D-1)	11	100
Novocastra NCL-CD15	7	29
Ventana 760 2504	67	94
Other	12	50
Dako Omnis GA062 RTU (Carb-3)	4	100
Dako M3631 (Carb-3)	30	100
Leica PA0039 RTU (Carb-1)	16	0
Leica PA0473 RTU (MMA)	3	100
BD Biosciences 332778 (MMA)	2	100
CellMarque 115M (MMA)	2	100
Dako IR062 RTU (Carb-3)	5	100
Dako IS062 RTU (Carb-3)	3	100

Lymphoma Run: 119

Heat Mediated Retrieval	BCL6 N	BCL6 %	CD15 N	CD15 %
_Ventana Benk CC1 (8mins)	0	0	1	0
Dako Omnis	12	92	10	100
Dako PTLink	14	71	14	100
Lab vision PT Module	1	100	1	100
Leica ER1 10 mins	1	0	1	100
Leica ER1 20 mins	0	0	7	100
Leica ER1 30 mins	2	50	6	100
Leica ER2 10 mins	0	0	1	100
Leica ER2 20 mins	36	92	32	28
Leica ER2 30 mins	22	82	7	71
Leica ER2 40 mins	4	100	2	100
Microwave	1	100	0	0
None	0	0	1	100
Pressure Cooker in Microwave Oven	0	0	1	100
Ventana CC1 24mins	1	0	2	50
Ventana CC1 32mins	8	75	10	90
Ventana CC1 36mins	1	100	3	100
Ventana CC1 40mins	4	100	3	100
Ventana CC1 48mins	8	88	7	100
Ventana CC1 52mins	1	100	1	100
Ventana CC1 56mins	5	100	5	100
Ventana CC1 64mins	42	95	36	94
Ventana CC1 72mins	2	100	0	0
Ventana CC1 76mins	4	75	3	100
Ventana CC1 80mins	1	100	0	0
Ventana CC1 88mins	3	100	0	0
Ventana CC1 8mins	0	0	1	100
Ventana CC1 92mins	6	83	2	100
Ventana CC1 extended	4	100	2	100
Ventana CC1 mild	0	0	1	100
Ventana CC1 standard	10	100	18	89
Ventana CC2 32mins	0	0	1	100
Ventana CC2 64mins	1	100	1	100
Water bath 95-98 OC	2	0	2	50

Lymphoma Run: 119

Enzyme Mediated Retrieval	BCL6 N	BCL6 %	CD15 N	CD15 %
AS PER KIT	4	100	3	67
Dako Protease (S2019)	1	0	1	0
NOT APPLICABLE	107	88	76	80
Ventana Protease	0	0	1	0

Lymphoma Run: 119	BCL6		CD15	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	13	77	20	70
BioGenex HRP (HK 519-06K)	1	100	0	0
Dako EnVision FLEX (K8000/10)	4	75	7	100
Dako EnVision FLEX+ (K8002/12)	15	80	10	100
Dako Envision+ HRP mouse K4004/5/6/7	1	100	1	100
Dako REAL HRP/DAB (K5001)	1	0	1	0
Leica Bond Polymer Define (DS9713)	1	0	1	0
Leica Bond Polymer Refine (DS9800)	57	86	42	60
NOT APPLICABLE	1	100	2	100
Other	4	100	3	100
Power Vision DPVB999 HRP	1	100	1	100
Ventana iView system (760-091)	1	0	1	100
Ventana OptiView (760-700) + Amp. (7/860-099)	10	80	3	100
Ventana OptiView Kit (760-700)	48	96	40	95
Ventana UltraView Kit (760-500)	34	97	43	91

Lymphoma Run: 119	BCL6		CD15	
Automation	**N**	**%**	**N**	**%**
Dako Autostainer Link 48	14	64	16	100
Dako Autostainer plus	0	0	1	100
Dako Autostainer Plus Link	2	50	0	0
Dako Omnis	12	92	10	100
LabVision Autostainer	0	0	1	100
Leica Bond Max	25	72	18	50
Leica Bond-III	41	93	41	59
Menarini - Intellipath FLX	1	0	1	0
None (Manual)	1	100	0	0
Other	1	100	1	100
Ventana Benchmark GX	2	100	1	100
Ventana Benchmark ULTRA	75	93	75	93
Ventana Benchmark XT	23	91	21	95

Lymphoma Run: 119	BCL6		CD15	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	26	96	38	79
BioGenex liquid DBA (HK-124-7K)	1	100	0	0
DAKO DAB+	1	100	2	100
Dako DAB+ Liquid (K3468)	1	100	1	100
Dako EnVision Plus kits	1	0	2	100
Dako FLEX DAB	23	78	18	100
Dako REAL K5001 DAB	1	0	1	0
Leica Bond Polymer Refine kit (DS9800)	59	83	45	58
Other	10	100	6	100
Ventana DAB	33	91	25	96
Ventana iview	1	100	2	100
Ventana Ultraview DAB	40	90	47	91

BEST METHODS - Gold Standard Antibody

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

CD15 - Method 1

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

Primary Antibody: Dako Omnis GA062 RTU (Carb-3) , 12 Mins, 32 ºC Prediluted
Automation: Dako Omnis
Method: Dako FLEX kit
Main Buffer: Dako Wash Buffer (S3006)
HMAR: Dako Omnis, PH: 9
EAR:
Chromogen: Dako FLEX DAB, 32 ºC., Time 1: 5 Mins
Detection: Dako EnVision FLEX (K8000/10) , 20 Mins, 32 ºC

CD15 - Method 2

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

Primary Antibody: Becton Dickinson 347420 (clone LEU M1) , 20 Mins, 21 ºC Dilution 1: 50
Automation: Dako Omnis
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako Omnis
EAR: NOT APPLICABLE
Chromogen: DAKO DAB+, 21 ºC., Time 1: 5 Mins
Detection: NOT APPLICABLE

CD15 - Method 3

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

Primary Antibody:	Ventana 760 2504 , 20 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.6
HMAR:	Ventana CC1 48mins, Buffer: CC1, PH: 7.8
EAR:	
Chromogen:	Other, Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 36 ºC Prediluted

CD15 - Method 4

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

Primary Antibody:	Ventana 760 2504 , 60 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 standard
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
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

BCL6 - Method 1

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

Primary Antibody:	Ventana 760-4241 (1GI191E/A8) , 8 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB, 36 ºC., Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 36 ºC Prediluted

BCL6 - Method 2

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

Primary Antibody:	Dako RTU Omnis (PG-B6p) GA625 , 12 Mins, 22 ºC Prediluted
Automation:	Dako Omnis
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis, Buffer: FLEX TRS HIGH
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 , 28 Mins, 36 ºC Dilution 1: 100
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 56mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB, RT ºC., Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, RT ºC Prediluted

BCL6 - Method 4

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

The Neuropathology Module Run 119

Neil Bilbe

	Gold Standard	Second Antibody
Antigens Assessed:	Ki67	EMA
Tissue Sections circulated:	Meningioma and metastatic large cell neuroendocrine tumour	Extra-skeletal myxoid chondrosarcoma and meningioma
Number of Registered Participants:	59	
Number of Participants this Run	58 (98%)	

Introduction

Gold Standard: MIB-1

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: EMA

Epithelial membrane antigen (EMA) labels epithelial cells in a wide variety of tissues and is a useful tool for the identification of neoplastic epithelia. EMA belongs to a heterogenous population of human milk fat globule (HMFG), proteins. HMFG is a complex secretory product of mammary epithelium and EMA can be recovered from the aqueous phase of skimmed milk following extraction in chloroform and methanol. Besides in milk, these proteins are present in a variety of epithelia of both normal and neoplastic types. A number of monoclonal and polyclonal antisera have been raised against these molecules and anti-EMA has been extensively studied in a large number of neoplastic conditions, most often in conjunction with other antibodies.
EMA is valuable as a marker in the detection of breast carcinoma metastases in histological sections of liver, lymph node, and bone marrow, and is useful for differentiating anaplastic carcinoma from malignant lymphomas, and for the recognition of spindle cell epithelial malignancies.

Features of Optimal Immunostaining:
- Specific staining in the tumour cells, and other epithelia
- Clean background with no non-specific staining.

Features of Sub-Optimal Immunostaining:
- Weak or uneven staining of the metastatic tumour
- Diffuse, poorly-localised staining.
- Excessive background or non-specific specific staining.

References:

1. Cordell, J., Richardson, T. C., Pulford, K. A. F., Ghosh, A. K., Gatter, K. C., Heyderman, E. and Mason, D. Y.: Production of monoclonal antibodies against human epithelial membrane antigen for use in diagnostic immunocytochemistry. Br. J. Cancer 52; 347-354, 1985
2. Heyderman E, Steele K, Ormerod MG (1979) A new antigen on the epithelial membrane: its immunoperoxidase localization in normal and neoplastic tissue. J Clin Pathol 32:35–39
3. Hitchcock E, Morris CS (1987) Cross reactivity of antiepithelial membrane monoclonal for reactive and neoplastic glial cells. J Neurooncol 4:345–352

Assessment Summary:
58 out of 59 participants submitted slides. One lab did not return any slides. A total of 232 slides/sections were assessed:
G=58, H=58, J=58 K=58

The overall pass rate was 88% (204 slides), borderline 10% (24), and the failed slides rate was 2% (4). The average score for all slides was 16/20. The NEQAS slides averaged 15/20, In-house slides 16/20.

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

Slide	Antigen	Pass	Borderline	Fail
G (NEQAS)	Ki67 (58)	78% (45)	19% (11)	3% (2)
H (In-House)	Ki67 (58)	98% (57)	2% (1)	0% (0)
J (NEQAS)	EMA (58)	83% (48)	16% (9)	1% (1)
K (In-House)	EMA (58)	93% (54)	5% (3)	2% (1)
Total (Average)	232	88%	10%	2%

MIB-1/Ki 67 (**G & H**)
The NEQAS (**G**) pass rate of 78% was down from the previous Run (83%), primarily because there were two failed slides; previously none. The sample this Run included a neuroendocrine tumour, which may have required a more robust protocol than the GBM that we normally pair with the meningioma. The two failed slides both had very weak staining reactions (see Report Image Fig. 4). Of the 11 slides which were assessed as borderline, all bar one (background staining) were due to weak staining (see Report Image Fig. 2).

Both failed slides used the Dako (M7240) MIB-1, diluted 1:100 and 1:75, with the Leica ER2 20 mins, Leica Bond Polymer Refine, on a Leica Bond III. Other participants using the same primary antibody gained excellent results, usually when combined with the Ventana platform, a less dilute solution; e.g. 1:50, and/or increased antigen retrieval times of at least 40 mins. The three slides which obtained scores of $^{20}/_{20}$ employed different primaries: Dako M7240, Leica/ Novocastra RTU (K2) PA0230, and Ventana RTU (30-9) 790-4286.

EMA (**J & K**)
The overall results for EMA were slightly better than for the Ki 67, with 83% passing. Only one NEQAS (**J**) slide was assessed as a fail; using a protocol that appears to be successfully employed by other participants: (see Report Image Fig. 11). The main problem labs had was obtaining sufficient staining in both the chondrosarcoma and meningioma (example Report Image Fig. 10); typically one or other or both were weakly stained, resulting in a borderline score ($^{10\text{-}12}/_{20}$).

Only one marker (Leica/Novocastra RTU (GP1.4) PA0035) achieved a 100% pass rate. Although this was used by only three participants, all using a Leica platform: ER1 or 2, Bond Polymer Refine on a Bond Max or Bond III. The single failed in-house slide (**K**) was due to poor choice of control material, a tonsil section with virtually no staining. The participant scored a low pass ($^{13}/_{20}$) for their NEQAS slide (**J**).

Selected Images showing Optimal and Sub-optimal immunostaining

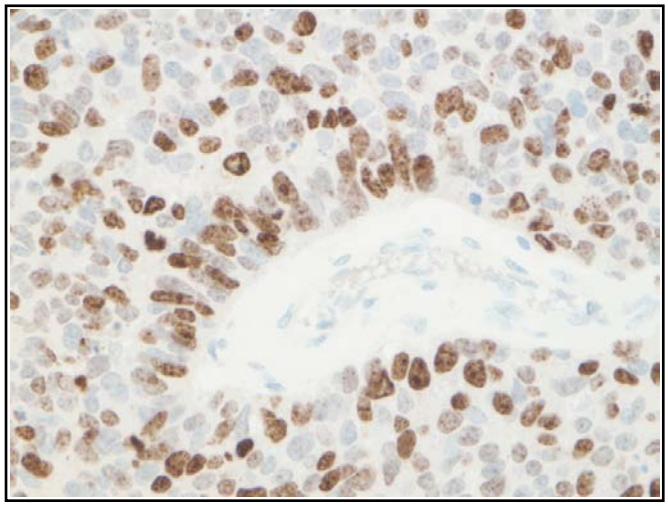


Fig 1. Sub-optimal Ki67 demonstration on the NEQAS neuroendocrine portion; staining is weak, and there is some background hue, but still assessed as a pass. NeoMarkers/Thermo Sci (SP6) RM 9106, 1:100, 15 mins; Leica ER2 20 mins; Leica Bond Polymer Refine (DS9800); on the Leica Bond-III.

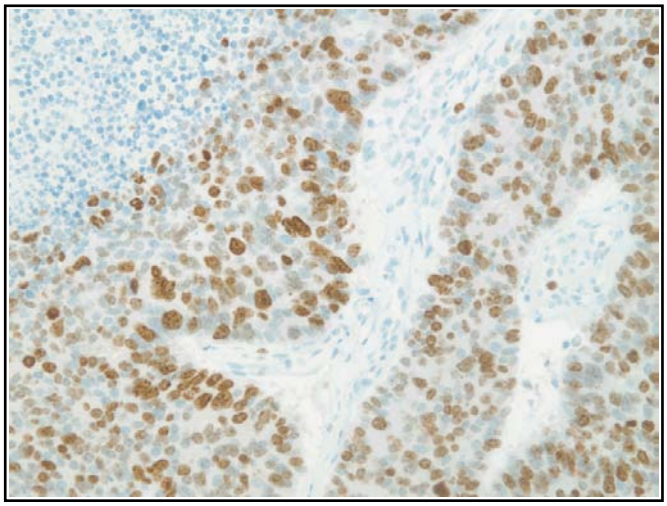


Fig 2. Sub-optimal Ki67 staining on the NEQAS neuroendocrine tissue. Tumours cells are a little weak, albeit adequately demonstrated. Dako M7240 (MIB1), 1;100, 32 mins; Ventana CC1 64mins; using the RTU Ventana UltraView Kit (760-500), 12 mins; and the Ventana Benchmark ULTRA.

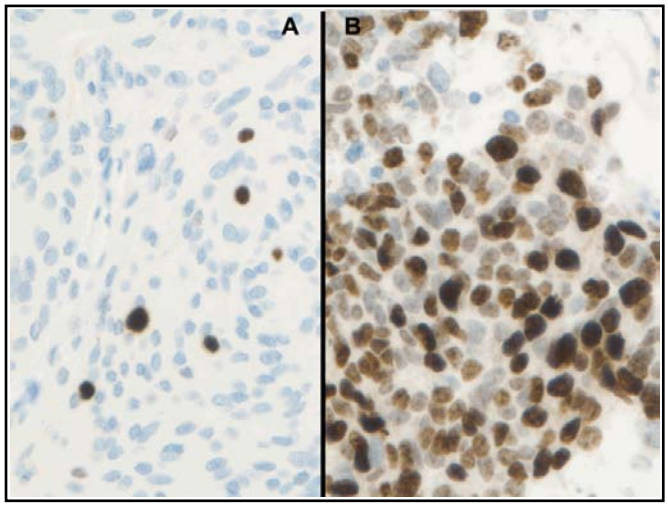


Fig 3. Optimal Ki67 demonstration on the NEQAS meningioma sample (A), and neuroendocrine tumour (B). It was rare to see crisp and abundant staining in the meningioma. Leica/Novocastra RTU (K2) PA0230, 15 mins; Leica ER2 20 mins; RTU Leica Bond Polymer Refine (DS9800), 8 mins; on the Leica Bond-III.

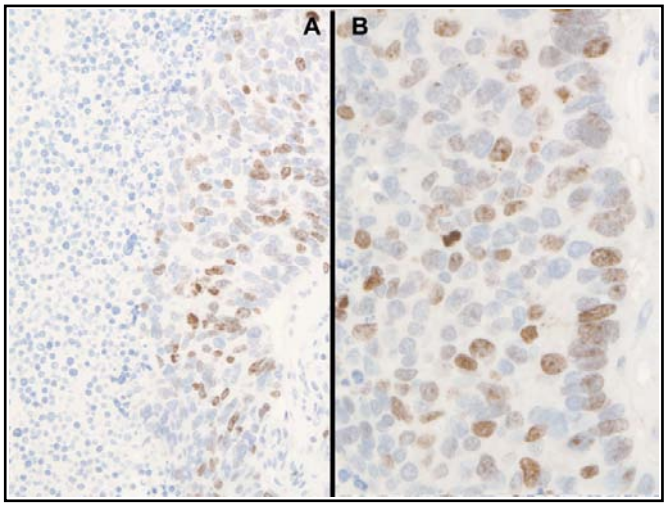


Fig 4. Poor Ki67 demonstration on the NEQAS NE sample. Low power (A), and high power (B). Staining is far too weak for diagnostic purposes, and the slide failed the assessment. Dako M7240 (MIB1), 1;100, 30 mins; Leica ER2 20 mins; Leica Bond Polymer Refine (DS9800), 8 mins; on a Leica Bond-III.

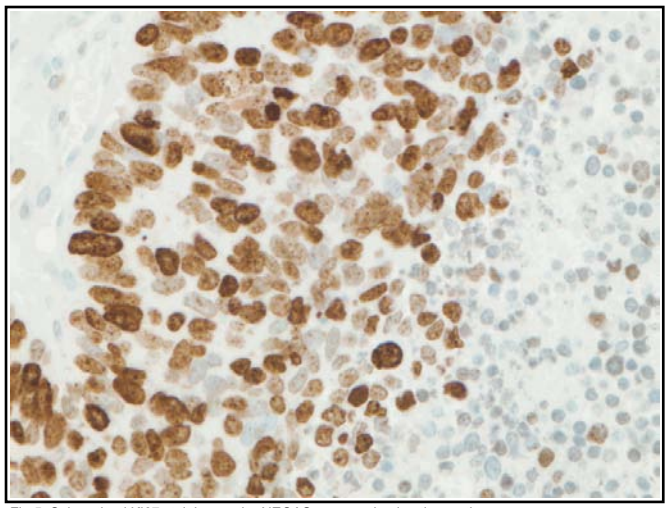


Fig 5. Sub-optimal Ki67 staining on the NEQAS neuroendocrine tissue, there are some non-specific reactions. Slide assessed as a low pass. Biocare CRM325C Ki67 (SP6), 1:30, 24 mins; Ventana CC1 64 mins; RTU Ventana UltraView Kit (760-500), 8 mins; on the Ventana Benchmark ULTRA.

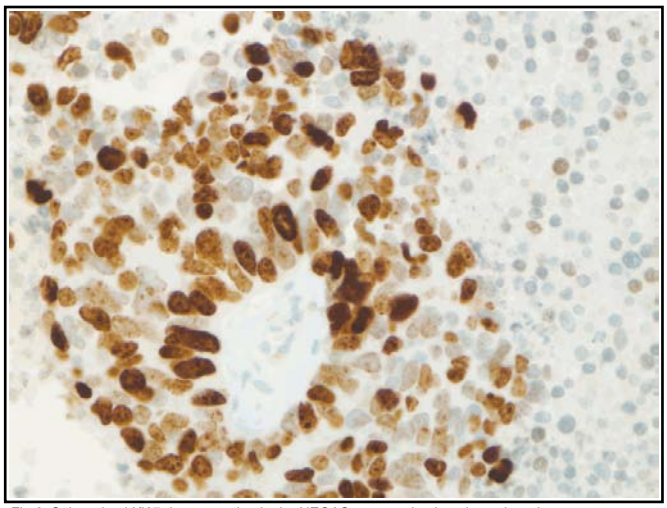


Fig 6. Sub-optimal Ki67 demonstration in the NEQAS neuroendocrine piece; there is pronounced background/non-specific staining in some areas. Again the slide assessed as adequate for diagnosis. Gennova AP10244C (SP6), 1:200, 72 mins; Ventana CC1 std; on the Ventana Benchmark XT.

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

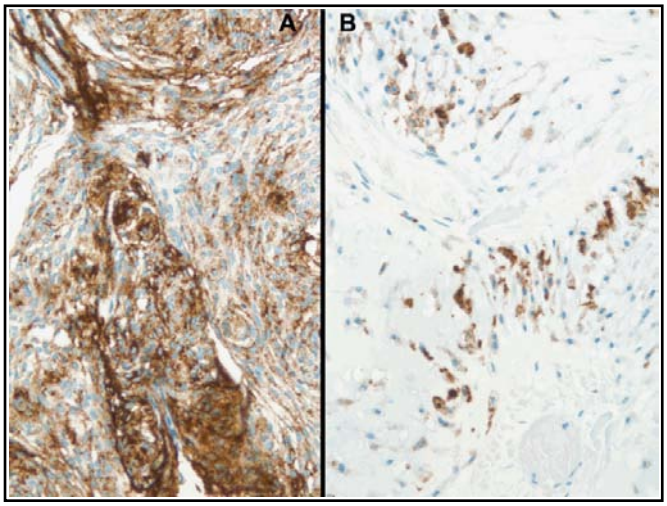


Fig 7. Optimal EMA demonstration on the NEQAS meningioma (A), and chondrosarcoma (B) samples. Both pieces show selective, clean staining of the tissues. Ventana RTU 790-4463, 20 mins; Ventana CC1 48 mins; RTU Ventana OptiView (760-700) + Amp. (7/860-099), 16 mins; on a Ventana Benchmark ULTRA.

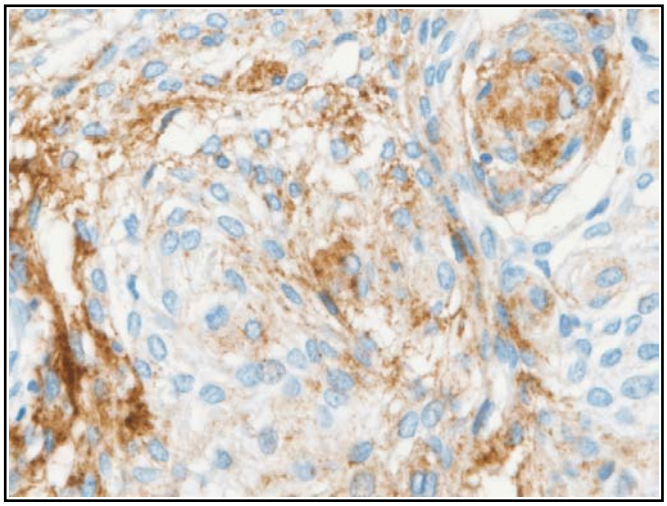


Fig 8. Sub- optimal EMA demonstration on the NEQAS meningioma portion. Staining is weak, and much of the tumour is negative. Slide assessed as borderline. Dako FLEX RTU (E29) IR629, 20 mins; Dako PTLink, 30 mins; with RTU Dako OMNIS Flex+, 20 mins; on the Dako Omnis platform.

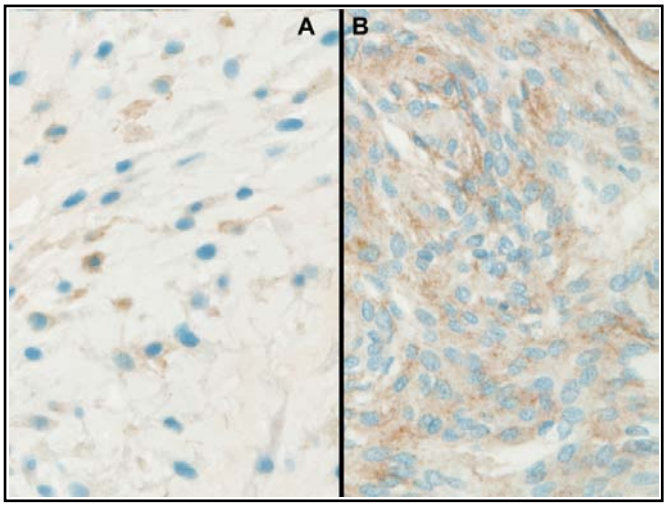
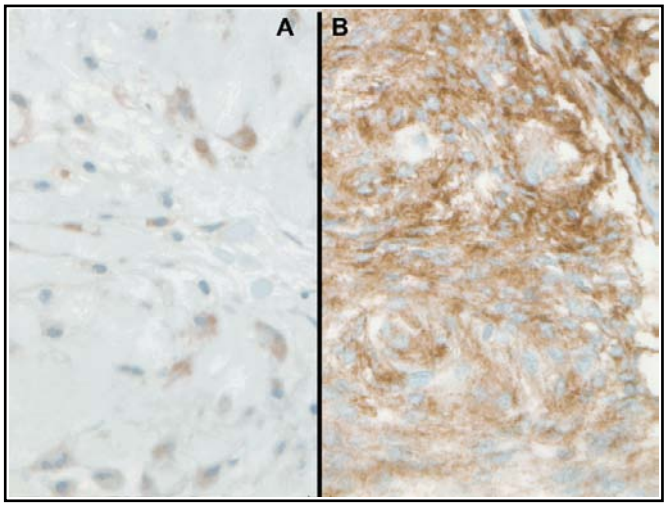


Fig 9. Sub-optimal EMA demonstration on the NEQAS meningioma (B), and chondrosarcoma (A), samples. Staining is feint and wispy with some background reaction, again scored as borderline. RTU Dako (E29) M0613, 32 mins; Ventana CC1 36 mins; RTU Ventana UltraView Kit (760-500), 12 mins; and the Ventana Benchmark ULTRA,



ig 10. Sub-optimal EMA demonstration on the NEQAS chondrosarcoma (A), and meningioma (B); staining could be more selective and intense. The slide gained a low pass in the assessment. Ventana Confirm anti-EMA (E29) 790-4463, RTU, 32 mins; CC1 32 mins; with Ventana OptiView Kit (760-700); on the Ventana Benchmark ULTRA.

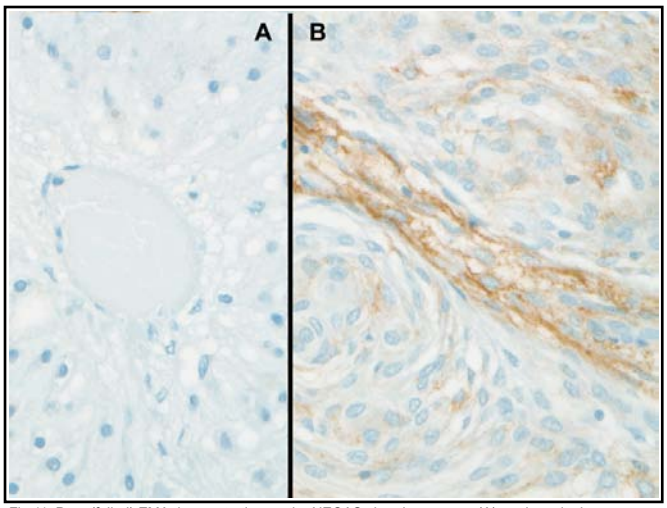


Fig 11. Poor (failed) EMA demonstration on the NEQAS chondrosarcoma (A), and meningioma (B). There is no staining in the sarcoma (A), and mainly non-tumour staining in the meningioma (B). Dako (E29) M0613, 1:75, 16 mins; CC1 20 mins; Ventana UltraView Kit (760-500); and the Ventana Benchmark XT.

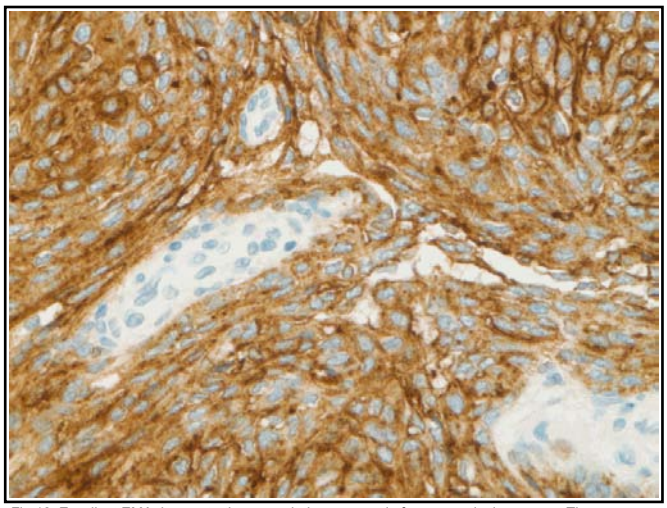
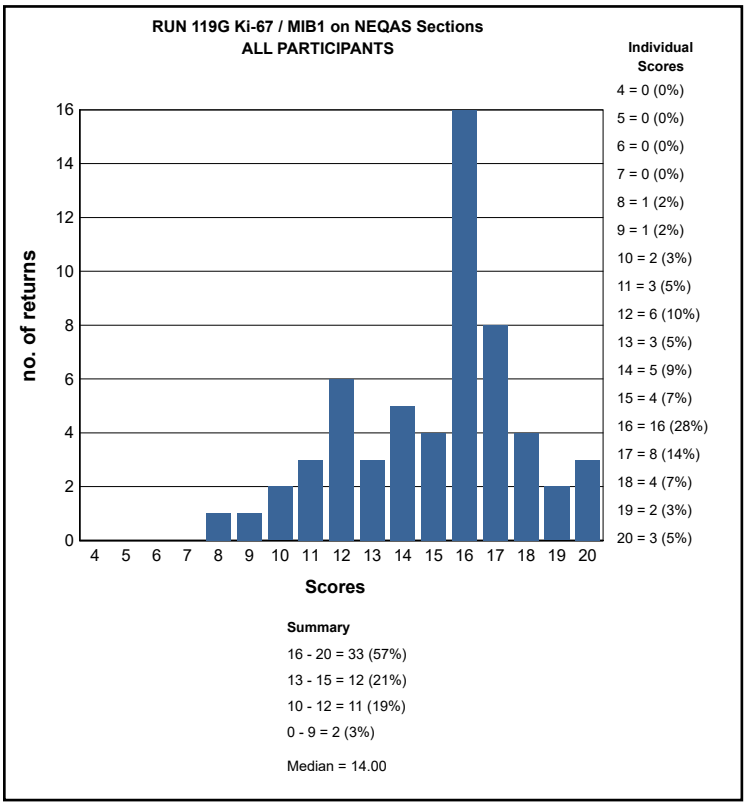
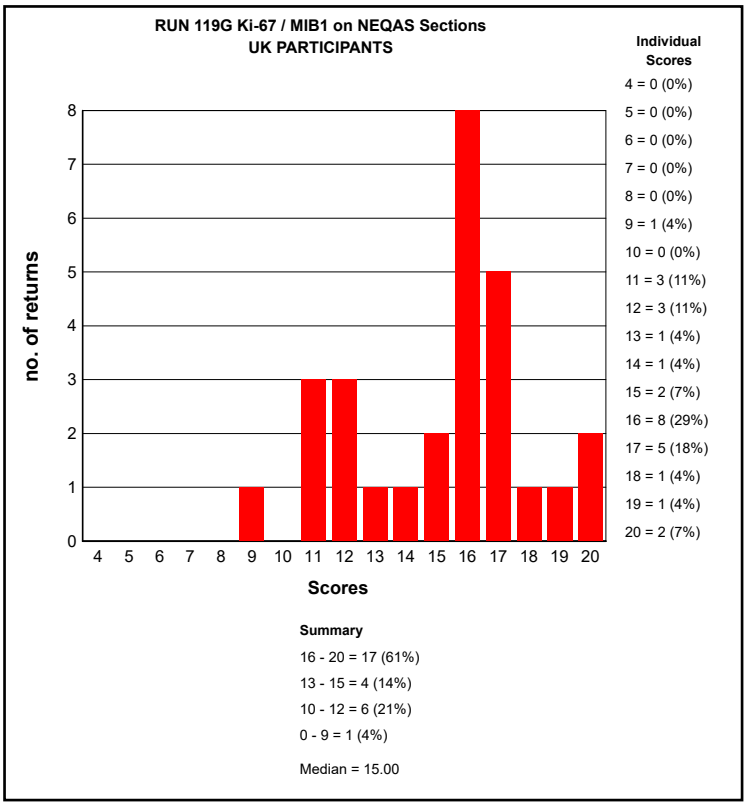
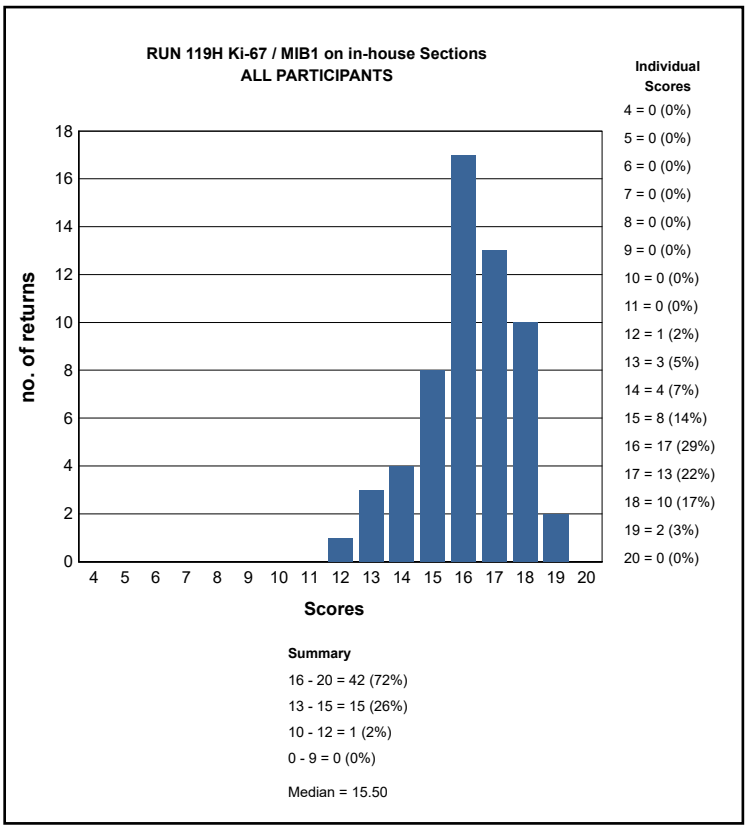


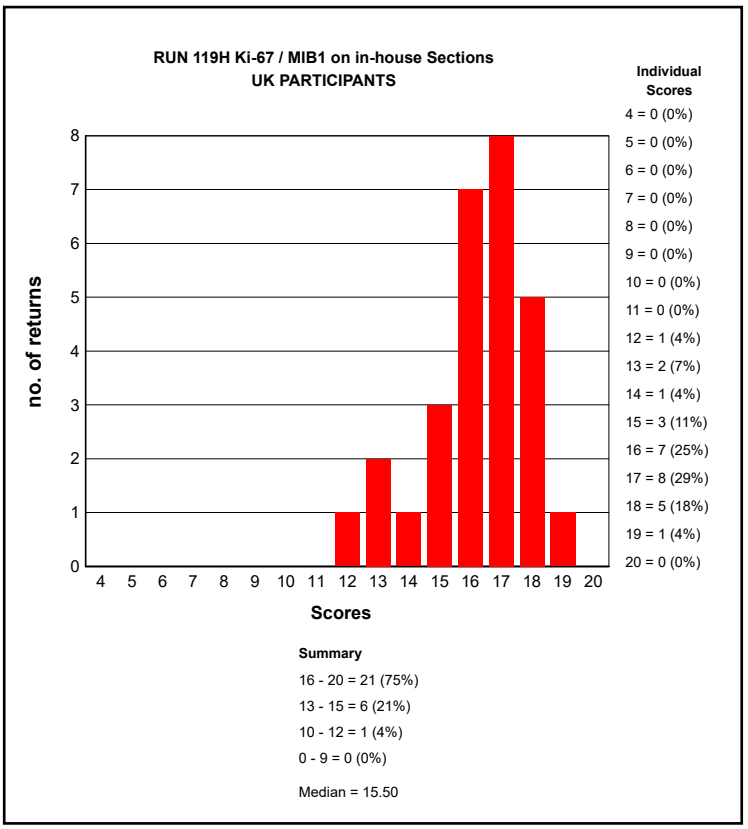
Fig 12. Excellent EMA demonstration on an in-house sample from a meningioma case. The staining is selective, and the background clean. Dako (E29) M0613, 1:300, 15 mins; Leica ER2 20 mins at pH9; RTU Leica Bond Polymer Refine (DS9800), 8 mins; on the Leica Bond Max platform.

GRAPHICAL REPRESENTATION OF PASS RATES

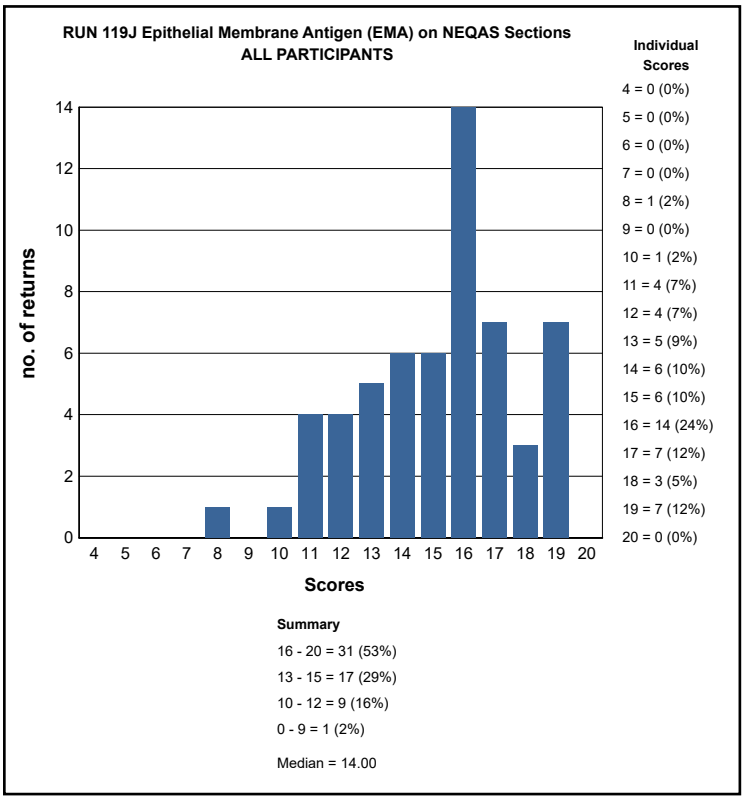


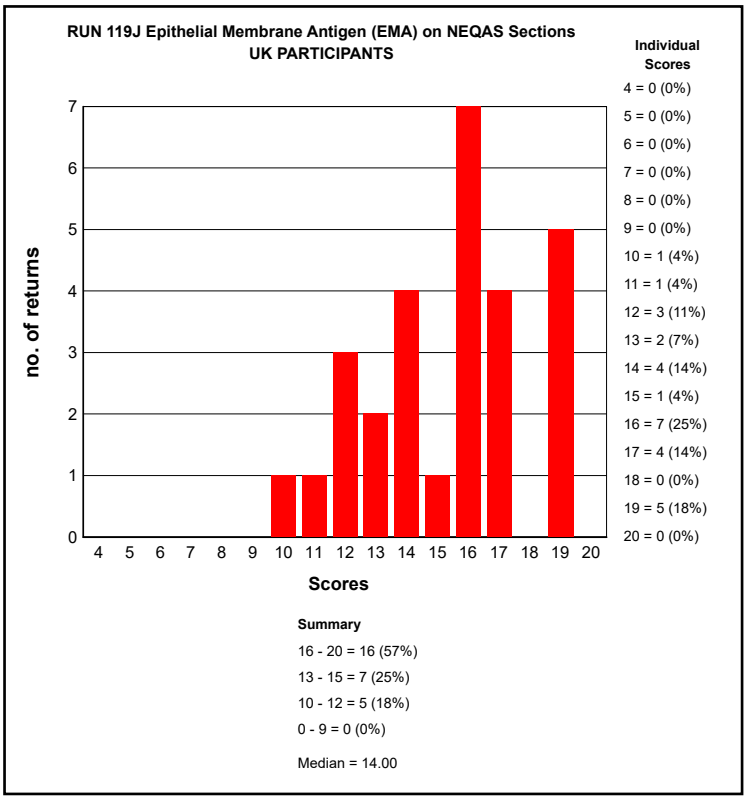


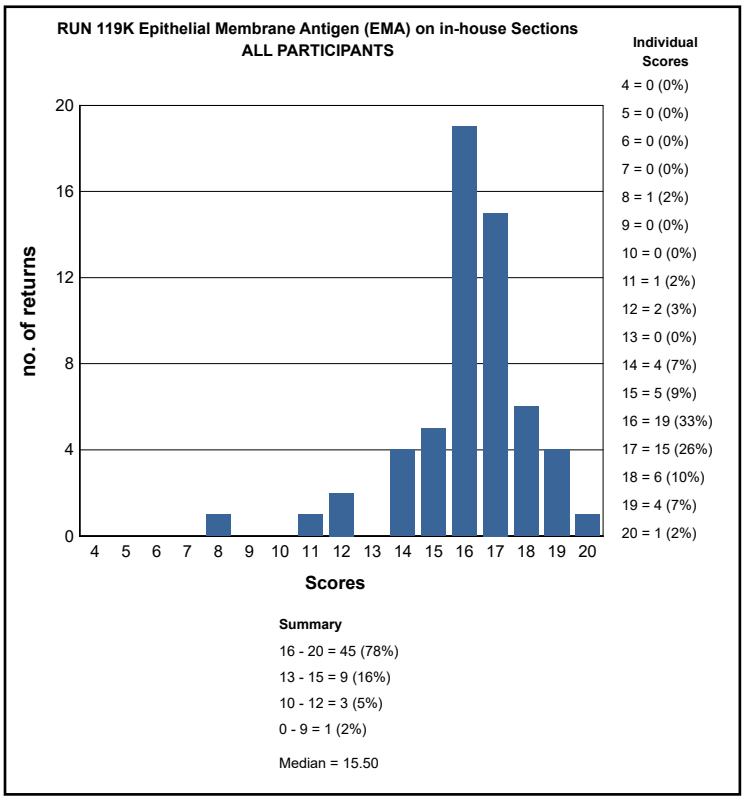


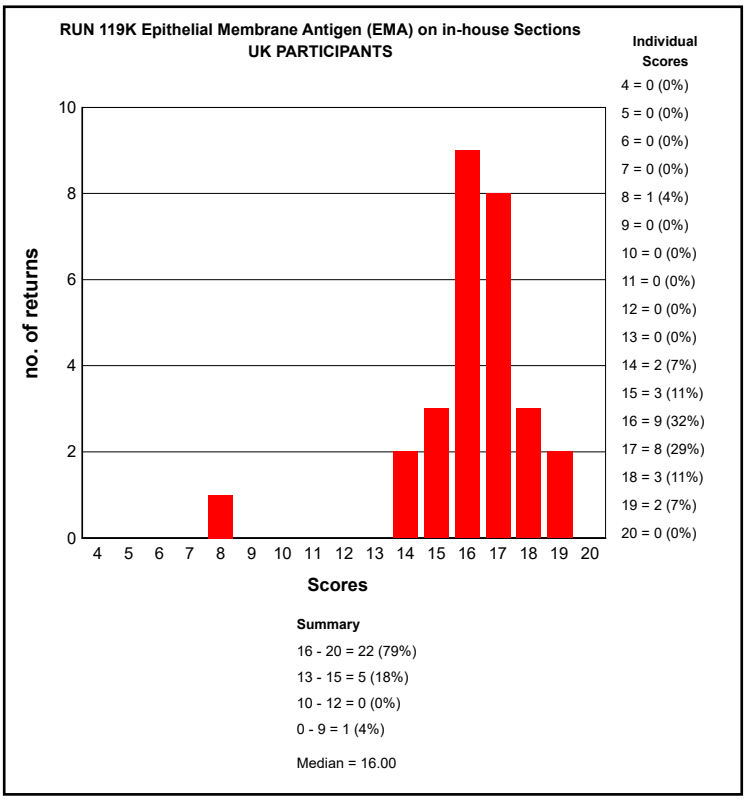


GRAPHICAL REPRESENTATION OF PASS RATES









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: 119

Primary Antibody : Ki-67 / MIB1

Antibody Details	N	%
Dako M7240 (MIB1)	30	63
NeoMarkers/Thermo Sci (SP6) RM 9106	1	100
Leica/Novocastra (MM1) NCL-Ki67-CE	1	100
Other	2	100
Leica/Novocastra RTU (MM1) PA0118	2	50
Leica/Novocastra RTU (K2) PA0230	3	100
Ventana RTU (30-9) 790-4286	9	100
Dako FLEX RTU (MIB1) IR626	2	100
DAKO FLEX RTU Omnis (MIB1) GA626	6	83
Gennova AP10244C (SP6)	1	100

Neuropathology Run: 119

Primary Antibody : Epithelial Membrane Antigen (EMA)

Antibody Details	N	%
Dako (E29) M0613	29	83
Leica/Novocastra (GP1.4) NCL-EMA	7	71
Other	7	86
Ventana (E29) 760-4259	4	75
Leica/Novocastra RTU (GP1.4) PA0035	3	100
Dako FLEX RTU (E29) IR629	7	86

Neuropathology Run: 119

Heat Mediated Retrieval	Epithelial Membrane Antigen (EMA) N	Epithelial Membrane Antigen (EMA) %	Ki-67 / MIB1 N	Ki-67 / MIB1 %
Dako Omnis	6	100	8	88
Dako PTLink	4	50	3	100
Lab vision PT Module	1	100	1	100
Leica ER1 20 mins	8	100	0	0
Leica ER2 20 mins	6	100	11	64
Leica ER2 30 mins	0	0	3	67
Leica ER2 40 mins	0	0	1	100
None	2	50	0	0
Other	1	100	2	100
Ventana CC1 16mins	1	0	0	0
Ventana CC1 20mins	1	0	0	0
Ventana CC1 24mins	0	0	1	100
Ventana CC1 32mins	4	100	5	60
Ventana CC1 36mins	4	25	2	50
Ventana CC1 40mins	1	100	0	0
Ventana CC1 48mins	2	100	2	100
Ventana CC1 56mins	0	0	1	100
Ventana CC1 64mins	8	88	9	78
Ventana CC1 72mins	1	100	0	0
Ventana CC1 mild	3	67	3	33
Ventana CC1 standard	2	100	2	100
Ventana CC2 64mins	0	0	2	100
Water bath 95-98 OC	1	100	1	100

Neuropathology Run: 119

Enzyme Mediated Retrieval	Epithelial Membrane Antigen (EMA) N	Epithelial Membrane Antigen (EMA) %	Ki-67 / MIB1 N	Ki-67 / MIB1 %
NOT APPLICABLE	29	90	36	75
VBS Bond Enzyme 1	1	100	0	0

Neuropathology Run: 119 Detection	Epithelial Membrane Antigen (EMA) N	%	Ki-67 / MIB1 N	%
AS PER KIT	5	100	8	75
Dako EnVision FLEX (K8000/10)	3	100	4	100
Dako EnVision FLEX+ (K8002/12)	4	75	4	100
Dako Envision HRP/DAB (K5007)	1	100	1	100
Leica Bond Polymer Refine (DS9800)	14	93	14	64
Other	1	0	1	100
Power Vision DPVB999 HRP	1	100	1	100
Ventana OptiView (760-700) + Amp. (7/860-099)	2	100	0	0
Ventana OptiView Kit (760-700)	9	89	10	80
Ventana UltraView Kit (760-500)	16	63	14	71

Neuropathology Run: 119 Automation	Epithelial Membrane Antigen (EMA) N	%	Ki-67 / MIB1 N	%
Dako Autostainer Link 48	3	67	3	100
Dako Autostainer Plus Link	1	100	0	0
Dako Omnis	7	86	8	88
Leica Bond Max	7	100	4	75
Leica Bond X	1	100	0	0
Leica Bond-III	8	88	11	73
None (Manual)	1	100	2	50
Other	1	100	1	100
Ventana Benchmark GX	1	100	0	0
Ventana Benchmark ULTRA	21	76	22	86
Ventana Benchmark XT	6	67	6	33

Neuropathology Run: 119 Chromogen	Epithelial Membrane Antigen (EMA) N	%	Ki-67 / MIB1 N	%
AS PER KIT	9	100	10	90
Dako DAB+ Liquid (K3468)	1	100	1	100
Dako DAB+ REAL Detection (K5001)	1	100	1	100
Dako EnVision Plus kits	2	50	1	0
Dako FLEX DAB	7	86	8	100
Leica Bond Polymer Refine kit (DS9800)	14	93	15	67
Other	2	100	2	50
Ventana DAB	5	80	4	100
Ventana Ultraview DAB	16	63	15	67

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 17/20 (In House slide) using this method.

Primary Antibody: Dako M7240 (MIB1)
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 40 mins
EAR:
Chromogen: Leica Bond Polymer Refine kit (DS9800)
Detection: Leica Bond Polymer Refine (DS9800)

Ki-67 / MIB1 - Method 2

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

Primary Antibody: Leica/Novocastra RTU (K2) PA0230 , 15 Mins, rt ºC Prediluted
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 20 mins
EAR: NOT APPLICABLE
Chromogen: Leica Bond Polymer Refine kit (DS9800), Time 1: 5 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins, rt ºC Prediluted

Ki-67 / MIB1 - Method 3

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

Primary Antibody:	Dako M7240 (MIB1) , 48 Mins, RT ºC Dilution 1: 50
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

Ki-67 / MIB1 - Method 4

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

Primary Antibody:	Ventana RTU (30-9) 790-4286 , 15 Mins Prediluted
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), Time 1: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins Prediluted

BEST METHODS - Secondary Antibody

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

Epithelial Membrane Antigen (EMA) - Method 1

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

Primary Antibody:	Leica/Novocastra RTU (GP1.4) PA0035 , 15 Mins, rt ºC Prediluted
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800), rt ºC., Time 1: 5 Mins
Detection:	Leica Bond Polymer Refine (DS9800)

Epithelial Membrane Antigen (EMA) - Method 2

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

Primary Antibody:	Dako (E29) M0613 , 32 Mins, 37 ºC Dilution 1: 25
Automation:	Ventana Benchmark GX
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 mild
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

Epithelial Membrane Antigen (EMA) - Method 3

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

Primary Antibody:	Dako (E29) M0613 , 15 Mins, RT ºC Dilution 1: 1/200
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800), Time 1: 10 Mins, Time 2: 5 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, RT ºC Prediluted

Epithelial Membrane Antigen (EMA) - Method 4

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

Primary Antibody:	Dako FLEX RTU (E29) IR629 , 20 Mins, 32 ºC Prediluted
Automation:	Dako Omnis
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis, Buffer: EnV FLEX TRS high PH
EAR:	
Chromogen:	Dako FLEX DAB, 32 ºC., Time 1: 5 Mins
Detection:	Dako EnVision FLEX (K8000/10) , 20 Mins Prediluted

Neil Bilbe and Irena Srebotnik Kirbis

	Gold Standard	Second Antibody
Antigens Assessed:	Ki 67	CD45
Sample circulated; cytospins and cell block sections:	Hypopharyngeal carcinoma cell line	Cell suspension from non-Hodgkin's lymphoma
Number of Registered Participants:	87-67 Cell block (77%), 20 Cytospins (23%).	
Number of Participants this Run	87 (100%)	

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 counter-stain

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

Second Antigen: CD45

Protein tyrosine phosphatase, receptor type, C also known as PTPRC is an enzyme that, in humans, is encoded by the *PTPRC* gene. PTPRC is also known as CD45 antigen, originally called leukocyte common antigen (LCA). It is a type I transmembrane protein that is in various forms present on all differentiated hematopoietic cells except erythrocytes and plasma cells that assists in the activation of those cells (a form of co-stimulation). It is expressed in lymphomas, B-cell chronic lymphocytic leukaemia, hairy cell leukaemia, and acute non-lymphocytic leukaemia. A monoclonal antibody to CD45 is used in routine immunohistochemistry to differentiate between lymphomas and carcinomas.

Features of Optimal Immunostaining:
- Strong, cell membrane staining of lymphocytes and NHL cells
- Clean background
- No non-specific staining of other cell types not expected to stain
- Adequate nuclear counterstain

Features of Sub-Optimal Immunostaining:
- 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 counterstain

References
1. Leong A, et al. Manual of Diagnostic Cytology (2nd ed.) Greenwich Medical Media Ltd. pp. 121-124.
2. Prasad RR, et al. Fine-needle aspiration cytology in the diagnosis of superficial lymphadenopathy: an analysis of 2,418 cases. Diagn Cytopathol. 1996; 15:382-386.
3. Hehn ST, et al. Utility of fine-needle aspiration as a diagnostic technique in lymphoma. J Clin Oncol. 2004; 22: 3046-3052.
4. Gong JZ, et al. Diagnostic impact of core-needle biopsy on fine-needle aspiration of non-Hodgkin lymphoma. Diagn Cytopathol. 2004; 31: 23-30.
5. Levien PH, et al. Role of fine-needle aspiration cytology in breast lymphoma. Diagn Cytopathol. 2004; 30: 332-340.

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

Assessment Summary:

All participants (87) submitted at least two slides. One lab did not submit any in-house control slides (**S & U**). This resulted in 346 slides for the assessors to score:
R = 87, S = 86, T = 87, U = 86.

Assessment Outcomes:

The overall pass rate was 93.1% (322 slides), 6.6% borderline (23), and a 0.3% failure rate (1).
The average score for all slides was 16/20.

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

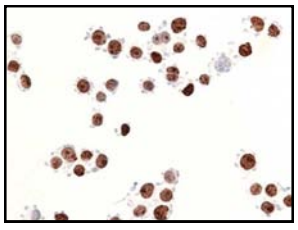


Fig.1 Ki-67 NEQAS Cytospin

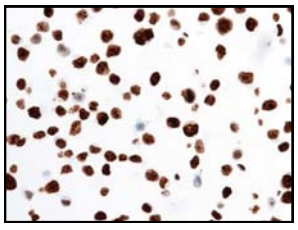


Fig.2 Ki-67 NEQAS Cell Block

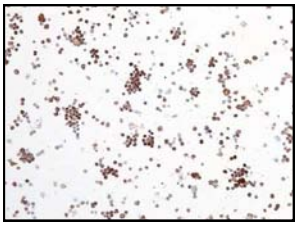


Fig.3 CD45 NEQAS Cytospin

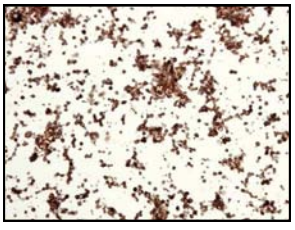


Fig.4 CD45 NEQAS Cell Block

(1) Summary Table - All Slides:

Slide	Antigen	Pass	Borderline	Fail
R (NEQAS)	Ki67 (87)	91% (79)	8% (7)	1% (1)
S (In-House)	Ki67 (86)	92% (79)	8% (7)	0% (0)
T (NEQAS)	CD45 (87)	94% (82)	6% (5)	0% (0)
U (In-House)	CD45 (86)	95% (82)	5% (4)	0% (0)
Average (Total)	346	93.1% (322)	6.6% (23)	0.3% (1)

Ki 67 (R & S)

The assessment outcome of a 91% pass rate for the NEQAS Ki 67 slides (**R**), was very similar to that for Run 118 (92% pass rate).

For this Run there was a single failed slide, which was on a NEQAS cytospin sample. The participant employed the Dako 7240 (MIB-1) at 1:50, without retrieval on the Ventana Benchmark XT platform. The staining was very weak, but additionally appears to show morphological damage (see Image Report Fig 5). Other cytospin users with similar protocols achieved excellent results, and overall the antibody (Dako M7240) had a 95% pass rate for all labs ($^{39}/_{41}$ users).

There were four slides which scored $^{20}/_{20}$, two were cytospins (See Images Report Fig 2) and two were cell block samples. The protocols employed on these slides are shown in the table below (2) and show very similar methodologies for each of the two sample types (CS and CB).

For the in-house controls (**S**), 16 participants (19%) submitted a sample, other than a FFPE section, which is close to the level of labs requesting cytospin preparations of 23%.

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

Sample	Antibody	Dilution	Antigen Retrieval	Platform
CS	Dako 7240 (MIB-1)	100	Ventana CC1 64mins	Ventana Benchmark ULTRA
CS	Dako 7240 (MIB-1)	100	Leica ER2 20 mins	Leica Bond-III
CB	Ventana RTU (30-9) 790-4286	RTU	Ventana CC1 standard	BioGenex GenoMX 6000i
CB	Ventana RTU (30-9) 790-4286	RTU	Ventana CC1 36mins	Ventana Benchmark ULTRA

CD45 (T & U)

The participants performed marginally better overall with the NEQAS CD45 (**T**) than with the Ki 67 NEQAS samples (**R**), with $^{82}/_{87}$ labs passing the assessment (94%), compared to 92% for the Ki 67 slides $^{79}/_{87}$.

What was more encouraging, was the improvement in pass rate from the last CD45 request in Run 116, where only 86% of participants passed the assessment. Additionally, there were three failed slides on the NEQAS samples in Run 116, whereas none failed for the Run 119 assessments.

The most significant finding when analysing the CD45 NEQAS slides (**T**) was that the best performing slides for this marker, were *all* on cell block sections (e.g. see Images Report Fig 11). No cytospin slide scored greater than $^{17}/_{20}$.

Unlike the Ki 67 methods, there was a spread of differing protocols. See the table below (3):

(3) Best Performing Methods CD45: NEQAS Samples (T):

Sample	Antibody	Dilution	Antigen Retrieval	Platform
CB	Dako M0701 (2B11+PD7/26)	400	Ventana CC1 mild	Ventana Benchmark XT
CB	Dako M0701 (2B11+PD7/26)	50	Ventana CC1 standard	Ventana Benchmark XT
CB	Dako RTU FLEX LINK IR751 (2B11 + PD7/26	RTU	Dako PTLink	Dako Autostainer Link 48
CB	Dako RTU FLEX LINK IR751 (2B11 + PD7/26	RTU	Dako PTLink	Dako Autostainer Plus Link
CB	Dako M0701 (2B11+PD7/26)	200	Ventana CC1 40mins	Ventana Benchmark ULTRA
CB	Ventana CONFIRM 760-2505 (RP2/18	RTU	Ventana CC1 32mins	Ventana Benchmark XT
CS	Dako M0701 (2B11+PD7/26)	500	None	Leica Bond-III

The number of labs who submitted an in-house sample (**U**) other than tissue sections was of a similar magnitude to the Ki 67 slides (**S**), with 17% doing so ($^{15}/_{86}$) for CD45.

Cell Block v Cytospins

(4) Summary Table - Average Scores NEQAS Samples:

Letter	Antigen	Type	Sample	Average All
R	CK	NEQAS	Cell Block	16
R	CK	NEQAS	Cytospin	15
T	CD45	NEQAS	Cell Block	16
T	CD45	NEQAS	Cytospin	15

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

It is worth stating, that on more than one occasion, the very best performing slides have been cytospins, it is only the average scores for all participants, where the cell blocks outperform cytospins.

There could be several reasons for this:

- Protocols, reagents and platforms designed and validated primarily for FFPE sections….
- ...particularly where antigen retrieval is important - many cytospin users routinely avoid or omit any HMAR steps
- The percentage of users from one or more regions and their preferred choice of sample in conjunction with their platforms, may also play a part

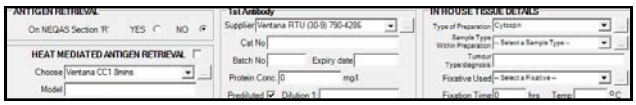
(5) Average Scores NEQAS Cytospin Samples w/wo Retrieval:

Letter	Antigen	Sample	Retrieval	Average
R	CK	Cytospin	Yes	16.0
R	CK	Cytospin	No	14.4
T	CD45	Cytospin	Yes	15.5
T	CD45	Cytospin	No	15.3

The first thing to emphasise is, as mentioned in previous write ups on the Cytopathology module, the accuracy as to whether a participant has used an antigen retrieval step on the NEQAS samples or not, requires them to use the Radio Buttons below the Antigen Retrieval section (YES or NO)*.

If the data is correct, then the table (5) shows that the retrieved NEQAS samples (**R & T**) performed better than the non-retrieved, but significantly more so for the Ki 67 slides (**R**)

*For example this Cytospin user has indicated that they have not used any retrieval method on the NEQAS cytospin sample (**R**) for the Ki 67 antigen, but they have entered a retrieval method from the HMAR drop down menu: Ventana CC1 8 mins.



Their in-house sample (**S**) is given as a cytospin. If all this data is taken literally, this means that antigen retrieval *has* been carried out the in-house sample, but *not* on the NEQAS sample!

The participant scored 16 for their NEQAS (**R**) cytospin (no retrieval), but only 11 on their in-house (**S**) cytospin (retrieved).

N.B. Laboratory scored 16 for *both* their CD45 slides (**T & U**), where retrieval was ? only used on an in-house (**U**) cytospin.

Selected Images showing Optimal and Sub-optimal Immunostaining

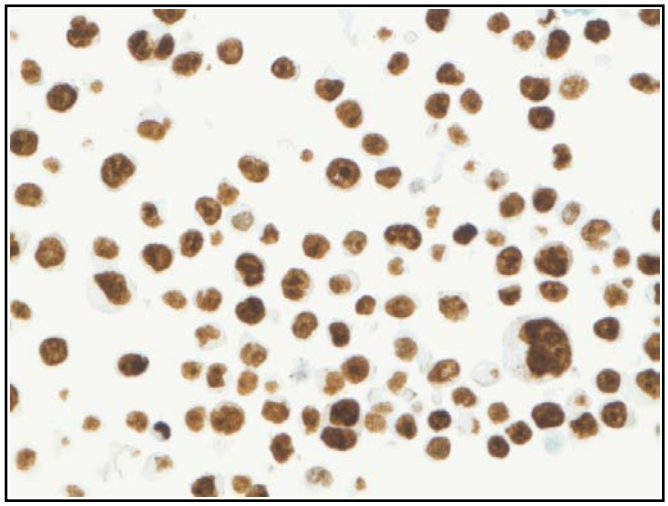


Fig 1. Optimal Ki67 demonstration on a NEQAS cell block. Nuclear staining of the tumour cells is clean, crisp and precise. Some negatives are also present. Dako 7240 (MIB-1), 1:100; with Ventana CC1 32 mins at pH9; using the RTU Ventana OptiView Kit (760-700) for 16 mins on the Ventana Benchmark ULTRA.

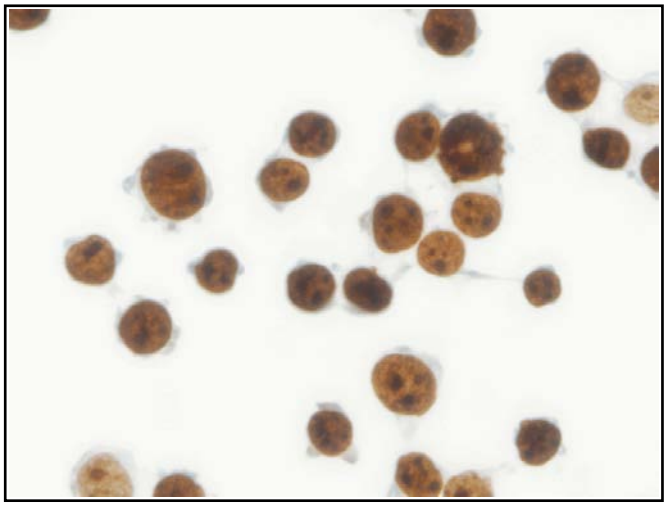


Fig 2. Optimal Ki67 demonstration on a NEQAS cytospin. Staining of the tumour nuclei is very precise, and prominent nucleoli are also seen. Dako 7240 (MIB-1), 1:100, 32 mins; Ventana CC1 64 mins, with the RTU Ventana UltraView Kit (760-500) 12 mins, on the Ventana Benchmark ULTRA.

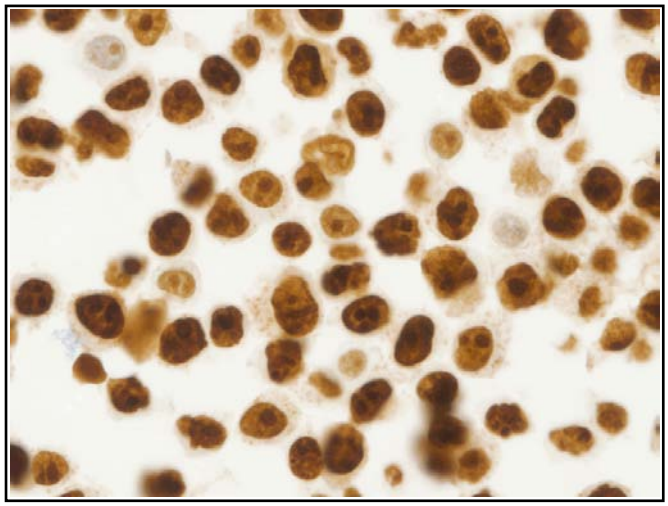


Fig 3. Sub-optimal demonstration of Ki67 on NEQAS cell block. Although nuclei are adequately stained, both diffuse and non-specific reactions are also visible. Slide assessed as low pass. RTU Leica/Novocastra (K2) PA0230; Leica ER1 10 mins; Leica Bond Polymer Refine (DS9800) on the Leica Bond III.

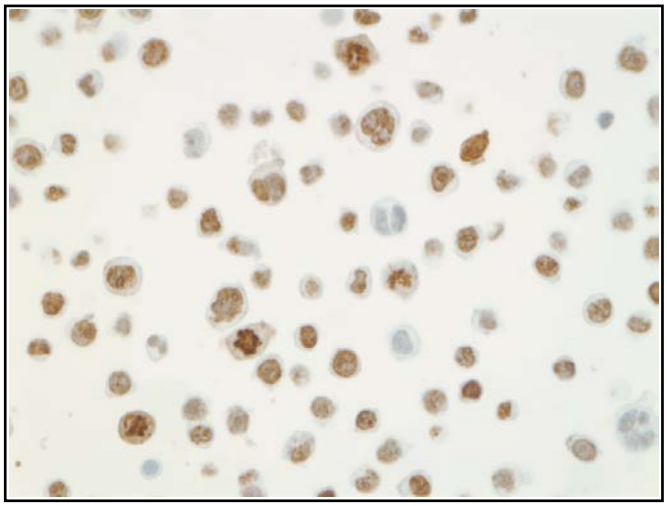


Fig 4. Sub-optimal Ki67 expression on a NEQAS cell block. Staining is weak, and some nuclei are not sufficiently demonstrated, resulting in a borderline score. Ventana RTU (30-9) 790-4286, 12 mins; Ventana CC1 16mins; with prediluted Ventana OptiView Kit (760-700); on the Ventana Benchmark ULTRA.

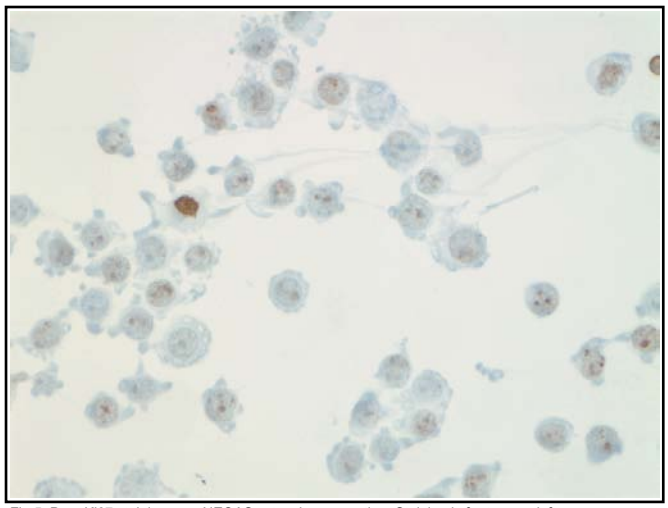


Fig 5. Poor Ki67 staining on a NEQAS cytospin preparation. Staining is far too weak for diagnostic proposes, and the sample also appears to have been damaged. Slide failed the assessment. Dako 7240 (MIB-1), 1:50; no retrieval performed; Ventana UltraView system and the Ventana Benchmark XT.

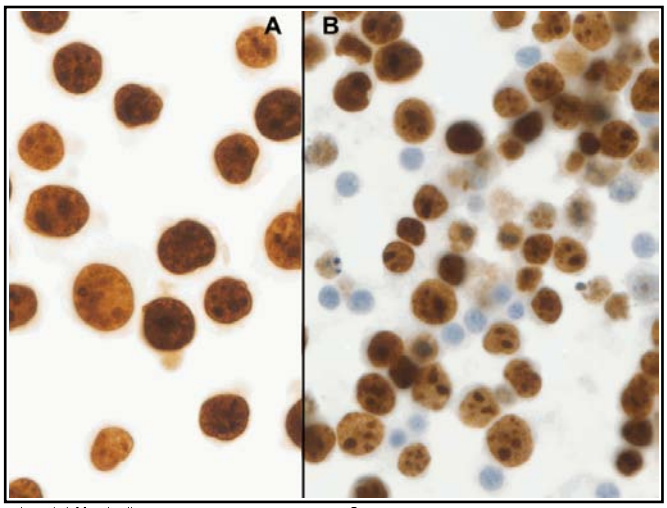


Fig 6. (A) Marginally sub-optimal staining on a NEQAS cytospin, (some negatives are stained); and (B) optimal in-house pleural effusion from a lymphoma. Ventana Confirm anti Ki67 (30-9), RTU, 16 mins; Ventana CC1 32 mins; Ventana OptiView Kit (760-700); on the Ventana Benchmark ULTRA.

Selected Images showing Optimal and Sub-optimal Immunostaining

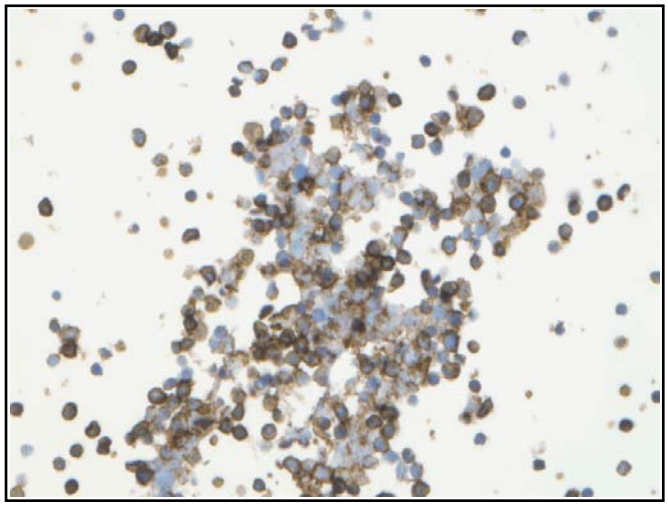


Fig 7. Sub-optimal CD45 on a NEQAS cell block, there some is morphological damage and the counterstain is a touch heavy; but adequate for diagnostic purposes. Dako M0701 (2B11+PD7/26), 1:1000, 15 mins; Leica ER2 10 mins; Leica Bond Polymer Refine (DS9800), 10 mins; on a Leica Bond-III.

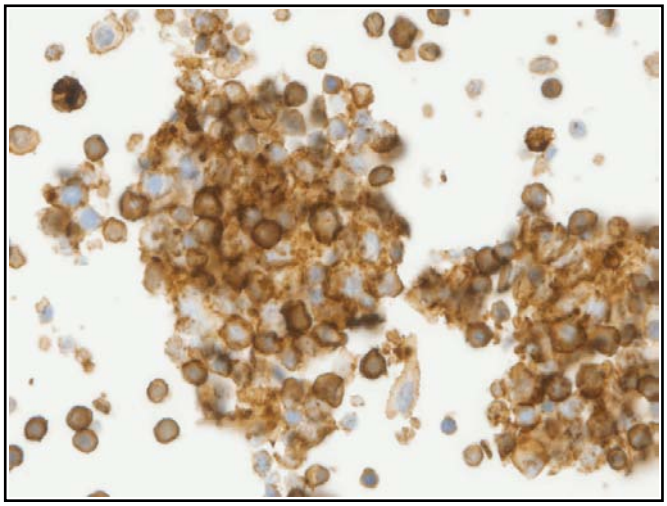


Fig 8. Optimal CD45 demonstration on a NEQAS cell block section. There is crisp cytoplasmic staining, with blue nuclei. Dako M0701 (2B11+PD7/26), 1:100, 32 mins; Ventana CC1 mild, 30 mins; RTU Ventana UltraView Kit (760-500), 8 mins; on the Ventana Benchmark XT.

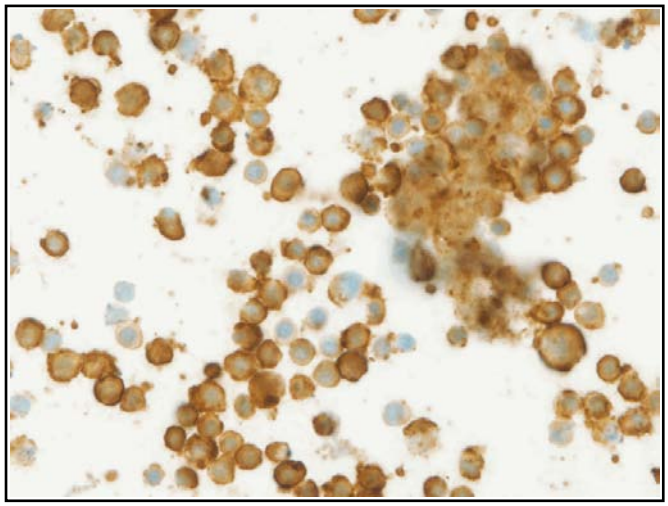


Fig 9. Sub-optimal CD45 on a NEQAS cytospin. Some of the (few) negative cells are weakly stained. Overall the slide is still satisfactory, and passed the assessment. Dako M0701 (2B11+PD7/26), 1:250, 32 mins; Ventana CC1 64 mins; the Ventana UltraView Kit (760-500) 12 mins; on a Benchmark ULTRA.

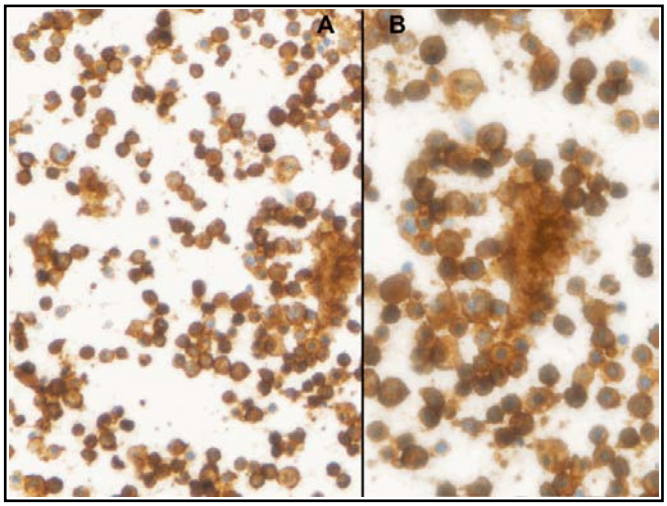


Fig 10. Sub-optimal CD45 on a NEQAS cytospin; lower power (A) and high power (B). Cells are heavily stained, although selectively so, throughout the sample. Dako M0701 (2B11+PD7/26), 30 mins, no dilution given; no retrieval; Leica Bond Polymer Refine (DS9800); on a Leica Bond-III.

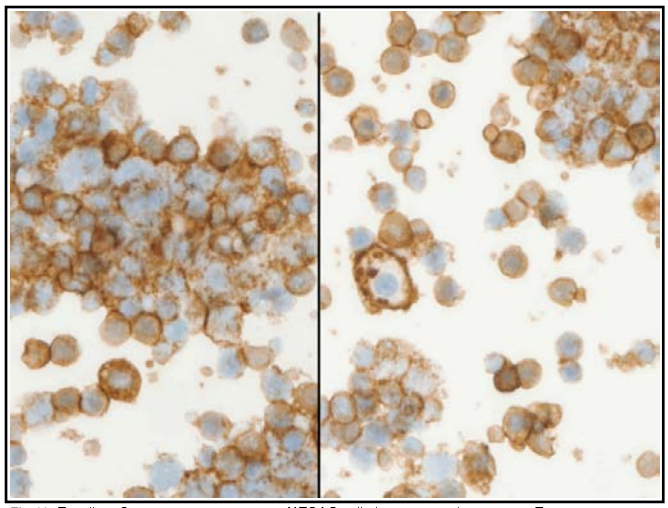


Fig 11. Excellent CD45 demonstration on a NEQAS cell block section (two views). The primary and counterstain staining are of optimal intensities. Dako RTU FLEX LINK IR751 (2B11 + PD7/26); Dako PTLink retrieval; with the Dako EnVision FLEX+ (K8002/12); on a Dako Autostainer Link 48.

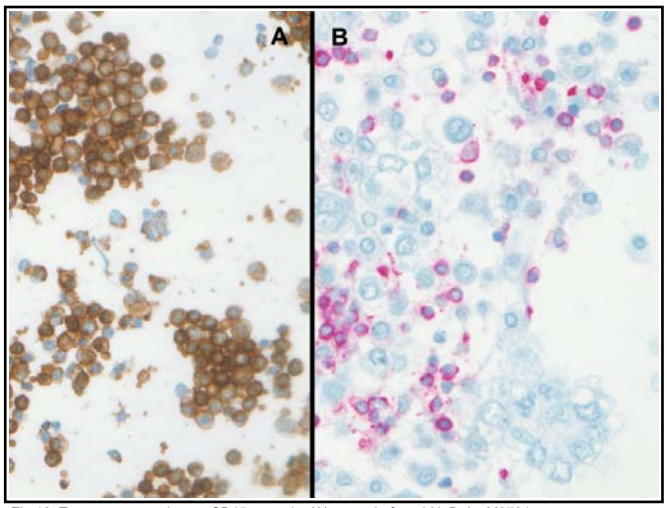
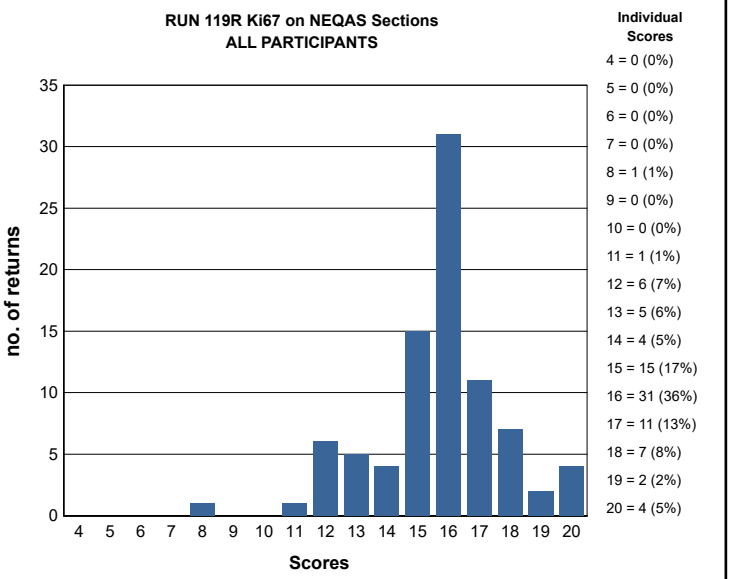
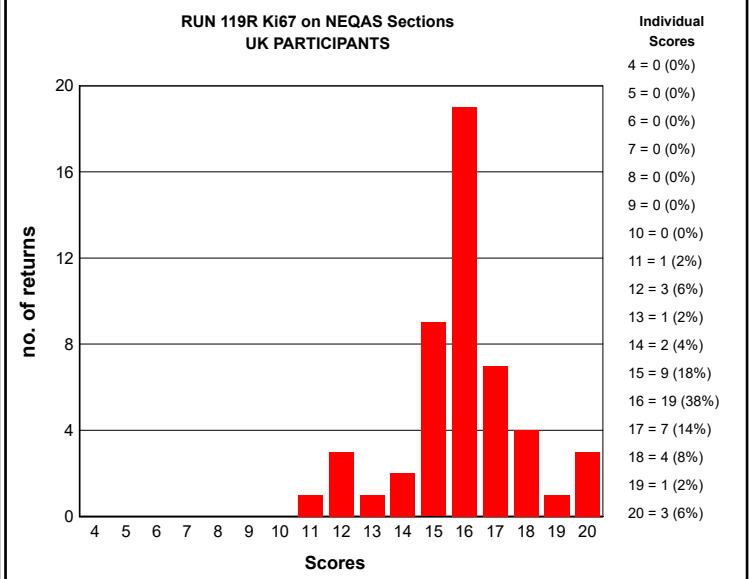
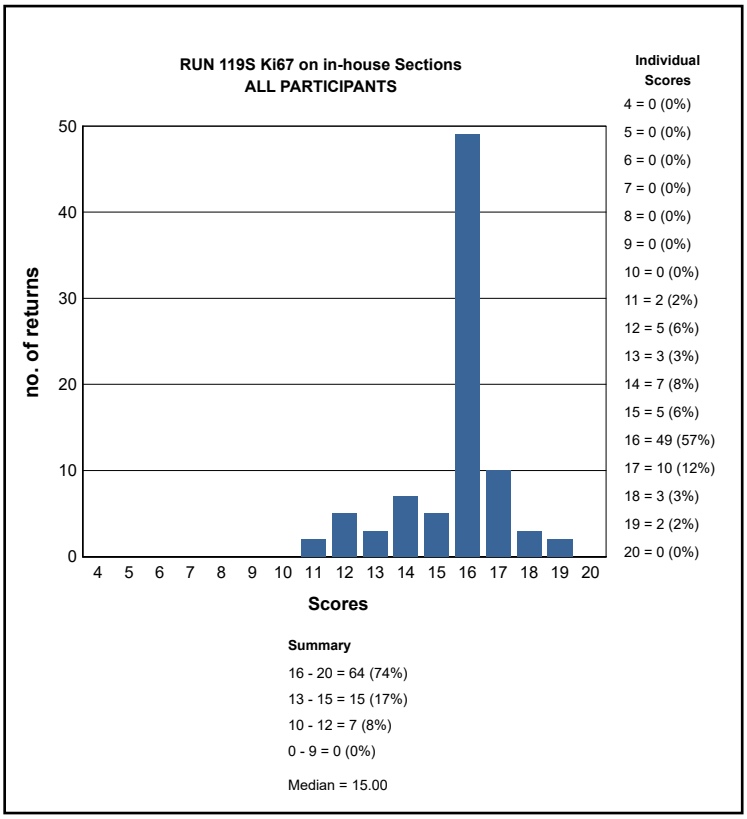


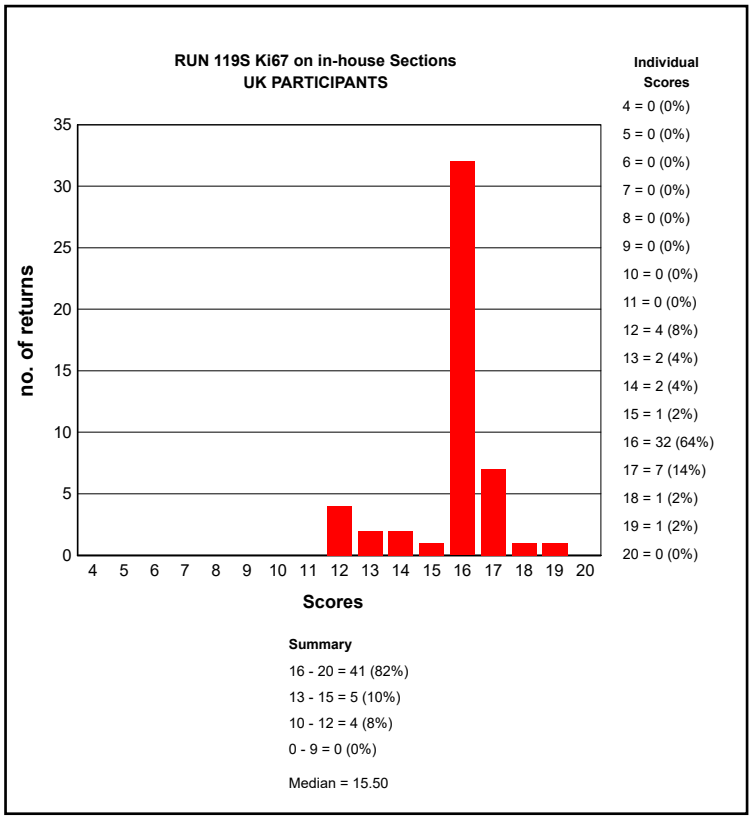
Fig 12. Two very nice in-house CD45 controls. (A) cytopsin from LN; Dako M0701 (2B11+PD7/26), 1:1000; no RT; Ventana iView system, on Ventana Benchmark GX. (B) cell block from pleural effusion; Diagnostic Biosystems LCA (PD7/26 + 2B11), 1:200; Ventana CC1 std; UV AP Red Detection Kit on Ventana Benchmark XT.

GRAPHICAL REPRESENTATION OF PASS RATES

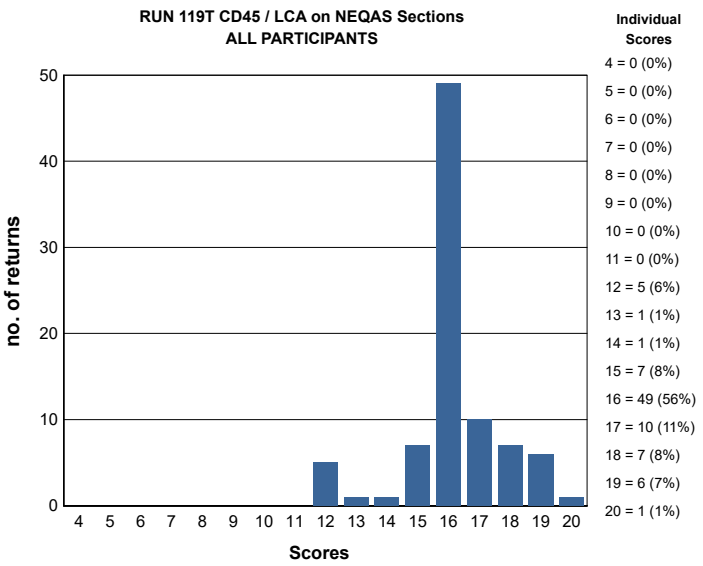


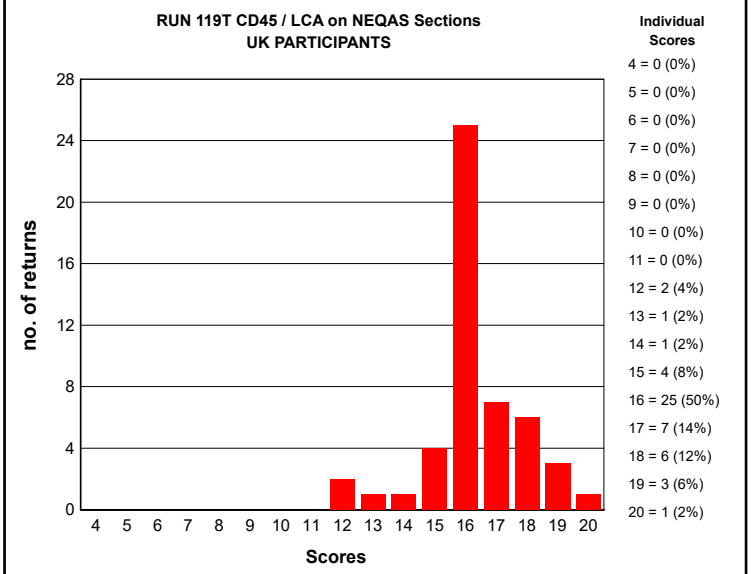


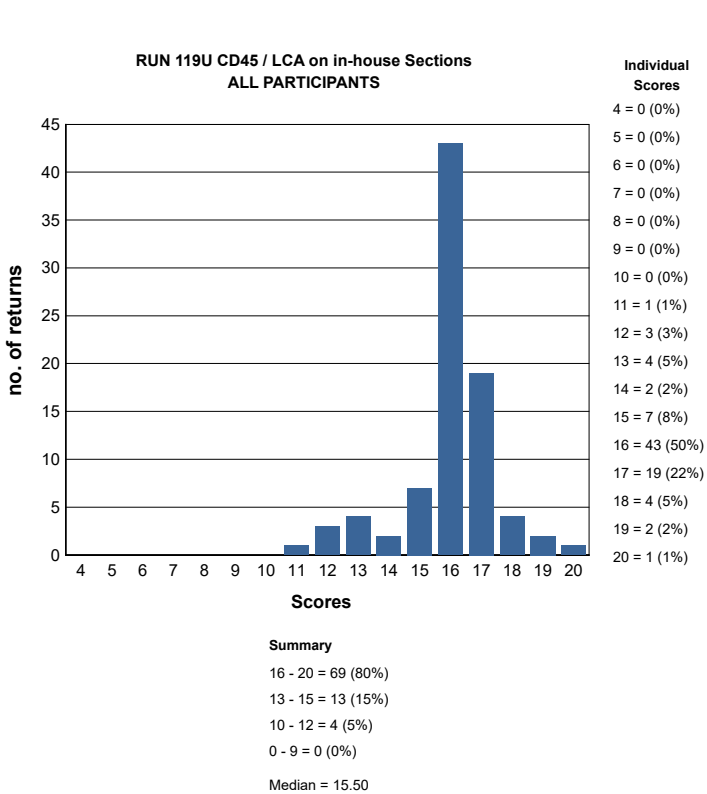


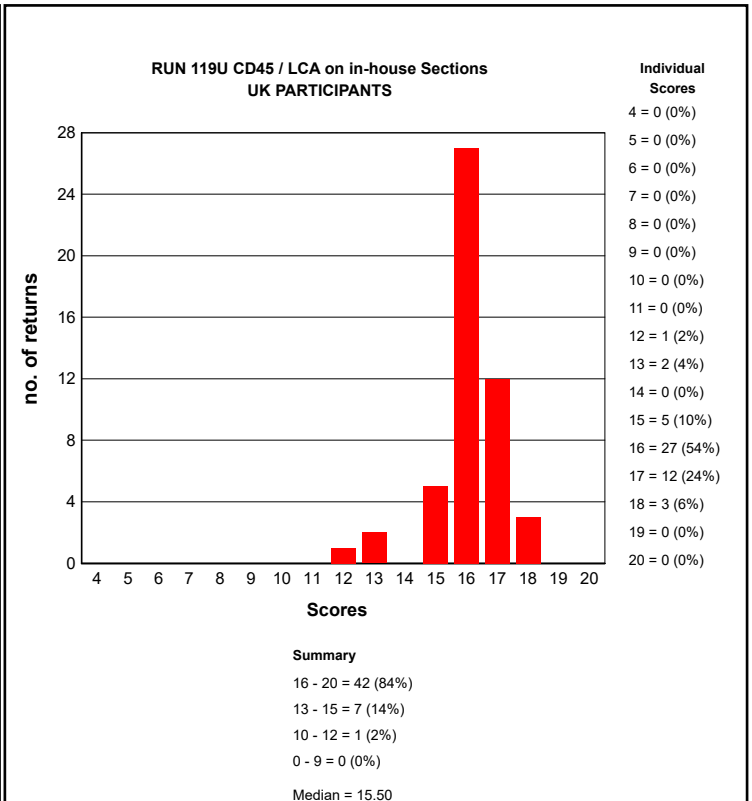


GRAPHICAL REPRESENTATION OF PASS RATES









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: 119

Primary Antibody : Ki67

Antibody Details	N	%
Dako 7240 (MIB-1)	41	95
Dako FLEX RTU IR626 (MIB-1)	6	83
Neomarkers/Thermo Sci (SP6) RM 9106	2	100
Leica/Novocastra RTU (MM1) PA0118	1	100
Leica/Novocastra RTU (K2) PA0230	10	70
Ventana RTU (30-9) 790-4286	22	91
Other	4	100

Cytology Run: 119

Primary Antibody : CD45 / LCA

Antibody Details	N	%
Dako M0701 (2B11+PD7/26)	52	94
Leica/Novocastra NCL-L-LCA (X16/99)	5	80
Ventana CONFIRM 760-2505 (RP2/18)	15	100
Other	1	100
Ventana 760-4279 (2B11 & PD7/26)	3	100
Cell Marque 145M-97	1	0
Leica/Novocastra Bond RTU PA0042 (X16/99)	2	100
Dako RTU FLEX LINK IR751 (2B11 + PD7/26)	4	100
Dako Omnis RTU GA751 (2B11+PD7/26)	3	100

Cytology Run: 119

Primary Antibody : Ki67

Antigen Retrieval	N	%
YES	27	31
NO	60	69
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	27	
Not Specified	0	

Cytology Run: 119

Primary Antibody : CD45 / LCA

Antigen Retrieval	N	%
YES	23	26
NO	64	74
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	23	
Not Specified	0	

Cytology Run: 119

Heat Mediated Retrieval

Cytology Run: 119

Heat Mediated Retrieval

Cytology Run: 119

Enzyme Mediated Retrieval

Cytology Run: 119

Enzyme Mediated Retrieval

Cytology Run: 119

Detection

	CD45 / LCA		Ki67	
	N	%	N	%
AS PER KIT	5	100	7	100
Dako EnVision FLEX (K8000/10)	1	100	3	100
Dako EnVision FLEX+ (K8002/12)	8	100	5	100
Dako Envision+ HRP mouse K4004/5/6/7	1	100	1	100
Leica Bond Polymer Define (DS9713)	1	100	0	0
Leica Bond Polymer Refine (DS9800)	22	100	19	79
Other	3	100	3	100
Power Vision DPVB999 HRP	1	0	1	100
Ventana iView system (760-091)	3	100	2	100
Ventana OptiView (760-700) + Amp. (7/860-099)	0	0	1	100
Ventana OptiView Kit (760-700)	20	100	19	84
Ventana UltraView Kit (760-500)	19	79	23	100

Cytology Run: 119

Automation

	CD45 / LCA		Ki67	
	N	%	N	%
BioGenex GenoMX 6000i	1	100	1	100
Dako Autostainer Link 48	7	100	8	100
Dako Autostainer Plus Link	2	100	1	100
Dako Omnis	3	100	3	100
Leica Bond Max	8	100	6	100
Leica Bond-III	18	100	16	75
Other	1	0	1	100
Ventana Benchmark GX	3	100	3	67
Ventana Benchmark ULTRA	30	97	36	94
Ventana Benchmark XT	13	77	11	91

Cytology Run: 119

Chromogen

	CD45 / LCA		Ki67	
	N	%	N	%
AS PER KIT	7	100	13	92
DAKO DAB+	1	100	1	100
Dako DAB+ Liquid (K3468)	1	0	1	100
Dako EnVision Plus kits	1	100	0	0
Dako FLEX DAB	9	100	8	100
Leica Bond Polymer Refine kit (DS9800)	23	100	18	78
Other	5	100	6	83
Ventana DAB	13	100	14	93
Ventana iview	4	100	3	67
Ventana Ultraview DAB	22	82	22	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 17/20 (In House slide) using this method.

Primary Antibody:	Dako 7240 (MIB-1) , 16 Mins, RT ºC Dilution 1: 100
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 32mins, Buffer: 0, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB, RT ºC., Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 16 Mins, RT ºC Prediluted

Ki67 - Method 2

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

Primary Antibody:	Neomarkers/Thermo Sci (SP6) RM 9106 , 15 Mins, ambient ºC Dilution 1: 200
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

Ki67 - Method 3

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

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

Ki67 - Method 4

Participant scored 20/20 (UK NEQAS Slide) and 18/20 (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)
HMAR:	Leica ER2 20 mins
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

BEST METHODS - Secondary Antibody

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

CD45 / LCA - Method 1

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

Primary Antibody:	Ventana CONFIRM 760-2505 (RP2/18) , 16 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.6
HMAR:	Ventana CC1 64mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB, 36 ºC., Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 36 ºC Prediluted

CD45 / LCA - Method 2

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

Primary Antibody:	Dako RTU FLEX LINK IR751 (2B11 + PD7/26) , 20 Mins, 20 ºC Prediluted
Automation:	Dako Autostainer Plus Link
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: high pH target retrieval solution
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 20 ºC., Time 1: 10 Mins, Time 2: 10 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 30 Mins, 20 ºC Prediluted

CD45 / LCA - Method 3

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

Primary Antibody:	Dako M0701 (2B11+PD7/26) , 16 Mins, 37 ºC Dilution 1: 200
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 40mins
EAR:	
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700)

CD45 / LCA - Method 4

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

Primary Antibody:	Dako M0701 (2B11+PD7/26) , 68 Mins, 37 ºC Dilution 1: 400
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.6
HMAR:	Ventana CC1 mild, PH: 8
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB, 37 ºC., Time 1: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC

The Alimentary Tract Module: GIST Run 119

Amy Newman

	First Antibody	Second Antibody
Antigens Assessed:	CD117	S100
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: **(Figs 1, 2 3 & 6)**

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

Features of Sub-optimal Immunostaining: **(Fig 4 & 5)**

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

Second Antibody: S100

S100 protein was first described as a nervous tissue protein, and was so named because of its solubility in 100% ammonium solution at neutral pH. It is made up of a mixture of S100A and S100B, and has been demonstrated in a wide variety of normal and abnormal tissues (Nakajima et al). The staining should be cytoplasmic as well as nuclear. S-100 is routinely used as part of a panel of antibodies in the differential diagnosis of GIST: Those tumours negative for CD117 and DOG-1 are further tested using a panel of antibodies, which includes S-100 along with SMA, desmin and CD34. Molecular analysis should also be considered.

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

- Strong intense staining of the nerve bundles, fibres and macrophages in the appendix.
- No staining of the GIST
- No staining of the desmoid tumour; only the nerve bundles should show strong distinct staining.

Features of Sub-optimal Immunostaining: (Figs 9 & 10)

- Weak and/or patchy staining of the nerve bundles, fibres and macrophages in the appendix (Fig 10)
- Weak and/or patchy staining of the nerve elements in the desmoid tumour section
- Excessive background or non specific staining
- Staining of the GIST
- Staining of the desmoid tumour

Tissue Distribution and Assessment Procedure

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

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

Assessment Summary:

CD117

Results from the **CD117** assessment showed an overall acceptable pass rate of 91%. This is a steady improvement from the previous Runs 117 & 118, where the pass rates were 75% & 84% respectively. The main reason for sub-optimal marks was due to very weak staining of the GIST. Non-specific and inappropriate false-positive staining was often observed in the desmoid tumour which should be negative for CD117 expression. Inappropriate antibody dilution factor and antigen retrieval were the main causes of these issues. The staining in the appendix TMA control showed to be the least variable in terms of expression. In-house tissues scoring 12 or less was predominantly due to not providing a multi-block with both normal appendix/gastric tissue together with a GIST. The most popular CD117 antibody choice still remains the Dako polyclonal, used by 84 participants and showed an acceptable pass rate of 90% in this assessment run.

NEQAS Pass Rates Run 118 v 119 CD117		
Run no	**118**	**119**
Acceptable	**84% (N=98)**	**91% (N=107)**
Borderline	**13% (N=15)**	**6% (N=7)**
Unacceptable	**3% (N=3)**	**3% (N=4)**

S100

Pass rates were similar for S100 with a pass rate of 92% on the NEQAS tissue. Pass rates for S100 I in-house control material was 96%. The main assessors comments on borderline and failing submissions was due to over staining of the S100 protein. The Dako Z0311 (polyclonal) antibody was most commonly used by 48% of participants who completed the on-line methods. The other antibody commonly employed was the Ventana (polyclonal) used by 22% 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. **Nakajima T, Watanabe S, Sato Y et al.** An immunoperoxidase study of S-100 protein distribution in normal and neoplastic tissue. Am J Surg pathol 1982; 6: 715-726.

Selected Images showing Optimal and Sub-optimal immunostaining

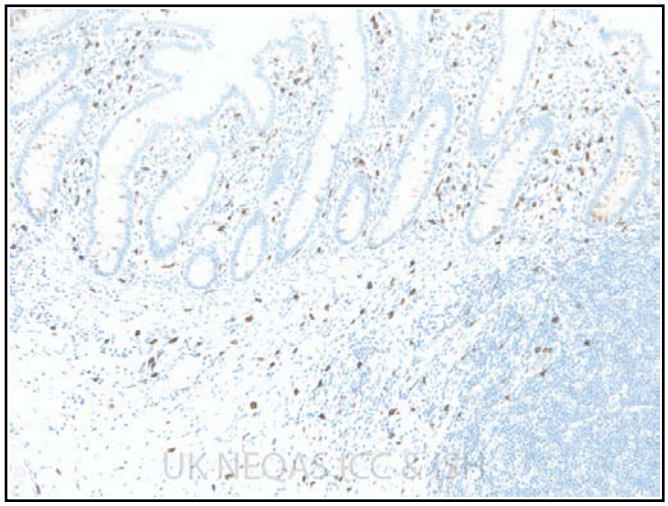


Figure 1: Excellent example of the NEQAS appendix. There is strong membrane staining in the mast cells and interstitial cells of Cajal with minimal background. Method: Leica RTU (EP10), 15mins; BOND III (ER2 20'; Refine detection).

Figure 2: Good demonstration of the Desmoid in the NEQAS sample. The mast cells are present and the negative tumour is free of background staining. Method: As in Figure 1.

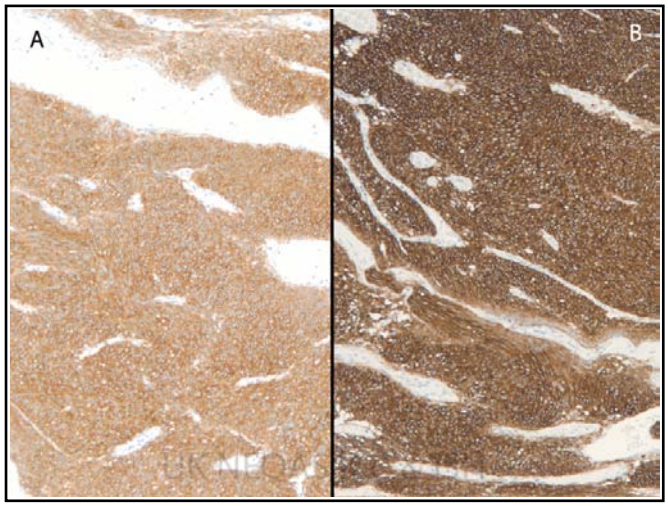


Figure 3: Two acceptable examples of CD117 positive GISTs in the NEQAS sample. Figure B appears to have had an enhancing agent added. Method (A): Dako A4502 (1:300, 48mins @ 37C); (Benchmark ULTRA; CC1 64mins; OptiView). Method (B): As in Figure 1.

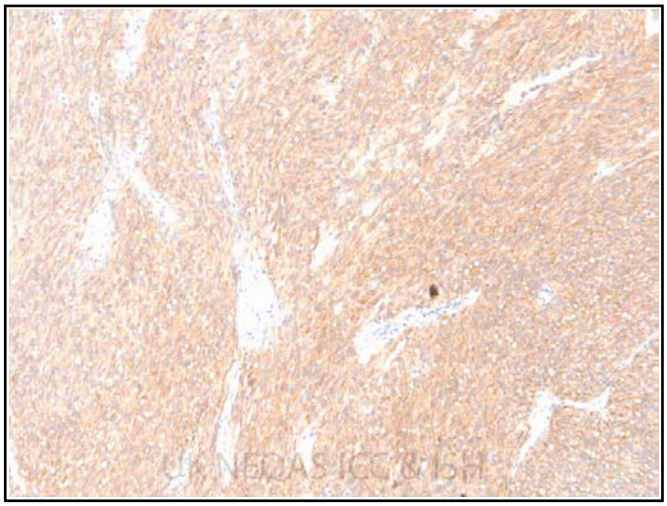


Figure 4: Sub-optimal staining in the GIST on the NEQAS sample. Although the staining is present, it is not well localised and may benefit for longer incubation with primary antibody or longer time in antigen retrieval. Method: Dako A4502 (1:100, 32mins); (Benchmark ULTRA; CC1 36mins; UltraView).

Figure 5: Poor demonstration of the NEQAS submitted Desmoid section. There is false-positive staining in the tumour cells which has the potential to cause a misdiagnosis. Refer to Figure 2 for optimal desmoid expression. Method: Dako A4502 (1:100; 20mins @21C); (PT Link, pH9.0; Autostainer Link 48; EnVision FLEX+).

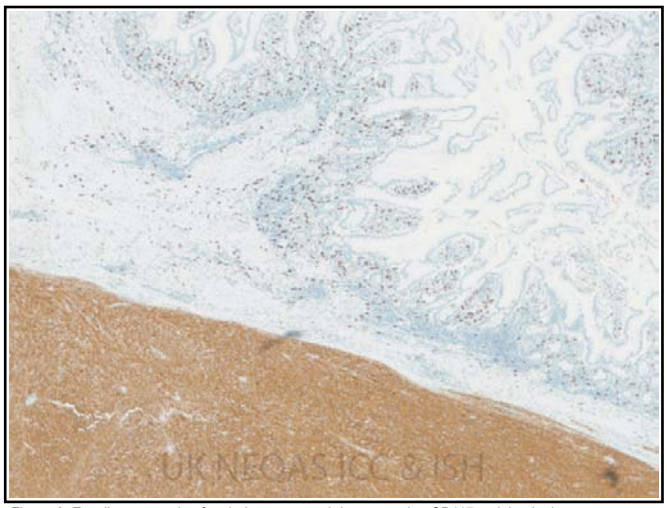


Figure 6: Excellent example of an in-house control demonstrating CD117 staining in the mast cells and Cajal cells in normal GI tissue and GIST in the attached tumour. This is an ideal control as it contains elements of normal and tumour tissue. Method: Epitomics (EP10) (1:200, 16mins @ 37C); (Benchmark ULTRA; CC1 40mins; OptiView).

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

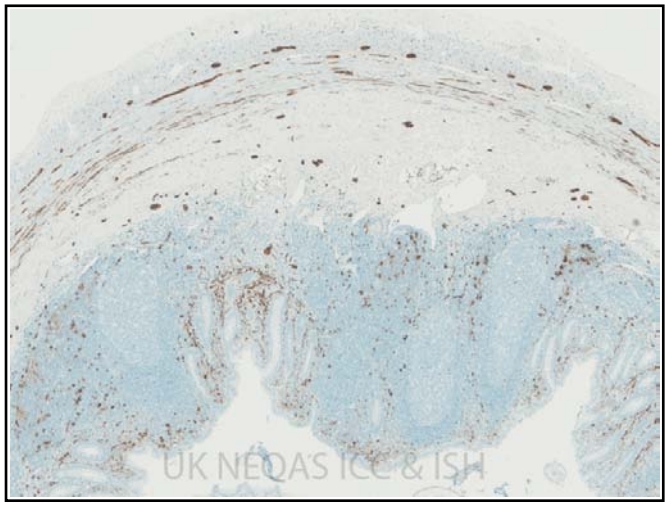


Figure 7: Optimal staining of S100 in the NEQAS appendix sample. The adipocytes, Schwann cells and dendritic cells are positive and the smooth muscle is clearly negative. Method: Ventana (S100 poly) RTU (4mins @ 36C); (Benchmark ULTRA, CC1 8mins, OptiView).

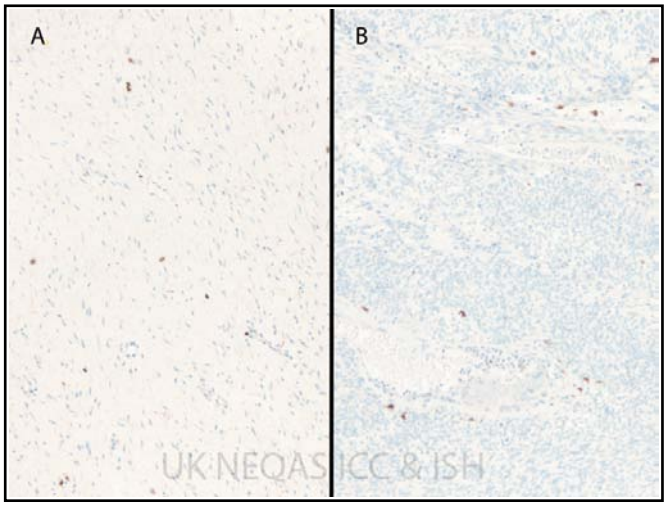


Figure 8: Optimal demonstration of the NEQAS desmoid (A) and GIST (B) samples with S100. Both tumours are negative with a scattering of positive nerve and dendritic cells. Method (A & B): Dako (S100 Poly); (Benchmark; UltraView).

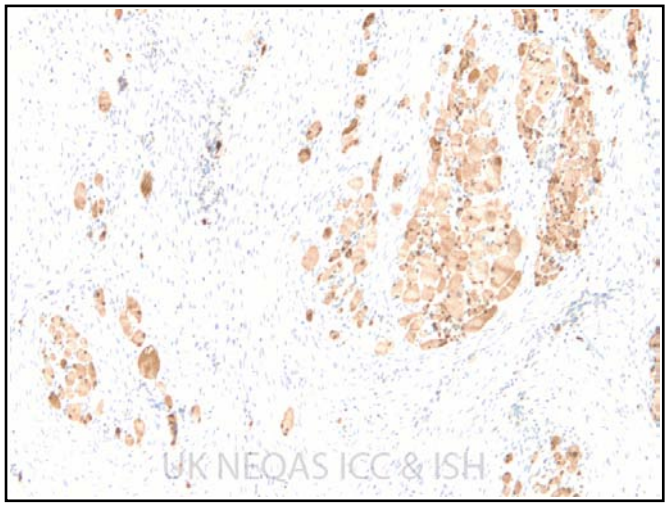


Figure 9: Sub-optimal staining on the desmoid tumour on the NEQAS sample exhibiting false-positive staining within the skeletal muscle. The method suggests that the S100 concentration is too strong. Method: Dako (S100 poly) 1:200, 30mins @ RT (PT Link + FLEX).

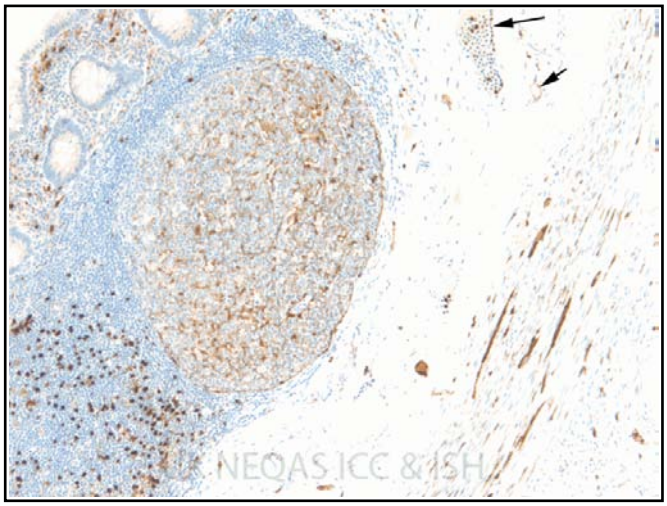


Figure 10: Slightly strong staining in the NEQAS appendix with S100. The follicular dendritic cells are staining strongly, which is acceptable, however, some non-specific background staining is becoming evident as indicated by the arrows. Method: Dako (S100 poly) 1:200, 30mins @ RT (PT Link + FLEX).

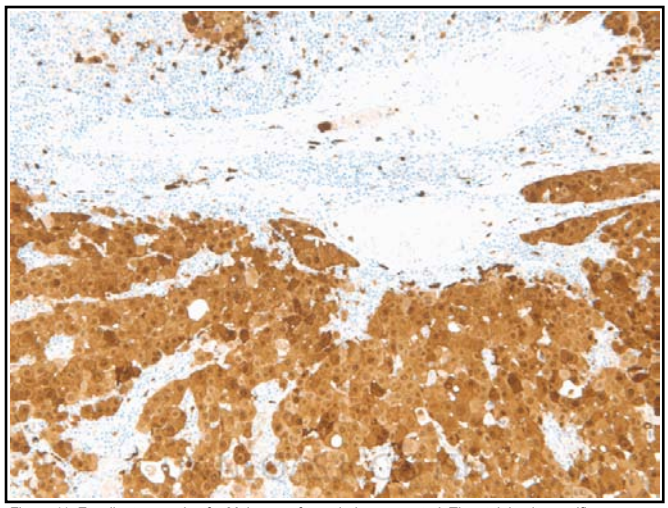


Figure 11: Excellent example of a Melanoma for an in-house control. The staining is specific within the tumour and also in the nerve and dendritic cells. Method: Dako (S100 poly) 1:3000 (15mins @20C); (ER2 20mins, BOND MaX; Refine).

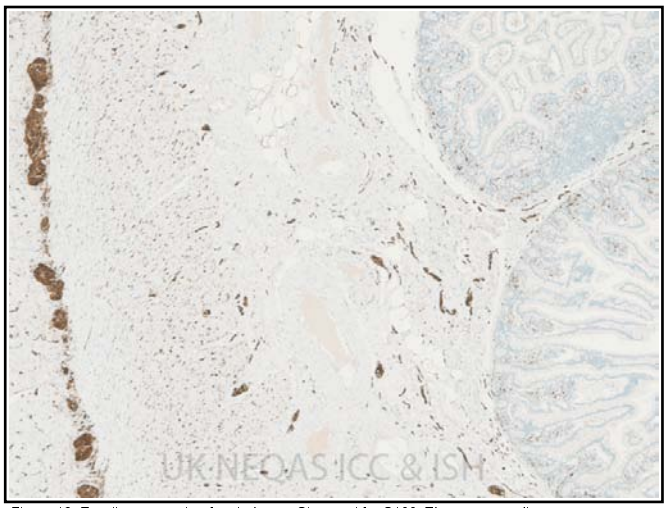
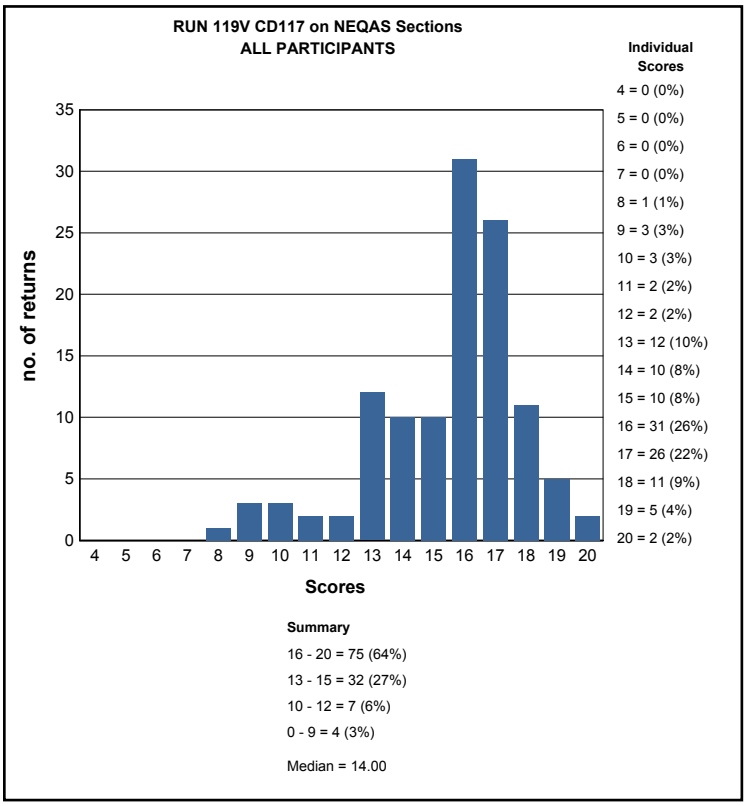
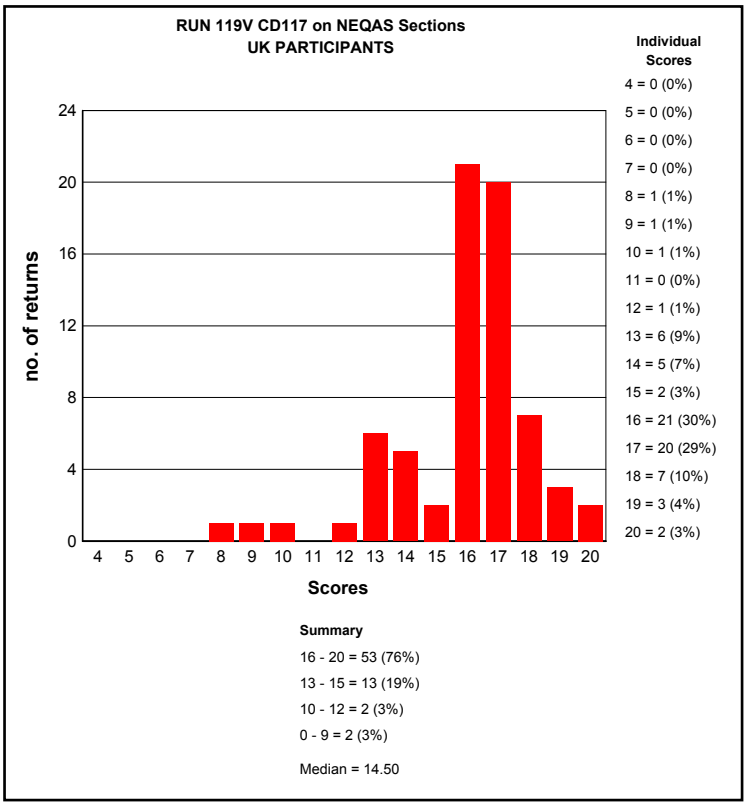
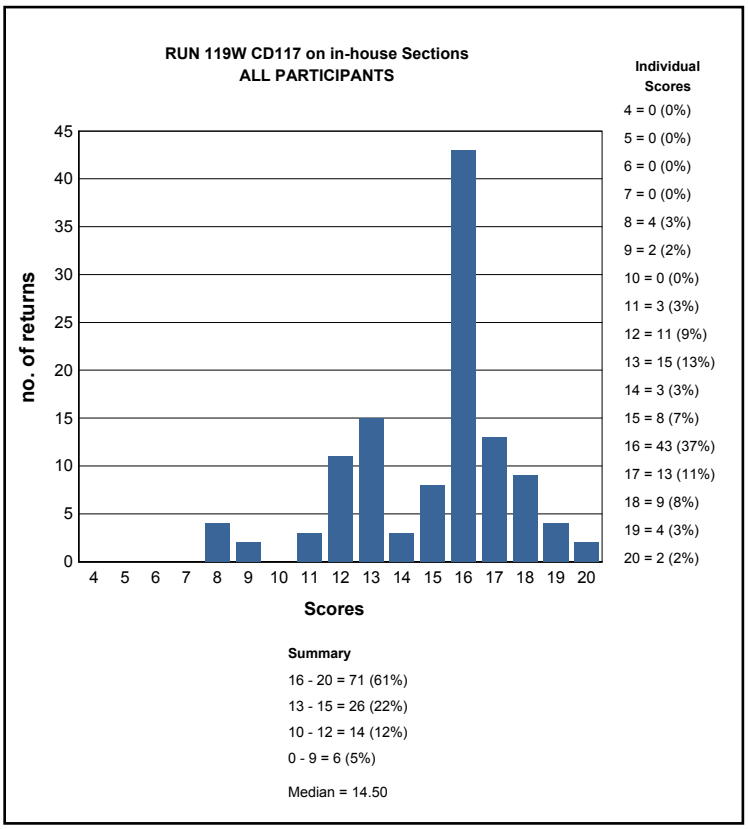


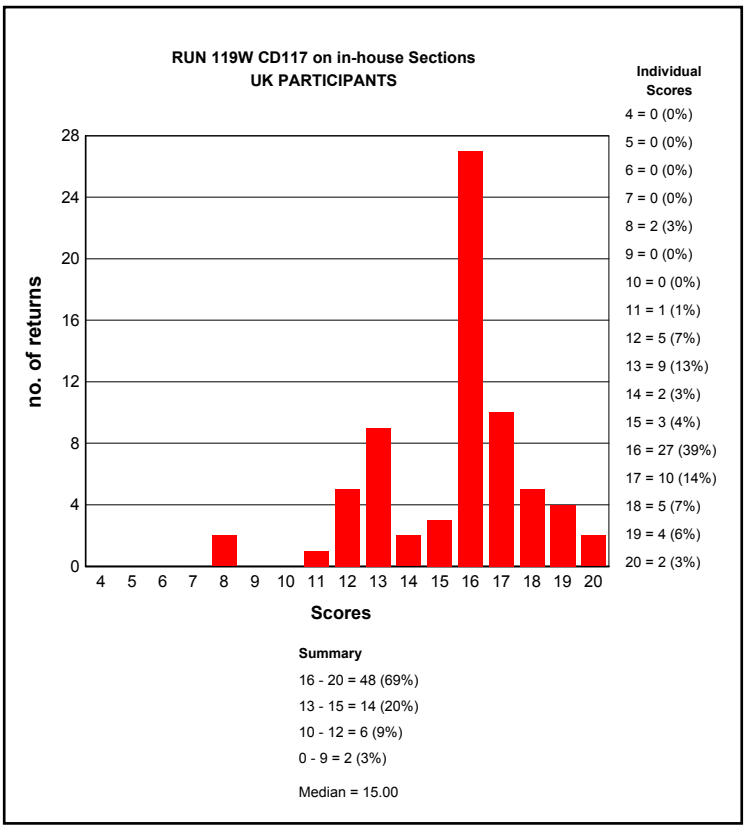
Figure 12: Excellent example of an in-house GI control for S100. The nerve bundles, adipocytes and interfollicular dendritic cells are clearly demonstrated. Method: Dako (S100 poly) 1:1000 (24mins @ 37C); (Benchmark ULTRA, CC1 24mins; OptiView).

GRAPHICAL REPRESENTATION OF PASS RATES

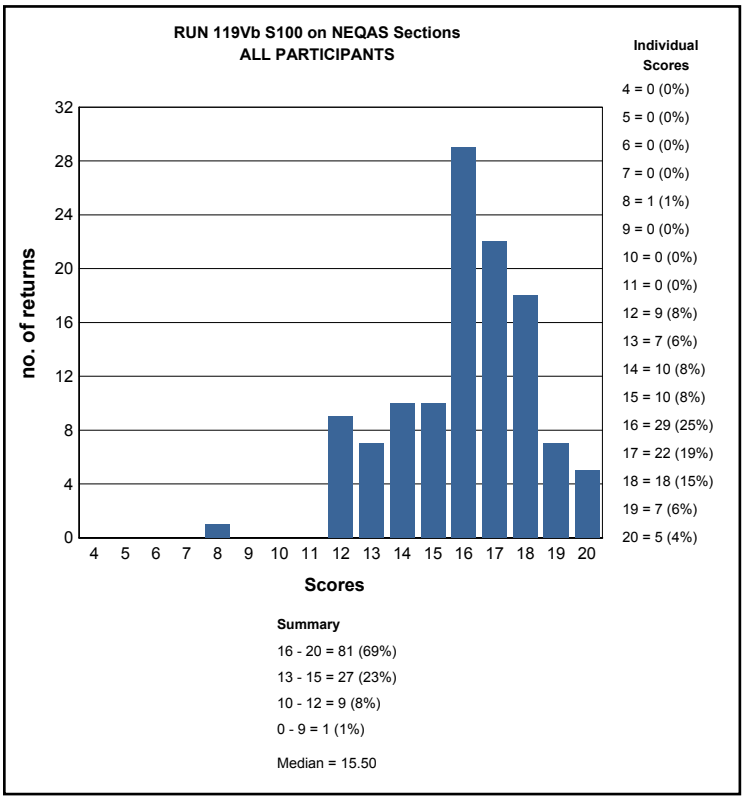


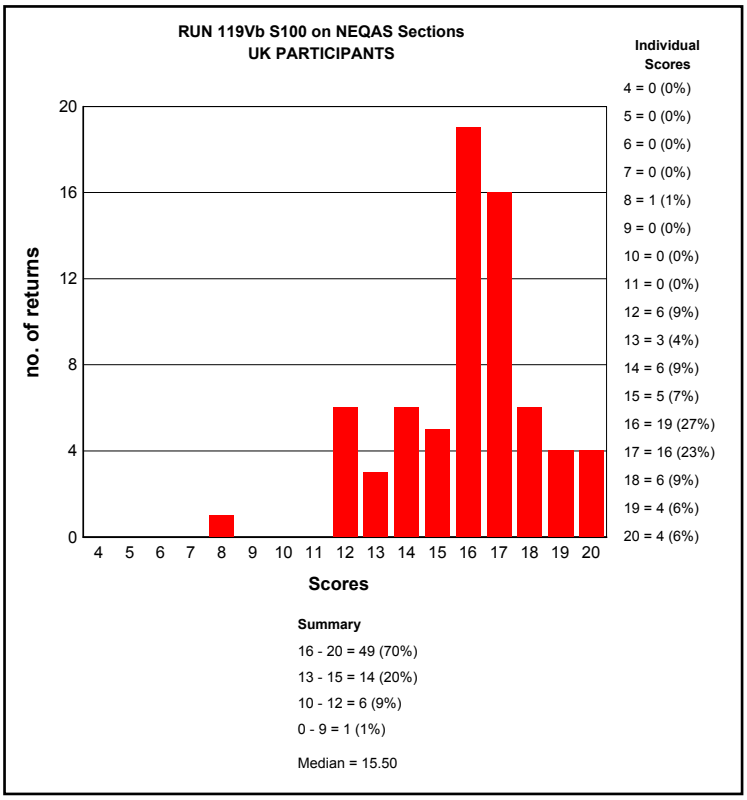


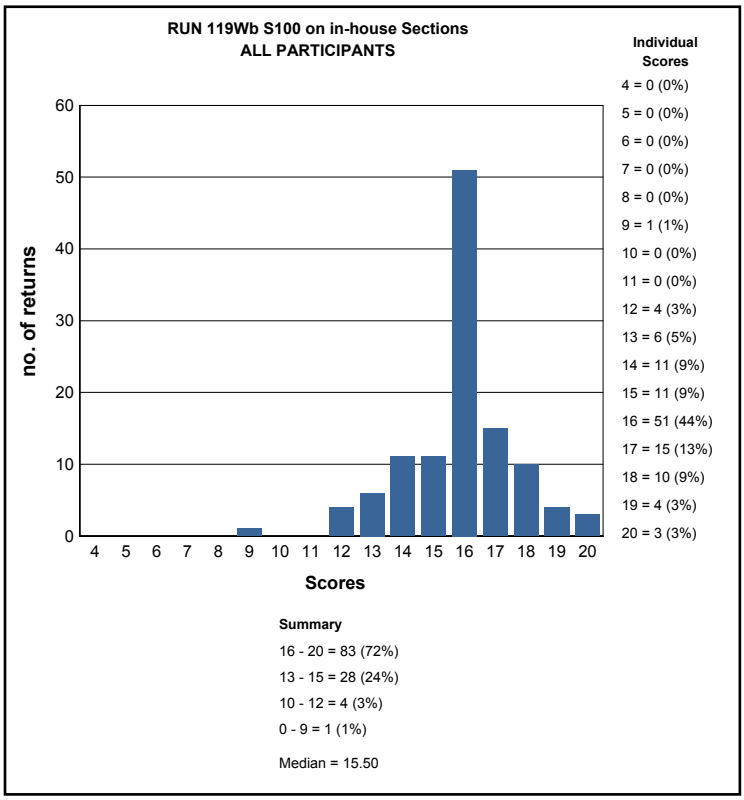


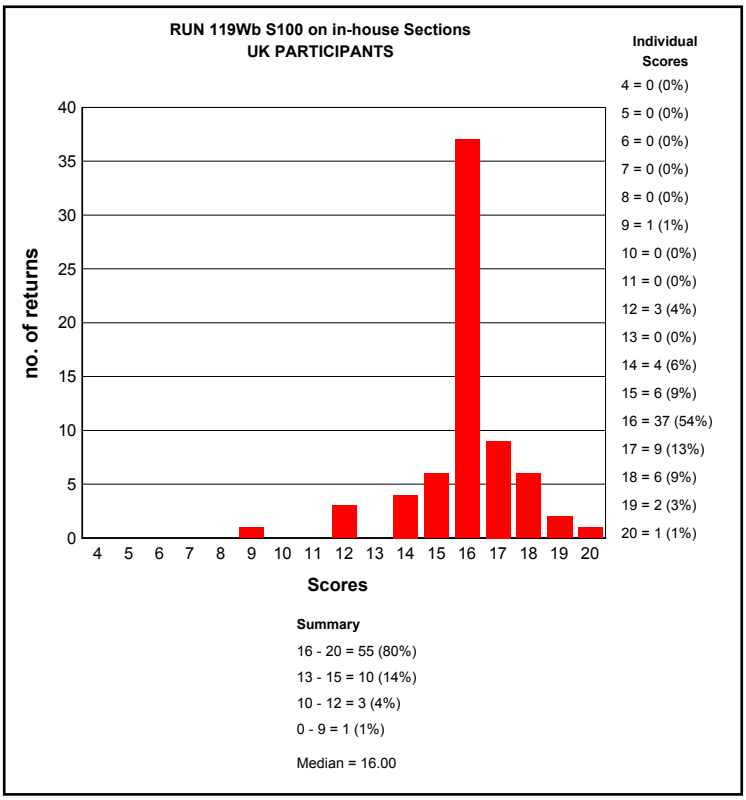


GRAPHICAL REPRESENTATION OF PASS RATES









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: 119

Primary Antibody : CD117

Antibody Details	N	%
Dako A4502 (rb poly)	84	90
Ventana 790-2939 (rb poly)	2	50
Cell Marque 117R/S-xx (YR145)	10	100
Leica/Novocastra NCL-L-CD117 (T595)	1	0
Ventana 790-2951 (9.7)	10	90
Epitomics AC-0029 (EP10)	1	100
Leica RTU (EP10) PA0007	9	100

Alimentary Tract Pathology Run: 119

Primary Antibody : S100

Antibody Details	N	%
Biogenex MU058-UC	2	100
Dako Z0311 (S100 poly)	57	93
Leica/Novocastra NCL--L-S100p (polyclonal)	14	79
Other	6	67
Ventana 760 2523 (S100 poly)	10	100
Ventana 790 2914	16	100
Leica/Novocastra PA0900 RTU (polyclonal)	1	100
Dako IR504 RTU FLEX (polyclonal)	2	100
Dako IS504 RTU Plus (polyclonal)	1	100
Dako Omnis GA504 RTU Flex (polyclonal)	5	100

Alimentary Tract Pathology Run: 119

Heat Mediated Retrieval	CD117 N	CD117 %	S100 N	S100 %
Dako Omnis	4	100	5	100
Dako PTLink	10	90	8	100
Leica ER1 10 mins	0	0	2	100
Leica ER1 20 mins	6	100	1	100
Leica ER1 30 mins	4	100	1	100
Leica ER2 10 mins	1	100	2	100
Leica ER2 20 mins	24	96	5	100
Leica ER2 30 mins	5	80	0	0
None	2	50	33	82
Pressure Cooker	0	0	1	100
Pressure Cooker in Microwave Oven	0	0	1	100
Ventana CC1 16mins	1	100	5	100
Ventana CC1 20mins	0	0	4	100
Ventana CC1 24mins	0	0	1	100
Ventana CC1 32mins	8	63	2	100
Ventana CC1 36mins	5	80	3	100
Ventana CC1 40mins	2	100	0	0
Ventana CC1 48mins	1	0	0	0
Ventana CC1 52mins	1	100	0	0
Ventana CC1 56mins	4	100	0	0
Ventana CC1 64mins	22	91	3	100
Ventana CC1 72mins	2	100	0	0
Ventana CC1 76mins	1	100	0	0
Ventana CC1 88mins	1	100	0	0
Ventana CC1 8mins	0	0	17	100
Ventana CC1 92mins	1	100	0	0
Ventana CC1 mild	4	100	9	89
Ventana CC1 standard	7	100	1	100
Ventana CC2 mild	1	100	0	0

Alimentary Tract Pathology Run: 119

Enzyme Mediated Retrieval	CD117 N	CD117 %	S100 N	S100 %
AS PER KIT	1	100	6	67
BD Trypsin Difco (215240)	0	0	1	100
NOT APPLICABLE	83	92	43	98
Other	0	0	2	50
VBS Bond Enzyme 1	0	0	14	79
VBS Bond Enzyme 2	0	0	1	0
Ventana Protease	0	0	1	100
Ventana Protease 1 (760-2018)	0	0	13	92

Alimentary Tract Pathology Run: 119	CD117		S100	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	7	100	8	100
Dako EnVision FLEX (K8000/10)	2	100	3	100
Dako EnVision FLEX+ (K8002/12)	7	100	6	100
Dako Envision+ HRP rabbit K4008/9/10/11	1	0	0	0
Leica Bond Polymer Refine (DS9800)	38	95	34	79
None	0	0	1	100
Other	2	100	4	100
Ventana iView system (760-091)	1	100	1	0
Ventana OptiView (760-700) + Amp. (7/860-099)	1	100	1	100
Ventana OptiView Kit (760-700)	28	82	23	100
Ventana UltraView Kit (760-500)	29	90	30	97

Alimentary Tract Pathology Run: 119	CD117		S100	
Automation	**N**	**%**	**N**	**%**
Dako Autostainer Link 48	8	88	5	100
Dako Autostainer plus	0	0	1	100
Dako Autostainer Plus Link	3	100	1	100
Dako Omnis	4	100	4	100
LabVision Autostainer	0	0	1	100
Leica Bond Max	11	91	9	78
Leica Bond-III	29	97	27	81
Ventana Benchmark GX	1	100	1	100
Ventana Benchmark ULTRA	45	87	40	100
Ventana Benchmark XT	15	87	15	87

Alimentary Tract Pathology Run: 119	CD117		S100	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	14	100	22	86
BioGenex Liquid DAB (HK153-5K)	0	0	1	100
DAKO DAB+	2	100	1	100
Dako EnVision Plus kits	1	100	0	0
Dako FLEX DAB	10	90	9	100
Leica Bond Polymer Refine kit (DS9800)	37	95	31	87
Other	4	75	4	100
Ventana DAB	17	76	12	100
Ventana Enhanced Alk. Phos. Red Detection Kit	0	0	2	100
Ventana iview	1	100	1	0
Ventana Ultraview DAB	31	90	33	94

BEST METHODS - Gold Standard Antibody

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

CD117 - Method 1

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

Primary Antibody: Dako A4502 (rb poly) , 95 Mins, RT ºC Dilution 1: 200
Automation: Ventana Benchmark ULTRA
Method: Ventana Optiview
Main Buffer: Ventana reaction buffer (950-300), PH: 7
HMAR: Ventana CC1 standard, Buffer: Ventana Reaction Buffer
EAR: NOT APPLICABLE
Chromogen: Ventana DAB, RT ºC., Time 1: 8 Mins
Detection: Ventana OptiView Kit (760-700) , 8 Mins, RT ºC Prediluted

CD117 - Method 2

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

Primary Antibody: Leica RTU (EP10) PA0007 , 15 Mins Prediluted
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 20 mins
EAR:
Chromogen: Leica Bond Polymer Refine kit (DS9800)
Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins

CD117 - Method 3

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

Primary Antibody:	Dako A4502 (rb poly) , 30 Mins, 20 ºC Prediluted
Automation:	Dako Autostainer Plus Link
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: High pH target retrieval solution
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, Time 1: 10 Mins, Time 2: 10 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 30 Mins, 20 ºC Prediluted

CD117 - Method 4

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

Primary Antibody:	Cell Marque 117R/S-xx (YR145) , 60 Mins, 37 ºC Dilution 1: 100
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.6
HMAR:	Ventana CC1 mild, PH: 8
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB, 27 ºC., 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

S100 - Method 1

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

Primary Antibody:	Leica/Novocastra NCL--L-S100p (polyclonal) , 15 Mins, ambient ºC Dilution 1: 300
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	None
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

S100 - Method 2

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

Primary Antibody:	Dako Omnis GA504 RTU Flex (polyclonal) , 15.5 Mins, 31 ºC
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 31 ºC.
Detection:	Dako EnVision FLEX+ (K8002/12)

S100 - Method 3

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

Primary Antibody:	Ventana 790 2914 , 4 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 8mins, Buffer: CC1 Ultra
EAR:	NOT APPLICABLE
Chromogen:	Other, 37 ºC., Time 1: 8 Mins, Time 2: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 37 ºC Prediluted

S100 - Method 4

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

Primary Antibody:	Dako Z0311 (S100 poly) , 30 Mins, RT ºC Dilution 1: 2000
Automation:	LabVision Autostainer
Method:	Dako FLEX kit
Main Buffer:	AS PER KIT
HMAR:	Pressure Cooker in Microwave Oven
EAR:	
Chromogen:	AS PER KIT, RT ºC., Time 1: 7 Mins, Time 2: 7 Mins
Detection:	Dako EnVision FLEX (K8000/10) , 30 Mins, RT ºC Prediluted

The Alimentary Tract Module: Lynch Syndrome/HNPCC Run 119

Keith Miller and Jamie Hughes

	Gold Standard	Second Antibody
Antigens Assessed:	MSH2	MSH6
Tissue Sections circulated:	Positive and negative colonic tumours plus normal appendix	
Number of Registered Participants:	96	
Number of Participants This Run:	89 (93%) (MSH2 & MSH6)	

General Introduction

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

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

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

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

Mismatch Repair Markers

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

Clinical Interpretation & Reporting Guidelines

We recommend reporting MMR IHC findings in tumours as either:

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

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

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

Clinical Reporting

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

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

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

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

Further Discussion on MMR proteins

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

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

Assessment Procedure:

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

Features of Optimal Immunostaining: (Figs 1A, 2A, 3A, 4, 5 7A, 8A, 9A, 10, 11, 12)
Appendix: (Figs 1A, 4, 7A, 10)
Strong nuclear staining of the epithelium at the base and lower third-half of the crypts with fading of nuclear staining intensity in the middle and upper thirds of the crypts, to negative or weak staining of epithelial nuclei at the luminal surface.
Tumour without loss of MMR protein: (Figs 2A, 8A, 11A)

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

Tumour with loss of MMR protein: (Figs 3A, 5, 9A, 12)

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

Features of Suboptimal Immunostaining: (Figs 1B, 2B, 3B, 6, 7B, 8B, 9B)
Appendix: (Figs 1B, 7B)

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

Tumour without loss of MMR protein: (Figs 2B, 6, 8B)

- 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 3B, 9B)

- 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:

MSH2
89 laboratories submitted slides for the MSH2 assessment. The results show an increase of 3% in acceptable results from Run 117, as shown in the table below:

MSH2 Pass Rates : NEQAS section		
	Run 117	Run 119
Acceptable	76%(N=63)	79%(N=71)
Borderline	16%(N=13)	7%(N=36
Unacceptable	7%(N=6)	13%(N=12)

The predominant reasons for a borderline or failed result in run 119 was due to either weak staining or background staining being observed.

The in-house MLH2 had a better result with 85% (N=75) achieving an acceptable result, 11% (N=10) receiving a borderline result and on 4% (N=4) receiving an unacceptable result. Predominant reasons again due to very weak demonstration of antigen and/or excessive background.

The most popular antibody used in this run was Ventana 760-4265 (G219-1129) used by 40 participants with a pass rate of 80%. The second most popular antibody was Dako M3639 (FE11) used by 14 participants with a pass rate of 100%.

The most common automated platform for MSH2 was the Ventana Benchmark Ultra used by 36 participants with a pass rate of 78%. The second most common was the Leica Bond III used by 17 participants with a 88% pass rate.

MSH6
89 laboratories submitted slides for the MSH2 assessment. The results show a decrease of 26% in acceptable results from Run 117, as shown in the table below:

MSH6 Pass Rates : NEQAS section		
	Run 117	Run 119
Acceptable	84%(N=71)	58%(N=52)
Borderline	12%(N=10)	20%(N=18)
Unacceptable	5%(N=4)	21%(N=19)

The predominant reasons for a borderline or failed result in run 119 was due to either weak staining or excessive background staining being observed, with some exhibiting cytoplasmic staining. For 30% of the unacceptable and borderline results, very weak demonstration of the antigen was the most common comment used by the assessors.

The in-house MLH6 had an improved result with 86% (N=77) achieving an acceptable result, 11% (N=10) receiving a borderline result and on 2% (N=2) receiving an unacceptable result. Predominant reasons for loss of marks were again due to very weak demonstration of antigen and/or excessive background.

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The most popular antibody used in this run was Ventana CONFIRM 790-4455 (44) used by 33 participants with a pass rate of 24%. The second most popular antibody was Dako M3646 (EP49) used by 21 participants with a pass rate of 100%.

The most common automated platform for MSH2 was the Ventana Benchmark Ultra used by 40 participants with a pass rate of 38%. The second most common was the Leica Bond III used by 17 participants with a 88% pass rate.

In-House Control Tissue Recommendations

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

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Selected Images showing Optimal and Sub-optimal immunostaining

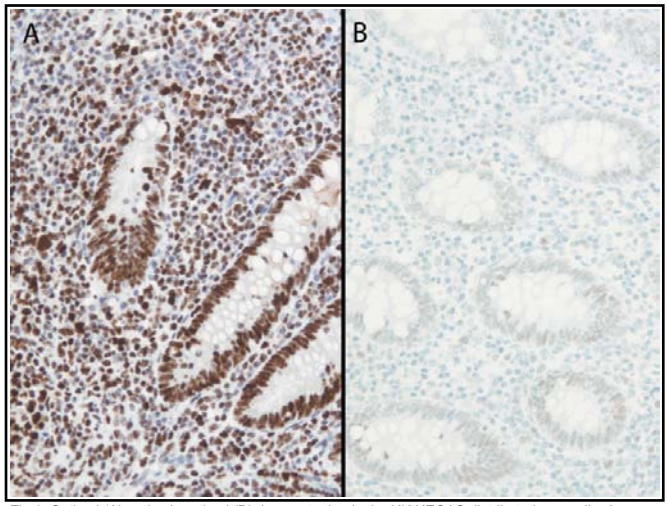


Fig 1: Optimal (A) and sub-optimal (B) demonstration in the UK NEQAS distributed appendix. A - shows the expected intense nuclear staining in the basal and lower half of the intestinal crypts. B - shows very weak to no staining. A) Dako (FE11) RTU; Ventana Ultra; CC2 80mins; OptiView + Amp. B) Ventana (G219-1129); Ventana XT; CC1 32mins; UltraView Kit.

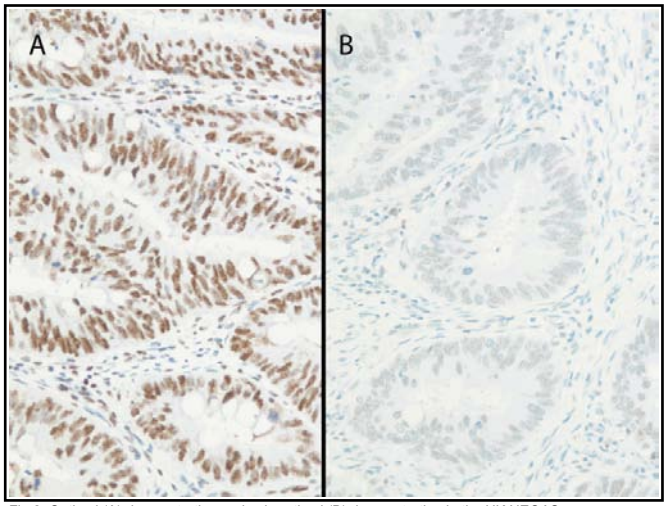


Fig 2: Optimal (A) demonstration and sub-optimal (B) demonstration in the UK NEQAS distributed positive tumour. A - shows the expected intense nuclear staining in the tumour cells. B - shows very weak to no staining. A) Dako (FE11) RTU; Leica Bond Max; ER2 30 mins; Leica Bond Polymer Refine. B) Ventana (G219-1129); Ventana XT; CC1 32mins; UltraView Kit.

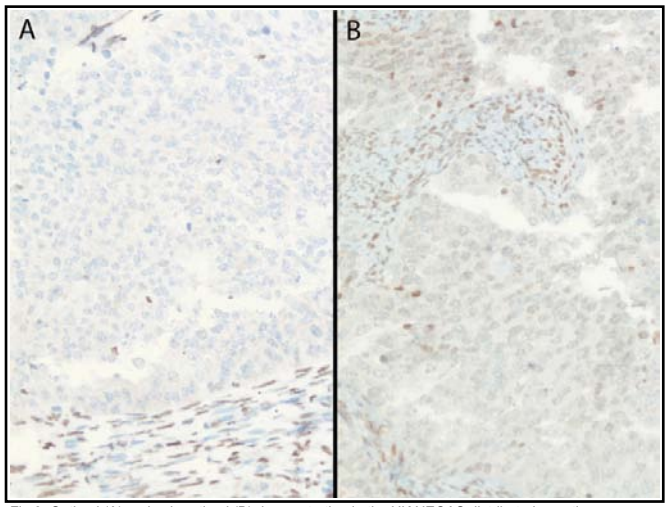


Fig 3: Optimal (A) and sub-optimal (B) demonstration in the UK NEQAS distributed negative tumour. A - shows absence of staining in the neoplastic cells with positive stromal cells. B - shows positive stromal cells and false positive nuclear staining of the negative tumour. A) As per fig. 2 A. B) Calbiochem (FE11) 1:20 60mins at 36°C; Ventana Ultra; CC1 92mins; UltraView

Fig 4: Shows optimal demonstration of MSH2 in the participant's in-house tissue, showing intense nuclear staining in the basal and lower half of the intestinal crypts, which fades towards the luminal surface. Ventana (G219-1129); Ventana Ultra; CC1 40mins; OptiView Kit.

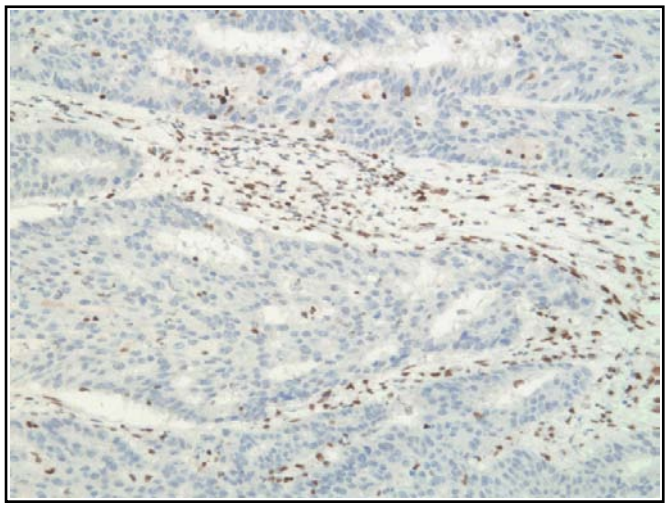


Fig 5: Shows optimal demonstration of MSH2 negative tumour on a participant's in-house tissue, showing absent nuclear staining in the neoplastic cells and intense staining in the stromal cells. Dako (FE11); Leica Bond Max; ER2 30 mins; Leica Bond Polymer Refine.

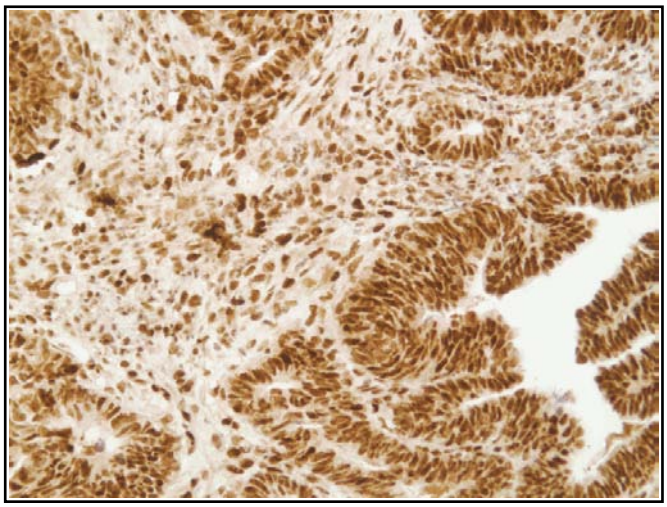


Fig 6: Shows sub-optimal demonstration of MSH2 on a participant's in-house tissue, showing excessive background staining. CellMarque (G219-1129) 1:300 30mins at RT; Dako Autostainer; Lab vision PT Module; Immunologic.

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

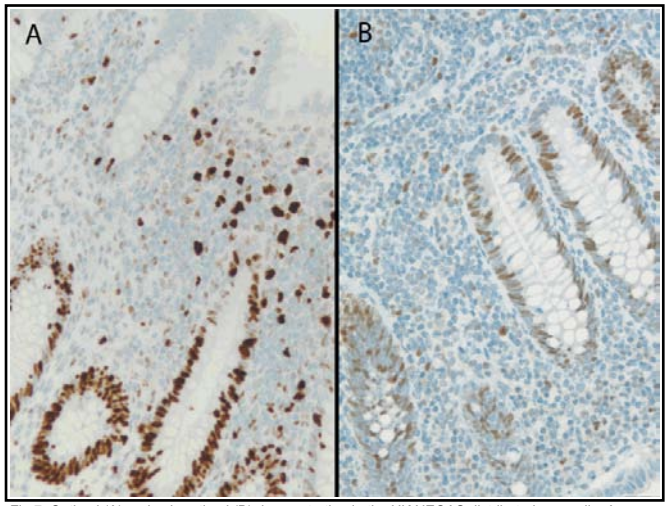


Fig 7: Optimal (A) and sub-optimal (B) demonstration in the UK NEQAS distributed appendix. A - shows intense nuclear staining in the basal and lower half of the intestinal crypts, which fades towards the luminal surface.B - shows weak and patchy staining. A) Ventana CONFIRM (44) 44mins 37°C; Ventana Ultra; CC1 92mins; As per kit. B) As per A (but incubated at 36°C)

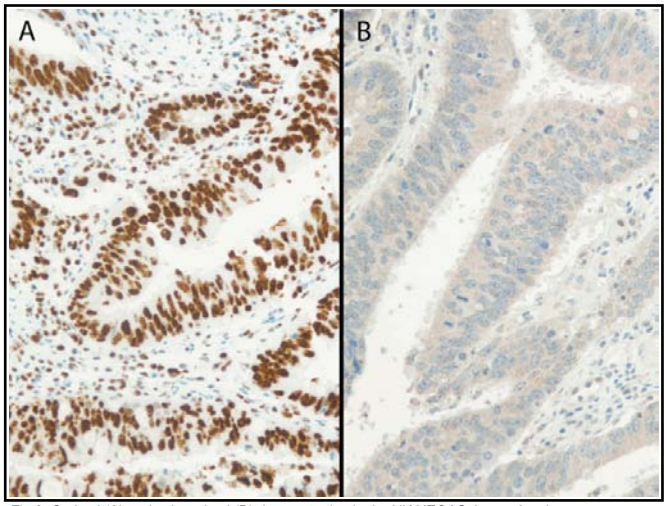


Fig 8: Optimal (A) and sub-optimal (B) demonstration in the UK NEQAS tissue. A - shows intense nuclear staining in the tumour cells. B - shows very weak to no nuclear staining and cytoplasmic staining. A) Abcam (EPR3945) 1:550 32mins 37°C; Ventana Ultra; CC1 64mins; OptiView Kit. B) BD BioSci (44) 1:100 15 mins 20°C; Leica Bond Max; ER2 30 mins; Refine kit.

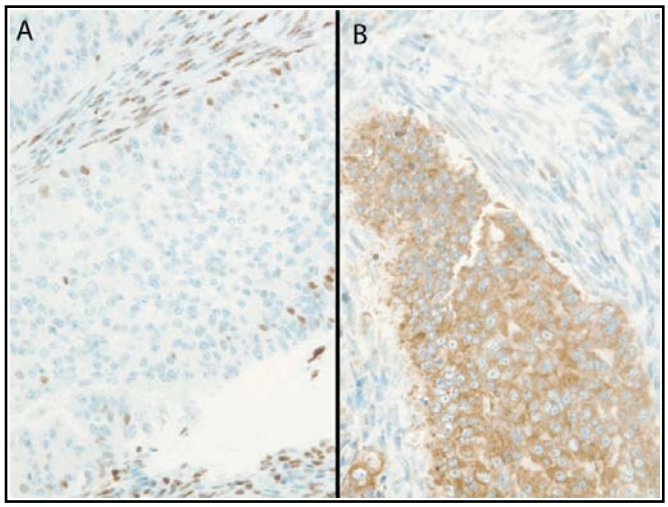


Fig 9: Optimal (A) and subotimal (B) demonstration in the UK NEQAS distributed negative tumour. A - shows the expected absence of staining in the neoplastic cells with positive stromal cells as the internal control. B - shows positive stromal cells and cytoplasmic staining of the negative tumour. A) Protocol as per fig. 8 A. B) Protocol as per fig. 8 B.

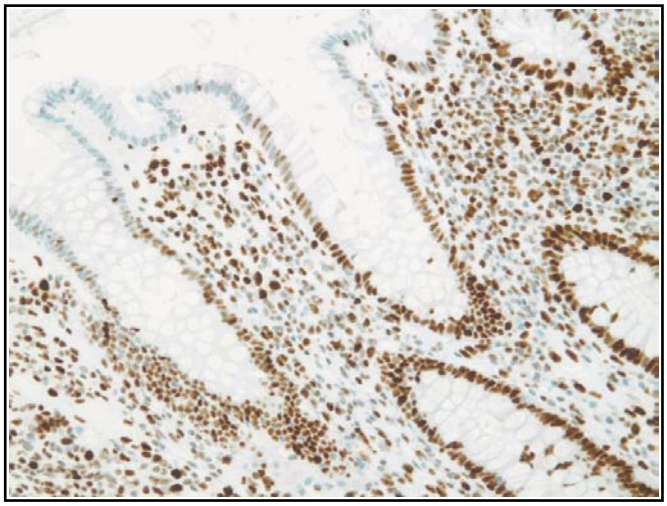


Fig 10: Shows optimal demonstration of MSH6 in the participant's in-house tissue, showing intense nuclear staining in the basal and lower half of the intestinal crypts, which fades towards the luminal surface. Protocol as per fig. 8 A.

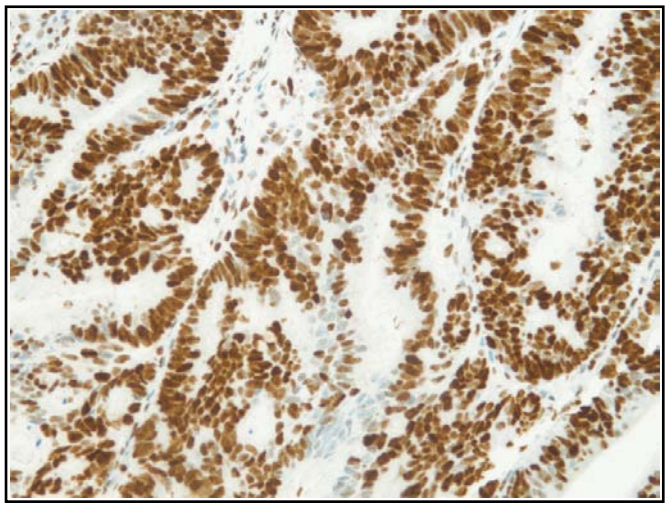


Fig 11: Shows optimal demonstration of MSH6 on a participant's in-house's tissue, showing intense nuclear staining in the neoplastic cells and positive stromal cells. Protocol as per fig. 8 A.

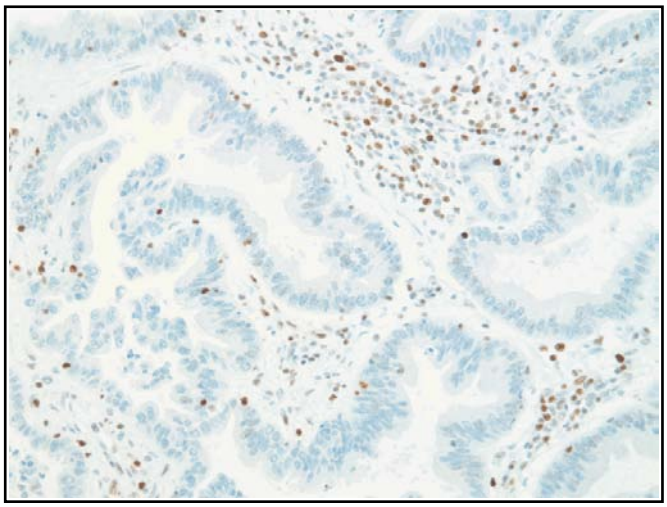
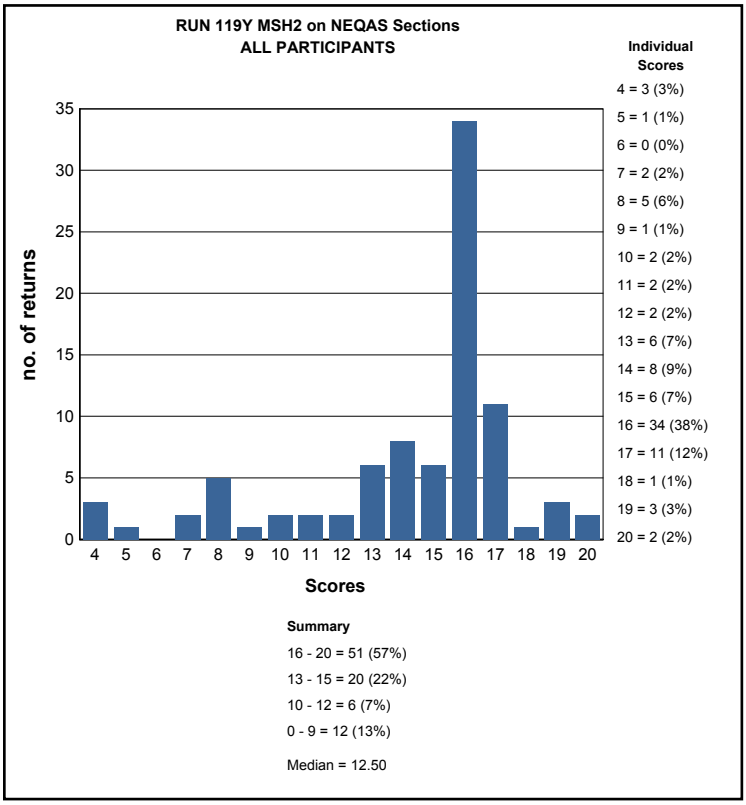
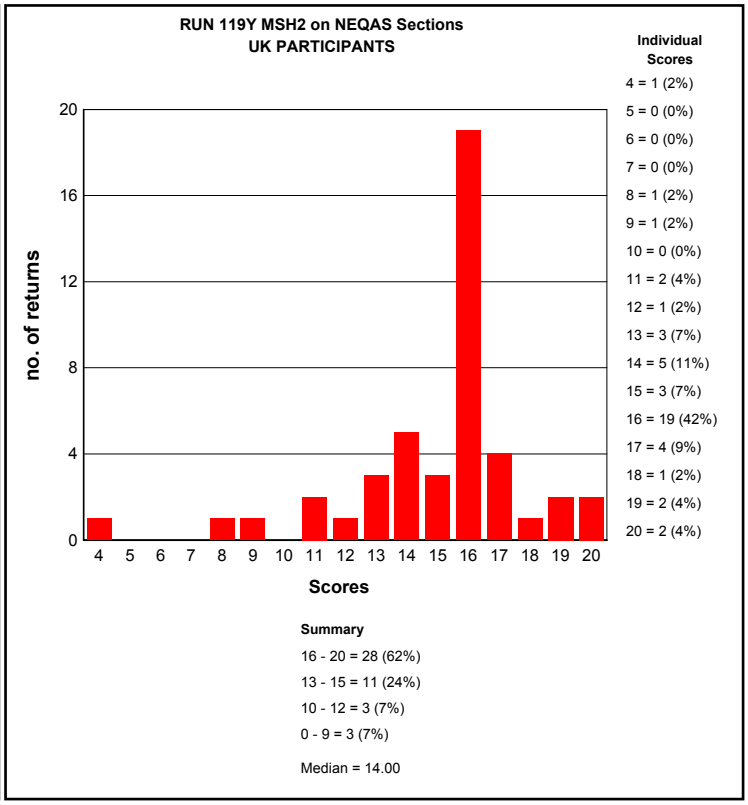
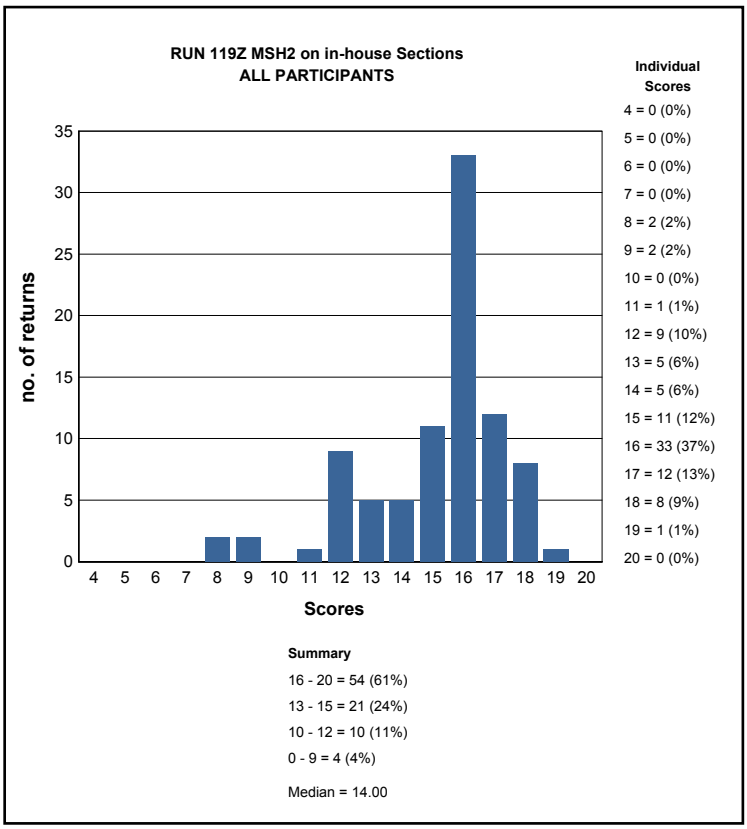


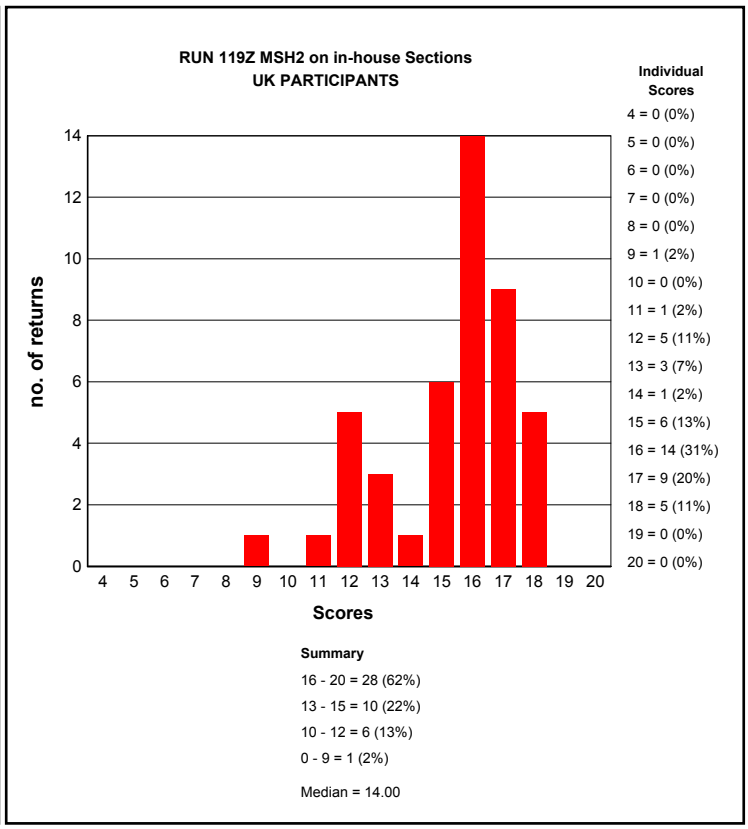
Fig 12: Shows optimal demonstration of MSH6 on a participant's in-house tissue, showing absent nuclear staining in the neoplastic cells and intense staining in the stromal cells. Protocol as per fig. 8 A.

GRAPHICAL REPRESENTATION OF PASS RATES

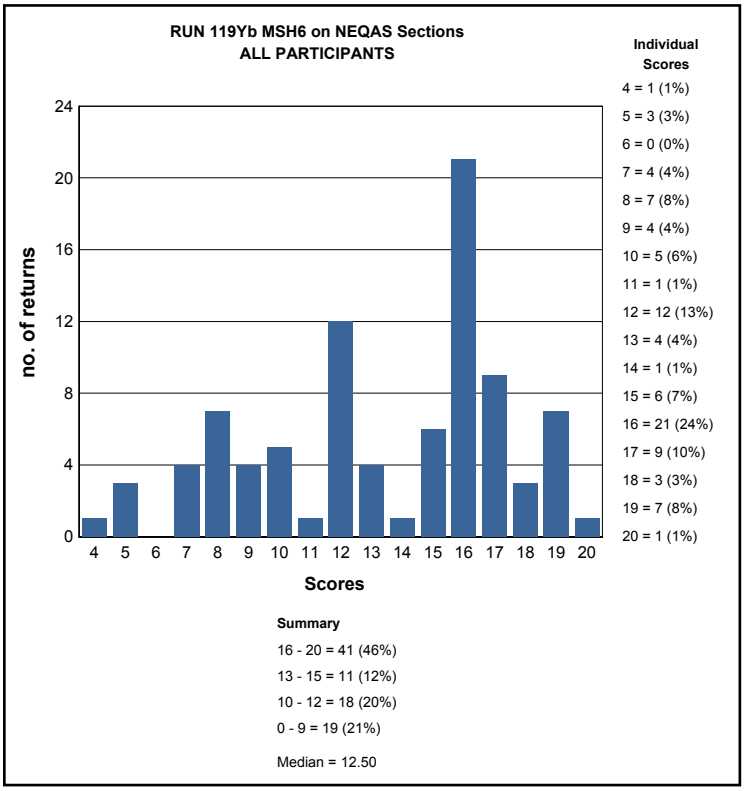


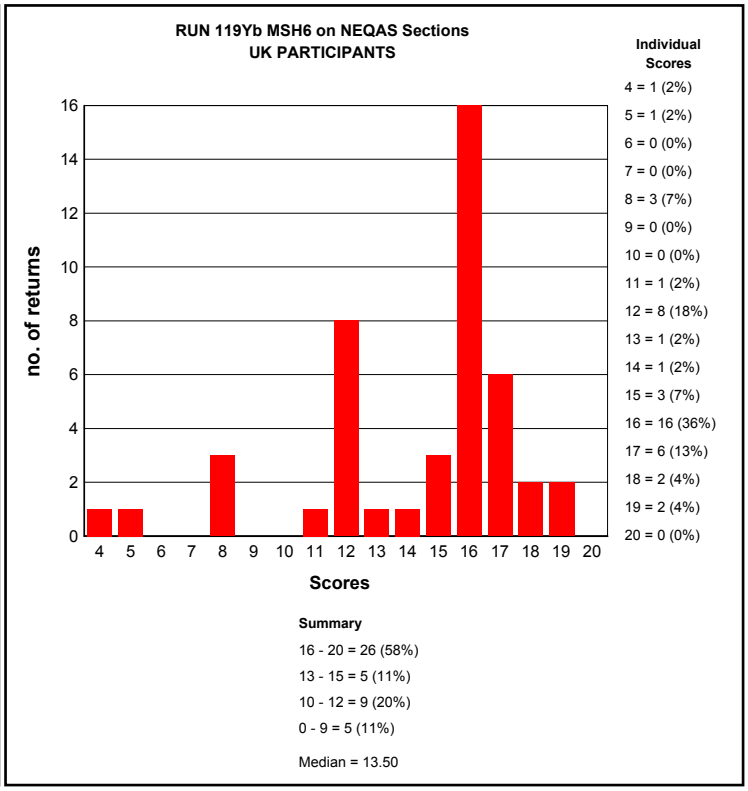


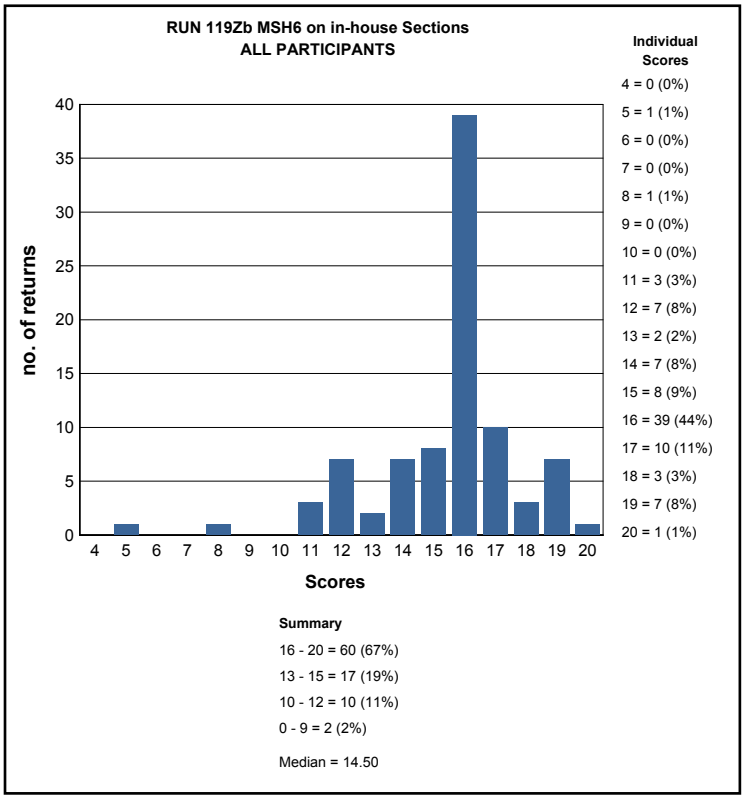


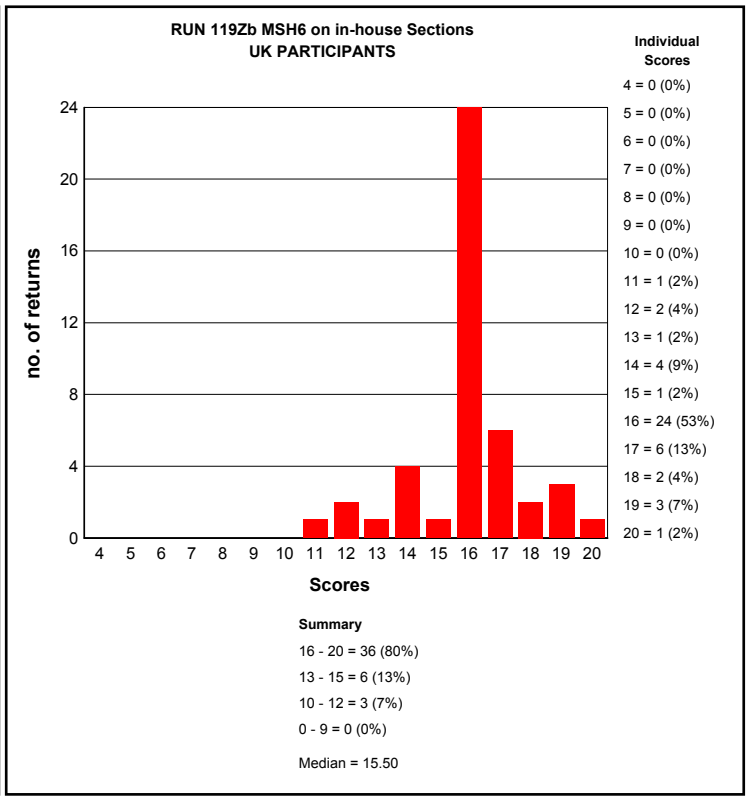


GRAPHICAL REPRESENTATION OF PASS RATES









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: 119

Primary Antibody : MSH2

Antibody Details	N	%
Other	3	0
BD Bio/Pharmingen 556349 (G219-1129)	3	100
Leica/Novocastra NCL-MSH2 (25D12)	7	71
BD Bio/Pharmingen 610360 (27)	1	0
Invitrogen 33-7900 (FE11)	1	100
Ventana 760-4265 (G219-1129)	40	80
Cell Marque CMAx/Cx (G219-1129)	5	60
Calbiochem NA27/T (FE11)	1	0
Dako FLEX RTU IR085 (FE11)	12	92
Dako M3639 (FE11)	14	100
Epitomics AC-0211 RED2	1	100

Primary Antibody : MSH6

Antibody Details	N	%
Other	2	50
Abcam AB92471 (EPR3945)	3	100
Leica/Novocastra NCL-L-MSH6 (PU29)	3	0
Ventana CONFIRM 790-4455 (44)	33	24
BD T. Labs/BioSci/Pharmingen 610919 (44/MSH6)	1	0
BD T. Labs/BioSci/Pharmingen 610918 (44)	4	0
Cell Marque 287R-24/25/26 (SP93)	4	50
Cell Marque 287R-27/28 RTU (SP93)	1	100
Epitomics AC-0047 (EP49)	2	100
Dako FLEX RTU IR086 (EP49)	14	93
Dako M3646 (EP49)	21	100

HNPCC Run: 119

Heat Mediated Retrieval	MSH2 N	MSH2 %	MSH6 N	MSH6 %
Biocare Decloaking Chamber	1	100	1	100
Dako Omnis	3	100	3	100
Dako PTLink	8	100	8	88
Lab vision PT Module	1	0	1	0
Leica ER1 30 mins	1	100	2	100
Leica ER2 20 mins	8	88	4	100
Leica ER2 30 mins	9	67	13	69
Leica ER2 40 mins	6	83	4	100
Ventana CC1 24mins	2	0	0	0
Ventana CC1 32mins	12	67	5	20
Ventana CC1 36mins	0	0	1	0
Ventana CC1 40mins	5	80	3	33
Ventana CC1 44mins	0	0	1	100
Ventana CC1 48mins	2	100	3	67
Ventana CC1 56mins	3	100	5	60
Ventana CC1 64mins	17	88	12	50
Ventana CC1 72mins	0	0	2	50
Ventana CC1 80mins	1	100	3	67
Ventana CC1 88mins	1	100	3	33
Ventana CC1 92mins	3	33	8	13
Ventana CC1 extended	0	0	1	100
Ventana CC1 standard	3	100	3	33
Ventana CC2 48mins	1	0	1	0
Ventana CC2 80mins	1	100	0	0
Ventana CC2 92mins	0	0	1	0

HNPCC Run: 119

Enzyme Mediated Retrieval	MSH2 N	MSH2 %	MSH6 N	MSH6 %
AS PER KIT	2	100	3	67
NOT APPLICABLE	53	79	55	62
Ventana Protease 1 (760-2018)	0	0	1	0

HNPCC Run: 119	MSH2		MSH6	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	4	50	4	75
Dako EnVision FLEX (K8000/10)	1	100	3	100
Dako EnVision FLEX+ (K8002/12)	8	100	7	86
Leica Bond Polymer Refine (DS9800)	22	86	22	86
NOT APPLICABLE	0	0	1	0
Other	3	33	2	0
Ventana OptiView (760-700) + Amp. (7/860-099)	14	86	16	44
Ventana OptiView Kit (760-700)	30	77	26	42
Ventana UltraView Kit (760-500)	4	50	5	20

HNPCC Run: 119	MSH2		MSH6	
Automation	**N**	**%**	**N**	**%**
Dako Autostainer	1	0	1	0
Dako Autostainer Link 48	6	100	6	83
Dako Autostainer plus	1	100	1	100
Dako Autostainer Plus Link	1	100	1	100
Dako Omnis	4	100	3	100
LabVision Autostainer	1	100	0	0
Leica Bond Max	7	57	6	67
Leica Bond-III	17	88	17	88
None (Manual)	1	100	1	100
Ventana Benchmark GX	1	100	1	0
Ventana Benchmark ULTRA	36	78	40	38
Ventana Benchmark XT	13	69	11	55

HNPCC Run: 119	MSH2		MSH6	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	17	65	18	50
Dako EnVision Plus kits	1	100	1	0
Dako FLEX DAB	9	100	9	100
Dako REAL EnVision K5007 DAB	1	100	1	100
Leica Bond Polymer Refine kit (DS9800)	23	83	22	86
Other	5	60	5	40
Ventana DAB	27	89	25	40
Ventana Ultraview DAB	5	40	7	14

BEST METHODS - Gold Standard Antibody

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

MSH2 - Method 1

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

Primary Antibody: Ventana 760-4265 (G219-1129) , 8 Mins, 37 ºC Prediluted
Automation: Ventana Benchmark ULTRA
Method: Ventana Optiview
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 64mins
EAR: NOT APPLICABLE
Chromogen: AS PER KIT
Detection: Ventana OptiView (760-700) + Amp. (7/860-099)

MSH2 - Method 2

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

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

MSH2 - Method 3

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

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

MSH2 - Method 4

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

Primary Antibody:	Dako FLEX RTU IR085 (FE11) , 30 Mins
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+ (K8002/12) , 30 Mins

BEST METHODS - Secondary Antibody

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

MSH6 - Method 1

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

Primary Antibody:	Abcam AB92471 (EPR3945) , 32 Mins, 37 ºC Dilution 1: 1:550
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB, 37 ºC., Time 1: 12 Mins, Time 2: 12 Mins
Detection:	Ventana OptiView Kit (760-700) , 12 Mins, 37 ºC Prediluted

MSH6 - Method 2

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

Primary Antibody:	Abcam AB92471 (EPR3945) Dilution 1: 150
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 30 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

MSH6 - Method 3

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

Primary Antibody:	Dako FLEX RTU IR086 (EP49) , 20 Mins, 20 ºC Prediluted
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)

MSH6 - Method 4

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

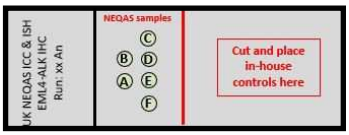
Primary Antibody:	Epitomics AC-0047 (EP49) , 32 Mins, 36 ºC Dilution 1: 50
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB, 36 ºC., Time 1: 8 Mins, Time 2: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 36 ºC Prediluted

The ALK for NSCLC Module Run 119

Suzanne Parry

Antibody Assessed:	ALK
Samples Circulated:	Cell lines and NSCLC tissue samples with varying ALK IHC expression & normal appendix (see table below)
Number 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.



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

Introduction

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

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

Assessment Criteria

NEQAS & In-house controls

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

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

Interpretation criteria incorporating staining intensity

The assessment 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.

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 staining - Lower level of staining than expected - Excessive background staining - Non-specific or inappropriate staining
Marks may also be deducted for reasons which will be shown on individual participant reports such as: morphology damage or poor quality/choice of in-house control tissue	

Features of Optimal Staining (Figures 1, 3, 5, 7A & 8)

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

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

Features of Sub-optimal Staining (Figures 2, 4, 6 & 7B)
- False negative or absence of ALK staining where tumour cells should be staining positive
- Lower expression level than expected
- Weaker staining than expected for the assay/method used
- Non-specific/excessive tyramide staining
- Absence of staining in the appendix ganglion and nerve cells

Results & Discussion

Distributed NEQAS Sample Results

Of the 63 currently participating laboratories, 61 laboratories (97%) returned both the NEQAS and their in-house samples for assessment. The results were very similar to the previous assessment, with 77% (N=47) of laboratories receiving an acceptable pass on the NEQAS samples, and a further 7% (N=4) achieved borderline passes, and 10 laboratories (16%) failed the assessment.

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

Examples of sub-optimal staining are shown in the subsequent images.

Figure 2 shows excessive teramide inappropriate staining in the ALK negative cell line. The Ventana D5F3 CDx assay uses an enhanced protocol with Optiview detection and an additional amplification step, and therefore often shows a granular staining pattern, but this level of additional teramide deposit is unacceptable. The image highlights how this level of inappropriate deposit makes the sample uninterpretable, as this could possibly be seen to be positive or may mask any true positive staining on clinical cases.

Figure 4 shows how the positive tumour sample C is staining weaker than expected for the Ventana D5F3 assay used. This was expected to be ALK IHC 3+ expression as seen on the Gold standard reference slides stained with the D5F3 assay. Although the example shown in Figure 4 is still ALK IHC positive, concerns would be if the staining was dropped on the lower expressing critical cases.

Figure 6 again shows lower staining than expected on the NEQAS ALK positive tumour sample D stained with the Ventana D5F3 CDx assay. The Gold standard reference slide stained this sample as ALK IHC 2+ with the D5F3, but as highlighted in the example in Figure 6, although still ALK IHC positive, the staining is much weaker than expected.

Figure 7B shows false positive staining in the NEQAS sample known to be ALK negative. In the clinical setting, this could potentially lead to the inappropriate treatment of a patient unlikely to respond to Crizotinib, Ceritinib or Alectinib therapy.

The most popular antibody of choice was the Ventana/Roche (D5F3), used by 48 participants and showed a 96% pass rate. The Cell Signalling Technology (D5F3) was used by 5 participants, but only 1 of these participants achieved an acceptable pass. Other antibodies included the Novocastra? Leica antibody was used by 5 laboratories, but none of these laboratories received acceptable passes on the NEQAS material.

In-house Control Results

Results on the participants in-house tissues were similar to that on the NEQAS samples, with 78% receiving an acceptable pass, 10% borderline and 11% failed the assessment. Some of the borderline passes were due to the laboratory not providing an ideal selection of controls, necessary to help gauge the sensitivity of their staining assay. (See section below on ALK IHC control recommendations). Failures on the in-house samples were due to no or uninterpretable staining due to excessive inappropriate teramide or background staining. An anaplastic large cell lymphoma tumour was used as a positive control by a single laboratory. This tumour type is not recommended as a control for ALK in the lung setting as it overexpresses the ALK protein significantly more than positive lung tumours.

ALK IHC Control Recommendations

The UK NEQAS ICC & ISH ALK IHC assessment panel recommend that the ideal control should consist of:
- NSCLC ALK IHC positive tumour: Gauges sensitivity
- NSCLC ALK IHC negative tumour: Gauges specificity

Laboratories that do not provide this composite control tissue are scored a maximum borderline mark of 12/20

- Appendix is also recommended alongside the lung tumour controls to gauge both the sensitivity & specificity, but should not be used 'alone' as a single control.
- Commercially available control material, such as cell lines or xenografts showing at least positive and negative ALK IHC expression may also be used in combination with an in-house tissue sample.
- It is also recommended that control material should be cut and placed alongside the clinical sample being tested.

References:

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Selected Images showing Optimal and Sub-optimal immunostaining

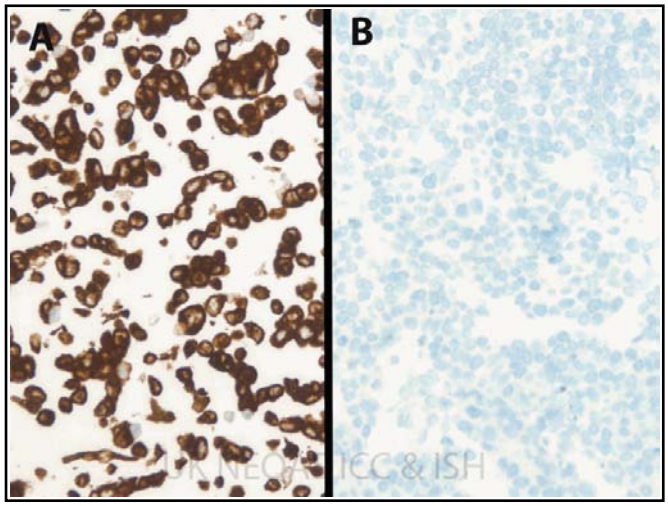


Fig 1. Good example showing the expected ALK expression in the UK NEQAS distributed cell lines. The positive cell line (sample B) shows strong, membranous and cytoplasmic staining, while there is no staining in the negative cell line (sample A). This sample remains clean and without background. Both stained with the Ventana Roche D5F3 ALK assay.

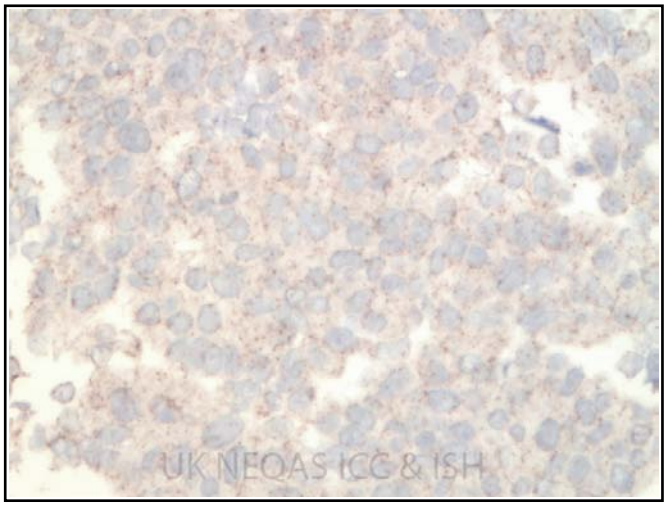


Fig 2. Sub-optimal ALK demonstration in the UK NEQAS distributed negative cell line (sample A) (compare to Fig 1B). The example shows excessive non-specific teramide deposit.

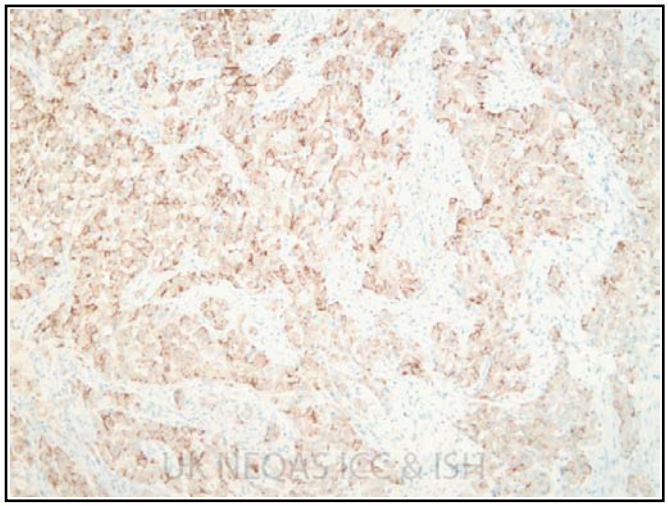


Fig 3. Expected level of staining in the UK NEQAS distributed positive tumour sample C. The example shows moderate to strong membranous and cytoplasmic staining of neoplastic cells. Stained using the Ventana D5F3 assay on the Benchmark with OptiView detection and amplification.

Fig 4. Sub-optimal staining of the UK NEQAS positive tumour sample C. Although the tumour is staining positive as expected, the reaction is much weaker than expected for the assay used. Stained with the Ventana D5F3 assay.

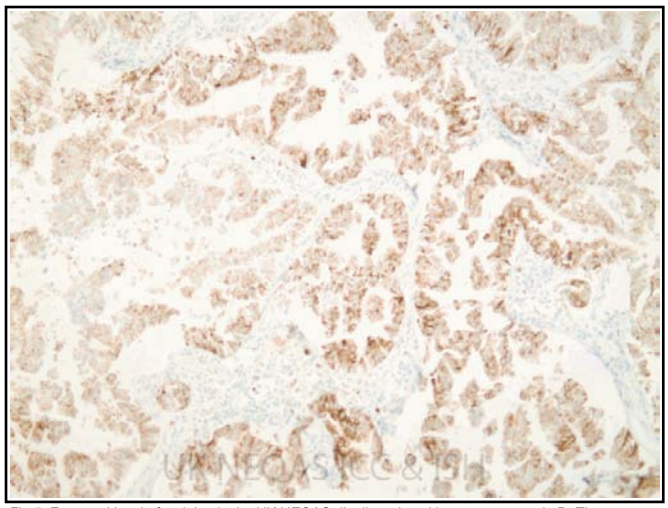


Fig 5. Expected level of staining in the UK NEQAS distributed positive tumour sample D. The section shows mostly high intensity, with some areas of moderate expression. Stained using the Ventana Roche D5F3 assay.

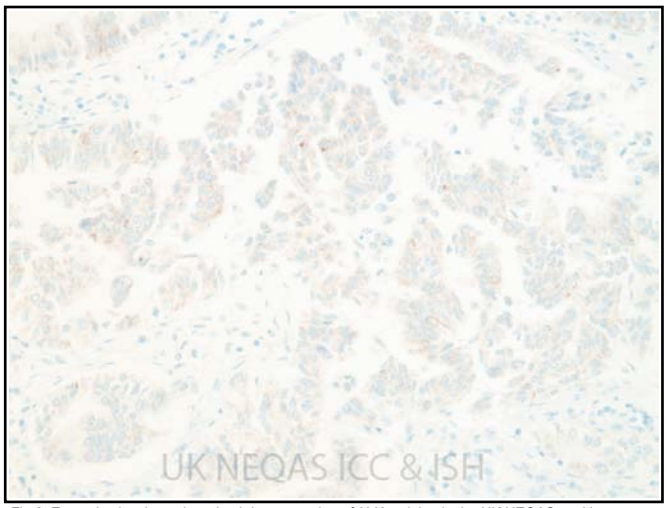


Fig 6. Example showing sub-optimal demonstration of ALK staining in the UK NEQAS positive tumour sample D. While the tumour is mostly staining, the expression is weak, with some areas barely staining at all. Stained with the Ventana D5F3 assay.

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

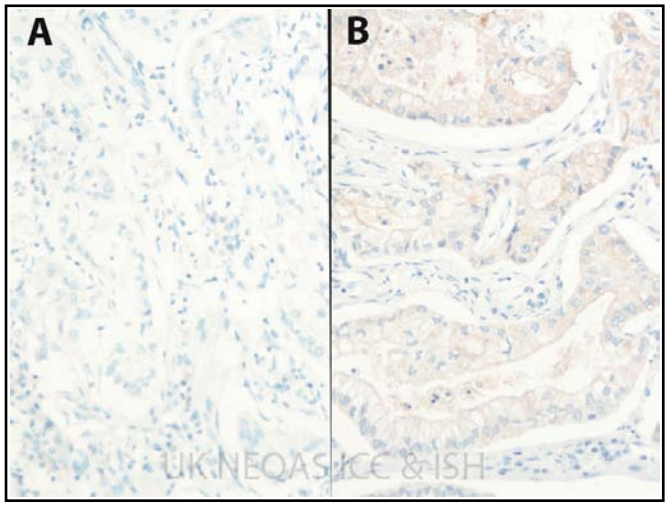


Fig 7. Two examples showing ALK IHC demonstration in the UK NEQAS negative tissue sample. As expected, sample A shows no staining. However, example B shows inappropriate staining of the tumour, and therefore appears to be weakly positive, therefore, clinically indicating a false-positive result.

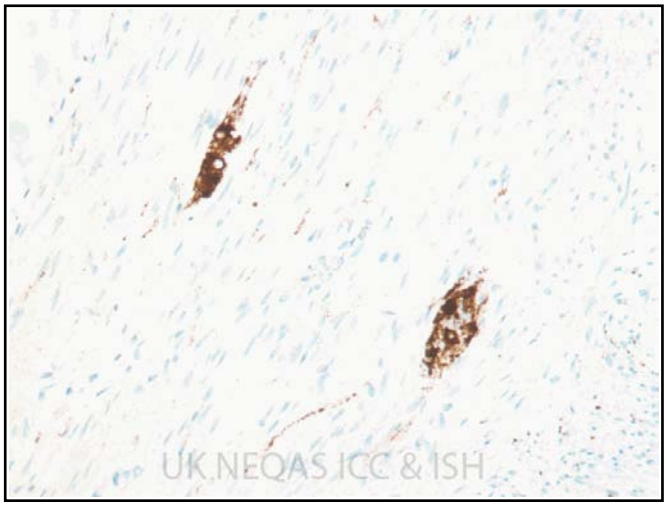
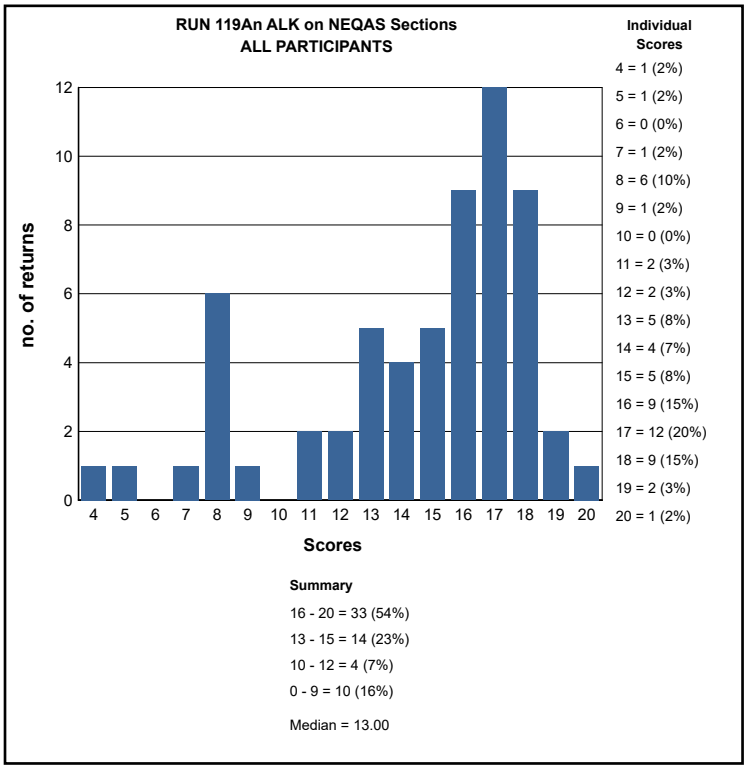
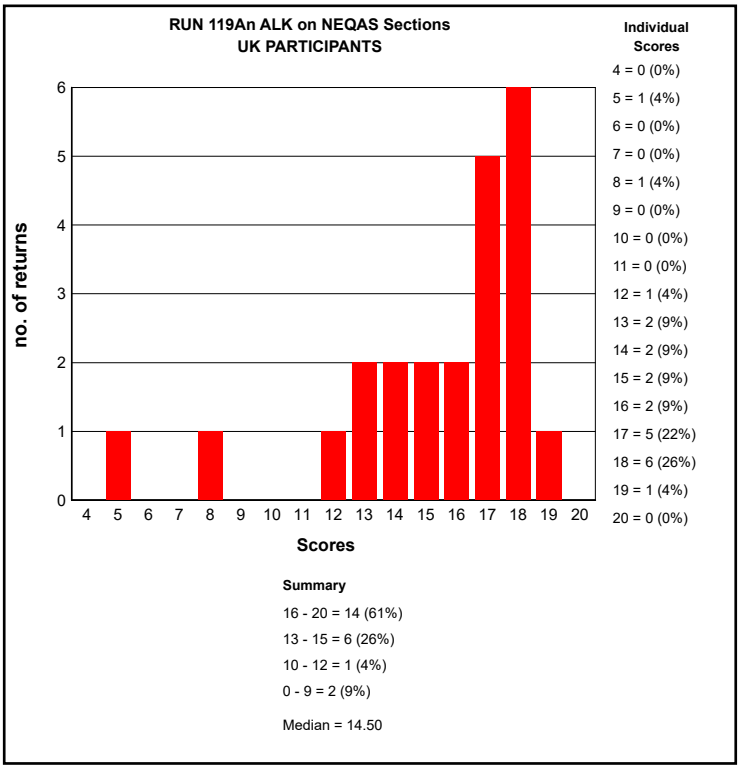
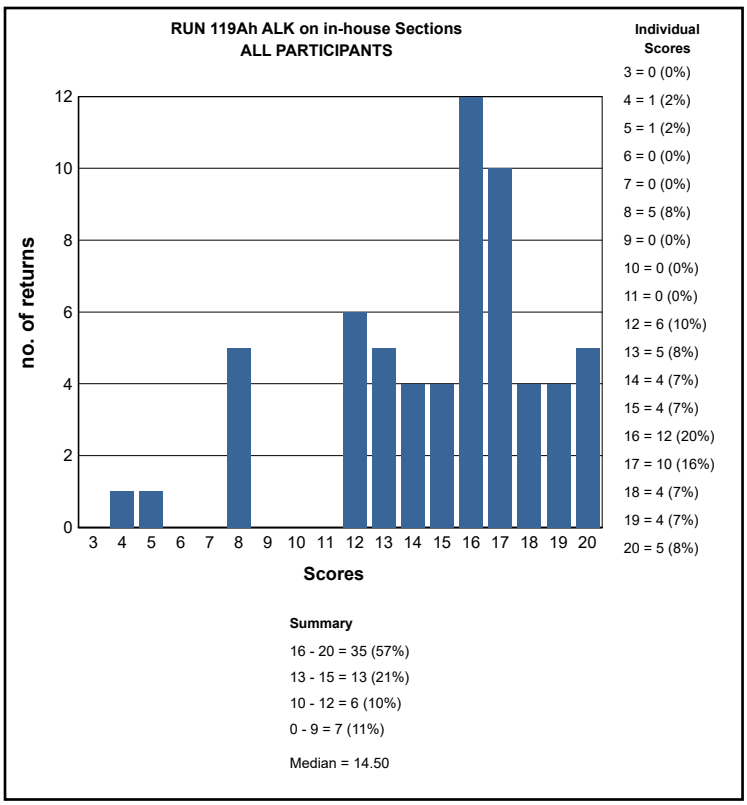


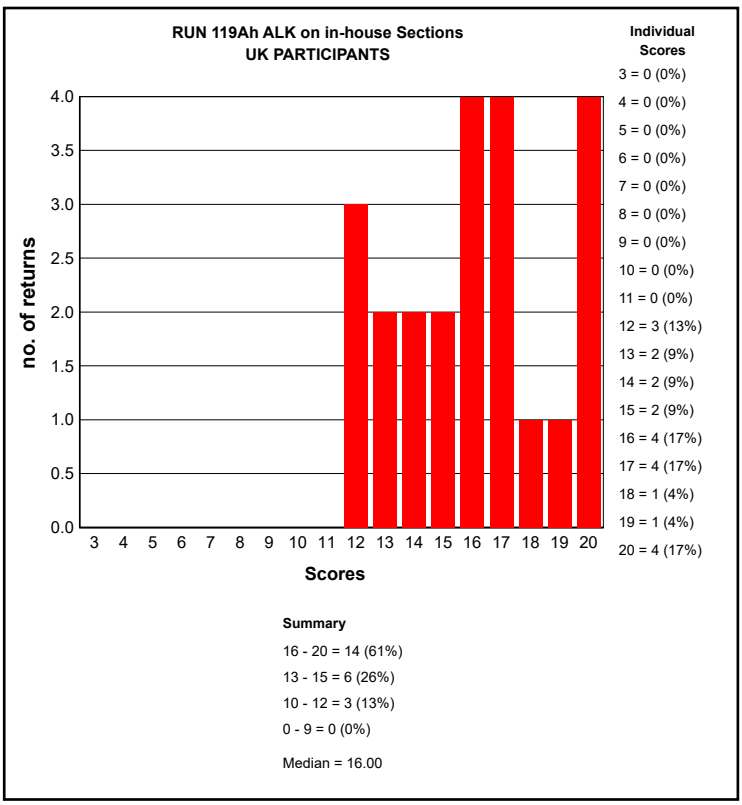
Fig 8. Example showing optimal ALK staining in the UK NEQAS appendix (sample F). The section shows strong positive staining of ganglion cells and axons. Appendix acts as a very good normal control to help gauge the sensitivity of the assay. Stained with the Ventana Roche D5F3 assay.

GRAPHICAL REPRESENTATION OF PASS RATES









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

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

ALK NSCLC Run: 119

Primary Antibody	N	%
Cell Sign. Tech. (D5F3)	5	20
Novocastra NCL-ALK (5A4)	4	0
Novocastra PA0306 (5A4)	1	0
Other	1	0
Ventana/Roche (D5F3)	48	96

ALK NSCLC Run: 119

Automation	N	%
Dako Autostainer Link 48	1	0
Dako Omnis	1	0
Leica Bond Max	1	0
Leica Bond-III	3	0
Ventana Benchmark GX	2	100
Ventana Benchmark ULTRA	30	83
Ventana Benchmark XT	21	95

ALK NSCLC Run: 119

Heat Mediated Retrieval	N	%
Dako Omnis	1	0
Dako PTLink	1	0
Leica ER2 20 mins	3	0
None	1	100
Other	3	67
Ventana CC1 36mins	1	100
Ventana CC1 64mins	1	100
Ventana CC1 88mins	2	100
Ventana CC1 92mins	38	97
Ventana CC1 extended	5	40
Ventana CC1 standard	3	33

ALK NSCLC Run: 119

Detection	N	%
AS PER KIT	1	0
Dako EnVision FLEX (K8000/10)	1	0
Dako EnVision FLEX+ (K8002/12)	1	0
Leica Bond Polymer Refine (DS9800)	3	0
Ventana OptiView (760-700) + Amp. (7/860-099)	36	92
Ventana OptiView Kit (760-700)	16	88
Ventana UltraView Kit (760-500)	1	0

ALK NSCLC Run: 119

Enzyme Retrieval	N	%
AS PER KIT	3	67
NOT APPLICABLE	31	77

ALK NSCLC Run: 119

Chromogen	N	%
AS PER KIT	18	78
DAKO DAB+	1	0
Dako EnVision Plus kits	1	0
Leica Bond Polymer Refine kit (DS9800)	4	0
Ventana DAB	31	94
Ventana Ultraview DAB	4	100

BEST METHODS

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

ALK - Method 1

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

Primary Antibody:	Ventana/Roche (D5F3)
Automation:	Ventana Benchmark ULTRA
Method:	
Main Buffer:	
HMAR:	Ventana CC1 92mins
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099)

ALK - Method 2

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

Primary Antibody:	Ventana/Roche (D5F3) Prediluted
Automation:	Ventana Benchmark XT
Method:	
Main Buffer:	
HMAR:	Ventana CC1 92mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700)

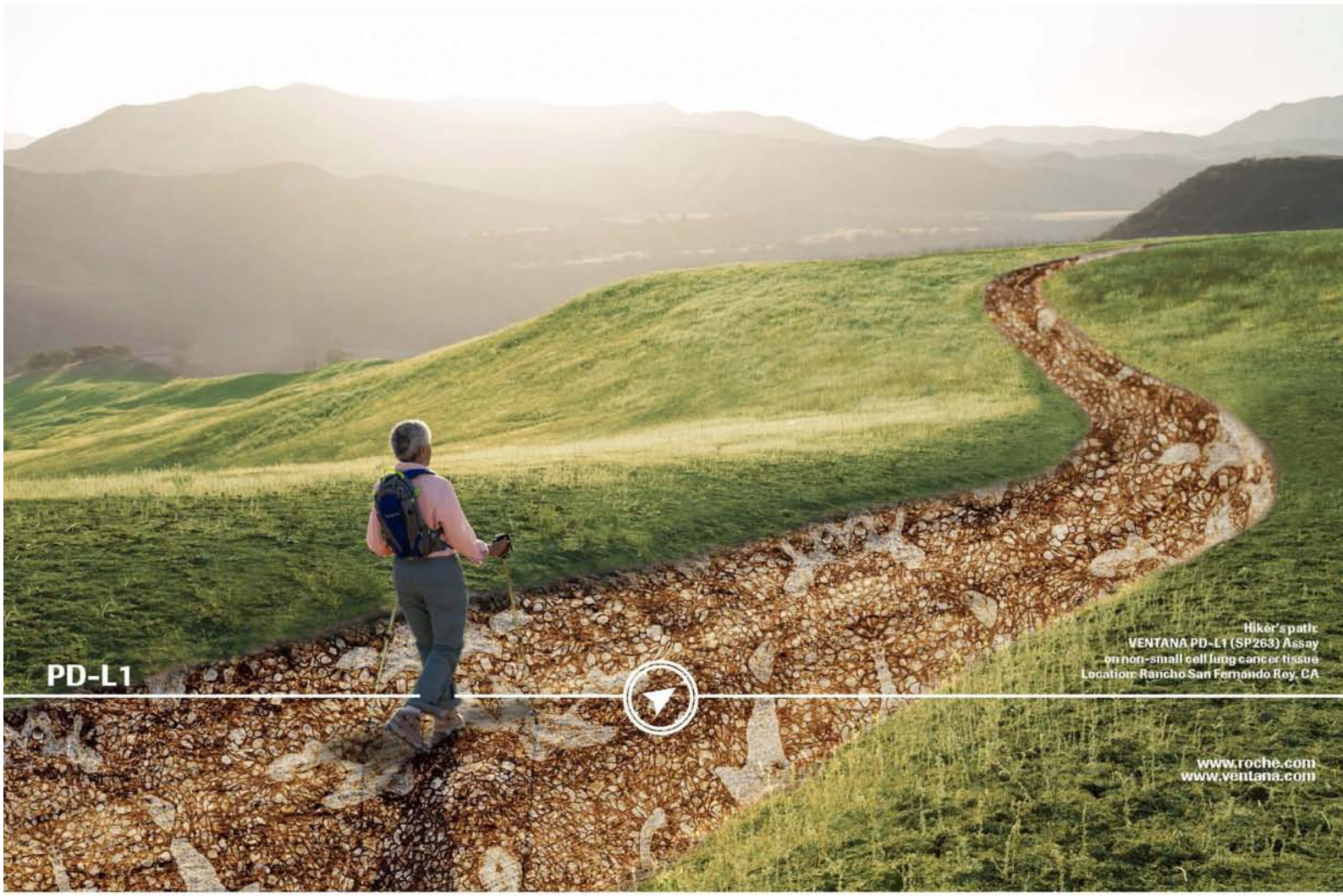
Guiding immunotherapy

VENTANA PD-L1 (SP263) Assay

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

Empowering pathologists to answer PD-L1 questions by

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



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Roche Diagnostics Limited. Charles Avenue, Burgess Hill, West Sussex, RH15 9RY, United Kingdom.

Registration number 571546. Date of Preparation: November 2016. Material No: 08176230001

The HER2 ISH Module - Interpretive Run 48

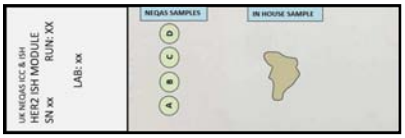
Suzanne Parry

Gene Assessed:	HER2
Sections Circulated:	Composite slide consisting of 4 breast carcinoma sections (see table below)
Number of Registered Participants:	153
Number of Participants Taking Part this Run	129 (84%)

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+**	Borderline/Non-amplified
C	**2+**	Non-amplified
D	**2+**	Non-amplified

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



Introduction

Breast cancer HER2 status should be determined in all newly diagnosed and recurrent 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 **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 recommendations regarding HER2 ISH testing including those by ASCO/CAP (USA) and the 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 prior to their introduction into the laboratory as a diagnostic test.

Assessment Procedure

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

Assessment Results

All laboratories employed a dual probe and a ratio scoring algorithm. 64% of participants achieved excellent or acceptable results. 26% received a borderline pass and 13 laboratories (10%) had an unacceptable interpretation result. Two of the unacceptable results were from UK laboratories. The most common brightfield method was the Ventana DDISH with 46 (36%) laboratories using this technique, and for the FISH method, the most popular assay was the Pathvysion Vysis Kit, used by 38 (29%) of laboratories. Acceptable pass rates with the Pathvysion Vysis Kit was slightly better than with the Ventana DDISH assay for this particular assessment run: 66% and 59% respectively. The most notable difference when comparing the interpretative results of run 48 to the previous run 47 is the drop in acceptable results, which has fallen from 74% in the previous run (47) to 64% in the current run (48).

HER2 ISH Method and Probe Enumeration

The subsequent table and graphs show the various ISH methods employed along with percentages of pass rates. 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.

Bibliography

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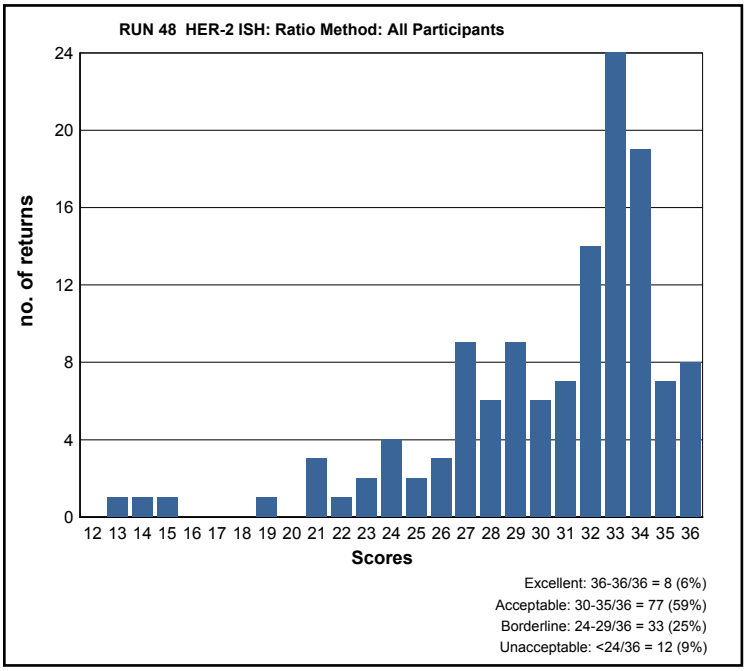
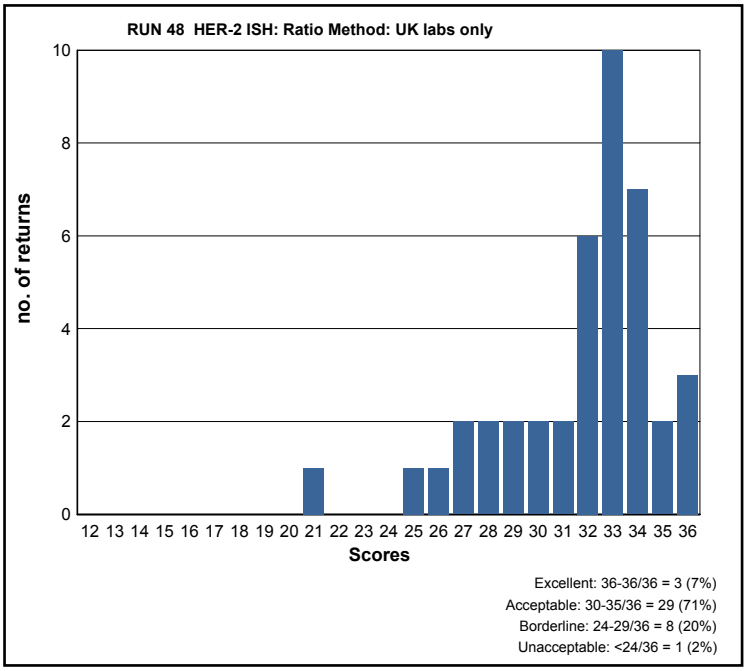
2. Bartlett, J., Starczynski, J., Atkey, N., Kay, E., O'Grady, A., Gandy, M., Ibrahim, M., Jasani, B., Ellis, I., Pinder, S. and Walker, R. (2011). HER2 testing in the UK: recommendations for breast and gastric in-situ hybridisation methods. *Journal of Clinical Pathology*, 64(8), pp.649-653.
3. Moore, 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

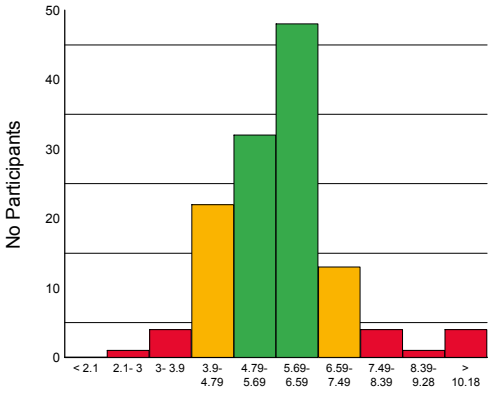
Ratio Method	N	% Pass (score ≥ 30/36)
Ratio: Dako IQFISH pharmDX	12	58%
Ratio: Dako Pharm Dx	1	0%
Ratio: In house FISH	3	67%
Ratio: Kreatech Probes	3	100%
Ratio: Leica HER2 FISH TA9217	8	88%
Ratio: Other - CISH	1	100%
Ratio: Other - FISH	4	75%
Ratio: Pathvysion Vysis Kit	38	68%
Ratio: Ventana BDISH 800-098/505	4	50%
Ratio: Ventana DDISH (780/800-4422)	47	60%
Ratio: Ventana Inform Silver ISH	2	100%
Ratio: Zytovision ZytoDot 2C	1	0%
Ratio: Zytovision ZytoLight	5	80%

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

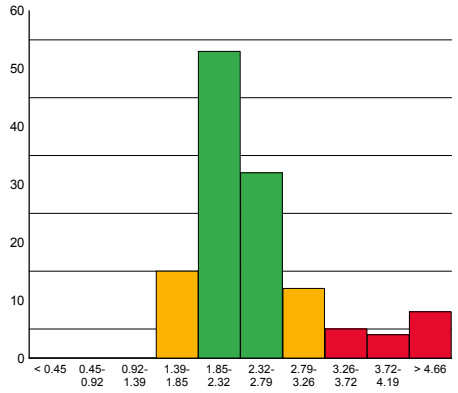
FREQUENCY HISTOGRAMS

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

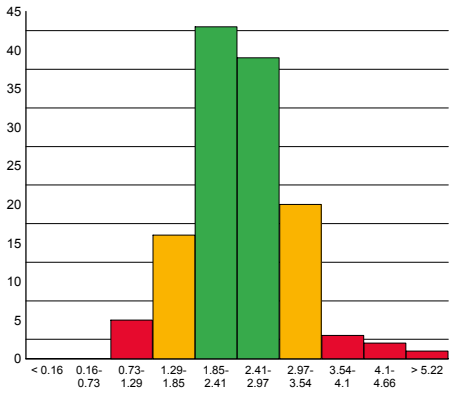
Green = mean +/- 1 Stdev Amber = mean +/- 2 Stdev Red = mean ≥ +/- 3 stdev



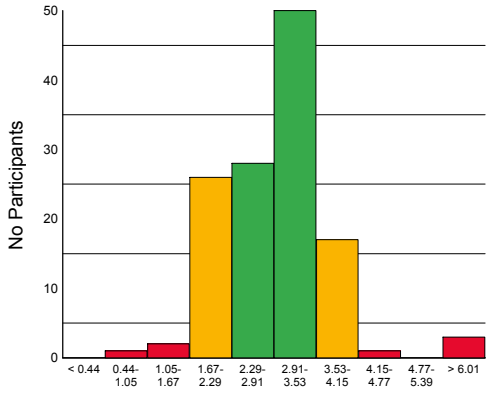
Sample A: Av. HER2 copy



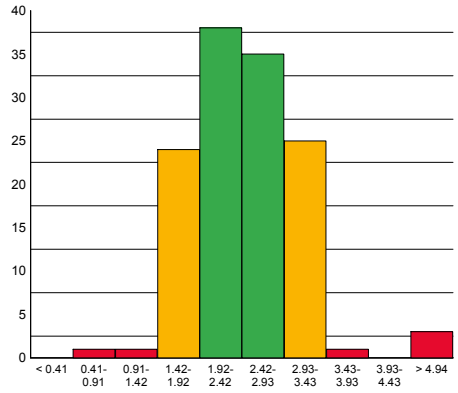
Sample A: Av. Chr17



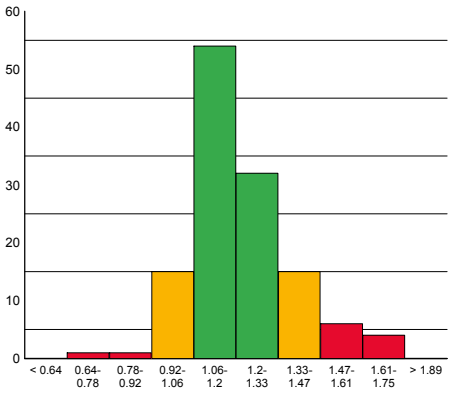
Sample A: Ratio



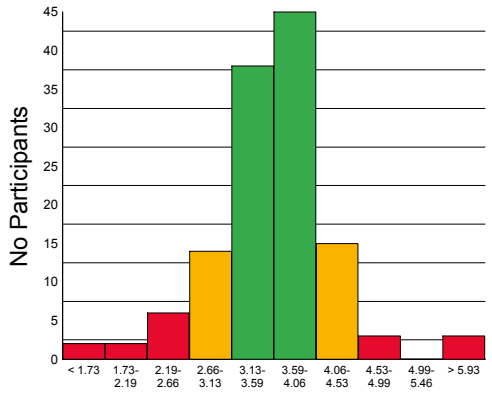
Sample B: Av. HER2 copy



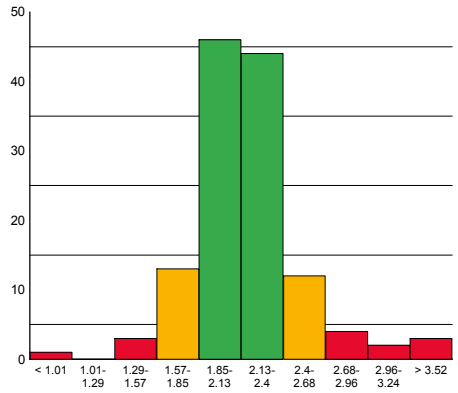
Sample B: Av. Chr17



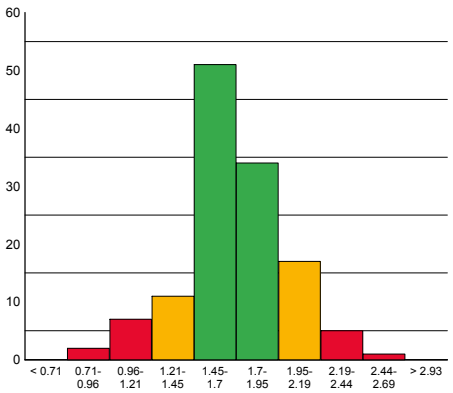
Sample B: Ratio



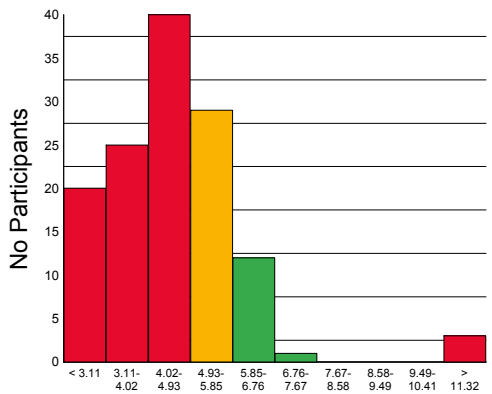
Sample C: Av. HER2 copy



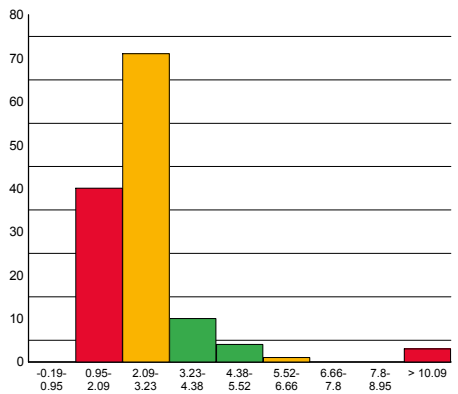
Sample C: Av. Chr17



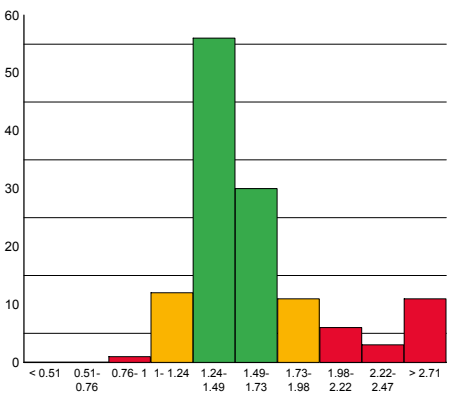
Sample C: Ratio



Sample D: Av. HER2 copy



Sample D: Av. Chr17



Sample D: Ratio

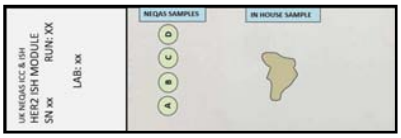
The HER2 ISH Module - Technical Run 48

Suzanne Parry and Jamie Hughes

Gene Assessed:	HER2
Sections circulated:	Composite slide consisting of 4 breast carcinoma sections (see table below)
Number of Registered Participants:	153
Number of Participants Taking Part this Run	126 (82%) (71 Fluorescent and 55 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+**	Borderline/Non-amplified
C	**2+**	Non-amplified
D	**2+**	Non-amplified



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

Assessment Procedure

Brightfield ISH (CISH / SISH / BDISH / DDISH) 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 were 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 for example, when using the Ventana/Roche SISH technique, which requires the Cen17 and HER2 probes to stained separately, the assessors will 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 also requested for this module and participants will be required to place them on the distributed NEQAS slides whenever possible. However, due to there sometimes being the requirement to alter the digestion times for the differences in pre-analytics, separate in-house samples are accepted. A single tissue only is required for the in-house sample.

Results Summary

CISH Results

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

The overall results showed slightly lower pass rates compared to the previous run (Run 47). 31 (56%) of laboratories achieved an acceptable pass rate on this Run (48) compared to 59% on previous run on the UK NEQAS distributed material. Again, the failures were predominantly due to weak or no Cen17 signals, as seen in previous assessment runs. 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. More recently the assessment is also seeing more red precipitate, sometimes obscuring the signals, and lack of staining in the normal cells. These observational results refer to the Ventana DDISH, Inform or BDISH methods. Another assay used in the assessment was the ZytoDot 2C, ZytoVision, which was used by one participant, but unfortunately this slide did not pass the assessment. Th Dako DuoCISH method was also used by 1 laboratory, and unfortunately also failed the assessment.

Important: Whichever Brightfield ISH methodology is being used, any laboratory experiencing staining problems should contact the relevant company for further support.

FISH Results

71 laboratories submitted slides for the FISH assessment. The results show an increase of 3% in acceptable results from run

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

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

47, as shown in the table below:

HER2 FISH Pass Rates : NEQAS section		
	Run 47	**Run 48**
Acceptable	71%(N=47)	79%(N=56)
Borderline	12%(N=8)	7%(N=5)
Unacceptable	17%(N=11)	14%(N=10)

There was an improvement in the current run 48 compared to the previous run (47) 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 handling (e.g. cleaning of slides with alcohol before sealing), storage or transport errors (e.g. temperature fluctuations), as it did not appear to be assay specific.r

The failure rate dropped from 17% (run 47) to 14% (run 48). This is reflected in the overall pass rate with an increase of acceptable pass rates from 71% (run 47) to 79% (run 47). Images of acceptable and unacceptable levels of staining are illustrated in **Figures 7-8**.

The Pathvysion Vysis kit was the most commonly used FISH method, used by 51% (N=38) of FISH participants. The acceptable pass rate using the Vysis kit for this run was 67%, and a further 8% achieved a borderline pass. The second most common assay was the Dako IQFISH used by 15% of FISH participants, and showed an acceptable pass rate of 91%. Zytovision (ZytoLight) was used by 7% of FISH participants and showed an acceptable pass rate of 100%. Other FISH assays/probes used include; Dako Pharm Dx, Leica HER2 FISH system and Kreatech Probes, all of which performed well overall, although the numbers of laboratories adopting these methods are low.

Validating ISH

It is crucial to emphasise the importance of undertaking a robust validation and verification protocol when introducing ISH into a diagnostic laboratory for the first time or a new assay/method is being introduced. For example, changing from a FISH assay to a brightfield ISH asssay, the new technique must be fully validated and staff must undergo extra training to gain expertise with the new method employed.

The UK NEQAS data indicates that there is a definite move towards the use of the Ventana DDISH method and laboratories introducing either this method or another ISH method should make sure that:

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

Recommendations for Returning FISH Slides for NEQAS Assessments

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

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

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

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

Selected Images showing Optimal and Sub-optimal staining

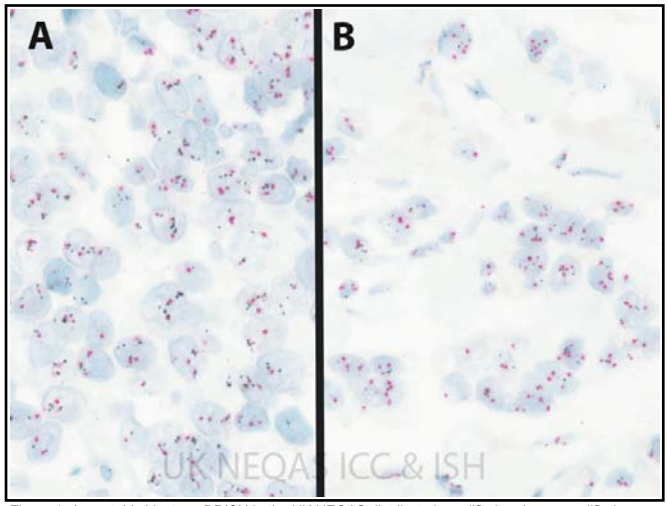


Figure 1. Acceptable Ventana DDISH in the UK NEQAS distributed amplified and non-amplified samples A and B. Both examples show distinct HER2 signals (black) and Chr17 signals (red) at the expected signal level.

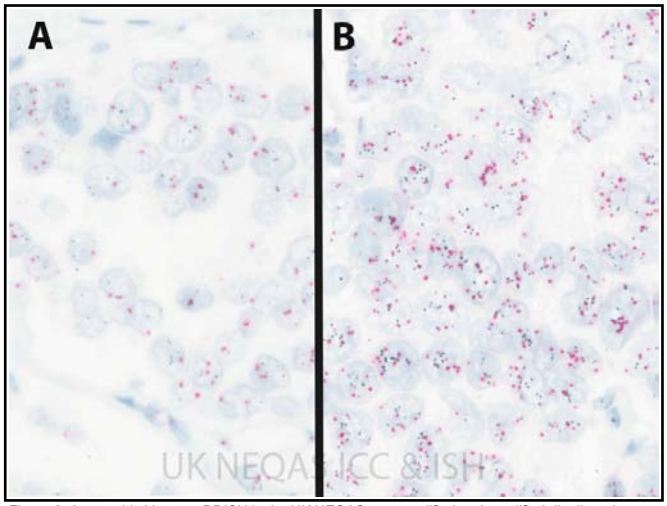


Figure 2. Acceptable Ventana DDISH in the UK NEQAS non-amplified and amplified distributed samples C and D. Both samples show strong HER2 signals (black) and Chr17 signals (red) with the expected average copy numbers per cell.

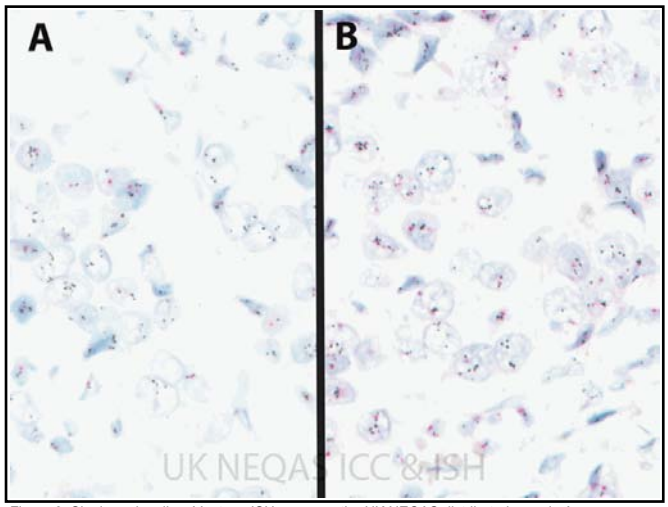


Figure 3. Single probe silver Ventana ISH assay on the UK NEQAS distributed sample A. Section A shows the Chr17 probe, while section B is the HER2 copy number. The section is non-amplified.

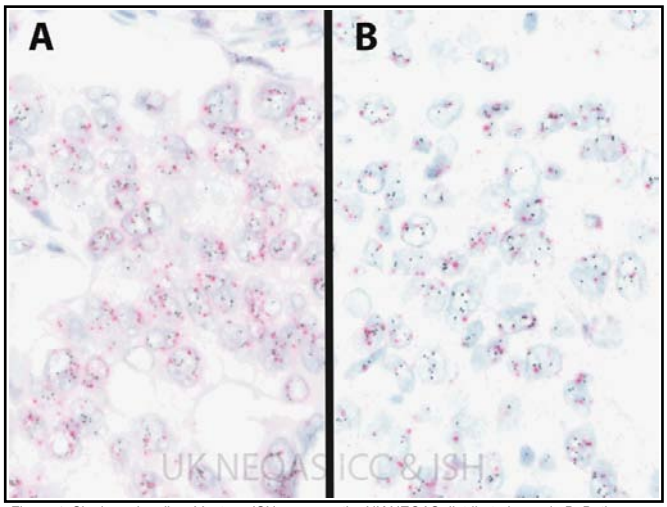


Figure 4. Single probe silver Ventana ISH assay on the UK NEQAS distributed sample B. Both the Chr17 (A) and the HER2 (B) probes show the good strong black signals at the expected level.

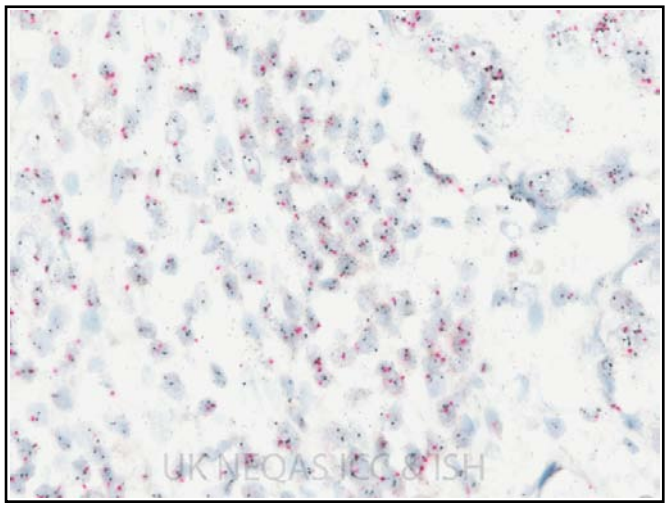


Figure 5. Two examples showing unacceptable Ventana DDISH staining in the UK NEQAS non-amplified samples (A and B): There are no Chr17 signals and therefore the samples are uninterpretable.

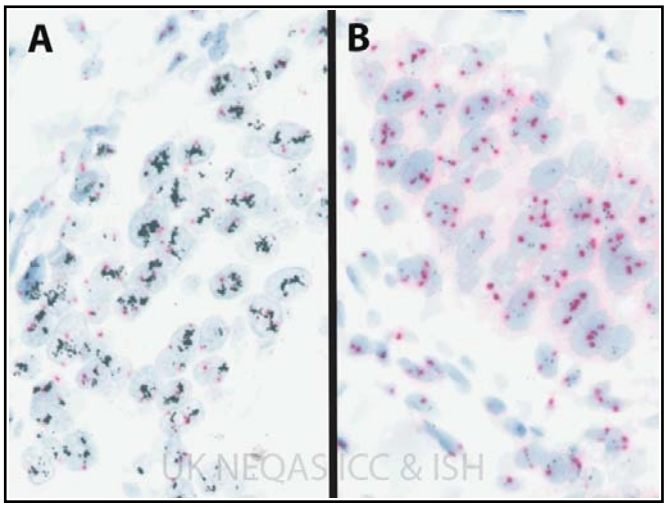


Figure 6. Unacceptable Ventana DDISH staining in the UK NEQAS non-amplified samples (C and D): Example A shows leaching of the Chr17 probe, and in example B there is excessive precipitate and background.

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

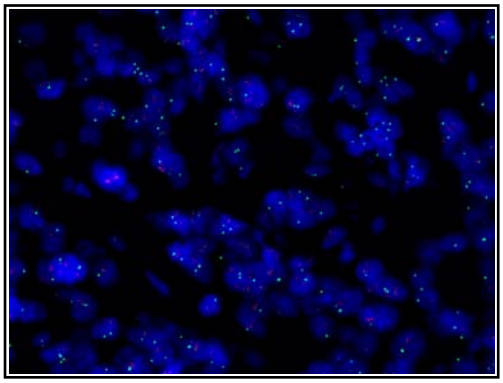


Figure 1: Excellent demonstration of HER2 gene and CEP17 on core A of the UK NEQAS distributed sample. Pathvysion Vysis Kit; Dako pre-treatment buffer; Pepsin 37°C for 5 mins

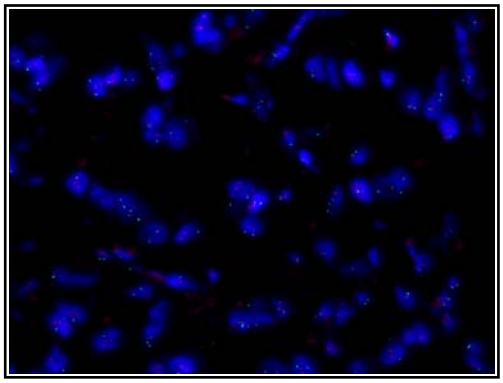


Figure 2: Excellent demonstration of HER2 gene and CEP17 on core B of the UK NEQAS distributed sample. Fibroblasts are clearly visible and appropriate number of signals are present. Pathvysion Vysis Kit; Dako pre-treatment buffer; Pepsin 37°C for 5 mins

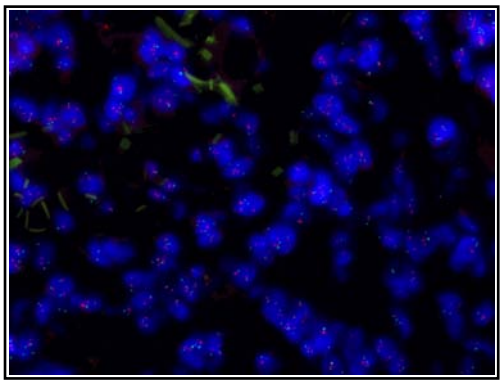


Figure 3: Excellent demonstration of HER2 gene and CEP17 on core C of the UK NEQAS distributed sample. Kreatech Probes; Other; Pepsin 37°C for 20 mins

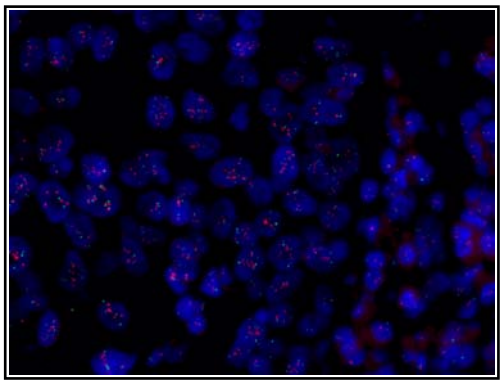


Figure 4: Excellent demonstration of HER2 gene and CEP17 on core D of the UK NEQAS distributed sample. Image shows clear amplified tumour with multiple single signals and cluster. Kreatech Probes; Other; Pepsin 37°C for 20 mins

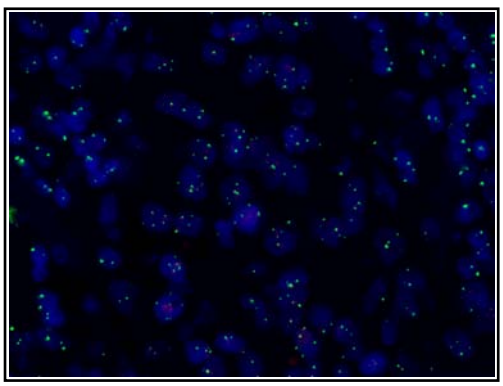


Figure 5: Poor example of HER2 gene and CEP17 demonstration on in-house control tissue. Excessive CEP17 signal inside and outside of the nucleus. Pathvysion Vysis Kit; Vysis (VP2000); Protease I 37°C for 60 mins

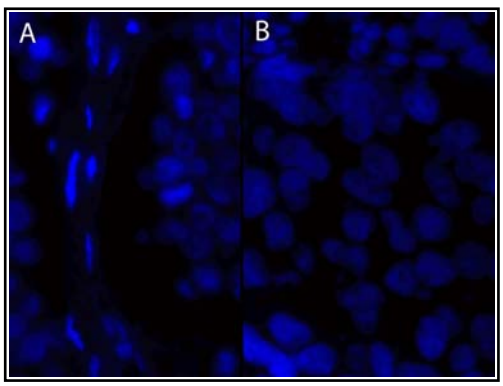
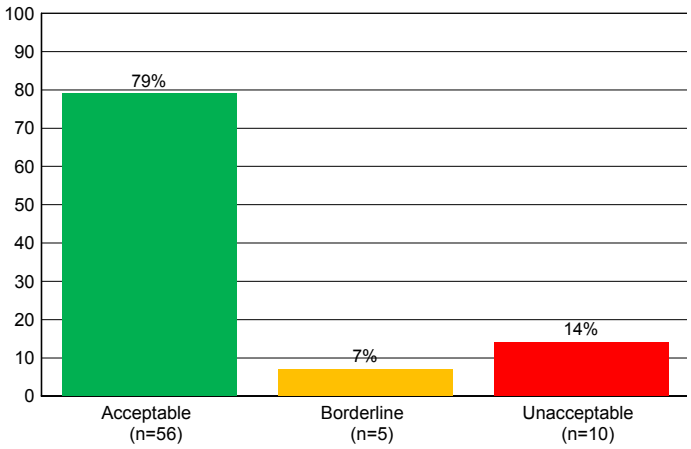


Figure 6: Poor example of HER2 gene and CEP17 demonstration on in-house control tissue. No signals observed. Likely due to fading before assessment date as participant scored acceptable in the interpretive module. Pathvysion Vysis Kit; Vysis kit I; Protease 37°C for 26 mins.

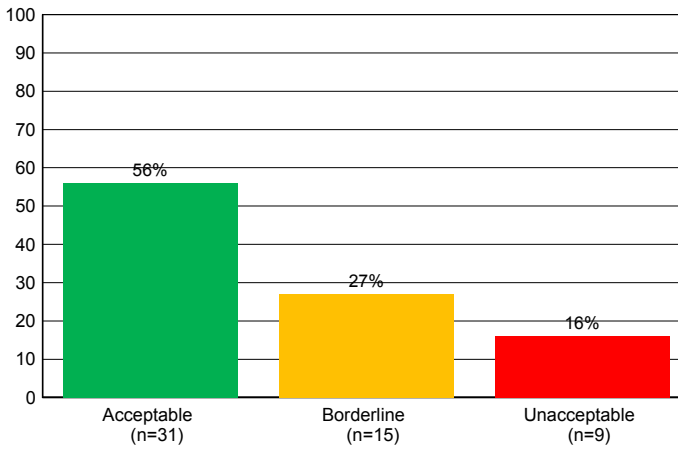
Technical ISH: Pass Rates and Methods

Overall Pass Rates

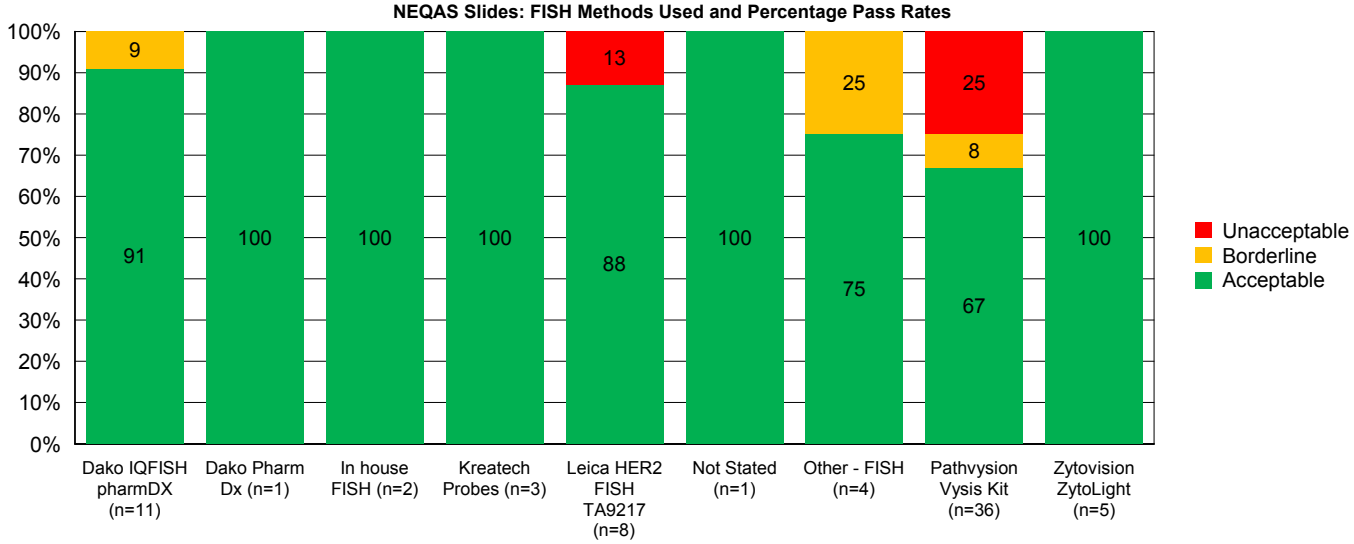
FISH NEQAS slide (n=71)



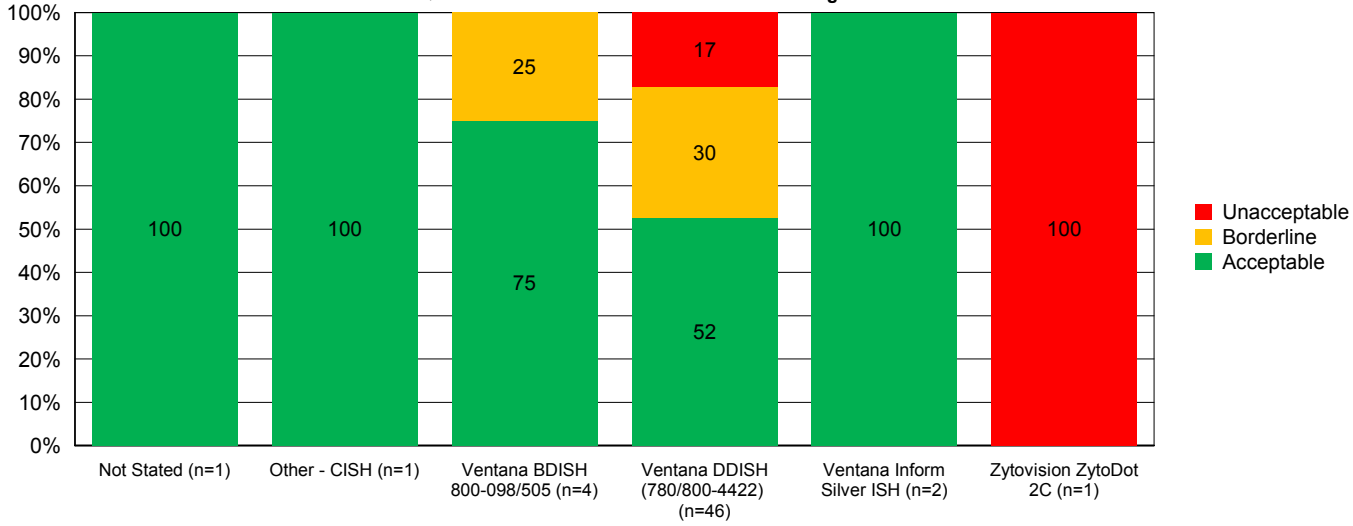
CISH NEQAS Slide (n=55)



NEQAS Slides: FISH Methods Used and Percentage Pass Rates



NEQAS Slides: CISH Methods Used and Percentage Pass Rates



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