UK NEQAS
International Quality Expertise

Immunocytochemistry

Improving Immunocytochemistry for Over 25 Years

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

Assessment Dates: 19th September — 7th October 2016

Articles / Reports

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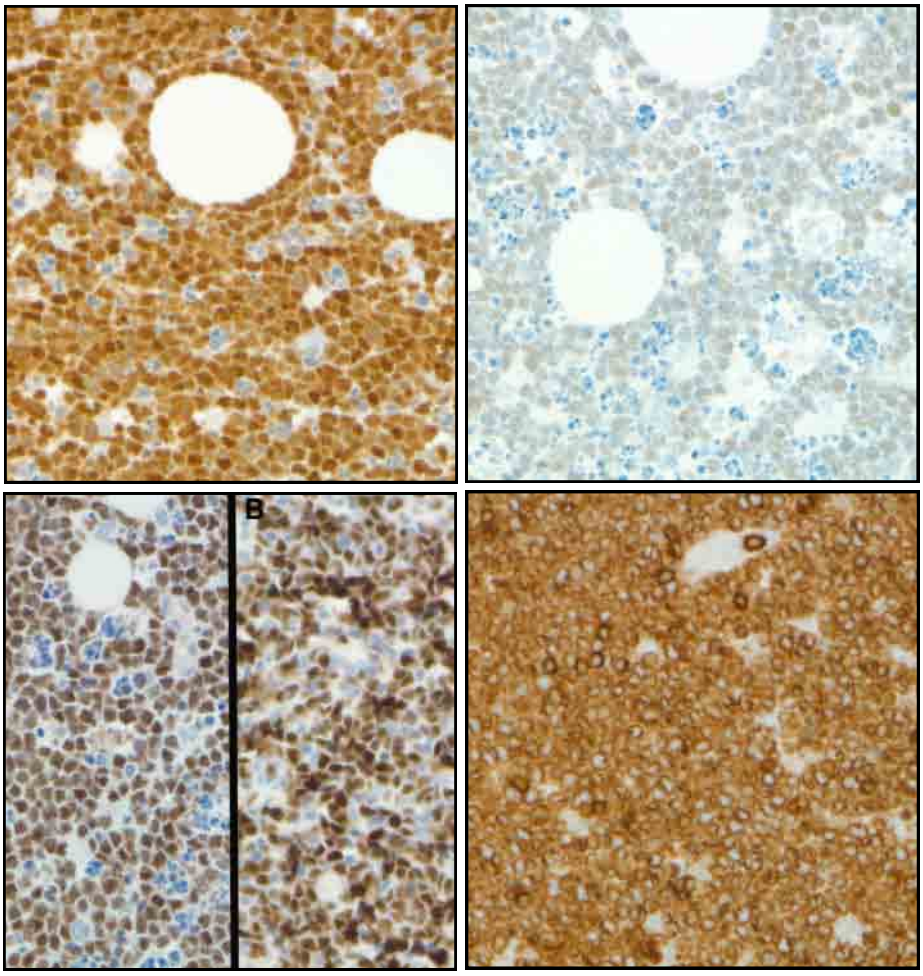

Cover Photo: Taken from the Lymphoma Module 2nd Antigen (TdT):

Top Left: Optimal TdT demonstration on the NEQAS tumour sample (N)
Top Right: Weak demonstration of TdT on the NEQAS sample (N)
Bottom Left: Excellent TdT staining NEQAS sample and an in-house control (P) image: B
Bottom Right: Good staining on alternative 2nd antigen CD3 on NEQAS tumour (N)

General Information

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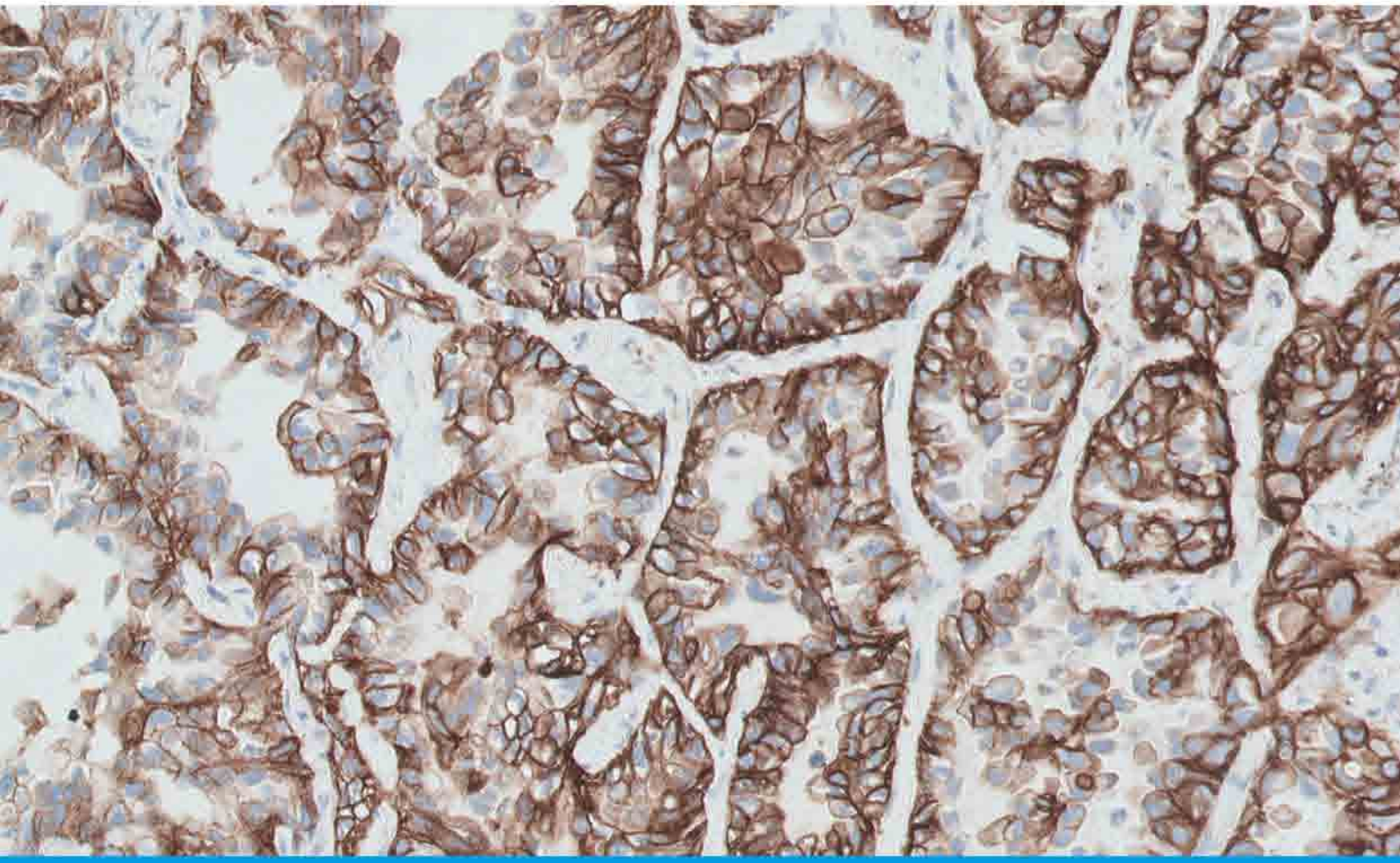

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The General Pathology Module Run 115

Gavin Rock and Suzanne Parry

	Gold Standard	Second Antibody
Antigens Assessed:	Ki-67	Melanoma Markers
Tissue Sections circulated:	Breast carcinoma x 2 and normal tonsil.	Normal skin and metastatic melanoma.
Number of Registered Participants:	315	
Number of Participants this Run	306 (97%)	

Introduction

Gold Standard: Ki-67

Ki-67 is a 345-395 kDa nuclear protein involved in the maintenance and up regulation during the cell division cycle. Ki-67 is present in the cell nuclei in all actively proliferating cells during late G1, S, M and G2 stages of the cell cycle and mitosis, but is not expressed in cells during the resting phase G0 (non-cycling cells). The Ki-67 antigen is used to measure the growth fraction of a given cell population (Ki-67 labelling index), i.e. the percentage of cells staining positive for Ki-67. The Ki-67 labelling index (LI) is used to assess the course of cancer in various solid tumours: It is known to correlate with tumour grade, survival and recurrence. For example, in the lymphoma setting the percentage nuclear staining with Ki-67 is used as a prognostic marker: Tumour grade is closely associated with the percentage of Ki-67 stained nuclei in non-Hodgkin's lymphoma (Brown et al, Hall et al.); with <20% Ki-67 expression seen in low grade lymphomas and >20% Ki-67 expression associated with high grade lymphomas. Furthermore, low grade lymphomas with a labelling index >5% have a worse prognosis than those with an index of <5%. In the breast cancer setting, the Ki-67 proliferative index is used as both a predictive and prognostic marker (Dowsett et al), and is suggested as a key prognostic indicator for risk recurrence in ER-positive breast cancer (Kantozoglou, Palla et al. 2013).

Features of Optimal Immunostaining:

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

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

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

Features of Sub-Optimal Immunostaining: (Figs 2, 5 & 6)
- Weak, uneven, partially missing staining of relevant cells.
- Fewer tumour cells staining than expected in the breast.

References:

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

Second Antigen: Melanoma Markers

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

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

Features of Optimal Immunostaining: (Figs 7, 8, 10, 11 & 12)
- Intense cytoplasmic staining of tumour cells.
- Clean background.
- No non-specific staining of other cell types.
- Adequate nuclear counterstain.

Features of Sub-optimal Immunostaining: (Fig 9)
- Weak, uneven or no staining of tumour cells.
- Excessive background staining.
- Non-specific staining of cell types or components not expected to stain.
- Excessive or very weak nuclear counterstain.

References

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

Gavin Rock and Suzanne Parry

Assessment Summary

Ki67

306 labs submitted slides for the Ki 67 assessment , and all but 2 laboratories submitted their in-house control sections for this run. The results show a decrease of 2% in acceptable results from Run 114, as shown in the table below:

The reason for failure was either due to weak/very weak staining or lower expression and percentage of cells staining than expected. The most popular clone used in this run was the DAKO MIB1, used by 143 participants and showed a pass rate of 68%. Another popular choice was the Ventana 30-9 clone, which was used by 70 participants and showed an acceptable pass rate of 97%. The acceptable pass rate on the in-house material was 93% compared to the NEQAS of 78%. Many laboratories are only using an appendix as their in-house positive controls, and unlike some of the other UK Neqas ICC & ISH modules, labs are not penalised for only using a single control. However, the advice from the scheme is to use a composite control to help gauge the sensitivity of the assay, as an appendix alone may not always highlight a slightly sub-optimal assay. It is best practice to use a known normal tissue and a known positive control to help measure the sensitivity of the assay.

The table below summarises the pass rates for the Ki-67 Run 115 assessment:

Ki-67 Pass Rates : NEQAS section		
	Run 114	Run 115
Acceptable	80%(N=240)	78%(N=237)
Borderline	12%(N=36)	12%(N=37)
Unacceptable	7%(N=22)	10%(N=32)

Melanoma Markers (HMB45 /Melan-A)

304 laboratories submitted slides for the melanoma marker assessment. Of these, all apart from 1 lab also submitted their in-house material. The acceptable pass rates on the NEQAS sections were not great with only 37% of laboratories achieving acceptable scores, compared to the in-house sections obtaining an acceptable score of 94%. The slides that failed the assessment showed very weak or no demonstration of staining in the melanoma and/or in the normal skin. The in-house results showed very different pass rates to the NEQAS distributed slides, with 94% receiving an acceptable pass, a further 4% achieved a borderline pass, and only 5 labs (2%) failed on the in-house compared to 88 labs (29%) in the NEQAS sections in this assessment.

The table below summarises the pass rates for the Melanoma Run 115 assessment:

Melanoma Pass Rates Run 115		
	NEQAS	IN-HOUSE
Acceptable	37% (N=114)	94%(N=287)
Borderline	34% (N=102)	4%(N=11)
Unacceptable	29% (N=88)	2%(N=5)

The discordance between the Neqas material scores and the In-house material did raise the question on the stability of the samples provided for this assessment run. This was not picked up during initial testing, where the Gold standard slides showed the expected level of staining. However, the Run 115 results showed a higher unacceptable rate than expected, which did suggest that the sections in some cases may not have retained the necessary antigenicity. This may have been in part due to incorrect handling and storage of the slides by laboratories before staining. This issue was taken into consideration and it was decided not to include this Melanoma assessment in the poor performance monitoring. The scheme felt that if a laboratory had performed well on their in house material despite the low mark on the Neqas material, this was an indication that the laboratory is using an acceptable protocol for their testing. There were a number of laboratories that did achieve a very good level of staining on both the Neqas material and their in-house material. Very weak staining was the main reason for failure on the participant's in-house material. Marks were also lost when a laboratory used inappropriate in-house control material.

168 participants used the Melan A marker compared to 120 participants using the HMB45 clone. The most popular Melan A antibody of choice amongst participants was the Dako A103 clone which was used by 69 participants. For the HMB45 clone, the Dako antibody was the most popular of sources. The Epitomics EP43 Melan A clone was used by 1 participant, and this slide received an excellent score of 19/20 at assessment on the Neqas material and also an excellent result on their own in-house material. This laboratory carried out staining on the Leica BondMax platform with an antigen retrieval protocol of ER2 for 20 minutes. Another participant using the Dako A103 Melan A antibody on the autostainer with pre-treatment in the PT link achieved the maximum score of 20/20 at assessment on both the Neqas material and their own in-house tissue.

Selected Images showing Optimal and Sub-optimal Immunostaining

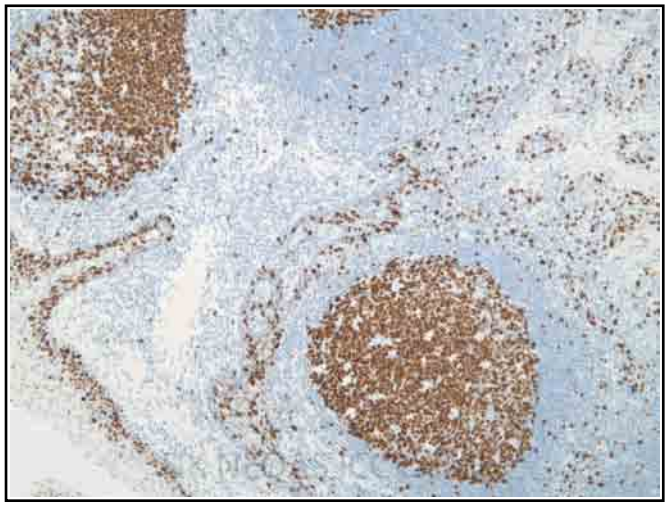


Fig 1. Good demonstration of Ki67 in the UK NEQAS distributed tonsil. Intense and well-localised staining of the germinal centre B-cells and supra-basal squamous epithelium. Clean background with no non-specific staining. Stained with the Ventana 30-9 prediluted antibody, on the Ventana Benchmark XT using CC1 standard protocol.

Fig 2. Poor demonstration of Ki 67 in the UK NEQAS distributed tonsil. (compare to Fig 1). The staining is weak and fewer cells are demonstrated than is expected. The corresponding breast tumours in this section had no staining. Stained with the DAKO M740 (MIB1) clone antibody (1:50) on the Leica Bond III with pre-treatment ER2 for 20 mins.

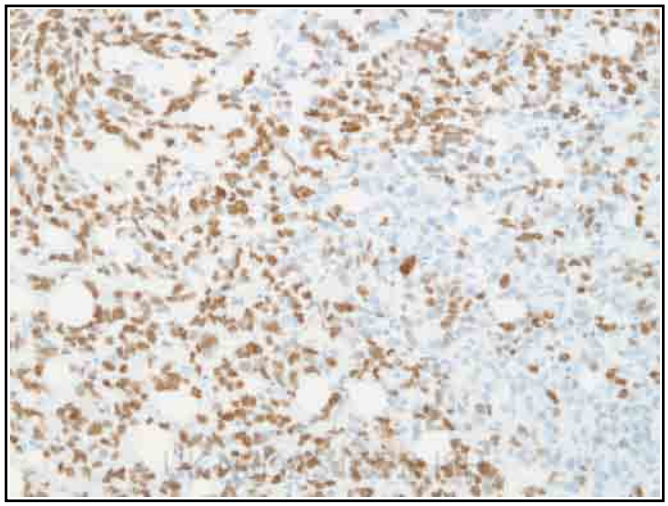


Fig 3. Optimal staining of Ki-67 in the UK NEQAS high expressing breast tumour. 70% of neoplastic cells show moderate distinct nuclear staining. Section is stained DAKO M740 (MIB1) clone antibody (1:50) on the DAKO auto-stainer with a PT link using a high pH buffer.

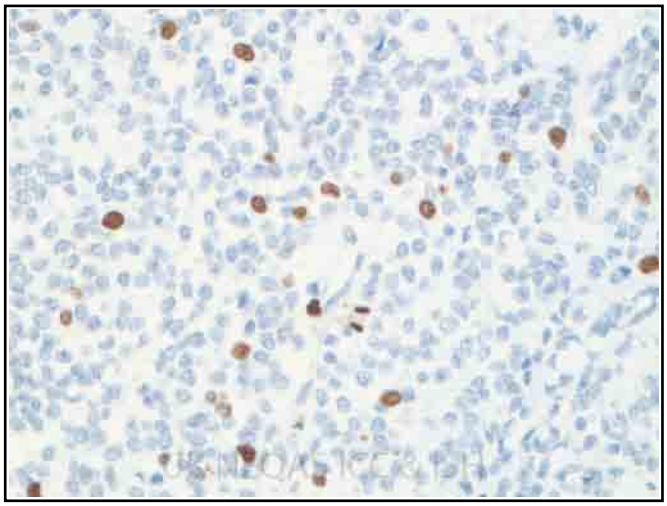


Fig 4. Good demonstration of Ki 67 in the UK NEQAS low expressing breast tumour approximately 5% of tumour cell staining with a moderate intensity. Same protocol used as in Fig 1.

Fig 5. Sub-optimal staining in the UK NEQAS distributed tonsil with weaker expression in the germinal centre B –cells than expected. This corresponds to the weak staining also seen in the UK NEQAS breast tumour samples on the same slide (shown in Fig 6). Stained with the DAKO MIB 1 clone (pre-dilute) on the DAKO auto-stainer with PT link (low pH buffer).

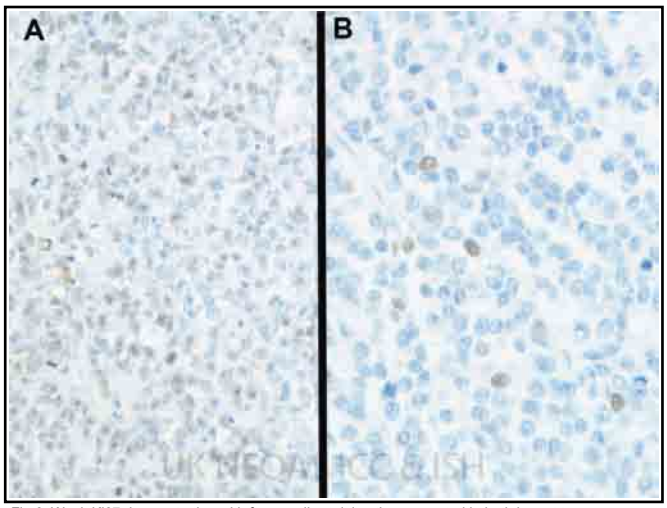


Fig 6. Weak Ki67 demonstration with fewer cells staining than expected in both breast tumours. (Same lab as Fig 5).

Selected Images showing Optimal and Sub-optimal Immunostaining

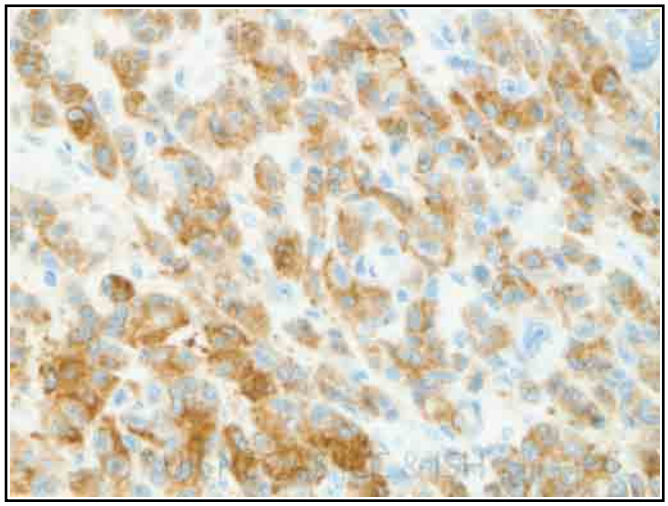


Fig 7.Optimal demonstration of the melanoma stained with the DAKO Melan A antibody. A 1:100 dilution was used on the DAKO auto-stainer with a PT link pre-treatment using a high pH buffer.

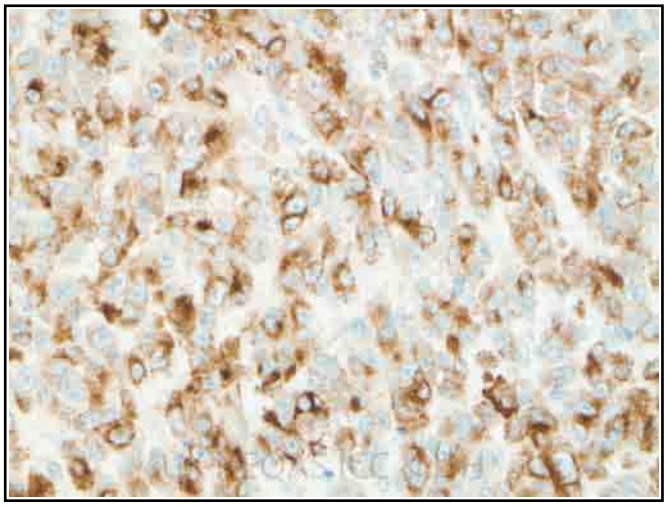


Fig 8. A good example of optimal staining in the melanoma section. The section was stained using the Ventana HMB45 pre-diluted antibody on the Benchmark Ultra with ultraview kit and CC1 pre-treatment for 32 mins.

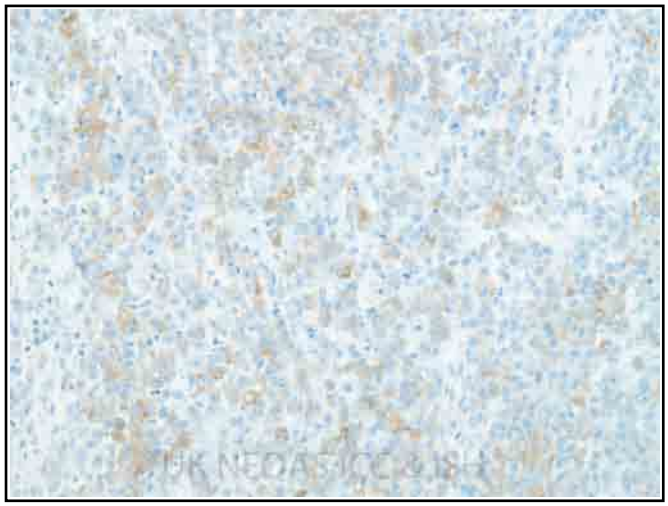


Fig 9. A poor example, of staining in the melanoma section. Even though the same protocol as the examples in Fig 7, the staining is muchweaker.

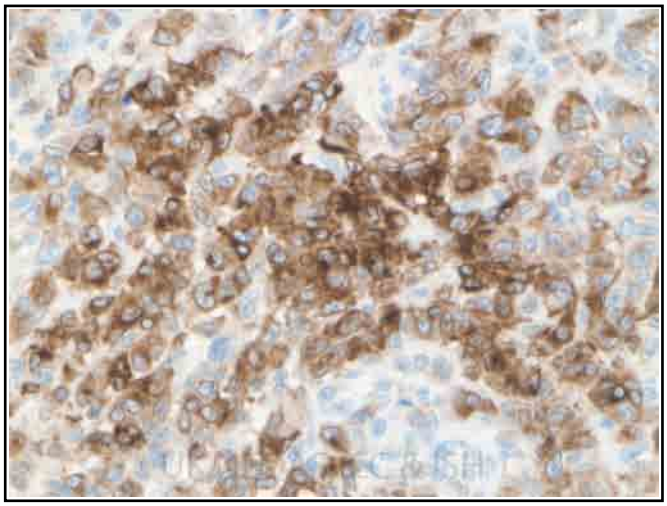


Fig 10. Good demonstration of the melanoma section stained with the DAKO Melan A antobody carried out on the Leica Bond III autostainer. A dilution of 1:50 was used with ER2 pretreatment for 30 minutes.

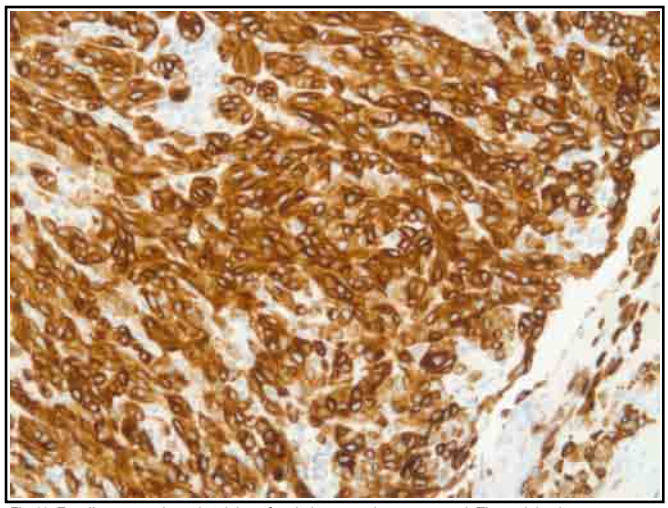


Fig 11. Excellent example and staining of an in house melanoma control. The staining is strong, while the background remains clean. (Same protocol as Fig 7).

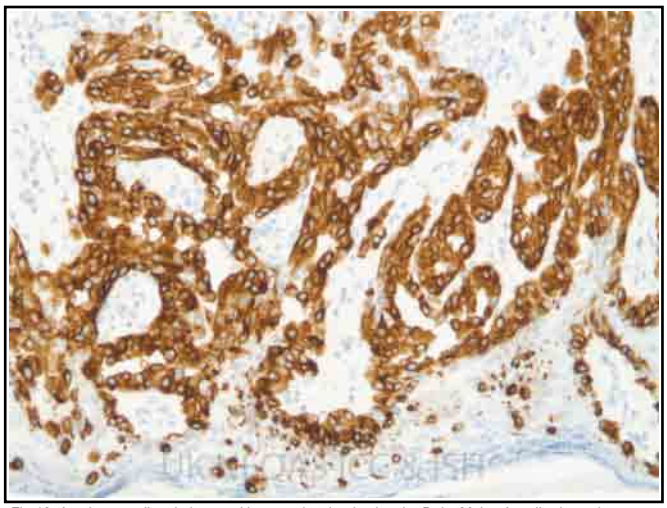
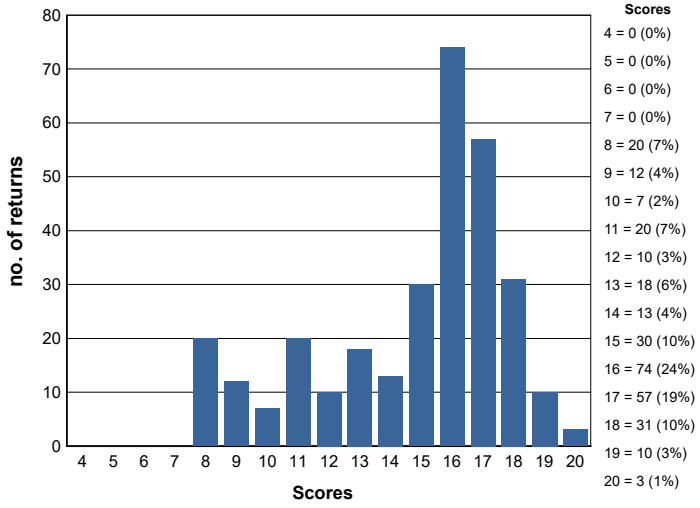


Fig 12. Another excellent in house skin control stained using the Dako Melan A antibody on the Dako Auotstainer, pre-treatement in the PT link with high pH buffer.

GRAPHICAL REPRESENTATION OF PASS RATES

RUN 115A Ki-67 / MIB1 on NEQAS Sections



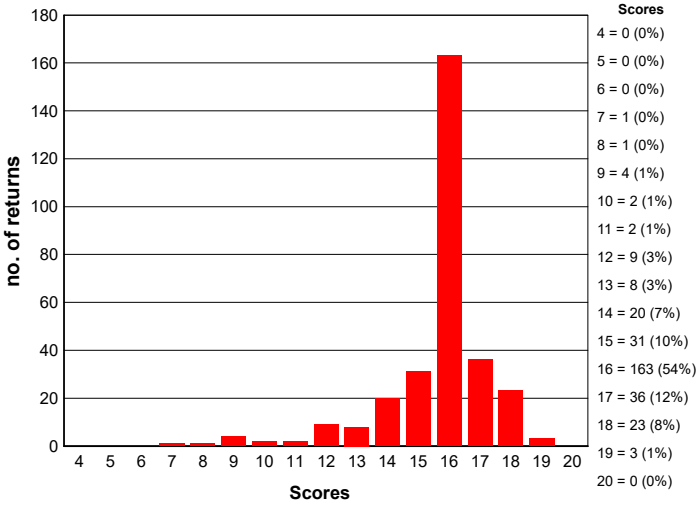
Individual Scores
4 = 0 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 0 (0%)
8 = 20 (7%)
9 = 12 (4%)
10 = 7 (2%)
11 = 20 (7%)
12 = 10 (3%)
13 = 18 (6%)
14 = 13 (4%)
15 = 30 (10%)
16 = 74 (24%)
17 = 57 (19%)
18 = 31 (10%)
19 = 10 (3%)
20 = 3 (1%)

Summary

16 - 20 = 175 (57%)
13 - 15 = 61 (20%)
10 - 12 = 37 (12%)
0 - 9 = 32 (10%)

Median = 14.00

RUN 115B Ki-67 / MIB1 on in-house Sections



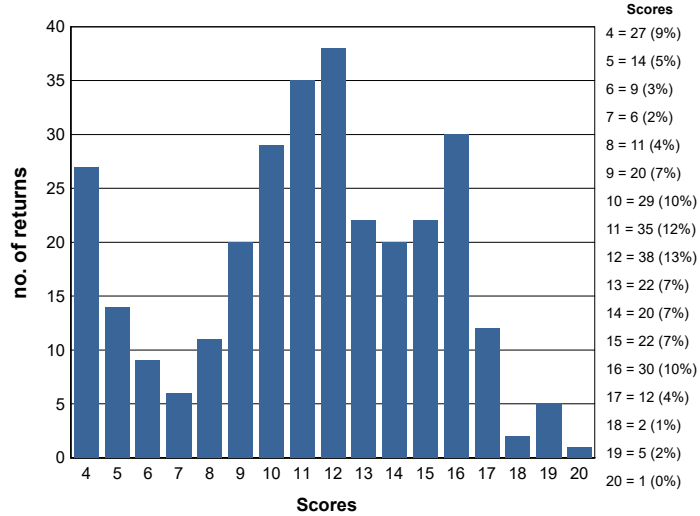
Individual Scores
4 = 0 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 1 (0%)
8 = 1 (0%)
9 = 4 (1%)
10 = 2 (1%)
11 = 2 (1%)
12 = 9 (3%)
13 = 8 (3%)
14 = 20 (7%)
15 = 31 (10%)
16 = 163 (54%)
17 = 36 (12%)
18 = 23 (8%)
19 = 3 (1%)
20 = 0 (0%)

Summary

16 - 20 = 225 (74%)
13 - 15 = 59 (19%)
10 - 12 = 13 (4%)
0 - 9 = 6 (2%)

Median = 13.00

RUN 115C HMB45 or Melan-A only on NEQAS Sections



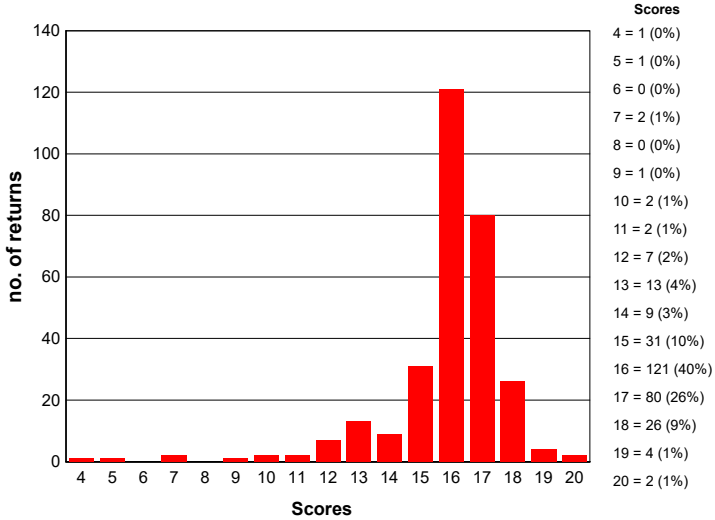
Individual Scores
4 = 27 (9%)
5 = 14 (5%)
6 = 9 (3%)
7 = 6 (2%)
8 = 11 (4%)
9 = 20 (7%)
10 = 29 (10%)
11 = 35 (12%)
12 = 38 (13%)
13 = 22 (7%)
14 = 20 (7%)
15 = 22 (7%)
16 = 30 (10%)
17 = 12 (4%)
18 = 2 (1%)
19 = 5 (2%)
20 = 1 (0%)

Summary

16 - 20 = 50 (17%)
13 - 15 = 64 (21%)
10 - 12 = 102 (34%)
0 - 9 = 87 (29%)

Median = 12.00

RUN 115D HMB45 or Melan-A only on in-house Sections



Individual Scores
4 = 1 (0%)
5 = 1 (0%)
6 = 0 (0%)
7 = 2 (1%)
8 = 0 (0%)
9 = 1 (0%)
10 = 2 (1%)
11 = 2 (1%)
12 = 7 (2%)
13 = 13 (4%)
14 = 9 (3%)
15 = 31 (10%)
16 = 121 (40%)
17 = 80 (26%)
18 = 26 (9%)
19 = 4 (1%)
20 = 2 (1%)

Summary

16 - 20 = 233 (77%)
13 - 15 = 53 (18%)
10 - 12 = 11 (4%)
0 - 9 = 5 (2%)

Median = 13.00

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: 115

Primary Antibody : Ki-67 / MIB1

Antibody Details	N	%
Dako M7240 (clone MIB1)	143	68
NeoMarkers RM 9106 (clone SP6)	4	100
Novocastra NCL-Ki67 (clone MM1)	3	33
Ventana 760-2910	3	100
Other	7	57
Dako IR/IS626 (clone MIB-1)	22	73
Leica NCL-Ki67-MM1 (MM1)	3	67
Leica RTU PA0410 (MM1)	3	67
Leica PA0118 (MM1)	4	25
Leica PA0230 (K2)	25	96
Vector VP-RM04 (clone SP6)	1	100
Ventana 790-4286 (clone30-9)	69	97
Cell Marque 275R-14/15/16/17/18 (SP6)	5	80
Dako Omnis GA626 (MIB-1)	10	70

General Pathology Run: 115

Primary Antibody : HMB45 or Melan-A only

Antibody Details	N	%
Cell Marque CMA710 (HMB45)	1	0
Dako M0634 (HMB45)	44	45
Dako M7196 (A103) Melan-A	69	46
Dako U7025 (HMB45)	1	0
Enzo 30930 (HMB45)	1	100
Novocastra NCL-HMB45 (HMB45)	9	33
Novocastra NCL-MELAN A	8	0
Ventana 760 2518 (HMB45)	2	0
Other	6	50
Ventana 790 2990 MART-1/MelanA	35	37
Novocastra NCL-RTU-MelanA (103)	1	0
Novocastra NCL-L-MelanA (103)	21	29
Novocastra PA0233 RTU MelanA (103)	3	33
Dako M0634 Melanosome (HMB45)	17	24
Cell Marque CM281M-9x MelanA (M2-7C10)	3	67
Biogenex AM361-5/10M (MelanA)	1	0
Biogenex Mu361-UC (MelanA)	2	50
Biogenex MU001A-UC (HMB45)	1	0
A. Menarini MU361-UC (MelanA)	1	100
Leica Bond PA0233 (A103) Melan A	12	42
Leica RTU-Melan A (A103)	4	0
Dako RTU Auto Link IR052 (HMB45)	10	30
Cell Marque 282M (HMB45)	1	0
Ventana 790-4366 (HMB45)	24	46
Neomarkers MART-1 Ab4 MS-799 (A103)	1	100
Epitomics AC-0041 (MART-1 EP43)	1	100
Dako RTU Omnis GA052 (HMB45)	4	25
Dako Melan A/MART-1 RTU Auto Link IR633	4	75
Dako Melan A/MART-1 RTU Auto Plus IS633	1	0
Novocastra PA0044 RTU MelanA (A103)	1	0
Novocastra PA0027 RTU HMB45	4	50

General Pathology Run: 115 Heat Mediated Retrieval	HMB45 or Melan-A only		Ki-67 / MIB1	
	N	%	N	%
_Ventana Benk XT CC1 (Mild)	1	0	0	0
Biocare Decloaking Chamber	3	67	2	100
Dako Omnis	12	25	12	58
Dako PTLink	31	68	34	82
Lab vision PT Module	3	67	3	100
Leica ER1 10 mins	2	50	0	0
Leica ER1 20 mins	14	50	7	71
Leica ER1 25 mins	1	0	0	0
Leica ER1 30 mins	7	43	1	0
Leica ER1 40 mins	1	0	0	0
Leica ER2 10 mins	2	0	1	0
Leica ER2 20 mins	40	40	57	58
Leica ER2 30 mins	11	55	15	60
Leica ER2 40 mins	0	0	3	67
Microwave	5	0	6	50
None	13	23	0	0
Other	3	0	3	67
Pressure Cooker	2	100	3	100
Pressure Cooker in Microwave Oven	1	0	0	0
Steamer	2	50	2	50
Ventana CC1 16mins	2	50	0	0
Ventana CC1 20mins	1	100	0	0
Ventana CC1 24mins	3	67	7	100
Ventana CC1 32mins	15	47	25	92
Ventana CC1 36mins	7	43	12	83
Ventana CC1 40mins	8	38	5	60
Ventana CC1 48mins	3	33	4	100
Ventana CC1 52mins	2	0	3	67
Ventana CC1 56mins	7	57	6	100
Ventana CC1 64mins	28	21	30	90
Ventana CC1 72mins	1	100	1	0
Ventana CC1 76mins	6	17	2	100
Ventana CC1 88mins	0	0	1	100
Ventana CC1 8mins	1	0	0	0
Ventana CC1 92mins	1	100	0	0
Ventana CC1 extended	2	50	0	0
Ventana CC1 mild	9	22	16	94
Ventana CC1 standard	24	25	30	90
Ventana CC2 24mins	0	0	1	100
Ventana CC2 44mins	0	0	1	0
Ventana CC2 64mins	0	0	2	100
Water bath 68 OC	1	0	1	100
Water bath 95-98 OC	3	33	3	33

General Pathology Run: 115 Enzyme Mediated Retrieval	HMB45 or Melan-A only		Ki-67 / MIB1	
	N	%	N	%
AS PER KIT	10	40	2	100
NOT APPLICABLE	131	43	163	72
VBS Bond Enzyme 1	2	0	0	0
Ventana Protease	1	0	1	100
Ventana Protease 1 (760-2018)	3	0	0	0

General Pathology Run: 115	HMB45 or Melan-A only		Ki-67 / MIB1	
Detection	**N**	**%**	**N**	**%**
A Menerini Polymer (MP-XCP)	0	0	1	100
AS PER KIT	18	44	17	88
Dako EnVision FLEX (K8000/10)	12	42	9	67
Dako EnVision FLEX+ (K8002/12)	22	59	27	74
Dako Envision HRP/DAB (K5007)	9	11	6	83
Dako Envision+ HRP mouse K4004/5/6/7	4	75	3	67
Dako rb-a-mo Ig (E0354)	0	0	1	100
Dako REAL (K5005)	1	100	0	0
Dako REAL HRP/DAB (K5001)	1	100	1	0
LabVision UltraVision ONE Polymer (TL-012/5-HDJ/T	1	0	1	100
Leica Bond Intense R Detection (DS9263)	1	100	0	0
Leica Bond Polymer AP Red Detection (DS9305)	1	100	0	0
Leica Bond Polymer Define (DS9713)	1	0	1	0
Leica Bond Polymer Refine (DS9800)	79	39	78	58
MenaPath X-Cell Plus (MP-XCP)	2	100	1	100
None	0	0	1	100
NOT APPLICABLE	1	0	0	0
Novocastra Novolink PDS (RE7-140/150/280/290-K)	1	100	1	100
Other	18	39	7	57
Ventana iView system (760-091)	7	29	3	100
Ventana OptiView (760-700) + Amp. (7/860-099)	4	50	5	80
Ventana OptiView Kit (760-700)	39	44	53	89
Ventana UltraView Kit (760-500)	68	25	82	88

General Pathology Run: 115	HMB45 or Melan-A only		Ki-67 / MIB1	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	1	0	0	0
Dako Autostainer	4	50	3	67
Dako Autostainer Link 48	26	65	32	81
Dako Autostainer plus	6	67	0	0
Dako Autostainer Plus Link	0	0	2	100
Dako Omnis	13	23	13	62
LabVision Autostainer	5	40	2	100
Leica Bond Max	49	51	32	44
Leica Bond-III	38	32	52	67
Menarini - Intellipath FLX	1	100	2	100
None (Manual)	12	17	10	60
Other	0	0	1	0
Shandon Sequenza	2	0	2	50
Ventana Benchmark GX	5	20	5	100
Ventana Benchmark ULTRA	84	37	96	88
Ventana Benchmark XT	50	28	50	92

General Pathology Run: 115	HMB45 or Melan-A only		Ki-67 / MIB1	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	28	32	34	82
BioGenex liquid DBA (HK-124-7K)	1	0	1	0
DAKO DAB+	2	0	1	0
Dako DAB+ Liquid (K3468)	2	50	4	100
Dako DAB+ REAL Detection (K5001)	1	0	0	0
Dako EnVision Plus kits	2	0	3	67
Dako FLEX DAB	34	59	37	76
Dako REAL EnVision K5007 DAB	8	13	5	80
Dako REAL K5001 DAB	2	100	1	0
LabVision DAB	1	100	0	0
Leica Bond Polymer Refine kit (DS9800)	82	38	78	58
menapath xcell kit DAB (MP-860)	1	100	2	100
NOT APPLICABLE	1	100	1	100
Other	16	88	13	92
Sigma DAB (D5637)	1	0	1	0
Sigma DAB (D5905)	0	0	1	100
Ventana DAB	28	43	34	91
Ventana Enhanced Alk. Phos. Red Detection Kit	15	40	0	0
Ventana iview	6	17	3	100
Ventana Ultraview DAB	65	22	83	87

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

Primary Antibody: Dako Omnis GA626 (MIB-1) , 30 Mins, 32 ºC Prediluted
Automation: Dako Omnis
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako Omnis, PH: 6
EAR:
Chromogen: Dako FLEX DAB, 32 ºC., Time 1: 5 Mins
Detection: Dako EnVision FLEX (K8000/10) , 20 Mins, 32 ºC

Ki-67 / MIB1 - Method 2

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

Primary Antibody:	Ventana 790-4286 (clone30-9) , 16 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 standard
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC Prediluted

Ki-67 / MIB1 - Method 3

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

Primary Antibody:	NeoMarkers RM 9106 (clone SP6)
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)

Ki-67 / MIB1 - Method 4

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

Primary Antibody:	Dako M7240 (clone MIB1) , 32 Mins Dilution 1: 100
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	
Chromogen:	Other
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099)

BEST METHODS - Secondary Antibody

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

HMB45 or Melan-A only - Method 1

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

Primary Antibody:	Epitomics AC-0041 (MART-1 EP43) , 20 Mins, amb ºC Dilution 1: 80
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	
Chromogen:	AS PER KIT, Time 1: 8 Mins, Time 2: 3 Mins
Detection:	Leica Bond Polymer AP Red Detection (DS9305) , 30 Mins, AMB ºC

HMB45 or Melan-A only - Method 2

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

Primary Antibody:	Novocastra PA0027 RTU HMB45 Prediluted
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER1 30 mins
EAR:	NOT APPLICABLE
Chromogen:	Other
Detection:	AS PER KIT

HMB45 or Melan-A only - Method 3

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

Primary Antibody:	Dako M7196 (A103) Melan-A , 30 Mins, 20 ºC Dilution 1: 100
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer, PH: 7.6
HMAR:	Dako PTLink, Buffer: High PH TRS, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 20 ºC., Time 1: 10 Mins, Time 2: 10 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 15 Mins, 20 ºC Prediluted

HMB45 or Melan-A only - Method 4

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

Primary Antibody:	Ventana 790-4366 (HMB45) , 20 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 32mins
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

The Breast Hormonal Receptor Module Run 115

Keith Miller and Suzanne Parry

Antigen Assessed:	Oestrogen Receptor (ER)
Tissue Sections circulated:	Composite slide consisting of 3 breast carcinomas with different levels of Oestrogen receptor expression and normal tonsil.
Number of Registered Participants:	281
Number of Participants This Run	264 (94%)

Circulated Tissue
The table below shows the staining characteristics of the tissue sections circulated during Run 115. This composed of three infiltrating ductal carcinomas (IDCs) with differing levels of oestrogen receptor expression along with a section of tonsil. The staining of the breast tumours was characterised using the Leica 6F11, Ventana SP1 and Dako EP1 clones.

Sections	% Positivity	Expected Staining Intensity	Allred/Quick Score
A. IDC	>95%	High	8
B. IDC	11-33%	Mid	4 (The oestrogen receptor expression level varied depending on the serial section received by the laboratory)
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 maximum 'borderline' pass of 12/20

Introduction

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

When examined by immunocytochemical staining, approximately 75% of primary breast cancers express ER-α, and of those approximately 50% co-express PR. Hormone receptor status is a good predictor of response to anti-

oestrogen based treatments such as Tamoxifen and aromatase-inhibitors (Fisher et al). PR expression is under the control of ER-α, and because of this it has been proposed that PR expression in a tumour indicates the presence of functional ER-α (Cui et al). Moreover, it potentially defines a subpopulation of patients with superior response to Tamoxifen (Osborne et al). Conversely, there is evidence that ER-α positive tumours that are PR negative are best treated with aromatase-inhibitors in preference to the ER-antagonists (Cui et al.). Finally, patients whose breast tumours are ER-α negative but PR positive respond equally well to anti-oestrogen based treatments as do those whose tumours are ER-α positive (Ciocca and Elledge). All these factors emphasise the importance of correct staining protocols and validated staining techniques to avoid false ER and/or PR staining (Rhodes et al. and Ibrahim et al.) which can have a direct impact on patient treatment regime.

Choice of Tissue for Assessments

The Run 115 assessment consisted of three invasive breast tumours of varying ER expression level (samples A-C). A tonsil (sample D) was also included and used as a further negative control to help gauge the specificity and sensitivity of the test.

NEQAS tissue samples were tested by staining every 50th serial section from the relevant tissue blocks using the Leica 6F11 clone. Any heterogeneity in the tissue samples was noted and taken into consideration during assessment. In addition samples were tested with the relevant commercial companies to further verify the expected level of staining. This included the Leica 6F11, Dako 1D5/ER-2-123 clones, Dako EP1 and Ventana SP1 clones.

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

Features of Optimal Immunostaining (Figs 1, 2, 3 & 4)

- 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 Sub-Optimal Immunostaining (Figs 5 & 6)

- 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 and fibroblasts

NEQAS Section Assessment Results

264 laboratories submitted their slides for the ER Run 115 assessment. The acceptable pass rate has fallen by 13% since the previous ER run (Run 113) but showed similar results to those of Run 111. Please see table below:

The borderline and failed marks for this assessment were mostly due to weak staining, particularly in the mid-expressing tumour. False positive staining in the negative control was also seen in some sections. These labs therefore failed the assessment. Several other labs failed due to inappropriate non-specific staining and excessive background.

In-House Tissue Assessment Results

99% of participants also submitted their in-house controls for assessment. Overall these showed a similar acceptable pass rate to the NEQAS section. However, more participants received a borderline pass and fewer laboratories failed on their in-house material. The borderline passes were marked down due to excessive cytoplasmic staining or because a mid -expressing tumour was not included as part of their in-house control.

Some laboratories received lower scores on the NEQAS tissue compared to their in-house material. This was mostly due to weak and low expression of the mid-expressing tumour. Several reasons may have caused this; for instance slides may not have been stained soon after receipt, and therefore the expression level of ER may have been reduced due to loss of antigenicity. UK NEQAS advises that slides are stained as soon as possible on arrival to the laboratory.

Another factor to take into consideration is that while the sensitivity of a participant's assay may be acceptable on their own tissue section, the sensitivity of the assay still may not be optimal. A protocol should be in place that will stain effectively on both the NEQAS samples and the laboratory's own samples.

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

Comparing NEQAS Sample Scores and In-house Control Scores.

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

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

Although it is not possible to know the expected staining levels of the participants' in-house controls, assessors do highlight to participants when there are conflicting observations between 'self-assessment' scores compared to the staining observed by the UK NEQAS assessors.

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Acknowledgments

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

Selected Images showing Optimal and Sub-optimal Immunostaining

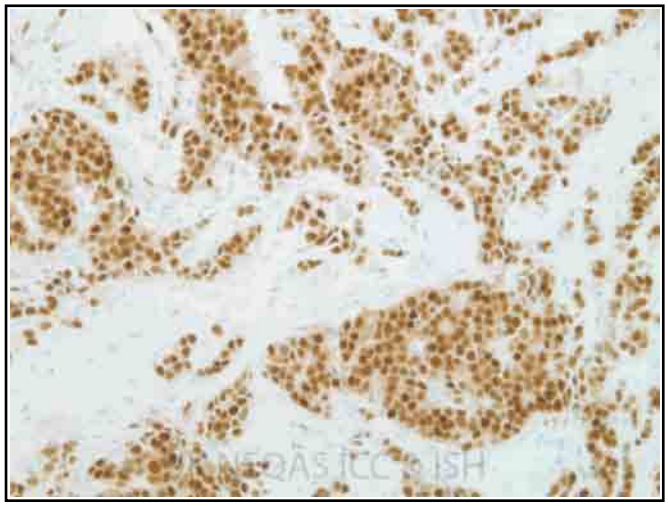


Fig 1. Good demonstration of the high expressing ER positive tumour. The nuclei are stained strongly in over 95% of the tumour cells. The method used was the Ventana (SP1) antibody pre-dilute on the Benchmark Ultra using CC1 standard per-treatment.

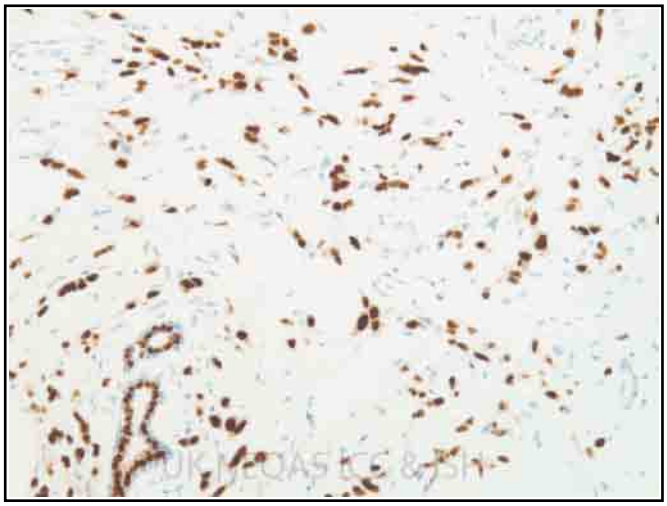


Fig 2. An excellent example demonstrating strong nuclear staining in the tumour cells and clean background in the high expressing ER positive tumour. Staining carried out on the Omnis platform.

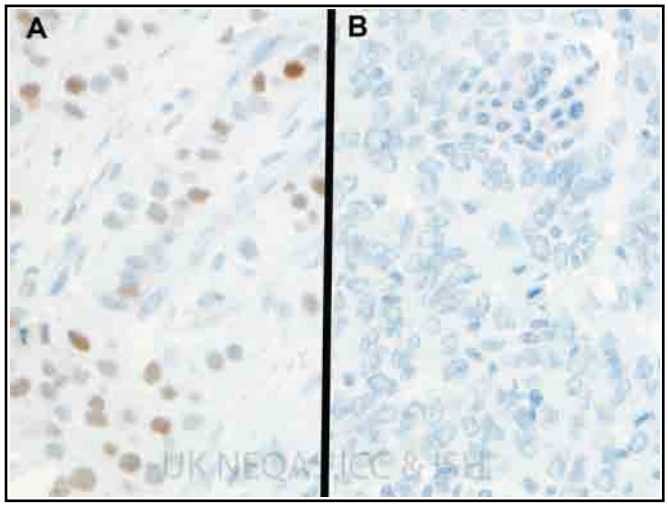


Fig 3 (A&B). Good demonstration of the mid expressing and negative expressing tumour. Staining of the expected proportion of invasive tumour nuclei with the anticipated staining intensity in the mid-expressing tumours and no staining of the tumour cells in the negative ER tumour. The primary antibody used was DAKO (EP1) 1:50 dilution on the Dako autostainer with

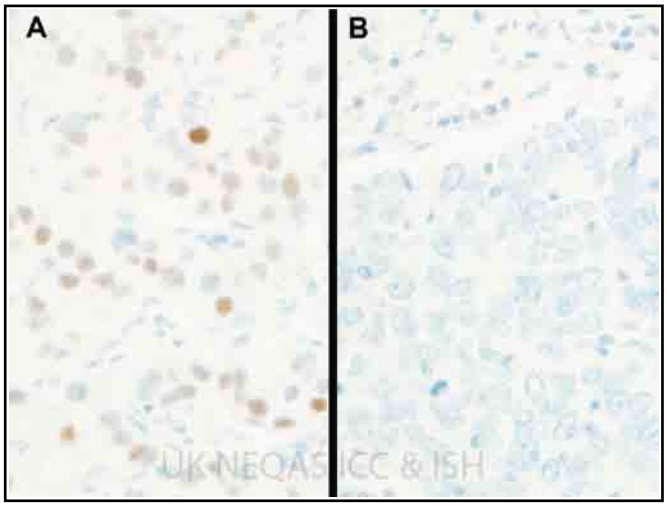


Fig 4 (A&B). Another good example of the correct level of staining in both the mid ER expressing tumour and the negative tumour. The Leica Bond (6F11) primary antibody was used on the Leica Bond Max with ER2 for 30 mins pre-treatment.

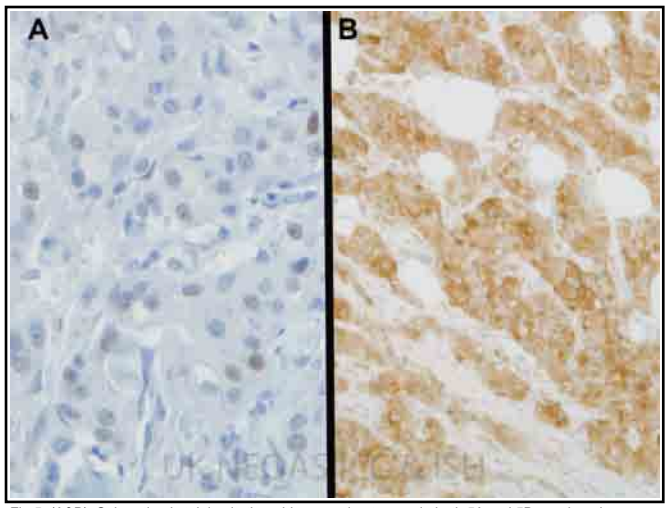


Fig 5. (A&B). Sub-optimal staining in the mid expressing tumour in both 5A and 5B, weak and less than expected number of tumour cells staining (A). Excessive/inappropriate cytoplasmic staining in the mid ER expressing tumour in (B). Section B was stained with the DAKO (1D5) clone 1:50 dilution on the Menarini-Intellipath FLX, pre-treated in Biocare decloaking chamber.

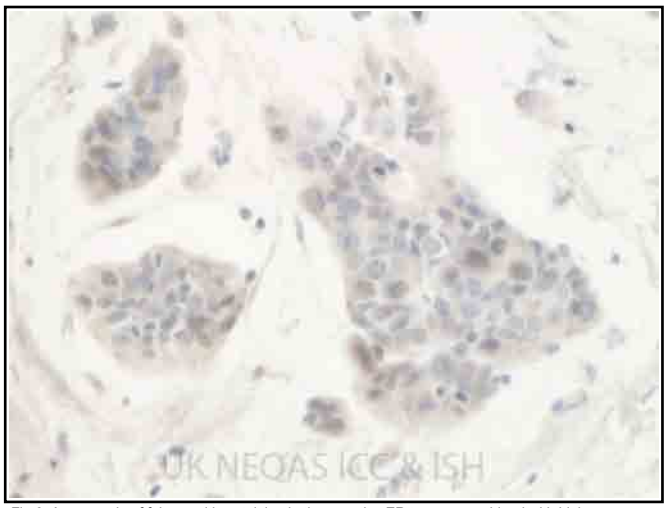
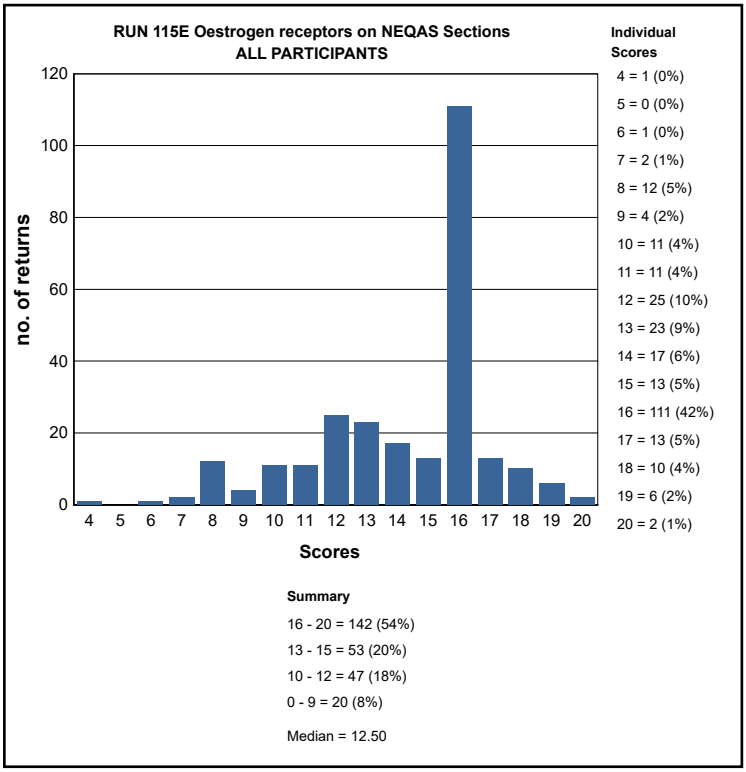
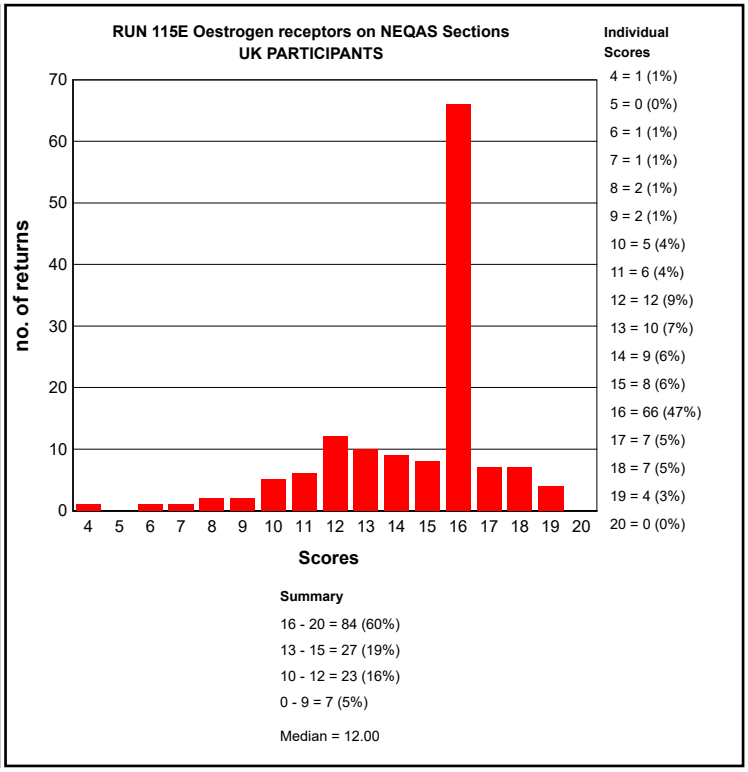
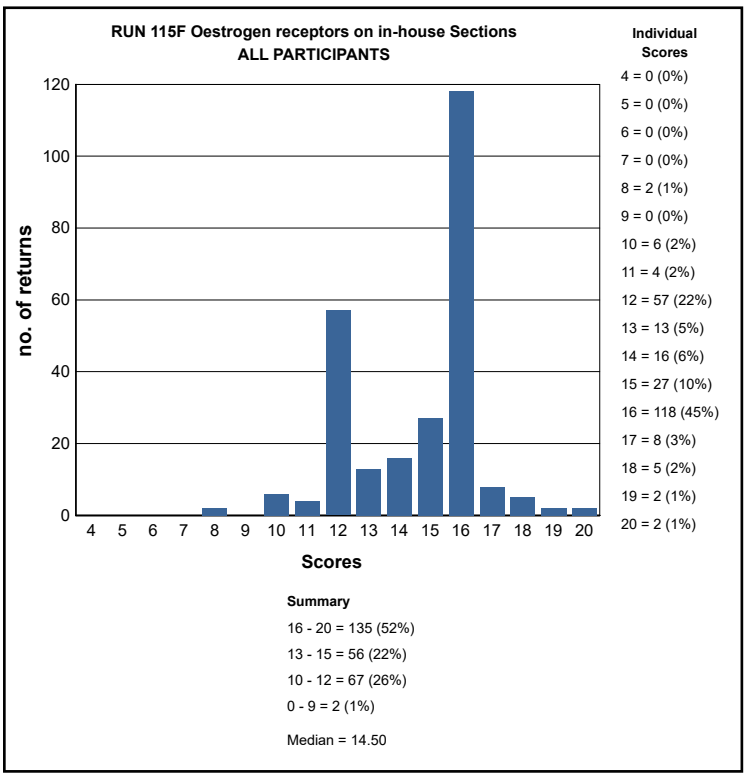


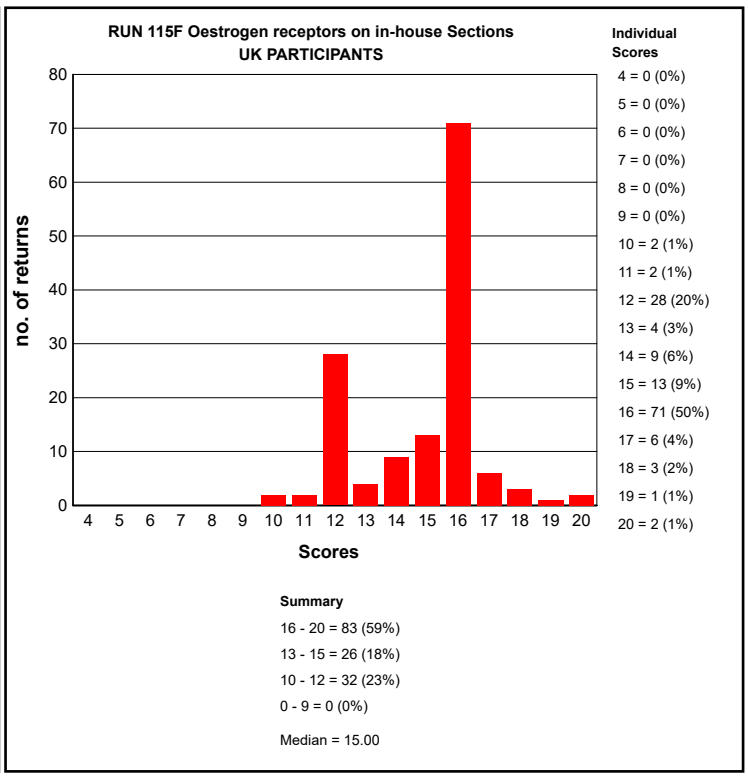
Fig 6. An example of false positive staining in the negative ER tumour, combined with high background staining. The section was stained using the Neomarkers ER (RM910) antibody at a 1:100 dilution on a dako auto-stainer, using a lab vision PT module for pre-treatment.

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: 115

Primary Antibody : Oestrogen receptors

Antibody Details	N	%
Dako M7047 ER (1D5)	4	0
Thermo Fisher/ Neomarkers RM 9101-S (SP1)	14	64
Leica/Novocastra NCL-ER-6F11 (6F11)	6	50
Vector VP-E613/4 (6F11)	2	100
Ventana 250- 2596 ER (6F11)	1	100
Ventana 790-4324 (SP1)	72	88
Leica Bond PA0151 (6F11)	5	60
Dako M3634 (SP1)	3	33
Ventana 790-4325 (SP1)	38	82
Leica/Novocastra NCL-L-ER- 6F11	32	63
Leica/Novocastra RTU-ER-6F11	4	0
Leica/Novocastra NCL-ER-6F11/2	7	57
Dako SK310 pharmDX kit (Link) (1D5 + ER-2-123)	1	100
Dako (EP1) RTU FLEX IR084	24	83
Dako (EP1) M3643	27	78
Dako FLEX (1D5) IR/IS657	1	0
Other	4	25
Dako (EP1) RTU Auto Plus IS084	2	100
Biocare Medical (SP1+6F11) RTU APA 308	1	100
Cell Marque 249-R (SP1)	3	67

Breast Steroid Hormone Receptor Run: 115

Automation	Oestrogen receptors N	Oestrogen receptors %
Dako Autostainer	1	0
Dako Autostainer Link 48	24	63
Dako Autostainer plus	1	0
Dako Autostainer Plus Link	3	67
Dako Omnis	7	86
LabVision Autostainer	3	33
Leica Bond Max	23	57
Leica Bond-III	44	75
Menarini - Intellipath FLX	1	0
None (Manual)	5	40
Shandon Sequenza	1	100
Ventana Benchmark GX	6	100
Ventana Benchmark ULTRA	88	85
Ventana Benchmark XT	51	73

Breast Steroid Hormone Receptor Run: 115

Heat Mediated Retrieval	Oestrogen receptors N	Oestrogen receptors %
Biocare Decloaking Chamber	1	0
Dako Omnis	7	86
Dako PTLink	26	62
Lab vision PT Module	4	25
Leica ER1 20 mins	13	54
Leica ER1 30 mins	15	67
Leica ER1 40 mins	5	80
Leica ER2 10 mins	3	100
Leica ER2 20 mins	25	76
Leica ER2 30 mins	4	25
Leica ER2 40 mins	2	100
Microwave	2	0
Other	2	100
Pressure Cooker	2	100
Ventana CC1 16mins	5	80
Ventana CC1 24mins	3	100
Ventana CC1 32mins	8	38
Ventana CC1 36mins	22	82
Ventana CC1 40mins	2	100
Ventana CC1 48mins	3	100
Ventana CC1 52mins	6	67
Ventana CC1 56mins	3	100
Ventana CC1 64mins	38	89
Ventana CC1 76mins	1	100
Ventana CC1 88mins	1	0
Ventana CC1 92mins	1	0
Ventana CC1 extended	4	50
Ventana CC1 mild	14	79
Ventana CC1 standard	27	85
Ventana CC2 64mins	1	100
Ventana CC2 mild	2	100
Water bath 95-98 OC	5	60

Breast Steroid Hormone Receptor Run: 115

Enzyme Mediated Retrieval	Oestrogen receptors N	Oestrogen receptors %
AS PER KIT	5	80
NOT APPLICABLE	167	74
Ventana Protease 1 (760-2018)	2	100

Breast Steroid Hormone Receptor Run: 115

Detection	Oestrogen receptors	
	N	%
AS PER KIT	10	60
Dako EnVision FLEX (K8000/10)	6	33
Dako EnVision FLEX+ (K8002/12)	18	72
Dako Envision HRP/DAB (K5007)	4	100
Dako Envision+ HRP mouse K4004/5/6/7	1	100
Dako REAL HRP/DAB (K5001)	1	0
LabVision UltraVision LP HRP (TL 125 HLJ)	1	0
Leica Bond Polymer Refine (DS9800)	63	70
MenaPath X-Cell Plus (MP-XCP)	1	0
None	1	100
NOT APPLICABLE	2	50
Novocastra Novolink PDS (RE7-140/150/280/290-K)	1	100
Other	6	33
Ventana iView system (760-091)	3	67
Ventana OptiView (760-700) + Amp. (7/860-099)	1	0
Ventana OptiView Kit (760-700)	18	78
Ventana UltraView Kit (760-500)	119	82

Breast Steroid Hormone Receptor Run: 115

Chromogen	Oestrogen receptors	
	N	%
AS PER KIT	19	74
BioGenex liquid DBA (HK-124-7K)	1	0
Dako DAB K3468	1	0
DAKO DAB+	2	100
Dako EnVision Plus kits	2	50
Dako FLEX DAB	24	63
Dako REAL EnVision K5007 DAB	3	100
Dako REAL K5001 DAB	1	0
Leica Bond Polymer Refine kit (DS9800)	64	70
menapath xcell kit DAB (MP-860)	1	0
Other	9	33
Ventana DAB	9	89
Ventana Enhanced Alk. Phos. Red Detection Kit	1	100
Ventana iview	3	67
Ventana Ultraview DAB	118	82

BEST METHODS

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

Oestrogen receptors - Method 1

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

Primary Antibody: Leica/Novocastra NCL-L-ER- 6F11 , 15 Mins Dilution 1: 1/50
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER1 30 mins
EAR: NOT APPLICABLE
Chromogen: Leica Bond Polymer Refine kit (DS9800)
Detection: Leica Bond Polymer Refine (DS9800)

Oestrogen receptors - Method 2

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

Primary Antibody: Ventana 790-4325 (SP1) , 20 Mins, 37 ºC Dilution 1: prefilled
Automation: Ventana Benchmark XT
Method: Ventana UltraView DAB
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 standard
EAR:
Chromogen: Ventana Ultraview DAB
Detection: Ventana UltraView Kit (760-500)

Oestrogen receptors - Method 3

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

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

Oestrogen receptors - Method 4

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

Primary Antibody:	Ventana 790-4324 (SP1) , 32 Mins, 23 ºC Prediluted
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) Prediluted

The Breast HER2 ICC Module Run 115

Keith Miller, Suzanne Parry and Dawn Wilkinson

Antigen Assessed:	HER2
Samples Circulated:	Xenografts and Human Breast Cancer Tumour Samples
Number of Registered Participants:	301
Number of Participants this Run	282 (93%)

Circulated Material:
The table below demonstrates the staining patterns looked for in the human breast xenograft and human breast tumour samples circulated during Run 115 assessments. This composed of 3 xenograft samples and 1 human breast tissue of differing Her2 expression level. The staining of the breast material was characterised using the Ventana 4B5, Leica Oracle and Dako HerceTest assays.

Xenograft / Human breast tumour position (slide label end)	Assessment of Xenograft & Human Breast Tumour Staining Pattern
A: 3+ IHC (Xenograft tissue) FISH amplified	Strong complete cell membrane staining
B: 2+ IHC (Human breast tumour) FISH equivocal	Weak to moderate complete cell membrane staining of over 10% of tumour cells
C: 2+ IHC (Xenograft tissue) FISH amplified	Weak to moderate complete cell membrane staining of over 10% of tumour cells
D: Negative IHC (Xenograft tissue) FISH non-amplified	No staining in the negative xenograft

Please Note: Multiple tissue blocks are compiled from the same cases, however, there may on occasion be variability in staining from the 'Her2 score shown above. This is taken into consideration when scoring participants slides and also noted prior to assessments when staining every 25th sample for quality control purposes.

Scoring Guideline Used in the Assessment of Slides

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 an overall score out of 20. See table below:

Score and Interpretation	Interpretation
16-20/20: Excellent	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 acceptable and suitable for interpretation
10-12/20: Borderline Acceptable	Overall the samples are borderline interpretable (still clinically relevant) indicating that technical improvements need to be made. Marks may have been deducted due to: Weaker/stronger staining than expected, cytoplasmic staining, morphological damage
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 membrane staining, excessive cytoplasmic staining, morphological damage, staining of normal glands

Introduction

HER2 immunohistochemistry has been routinely used as a predictive marker in breast cancer for more than 10 years. Patients with HER2 positive tumours generally have a poor overall prognosis. However, patients with metastatic disease showed an improved rate of survival when the humanized monoclonal antibody Trastuzumab (Herceptin) was given alone or added with chemotherapy treatment (Slamon et al., 1998). Herceptin, in the metastatic setting was made available in the UK in 2000 by the UK National Institute for Clinical Excellence (NICE). In 2005, Herceptin in the adjuvant setting was also shown to be effective in primary breast cancers by reducing the risk of recurrence and mortality (Wolff et al., 2006, and Walker et al., 2008. 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 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 carried out by laboratories and provides feedback to help improve the analytical phase of patient diagnosis.

Recommended HER2 testing guidelines are: The ASCO/CAP guidelines by Wolff et al.(2007), UK guidelines by Walker et al., (2008), 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 are having problems in finding appropriate invasive control material. It is therefore acceptable to submit DCIS tissue showing differing levels of membrane staining as an alternative. However, laboratories must indicate on their datasheet which component they have scored otherwise the invasive component (if present) will be assessed.

Choice of Material For Run 115

For the Run 115 assessment it was decided to use validated human breast tumour xenograft samples alongside a Her2 positive human breast tumour tissue. These samples were used as an alternative to the UK Neqas Her2 cell lines which are traditionally used for the assessment. The reason for using the xenorgrafts and human breast tissue for this run was because there have been a number of requests from both participants and assessors to use tissue to more accurately reflect what is being tested in the clinical setting.

As part of the validation of the material chosen for Run 115, the xenograft samples had previously been tested alongside the cell lines in the Run 113 assessment. Results showed there to be a good correlation with the expression levels of the gold standard cell lines. The validation process also included staining the material with the commercial company assays, such as the Ventana 4B5 pathway, Leica Oracle and the Dako HercepTest assay. Stability studies of these samples also showed no change of HER2 expression level compared to the Gold standard when the slides had been handled and stored correctly. This includes staining the sections soon on arrival to the laboratory as recommended for all biomarker testing.

FISH testing was also carried out on the samples at differing levels throughout the blocks used: The 2+ IHC xenograft sample was amplified, while the human breast tumour 2+ IHC samples was an equivocal case by FISH. Every 25th serial section was stained by our host laboratory and validated before the unstained slides were sent to participants. Gold standards were also stained by the commercial companies with their FDA-/CE-IVD approved assays for Her2.

NEQAS Section Assessment Results

For the run 115 assessment 63% (n=166) of participants obtained an acceptable pass rate, lower than the past 2 runs, but comparable to the average pass rate of 65% over the past 5 runs. The borderline rate was 19% (n=50), which is slightly higher than the average borderline pass of 17% over the past 5 runs. 18% (n=47) failed the assessment for run 115, which is comparable to the unacceptable average rate over the previous 5 runs for the cell lines (18%). See summary table below:

Summary

NEQAS Pass Rates Run 110-115:						
	Run 115	Run 114	Run 113	Run 112	Run 111	Run 110
Acceptable	63%	70%	71%	62%	56%	68%
Borderline	19%	15%	11%	19%	20%	18%
Unacceptable	18%	14%	18%	19%	24%	14%

The UK Neqas ICC scheme has been using traditional cell lines since the inception of the Her2 module, and although the cell lines are a robust and standardised model. It is important for the scheme to build on it's biobank and to ensure it has a choice of relevant samples that can reflect primary tumours in nature. Such samples are required to provide Her2 assessment, enabling UK Neqas ICC & ISH to accurately monitor laboratories who are routinely carrying out bio-marking for the prediction of tumour response to patient treatment. It is becoming increasingly more difficult to acquire appropriate invasive human control material with the range of Her2 expression levels. So UK Neqas ICC & ISH has been looking into other tumour models that will also conserve the original tumour characteristics, such as the architecture, tumour vasculature, heterogeneity, bio-molecular signature, and malignant pheno- and genotypes.

Evidence gathered from the validation of the samples used in run 115 against the traditional cell lines suggested that the human breast tumour and xenograft sections were robust enough to use for EQA purposes. The stability testing also showed the xenografts and human breast tumour sections to be stable if the correct storing and handling procedures were carried out by laboratories. However, at assessment it was felt that some laboratories showed signs of lower antigenicity and a decrease in their standard of staining compared to previous assessment runs. This could be due to laboratories not staining their slides soon on arrival to the laboratory: Neqas ICC and ISH recommend staining slides as soon as possible on receipt into the laboratory and to treat the Neqas material as a routine diagnostic case, adhering to the tight turnaround times you have for your diagnostic biomarker cases. Loss of antigenicity is well documented in the literature with certain antigens. Furthermore, slides should not be left on the bench for several weeks before staining. If slides need to be stored for a few days they should be stored in a fridge, ideally within a box with a desiccant to avoid moisture to the uncut sections. UK Neqas ICC and ISH follow strict timelines for cutting and sending out 'biomarker material' and ensure correct handling and storage procedures are carried out before dispatch. The scheme also continually monitors the stability of the Neqas material and carry out regular audits, introducing quality improvements where necessary.

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

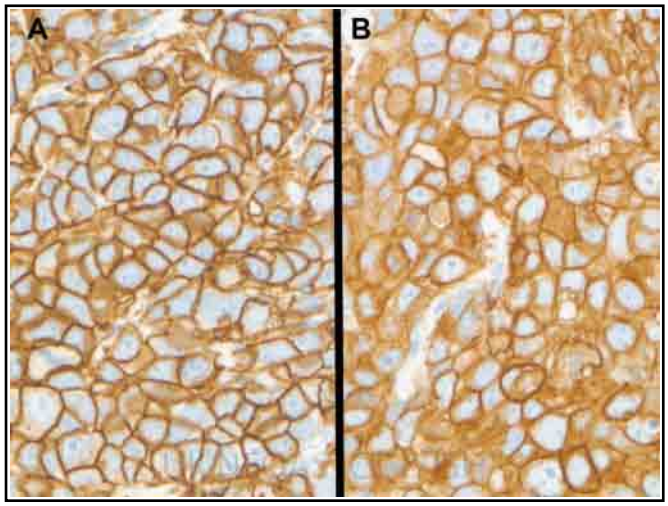


Fig 1. Two examples showing optimal staining of the UK NEQAS 3+ expressing xenograft sample (A). Both sections show complete strong membrane staining. (A) stained with the Ventana 4B5 Confirm on the Benchmark XT, CC1 retrieval. (B) stained with the Dako HerCepTest on the Autostainer with retrieval in the PT Link.

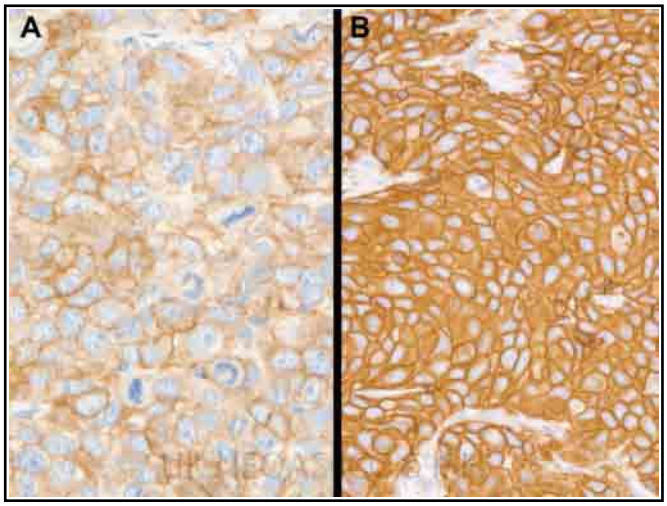


Fig 2. Two examples of sub-optimal staining of the UK NEQAS 3+ xenograft sample (A). Section A shows weak diffuse staining, while the staining in B has excessive cytoplasmic staining. A stained with the Leica Oracle platform, and B stained with the Novocastra CB11 clone on the Labvision autostainer, with pre-treatment in the PT module.

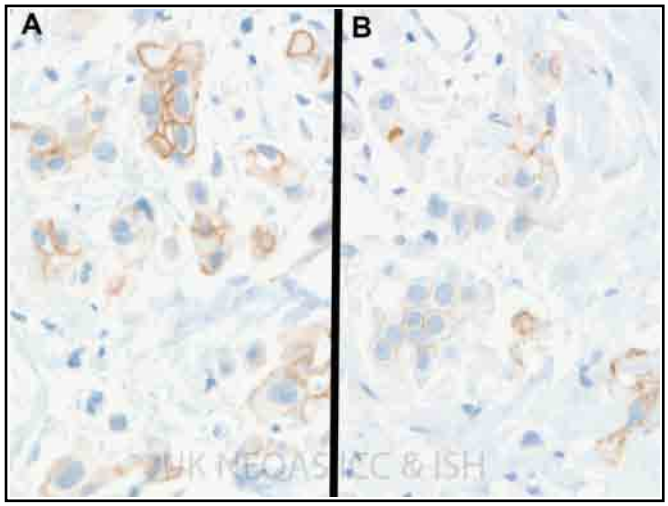


Fig 3. Two examples of acceptable staining on the UK NEQAS 2+ human breast tumour sample (B). Both sections show complete weak to moderate membrane staining . A stained with the Ventana 4B5, and B stained with the Dako HerCepTest (same protocols as Fig 1).

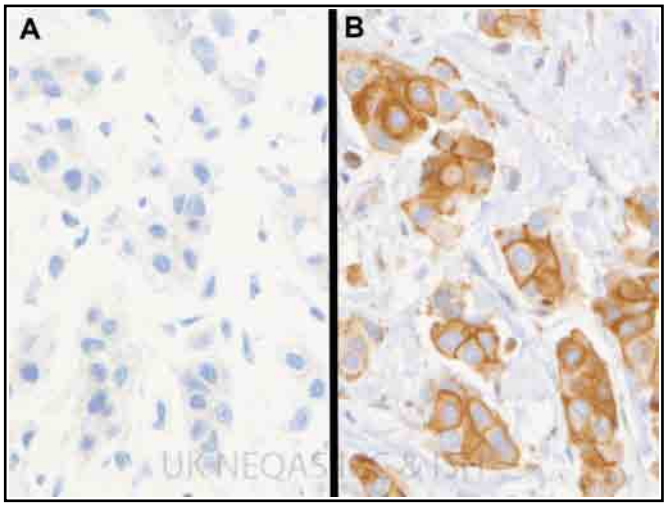


Fig 4. Two examples showing poor demonstration of the UK NEQAS 2+ human breast tumour sample (B) . The staining in A is very weak and therefore appears as negative, while the staining in B shows excessive cytoplasmic staining (same protocols as Fig 2).

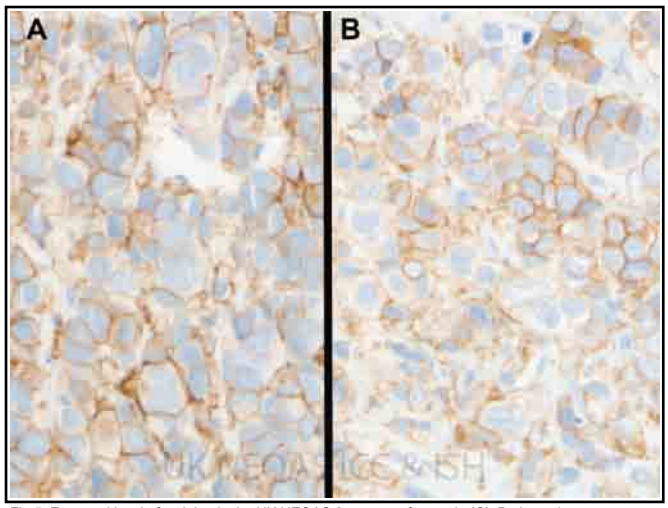


Fig 5. Expected level of staining in the UK NEQAS 2+ xenograft sample (C). Both sections show complete membrane staining with moderate intensity. A stained with the Ventana 4B5, and B stained with the Dako HerCepTest.

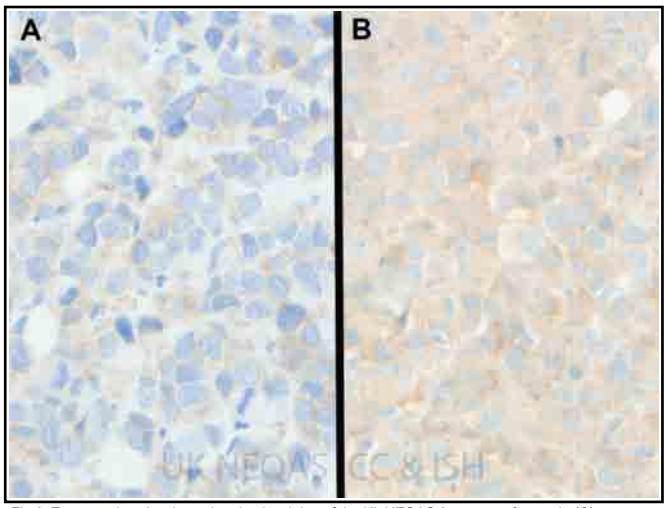


Fig 6. Two samples showing suboptimal staining of the Uk NEQAS 2+ xenograft sample (C). The staining in A is weak, while the staining in B shows poor localisation. Both sections stained using lab-devised methods.

Selected Images showing Optimal and Sub-optimal Immunostaining

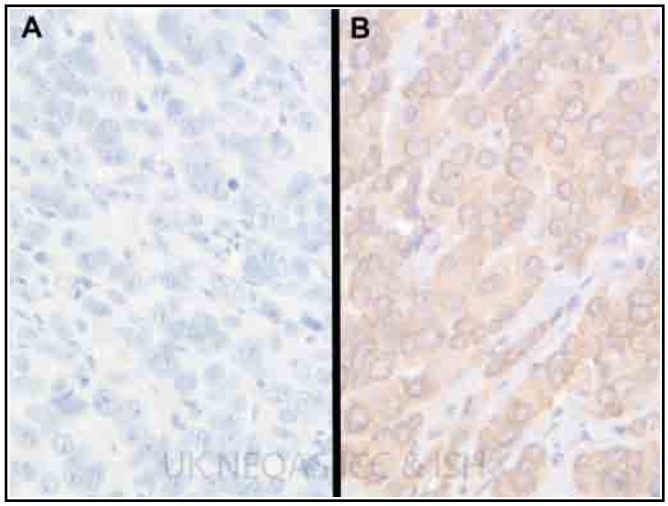


Fig 7. Section A shows the expected level with no staining in the UK NEQAS negative sample (D); stained with the Ventana 4B5 assay. Section B shows unacceptable non-speicifc staining of the negative UK NEQAS sample (D); stained using the Novocastra CB11 antibody on the Labvision autostainer,pretreatment in the PT link.

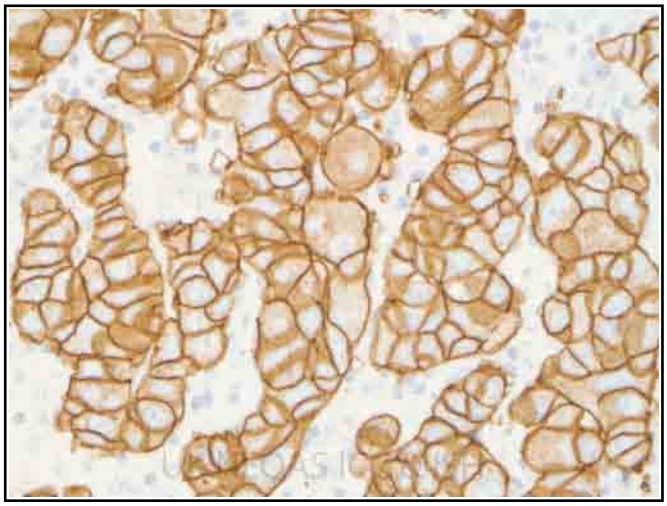


Fig 8. Good example of an in house 3+ sample stained with HER2. The section shows strong crisp membranous staining; carried out on the Ventana ULTRA platform with the 4B5 Pathway assay.

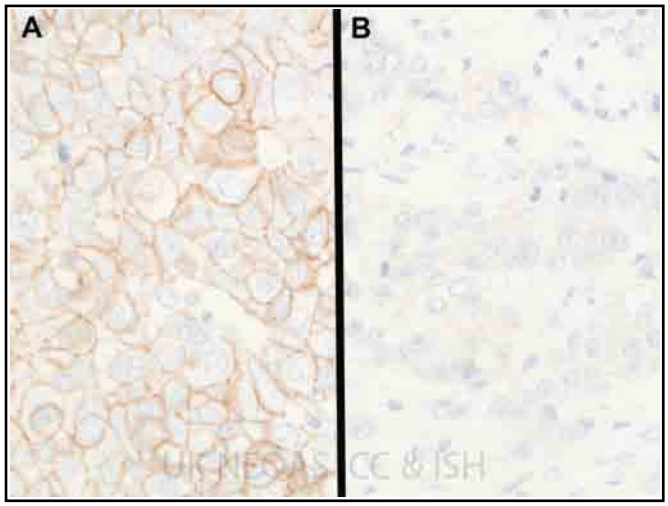


Fig 9. Good example of in-house controls and staining with the Ventana 4B5 assay. A shows 2+ expression, while b is negative for HER2 (same particiapnt as Fig 8).

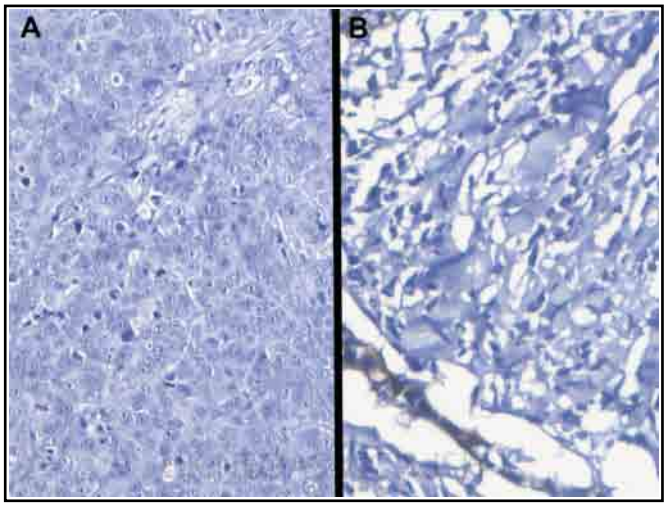
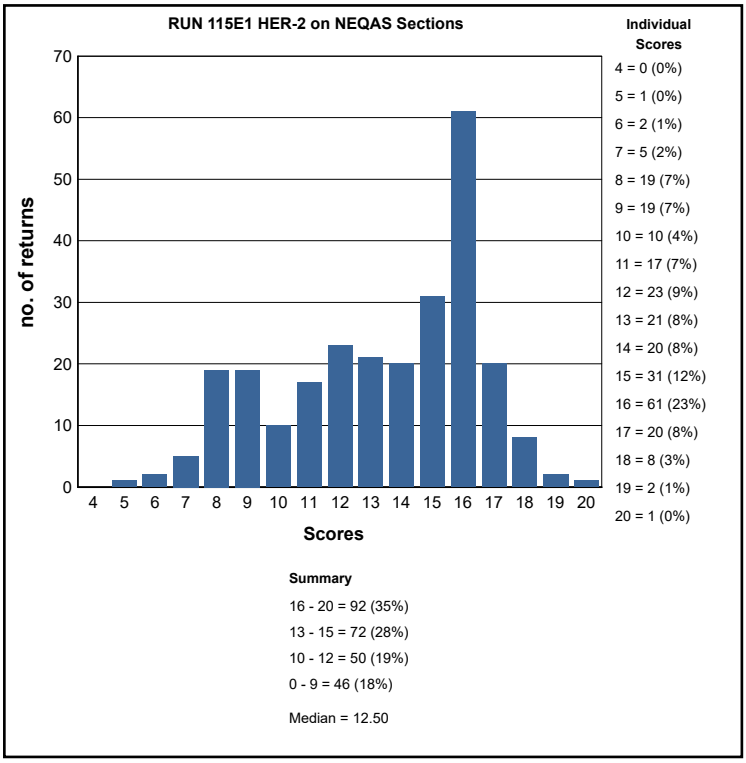
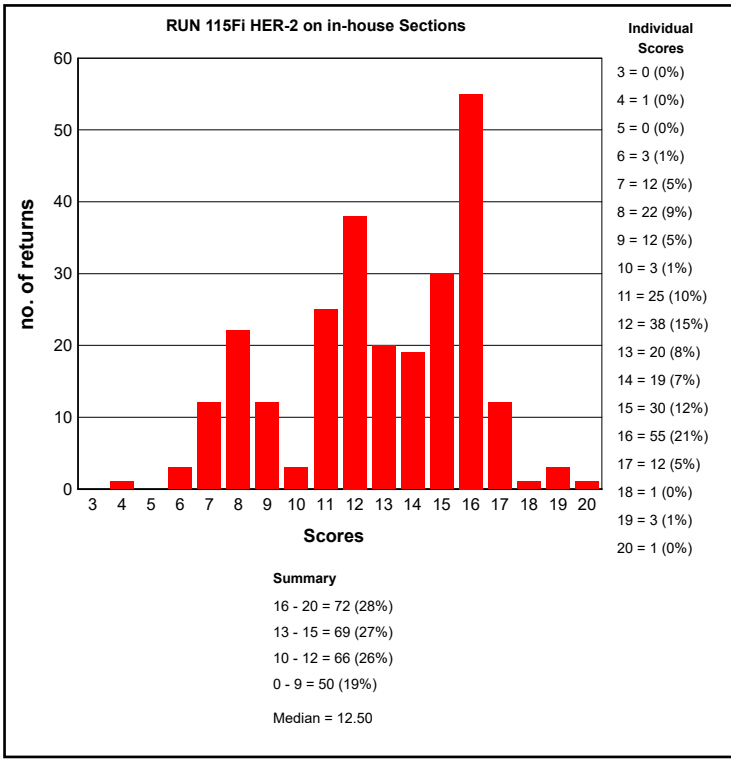


Fig 10. Examples of poor tissue and staining on a partaiciapnts in-house material: The counterstain is excessive, and therefore masking any membrane staining. The tissue is also poorly fixed, making a interpretation unreliable.

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: 115

Primary Antibody	N	%
Dako HercepTest K5204 (poly)	3	100
Dako HercepTest K5205 (poly)	1	0
Dako HercepTest K5207 (poly)	1	0
Dako A0485 C-erB-2 (poly)	23	39
Cell Marque CMA 601 (CB11)	2	0
Ventana Pathway 790-100 (4B5)	13	77
Labvision / Neomarkers RM-9103 (SP3)	2	50
Biogenex AM134-5M (CB11)	1	0
Leica Oracle HER2 Bond IHC (CB11)	14	21
Dako Link HercepTest SK001 (poly)	8	88
BioGenex (EP1045Y) rb mono	1	100
Ventana Confirm 790-4493 (4B5)	43	72
Ventana Pathway 790-2991 (4B5)	127	75
Novocastra NCL-L-CB11 (CB11)	4	0
Biocare CME 342 A,B (EP1045Y)	5	40
Other	5	20
Cell Marque 237R (SP3)	1	0

Breast HER2 ICC Run: 115

Automation	N	%
BioGenex GenoMX 6000i	1	0
Dako Autostainer	1	0
Dako Autostainer Link 48	13	62
Dako Autostainer plus	2	50
Dako Autostainer Plus Link	3	67
Dako Omnis	2	50
LabVision Autostainer	1	0
Leica Bond Max	13	23
Leica Bond-III	15	33
None (Manual)	11	18
Other	2	50
Shandon Sequenza	1	100
Ventana Benchmark GX	13	77
Ventana Benchmark ULTRA	93	76
Ventana Benchmark XT	82	70

Breast HER2 ICC Run: 115

Heat Mediated Retrieval	N	%
Biocare Decloaking Chamber	2	50
Dako Omnis	3	67
Dako Pascal	1	0
Dako PTLink	15	60
Lab vision PT Module	2	50
Leica ER1 10 mins	2	50
Leica ER1 20 mins	9	22
Leica ER1 25 mins	12	17
Leica ER1 30 mins	1	0
Leica ER1 40 mins	1	100
Leica ER2 10 mins	1	100
Leica ER2 30 mins	1	100
Microwave	5	0
None	4	50
Other	3	67
Pressure Cooker	1	0
Ventana CC1 16mins	5	60
Ventana CC1 20mins	3	0
Ventana CC1 24mins	1	0
Ventana CC1 32mins	16	81
Ventana CC1 36mins	46	76
Ventana CC1 40mins	2	100
Ventana CC1 48mins	1	0
Ventana CC1 52mins	3	100
Ventana CC1 56mins	7	57
Ventana CC1 64mins	6	100
Ventana CC1 72mins	1	100
Ventana CC1 8mins	3	67
Ventana CC1 92mins	1	100
Ventana CC1 mild	67	67
Ventana CC1 standard	15	87
Ventana CC2 36mins	1	100
Water bath 95-98 OC	8	63

Breast HER2 ICC Run: 115

Detection	N	%
AS PER KIT	17	53
Biocare SLAB (STU HRP 700H,L10)	1	0
BioGenex SS Polymer (QD 420-YIKE)	1	0
Dako HerCep Test (K5204)	2	100
Dako EnVision FLEX (K8000/10)	4	25
Dako EnVision FLEX+ (K8002/12)	8	25
Dako Envision HRP/DAB (K5007)	4	50
Dako HerCep Test Autor (SK001)	6	83
LabVision UltraVision ONE Polymer (TL-012/5-HDJ/T	1	0
Leica Bond Polymer Define (DS9713)	1	100
Leica Bond Polymer Refine (DS9800)	16	31
Other	7	43
Ventana iView system (760-091)	5	60
Ventana OptiView Kit (760-700)	10	60
Ventana UltraView Kit (760-500)	163	75

Breast HER2 ICC Run: 115

Enzyme Retrieval	N	%
AS PER KIT	17	41
NOT APPLICABLE	132	66
Ventana Protease	1	0
Ventana Protease 1 (760-2018)	2	100

Breast HER2 ICC Run: 115

Chromogen	N	%
AS PER KIT	38	53
BioGenex Liquid DAB (HK153-5K)	1	0
BioGenex liquid DBA (HK-124-7K)	1	0
DAKO DAB+	1	100
Dako DAB+ Liquid (K3468)	1	0
Dako DAB+ REAL Detection (K5001)	1	0
Dako EnVision Plus kits	1	0
Dako FLEX DAB	13	54
Dako REAL EnVision K5007 DAB	4	50
Leica Bond Polymer Refine kit (DS9800)	19	32
Other	8	38
Ventana DAB	7	43
Ventana iview	3	100
Ventana Ultraview DAB	156	76

BEST METHODS

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

HER-2 - Method 1

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

Primary Antibody:	Dako Link HercepTest SK001 (poly) , 30 Mins Prediluted
Automation:	Dako Autostainer Plus Link
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: Dako Citrate buffer, PH: 6
EAR:	
Chromogen:	Dako FLEX DAB, Time 1: 10 Mins, Time 2: 10 Mins
Detection:	Dako HerCep Test Autor (SK001) , 30 Mins Prediluted

HER-2 - Method 2

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

Primary Antibody:	Ventana Pathway 790-2991 (4B5) , 16 Mins 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)

HER-2 - Method 3

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

Primary Antibody:	Ventana Pathway 790-2991 (4B5) , 16 Mins
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.5
HMAR:	Ventana CC1 mild
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB, Time 1: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins

HER-2 - Method 4

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

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

The Gastric HER2 ICC Module Run 115

Suzanne Parry and Dawn Wilkinson

Antigen Assessed:	**HER2 for IHC**
The NEQAS distributed sections consisted of a composite slide containing 4 surgical resection samples of intestinal gastric carcinoma:	**The expected IHC expression levels of each tissue sample were:** **A.** 0 **B.** 1+ or 0 or 2+ depending on the serial section received **C.** 1+ or 0 **D.** 3+
Number of Registered Participants	74
Number of Participants this Run	58 (78%)

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

Score	Surgical resection samples: 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).

Table 2: HER2 IHC Staining & ISH Results

Section From Slide Label End	Staining Pattern with IHC	HER2 status by ISH
A	**0**	Non-Amplified
B	**1+ or 0 (or 2+)**	Non-Amplified (Amplified)
C	**2+**	Amplified
D	**3+**	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 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. Patients. More recently the Trastuzumab for Gastric Cancer (ToGA) study, which investigated Trastuzumab in HER2 positive advanced gastric cancer (Bang et al., 2010) showed overall median survival of nearly 3 months. Similar to breast cancer, the ToGA trial showed an increased benefit from Trastuzumab treatment for patients showing 3+ IHC and IHC2+/FISH+ expression. The initial development of the HER2 scoring criteria was developed as a precursor to the ToGA trial (Hoffman et al., 2008) with the study group modifying the breast HER2 IHC scoring algorithm to compensate for the incomplete membrane staining and greater tumour heterogeneity seen in gastric cancers. A different scoring system was also established for resection and core biopsies as illustrated in table 1. A more recent article by Rüschoff et al., (2010) has validated the scoring procedure further with a detailed approach to

'stepwise' HER2 IHC scoring in gastric cancers. The article also compared the Ventana 4B5 assay with the HercepTest and indicated "a tendency towards higher sensitivity for 4B5 detecting positive HER2 amplification" but "there was no difference between both test platforms with respect to the ISH-negative cases, with the one equivocal case testing negative at some sites and as positive at others". Rüschoff and colleagues also used both fluorescent (Dako PharmDX or PathVysion Vysis) and chromogen (Ventana DDISH) to confirm their IHC findings.

Assessment Results

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

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

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

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

Additional Comment:

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

Pass Rates

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

NEQAS Pass Rates Run 115:	
Acceptable	85% (N=49)
Borderline	9% (N=5)
Unacceptable	7% (N=4)

There is continued improvement in acceptable pass rate of 85% for Run 115 up from Run 114 (83%) Run113 of 73% and a vast improvement from Run 112 which was 65%. The borderline rates are back in line with runs 110 and 111, compared to the last 2 runs which saw approx. 10% increase for Run 112 &113. 4 labs (7%) still failed the assessment, and this was due to either weak membrane staining or over-staining. In the clinical setting false negative results will lead to suitable patients not being put forward for Herceptin therapy, and equally concerning is that many patients may be over-treated: For instance, a HER2 IHC result of 3+ will automatically mean that the patient is put forward for Herceptin therapy. Also, as IHC 2+ cases are also reflex tested, incorrect over-staining could mean that more samples than necessary are being put forward for in-situ hybridisation reflux 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 been the cause of either weaker or stronger membrane staining than expected.

All participants apart from 2 labs also submitted their in-house controls. The in-house results are summarised in the following table:

In-House Pass Rates Run 115:	
Acceptable	78% (N=43)
Borderline	22% (N=12)
Unacceptable	0% (N=0)

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

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 90% of participants with an average acceptable pass rate of 81%, 5 % of the participants used the Dako Hercept test with 100% acceptable pass rate.

Control Tissue and Recommendations

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

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

References:

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

Acknowledgments

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

Selected Images showing Optimal and Sub-optimal Immunostaining

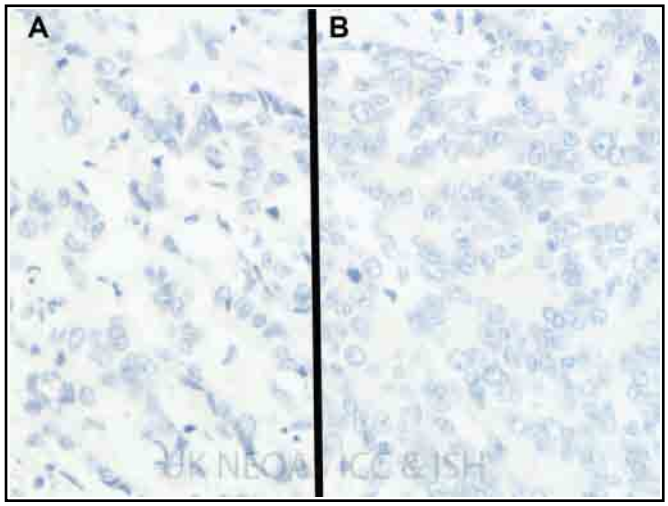


Fig 1. Expected HER2 negative UK NEQAS gastric tumour samples A and B. Sample B showed features of 1+ membrane staining. Both sections stained on the with the Ventana 4B5 assay on the ULTA Benchmark with CC1 reteival for 64 minutes.

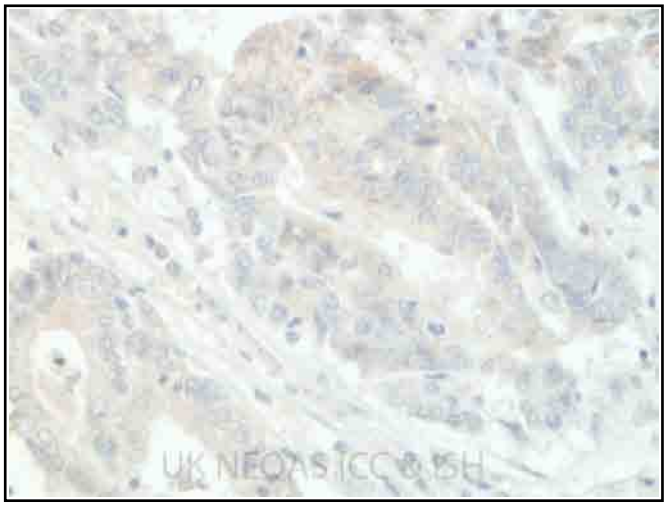


Fig 2. Inappropriate non-specific and cytoplasmic staining seen in the UK NEQAS negative sample A. This assay was carried out using a lab-devised method with the Neomarlers SP3 antibody and pre-treated in the Labvision PT Link.

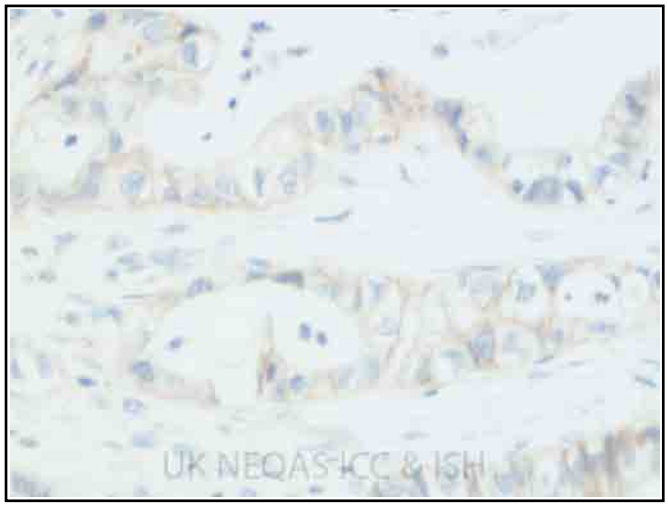


Fig 3. UK NEQAS distributed 2+ gastric tumour (Sample C), showing the expected level of 2+ membrane staining. (Stained with the same method in Fig 1).

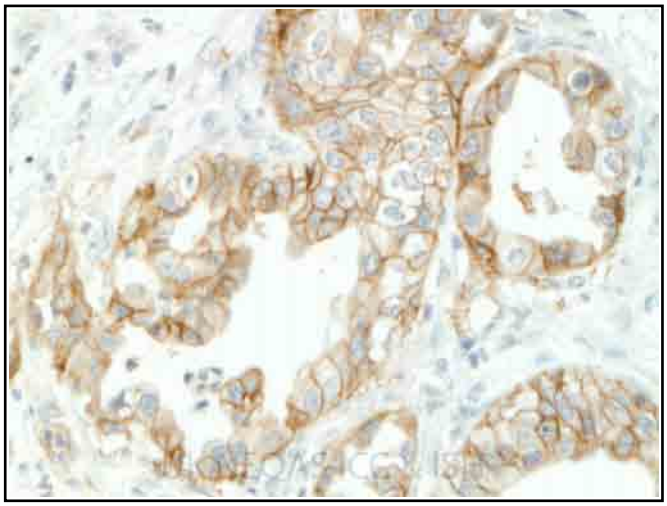


Fig 4. Unacceptable deomstration of HER2 in the UK NEQAS distributed sample C (compare to Fig 3). The sample should be 2+, but the stainin in the example is too high and more representative of 3+ staining. (Same method as Fig 2).

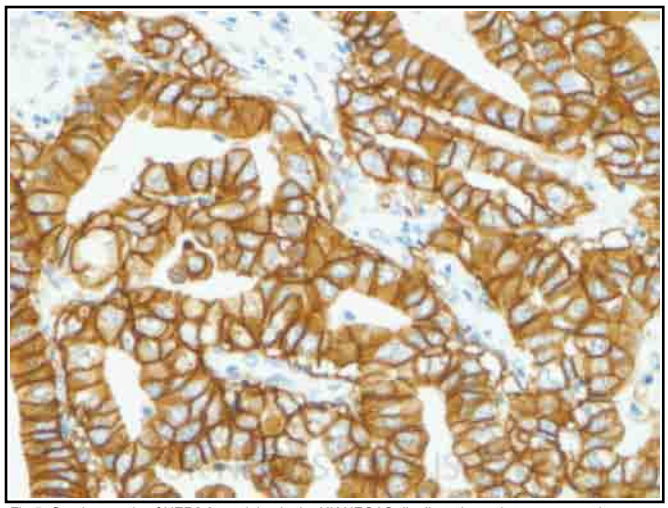


Fig 5. Good example of HER2 3+ staining in the UK NEQAS distributed gastric tumour sample D, showing the expected level of complete intense membrane staining. Section stained with Ventana Pathway 4B5 following recommended protocols.

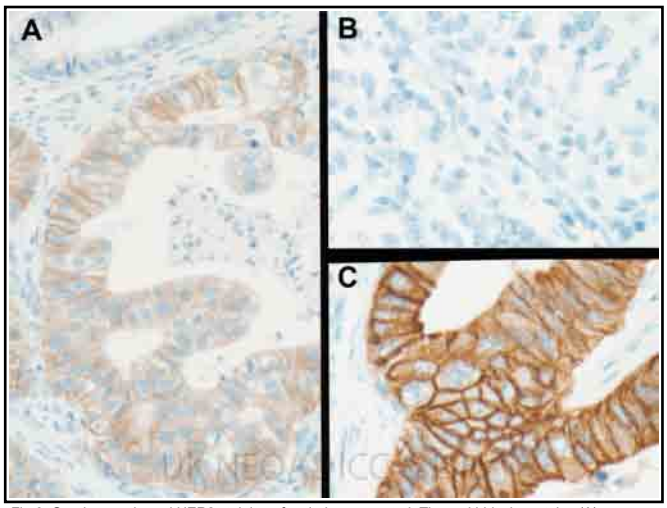
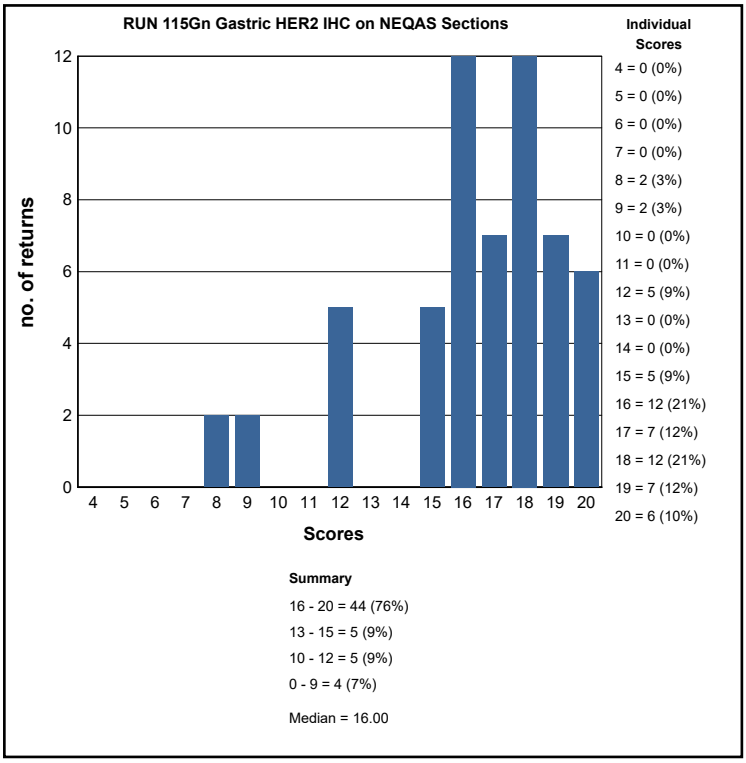
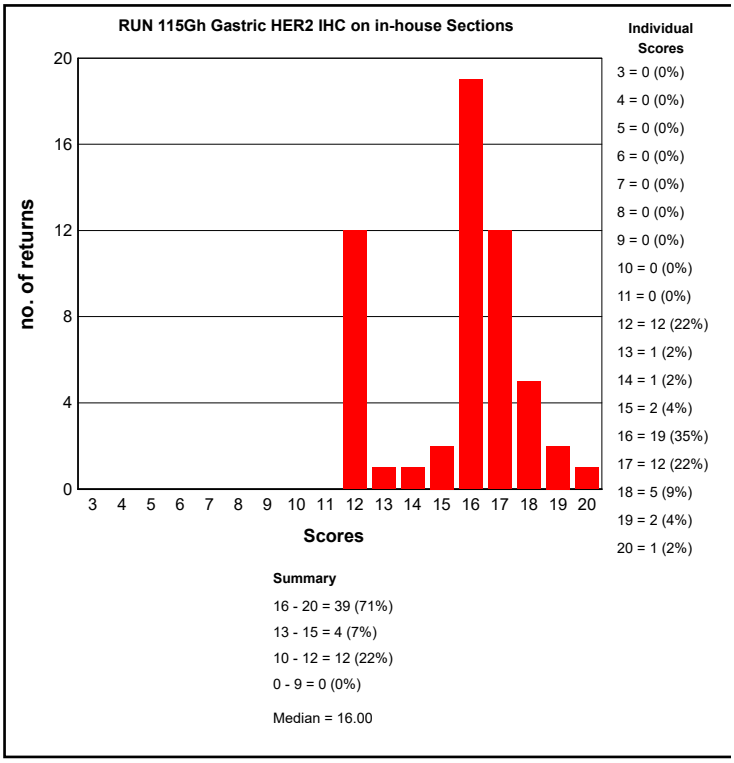


Fig 6. Good example and HER2 staining of an in-house control. The multi-block contains (A) 2+, (B) negative, and (C) 3+ expressing gastric tumours. Stained with the Ventana 4B5 assay on the Benchmark XT, CC1 mild retrieval.

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: 115

Primary Antibody	N	%
Dako A0485 C-erB-2 (poly)	1	100
Labvision / Neomarkers RM-9103 (SP3)	1	0
Dako Link HercepTest SK001 (poly)	3	100
Ventana Pathway 790-100 (4B5)	3	67
Ventana Pathway 790-2991 (4B5)	35	83
Ventana Confirm 790-4493 (4B5)	14	93

Gastric HER2 ICC Run: 115

Automation	N	%
Dako Autostainer Link 48	1	100
Dako Autostainer plus	1	0
Dako Autostainer Plus Link	2	100
Dako Omnis	1	100
Leica Bond Max	1	100
Ventana Benchmark GX	2	100
Ventana Benchmark ULTRA	27	85
Ventana Benchmark XT	22	82

Gastric HER2 ICC Run: 115

Heat Mediated Retrieval	N	%
Dako Omnis	1	100
Dako PTLink	3	100
Lab vision PT Module	1	0
Leica ER2 30 mins	1	100
Ventana CC1 16mins	1	0
Ventana CC1 20mins	1	0
Ventana CC1 24mins	1	100
Ventana CC1 32mins	6	83
Ventana CC1 36mins	12	83
Ventana CC1 56mins	2	100
Ventana CC1 64mins	5	100
Ventana CC1 mild	18	83
Ventana CC1 standard	5	100

Gastric HER2 ICC Run: 115

Detection	N	%
AS PER KIT	3	33
Dako EnVision FLEX+ (K8002/12)	1	100
Dako HerCep Test Autor (SK001)	2	100
Leica Bond Polymer Refine (DS9800)	1	100
Other	1	0
Ventana iView system (760-091)	2	50
Ventana OptiView Kit (760-700)	2	100
Ventana UltraView Kit (760-500)	44	89

Gastric HER2 ICC Run: 115

Enzyme Retrieval	N	%
AS PER KIT	3	67
NOT APPLICABLE	26	88
Ventana Protease 1 (760-2018)	1	100

Gastric HER2 ICC Run: 115

Chromogen	N	%
AS PER KIT	8	75
DAKO DAB+	1	100
Dako FLEX DAB	2	100
Leica Bond Polymer Refine kit (DS9800)	1	100
Other	1	0
Sigma DAB (D4168)	1	100
Ventana DAB	2	100
Ventana iview	1	100
Ventana Ultraview DAB	40	85

BEST METHODS

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

Gastric HER2 IHC - Method 1

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

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

Gastric HER2 IHC - Method 2

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

Primary Antibody:	Ventana Pathway 790-2991 (4B5) , 20 Mins, RT ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 36mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

Gastric HER2 IHC - Method 3

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

Primary Antibody:	Ventana Pathway 790-2991 (4B5)
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)

Gastric HER2 IHC - Method 4

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

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

David Blythe and Suzanne Parry

	Gold Standard	Second Antibody
Antigens Assessed:	BCL-2	Tdt
Tissue Sections circulated:	Follicular Lymphoma and Reactive Tonsil	Lymphoblastic Leukaemia
Number of Registered Participants:	214	
Number of Participants this Run	211(99%)	

Introduction

Gold Standard: BCL-2

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

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

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

Features of Sub-optimal Immunostaining (Fig 3, 4 & 6):

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

References:

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

Second Antigen: Terminal Deoxynucleotidyl Transferase (Tdt)

Terminal deoxynucleotidyl transferase (TdT), also known as DNA nucleotidylexotransferase (DNTT) or terminal transferase, is a specialized DNA polymerase expressed in immature, pre-B, pre-T lymphoid cells, and acute lymphoblastic leukaemia/lymphoma cells. The enzyme family of DNA polymerases plays a fundamental role in the replication, repair, and recombination of nucleic acid. Its members include DNA Polymerase b (Pol b), DNA Polymerase g (Pol g), and DNA Polymerase m (Pol m). TdT is a very unique and fascinating member of this family because, unlike all other DNA polymerases, TdT synthesizes DNA from only single-stranded DNA. It is now evident that such random nucleotide addition allows V(D)J recombination and therefore drives the evolution n, flexibility, and diversity of the vertebrate immune system. Without TdT, the body could not generate the sophisticated multitude of immunoglobulins and T-cell antigen receptors required for innate immunity. Uniform and strong expression is typical for pre-B and pre-T acute lymphoblastic leukaemia/lymphoblastic lymphoma (ALL/LBL). Very weak expression is also sometimes seen in Burkitt lymphoma. All other mature (peripheral) malignant lymphomas are negative. Acute myeloid leukaemia (AML) may show expression of TdT, which is not unusual finding in AML-M0 or AML with multilineage dysplasia, but rarely can be seen in other types of AML.

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

- Majority of neoplastic cells of the T Lymphoblastic Leukaemia
- No background or non-specific staining of cell types not expected to stain.
- A high signal to noise ratio is obtained.

Features of sub-optimal immunostaining (Figs 9 & 10)

- Weak, nuclear staining of relevant cells.
- Lower intensity and /or lower proportion of cells staining than expected.
- Diffuse staining
- High background or non-specific staining of cell types not expected to stain.

References:

1. Vardiman JW, Thiele J, Arber DA, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood. 2009;114:937–951.
2. Bennett JM, Catovsky D, Daniel MT, et al. French-American-British (FAB Cooperative Group) Proposals for the classification of the acute leukaemias. Br J Haematol. 1976;33:451–458.
3. Bennett JM, Catovsky D, Daniel MT, et al. The morphological classification of acute lymphoblastic leukaemia: concordance among observers and clinical correlations. Br J Haematol. 1981;47:553–561.
4. Jaffe ES, Harris NL, Stein H, et al. Introduction an overview of the classification of the lymphoid neoplasms. In: Swerdlow SH, Campo E, Harris NL, et al., editors. WHO Classification of tumours of haematopoietic and lymphoid tissue. IARC; Lyon: 2008. pp. 158–166.

Assessment Summary

BCL-2

204 laboratories submitted their slides for the BCL-2 assessment. Similarly to previous results, weak staining or poor localisation with diffuse uneven staining were the main reasons for receiving a borderline pass or in some cases, where there was very little staining at all, the labs received a failed score. This was not particularly attributable to a particular antibody clone, but mostly due to an inappropriate dilution or antigen retrieval protocol. All laboratories submitted an in-house control with a higher acceptable pass rate of 89% compared to the NEQAS scores for this run, with only 2 laboratories failing on their in-house material.

Pass rates shown in the table below:

NEQAS Pass Rates Run 115:BCL-2	
Acceptable	70% (N=143)
Borderline	26% (N=54)
Unacceptable	3% (N=7)

A variety of antibodies and automated platforms were used in the assessment. However, overall the most popular antibody clone was the Dako M0887, with 82 participants using this clone with a 70% acceptable pass rate.

David Blythe and Suzanne Parry

Terminal Deoxynucleotidyl Transferase (Tdt)

It was the first time that the UK NEQAS scheme had requested Tdt for assessment. 185 laboratories submitted slides, which showed an acceptable pass rate of 68% for the NEQAS sections and 70% for the in-house sections. The results are summarised in the table below:

NEQAS and In-house Pass Rates Run 115:Tdt		
	NEQAS	In-house
Acceptable	68% (N=125)	70%(N= 166)
Borderline	26% (N=49)	9%(N=16)
Unacceptable	6% (N=11)	2%(N=3)

It was noted that the borderline passes were mostly due to weak demonstration of the antibody. The laboratories (6%) that failed had a combination of very weak, uneven staining with a lower percentage of cells staining than expected . Looking through the data we were not able to pinpoint a particular issue relating to the dilution or antigen retrieval, or a combination of factors. This was also hindered by the fact that some of the methodology information was not complete. It is imperative to fill in our data entry forms as it enables us to assist when a laboratory has received a poor score and may require help with troubleshooting. The most popular primary antibody for this run was the Novocastra/Leica SEN 28 antibody with 66 participants using this clone. This received an average acceptable pass rate of 82%. Another popular choice of antibody was the Dako EP266, with 32 participants using this clone and showed an average acceptable pass rate of 85%.

CD3 (Alternative Antibody)

UK NEQAS ICC & ISH suggested CD3 as an alternative antibody for this run if the laboratory did not stock the Tdt antibody . 12 laboratories carried out CD3 staining. 5 of the laboratories achieved acceptable scores of 13 and above, however 7 out of 12 (58%) of the laboratories achieved borderline scores, mostly due to weak staining. This indicates a need for improvement for some of these laboratories carrying out CD3 staining.

Selected Images showing Optimal and Sub-optimal Immunostaining

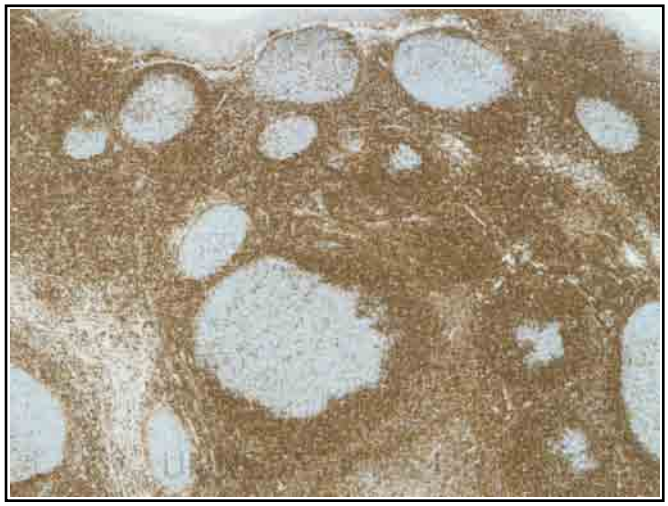


Fig 1. Optimal demonstration of BCL2 in the reactive tonsil section, showing strong cytoplasmic staining of the peripheral B-cells and intra-follicular T-cells. Section stained with the Novocastra 2-486 (3.1) antibody, 1:50, on the Leica Bond III, ER2 20 minutes.

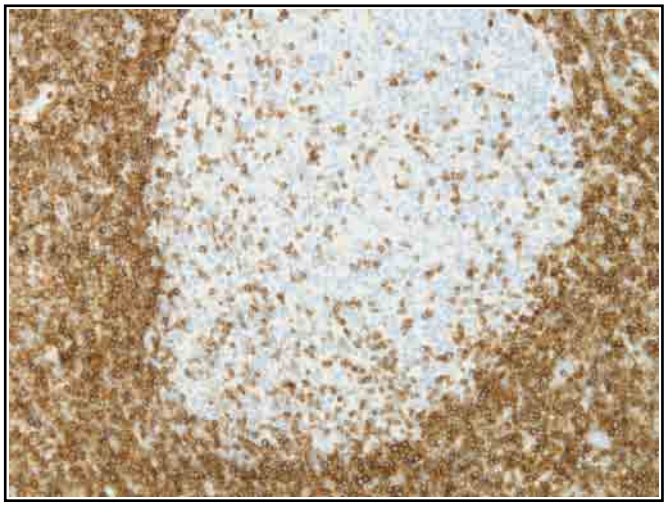


Fig 2. Higher power image of the UK NEQAS reactive tonsil stained with BCL2. The example demonstrates the good strong crisp staining of the peripheral B-cells and intra-follicular T-cells. Stained with the Dako 124 antibody, 1:20, on the Dako autostainer with pretreatment on the PT link.

Fig 3. Poor demonstration of BCL2 on the UK NEQAS reactive tonsil (compare to Fig 2). Although most of the cells expected to stain are demonstrated, the staining is weak. Section stained with the Dako 124 antibody, 1:50 on the Ventana ULTRA with 64 minutes antigen retrieval in CC1 buffer.

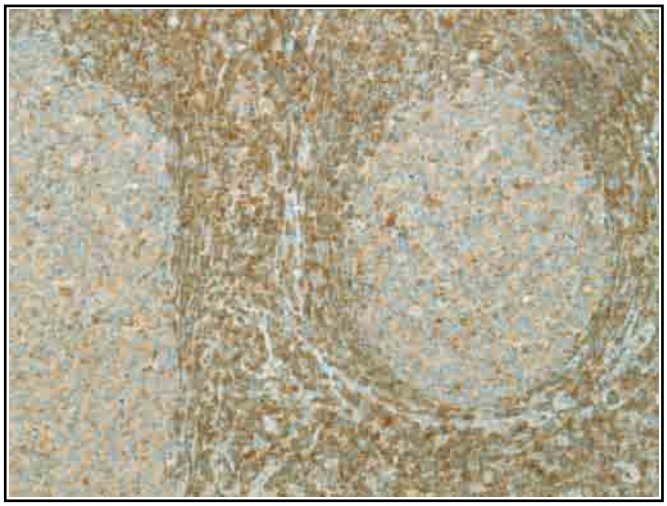


Fig 4. Borderline pass staining of BCL2 on the UK NEQAS reactive tonsil. Although the expected cells are staining, the section also shows excessive non-specific background staining. This may be due to a combination of the antibody dilution being to concentrated along with a high retrieval. Stained with the Neomarkers antibody, 1:25, Benchmark, CC1 extended.

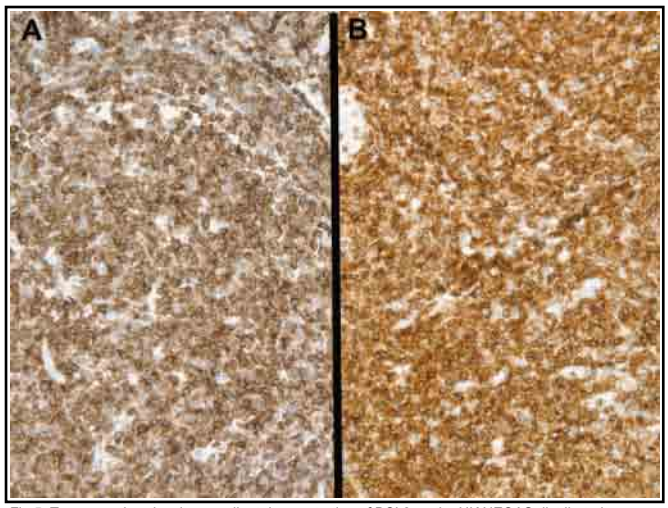


Fig 5. Two examples showing excellent demonstration of BCL2 on the UK NEQAS distributed follicular lymphoma. Both sections show strong crisp and well localised cytoplasmic staining (A & B using the same protocols as Figs 1 and 2 respectfully).

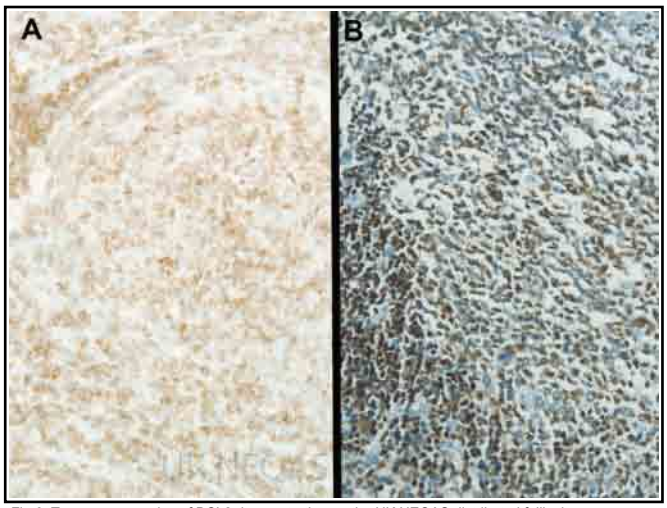


Fig 6. Two poor examples of BCL2 demonstration on the UK NEQAS distributed follicular lymphoma. The staining in section (A) is much weaker than expected, while section (B) appears to have been over digested. (B) was pretreated in the waterbath with high pH buffer.

Selected Images showing Optimal and Sub-optimal Immunostaining

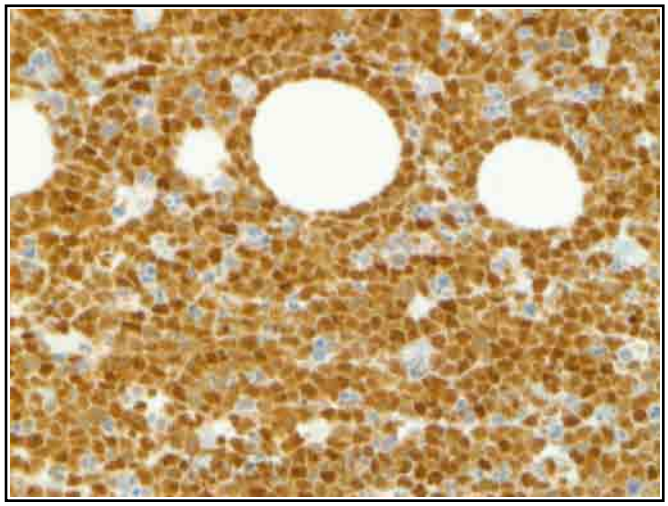


Fig 7. Good demonstraion of Terminal deoxynucleotidyle transferase (Tdt) on the UK NEQAS Lymphoblastic Leukaemia sample, showing good strong nuclear staining. Section stained with the Novocastra SEN28 antibody, 1:50, on the Leica BondMax and ER2 retrieval for 30 minutes.

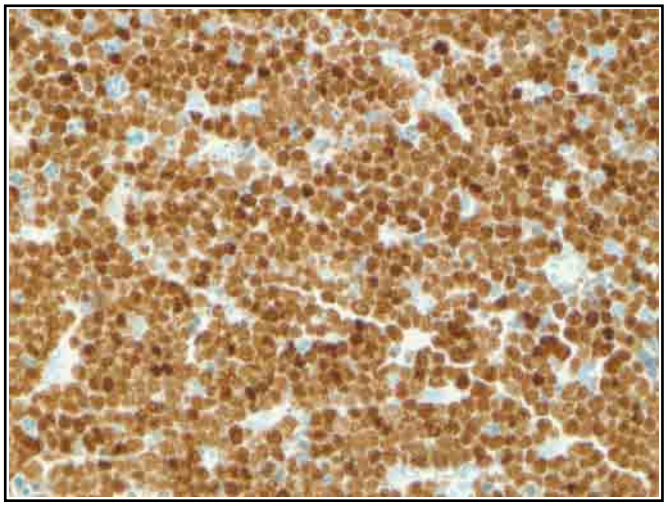


Fig 8. Optimal staining with Tdt on the UK NEQAS Lymphoblastic Leukaemia sample, showing strong crisp nuclear staining. Section stained with the Novocastra SEN28 antibody, 1:50, on the Ventana Benchmark XT, Optiview detection and 88 minutes retrieval in CC1 buffer.

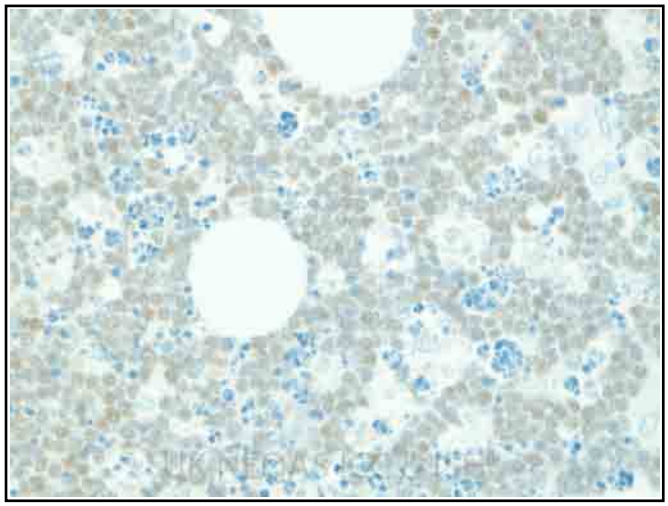


Fig 9. Suboptimal demonstraton of Tdt on the UK NEQAS Lymphoblastic Leukaemia (compare to Figs 7 & 8). The stainining is much weaker than expected, most likely due to insufficient antigen retrieval. Stained with the Novocastra SEN28 antibody, 1:50, on the Dako autostainer with pretreatment in the PT link for 20 minutes with low pH buffer.

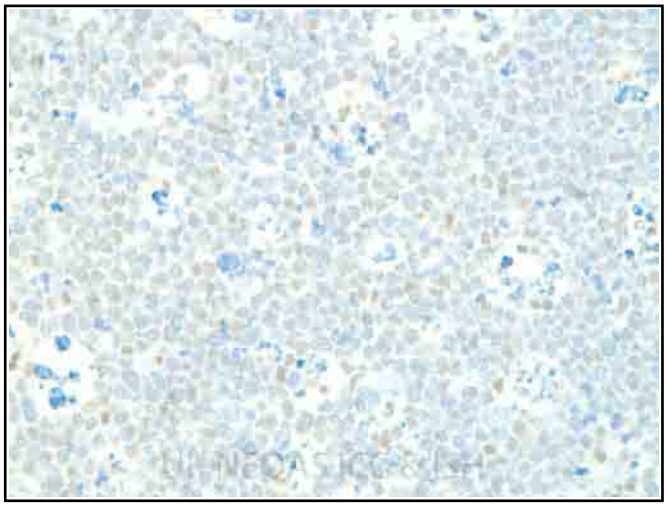


Fig 10. Another weak demonstration of Tdt on the UK NEQAS Lymphoblastic Leukaemia (compare to Figs 7 & 8). The slide received a fail at assessmet as many of the cells expected to stain appeared negative. The participants in house sample on the same slide was also falsely negative.

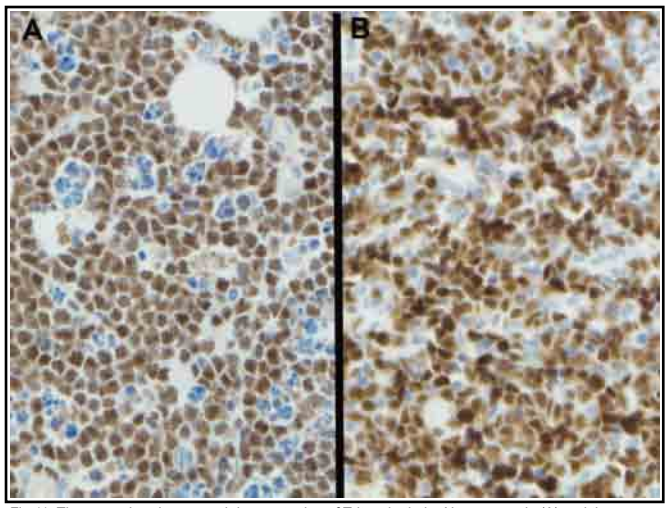


Fig 11. The examples shows good demonstration of Tdt on both the Neqas sample (A) and the participants in house control on the same slide (B). Stained with the Novocastra SEN28 antibody, 1:100, on the Leica Bond III and ER2 retrieval for 20 minutes.

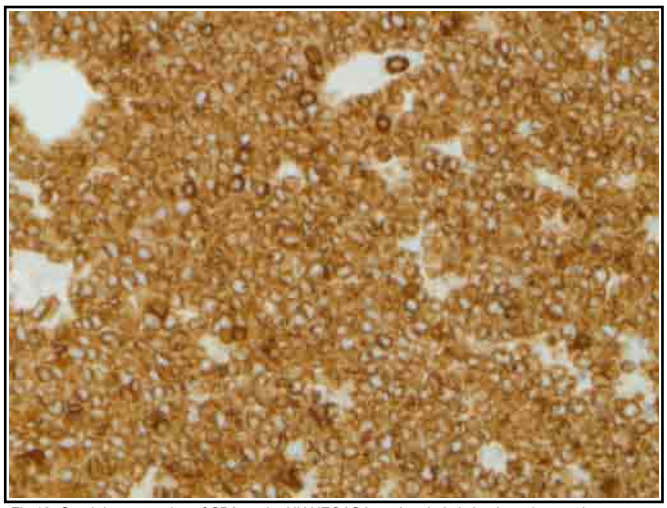
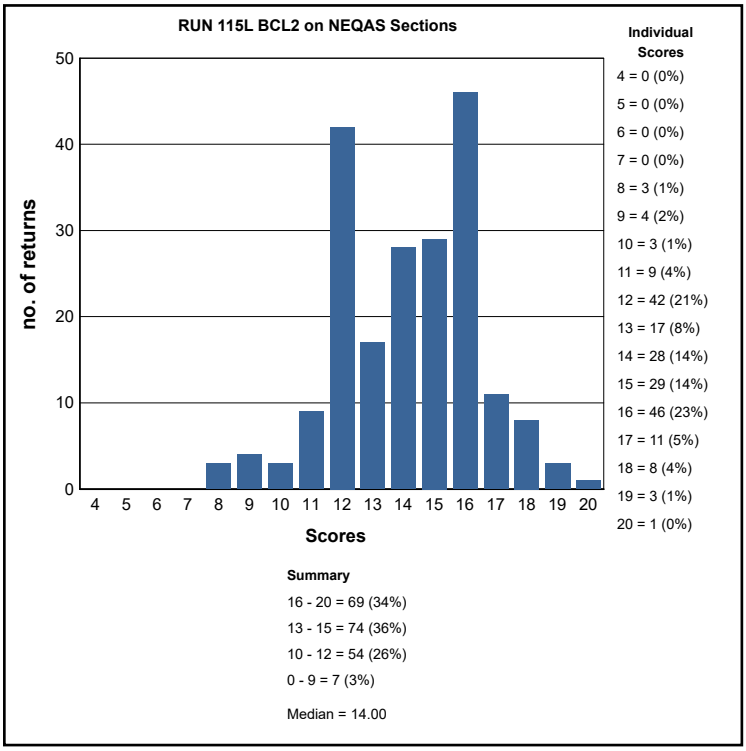
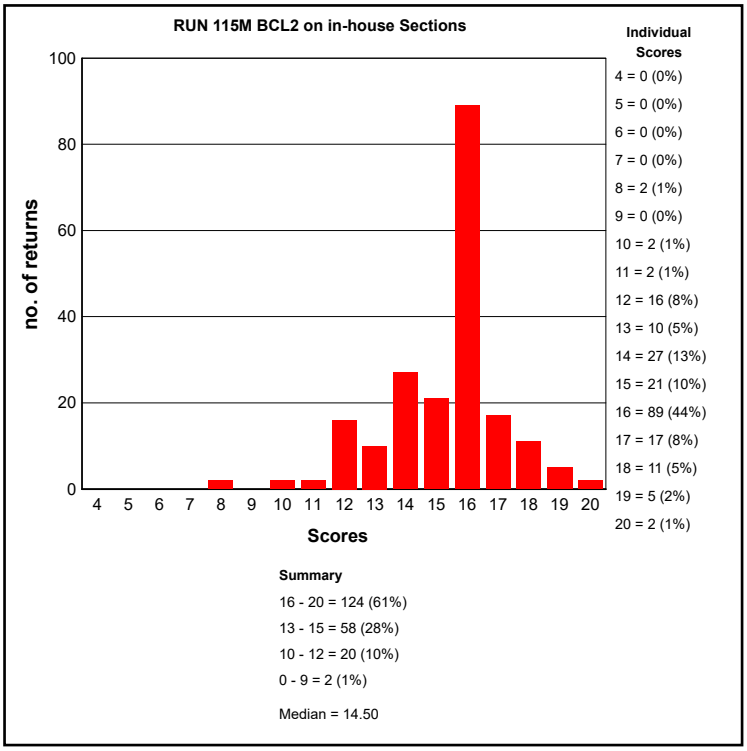
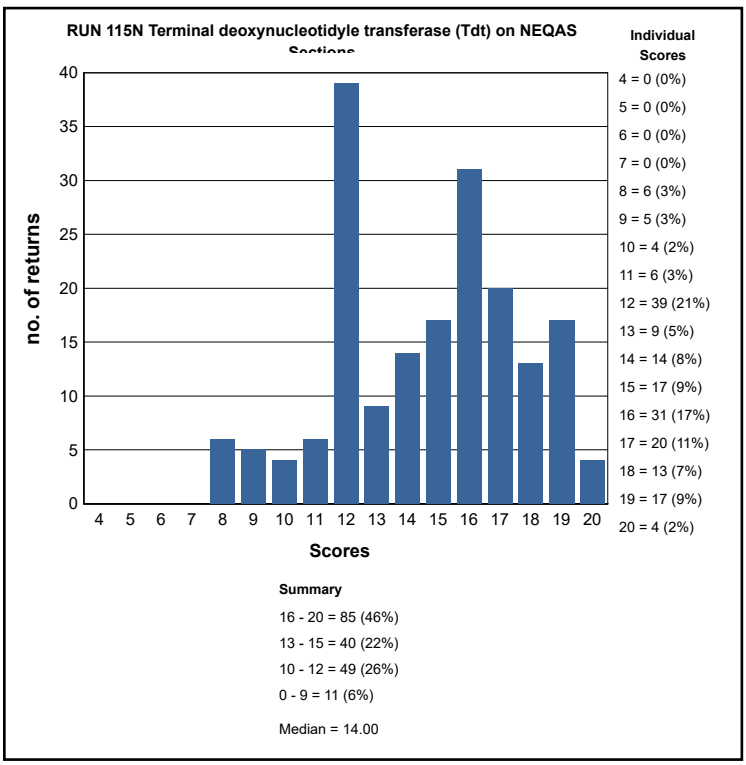


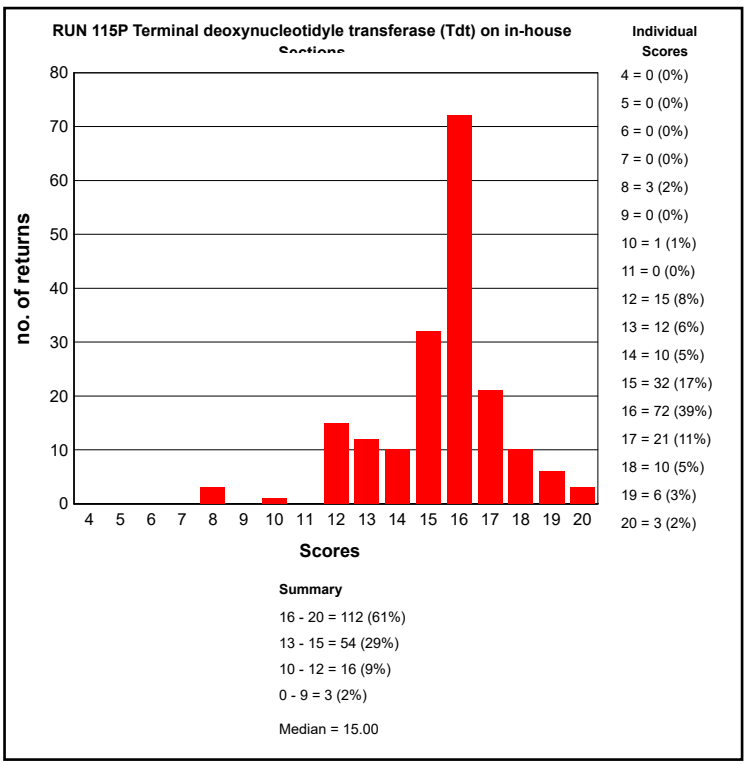
Fig 12. Good demonstration of CD3 on the UK NEQAS Lymphombalstic Leukaemia sample. This marker was provided as an alternative to stain with if the antibody is not stocked. The section shows strong distict stainin of the tumour cells.

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: 115

Primary Antibody : BCL2

Antibody Details	N	%
Dako M0887 (124)	82	70
Labvision/Neomarkers MS-123-P (100/D5)	2	50
Leica/Novocastra NCL-BCl-2 (BCl2/100/D5)	14	71
Ventana (SP66) 790-4604	21	81
Ventana 760-4240 (124)	3	100
Other	2	50
Cell marque CMC329 (124)	3	67
Dako FLEX IR614 (124)	16	56
Leica/Novocastra NCL-BCl-2-486 (3.1)	8	75
Leica RTU (BCl2/100/D5) PA0117	19	84
Ventana (124) 790-4464	23	61
Cell Marque 226R-26 (SP66)	7	71
Abcam ab32124 (E17)	1	100

Lymphoma Run: 115

Primary Antibody : Terminal deoxynucleotidyle transferase (Tdt)

Antibody Details	N	%
Biogenex AM373 (TdT88)	1	100
Cell Marque 338A-78 (Polyclonal)	8	63
Dako M3651 (EP266)	17	76
Dako IR093 RTU Link (EP266)	14	93
Novocastra/Leica NLC-L-TdT-399 (SEN28)	53	64
Novocastra/Leica RTU PA0339 (SEN28)	13	100
Ventana 760-2670 (Polyclonal)	25	64
Other	31	48

Lymphoma Run: 115

Heat Mediated Retrieval	BCL2 N	BCL2 %	Terminal deoxynucleotidyle transferase (Tdt) N	Terminal deoxynucleotidyle transferase (Tdt) %
Biocare Decloaking Chamber	1	100	1	100
Dako Omnis	7	43	6	50
Dako PTLink	23	87	22	77
Lab vision PT Module	1	100	1	100
Leica ER1 10 mins	1	0	0	0
Leica ER1 20 mins	3	67	1	100
Leica ER1 30 mins	6	50	5	60
Leica ER2 10 mins	1	100	1	100
Leica ER2 20 mins	43	81	28	71
Leica ER2 30 mins	8	88	10	70
Leica ER2 40 mins	1	0	3	100
Microwave	3	67	2	50
Other	1	100	0	0
Pressure Cooker	1	100	2	50
Pressure Cooker in Microwave Oven	1	0	0	0
Ventana CC1 24mins	2	100	2	50
Ventana CC1 32mins	7	71	15	67
Ventana CC1 36mins	3	33	3	0
Ventana CC1 40mins	4	50	0	0
Ventana CC1 48mins	6	100	7	100
Ventana CC1 52mins	1	0	3	67
Ventana CC1 56mins	7	71	5	80
Ventana CC1 64mins	31	65	19	74
Ventana CC1 72mins	2	50	4	50
Ventana CC1 76mins	6	50	0	0
Ventana CC1 88mins	1	100	1	100
Ventana CC1 92mins	3	67	2	100
Ventana CC1 extended	6	17	1	100
Ventana CC1 mild	1	100	5	40
Ventana CC1 standard	14	79	14	57
Ventana CC2 48mins	1	100	0	0
Ventana CC2 56mins	1	100	0	0
Water bath 95-98 OC	3	33	3	33

Lymphoma Run: 115

Enzyme Mediated Retrieval	BCL2 N	BCL2 %	Terminal deoxynucleotidyle transferase (Tdt) N	Terminal deoxynucleotidyle transferase (Tdt) %
AS PER KIT	3	67	6	67
NOT APPLICABLE	118	70	56	70

Lymphoma Run: 115	BCL2		Terminal deoxynucleotidyle	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	11	73	16	50
BioGenex SS Polymer (QD 420-YIKE)	1	100	1	0
Dako EnVision FLEX (K8000/10)	4	25	6	67
Dako EnVision FLEX+ (K8002/12)	19	84	16	75
Dako Envision HRP/DAB (K5007)	3	67	2	0
Dako Envision+ HRP mouse K4004/5/6/7	2	100	0	0
Dako rb-a-mo Ig (E0354)	0	0	1	100
Dako REAL HRP/DAB (K5001)	1	0	1	0
Leica Bond Polymer Define (DS9713)	2	100	0	0
Leica Bond Polymer Refine (DS9800)	57	75	42	74
MenaPath X-Cell Plus (MP-XCP)	1	100	1	100
NOT APPLICABLE	0	0	1	100
Other	5	60	4	50
Ventana iView system (760-091)	1	0	3	67
Ventana OptiView (760-700) + Amp. (7/860-099)	5	80	4	75
Ventana OptiView Kit (760-700)	45	71	28	75
Ventana UltraView Kit (760-500)	43	60	35	66

Lymphoma Run: 115	BCL2		Terminal deoxynucleotidyle transferase	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	1	100	1	0
Dako Autostainer	0	0	1	0
Dako Autostainer Link 48	23	78	20	75
Dako Autostainer Plus Link	3	100	3	100
Dako Omnis	6	50	7	43
LabVision Autostainer	2	50	1	100
Leica Bond Max	27	67	18	78
Leica Bond-III	38	84	30	70
Menarini - Intellipath FLX	2	50	2	50
None (Manual)	3	67	3	67
Ventana Benchmark GX	2	50	1	100
Ventana Benchmark ULTRA	67	69	54	67
Ventana Benchmark XT	27	59	26	62

Lymphoma Run: 115	BCL2		Terminal deoxynucleotidyle transferase	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	25	76	27	74
BioGenex DAB (QD430)	0	0	1	0
BioGenex Liquid DAB (HK153-5K)	1	100	0	0
BioGenex liquid DBA (HK-124-7K)	1	0	1	100
DAKO DAB+	1	0	2	100
Dako EnVision Plus kits	2	50	2	100
Dako FLEX DAB	24	83	21	62
Dako REAL EnVision K5007 DAB	2	50	1	0
Dako REAL K5001 DAB	1	0	2	0
Leica Bond Polymer Refine kit (DS9800)	56	77	42	69
menapath xcell kit DAB (MP-860)	1	100	1	100
NOT APPLICABLE	1	100	1	100
Other	14	79	9	78
Sigma DAB (D5905)	0	0	1	100
Ventana DAB	19	63	16	63
Ventana iview	3	33	2	100
Ventana Ultraview DAB	49	61	40	63
Vision BioSystems Bond X DAB	1	100	0	0

BEST METHODS - Gold Standard Antibody

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

BCL2 - Method 1

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

Primary Antibody: Dako M0887 (124) , 15 Mins Dilution 1: 50
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 20 mins
EAR: NOT APPLICABLE
Chromogen: Leica Bond Polymer Refine kit (DS9800)
Detection: Leica Bond Polymer Refine (DS9800)

BCL2 - Method 2

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

Primary Antibody: Dako M0887 (124) , 20 Mins, 20 ºC Dilution 1: 200
Automation: Dako Autostainer Link 48
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako PTLink
EAR: NOT APPLICABLE
Chromogen: Dako EnVision Plus kits
Detection: Dako EnVision FLEX+ (K8002/12)

BCL2 - Method 3

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

Primary Antibody:	Leica/Novocastra NCL-BCl-2-486 (3.1) , 15 Mins, 25 ºC Dilution 1: 80
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, 25 ºC

BCL2 - Method 4

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

Primary Antibody:	Ventana (SP66) 790-4604 Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 48mins
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	Ventana OptiView Kit (760-700) Prediluted

BEST METHODS - Secondary Antibody

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

Terminal deoxynucleotidyle transferase (Tdt) - Method 1

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

Primary Antibody:	Dako M3651 (EP266) , 20 Mins, 32 ºC Dilution 1: 50
Automation:	Dako Omnis
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis
EAR:	
Chromogen:	Dako FLEX DAB, 32 ºC., Time 1: 5 Mins
Detection:	Dako EnVision FLEX (K8000/10) , 20 Mins, 37 ºC

Terminal deoxynucleotidyle transferase (Tdt) - Method 2

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

Primary Antibody:	Novocastra/Leica NLC-L-TdT-399 (SEN28) , 15 Mins Dilution 1: 50
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 30 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

Terminal deoxynucleotidyle transferase (Tdt) - Method 3

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

Primary Antibody:	Novocastra/Leica NLC-L-TdT-399 (SEN28) , 60 Mins, 37 ºC Dilution 1: 50
Automation:	Ventana Benchmark XT
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 88mins, Buffer: CC1
EAR:	
Chromogen:	AS PER KIT, 37 ºC., Time 1: 8 Mins
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099) , 8 Mins, 37 ºC Prediluted

Terminal deoxynucleotidyle transferase (Tdt) - Method 4

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

Primary Antibody:	Dako M3651 (EP266) , 32 Mins, 42 ºC Dilution 1: 50
Automation:	Ventana Benchmark XT
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.6
HMAR:	Ventana CC1 32mins, Buffer: Ventana CC1, PH: 7.8
EAR:	
Chromogen:	Other, 42 ºC., Time 1: 8 Mins, Time 2: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 12 Mins, 42 ºC Prediluted

Neil Bilbe

	Gold Standard	Second Antibody
Antigens Assessed:	GFAP	ACTH
Tissue Sections circulated:	Glioblastoma.	Normal pituitary.
Number of Registered Participants:	61	
Number of Participants this Run	60 (98%)	

Introduction

Gold Standard: GFAP

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

Features of Optimal Immunostaining:

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

Features of Sub-optimal Immunostaining:

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

References:

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

Second Antigen: ACTH

Adrenocorticotropic hormone (ACTH or Corticotropin) is a polypeptide tropic hormone produced and secreted by the anterior pituitary gland. It is an important component of the hypothalamic-pituitary-adrenal axis and is often produced in response to biological stress (along with corticotrophin releasing hormone from the hypothalamus). Its principal effects are increased production of androgens and as its name suggests, cortisol from the adrenal cortex. The antibody labels corticotrophs in the adenohypophysis and is useful in the classification of pituitary adenomas; it also may react with other tumours (e.g., some small cell carcinomas of the lung) causing paraneoplastic syndromes by secreting ACTH.
This antibody stains cytoplasm in positive cells in formalin-fixed, paraffin embedded tissue sections.

Features of Optimal Immunostaining

- Specific cytoplasmic staining in the corticotrophin producing cells
- Clean background with no non-specific staining
- Good contrast with counterstain

Features of Suboptimal Immunostaining

- Weak or uneven staining of the hormone
- Diffuse, poorly-localised or nuclear staining
- Excessive background or non-specific specific staining
- Weak or overtly heavy counterstain

1.White, et al. Characterization of monoclonal antibodies to adrenocorticotropin. J Immunol Meth 1985; 79:185
2. Heitz PU. Multihormonal pituitary adenomas. Horm Res 1979; 10:1
3. Vrontakis ME; Sano T; Kovacs K; Friesen HG. Presence of galanin-like immunoreactivity in nontumorous corticotrophs and corticotroph adenomas of the human pituitary. Journal of Clinical Endocrinology and Metabolism, 1990, 70(3):747-51.
4. Berg KK; Scheithauer BW; Felix I; Kovacs K; Horvath E; Klee GG; Laws ER Jr. Pituitary adenomas that produce adrenocorticotropic hormone and alpha-subunit: clinicopathological, immuno-histochemical, ultrastructural, and immunoelectron microscopic studies in nine cases. Neurosurgery, 1990 Mar, 26(3):397-403.
5. Kovacs K, et al. Immunocytology of the human pituitary. In: DeLellis (ed.). Diagnostic Immunochemistry. New York: Masson Publ. 1980;3

Assessment Summary:

One lab failed to submit any slides. Seven participants requested an alternative antibody for ACTH (CD34) and six labs did not submit any slides for ACTH (**J&K**). This left a total of 228 slides for assessment: **G=60 H=60 J=54 K=54**.
The overall pass rate was 93% (211/228 ≥ 13/20).

GFAP (**G&H**)

No NEQAS (**G**) slides failed the assessment, and only a single in-house (**H**) scored less than 9. This lab used the Dako monoclonal 6F2 clone, with CC1 retrieval for 36 mins, on a Ventana Benchmark ULTRA. The staining was unacceptably weak.
Five GFAP slides were assessed as borderline; two NEQAS (Figs 2 and 5) and 3 in-house (**H**). One of the 5 labs were assessed as borderline for both (**G&H**), excessive haematoxylin counterstain (Fig 5) was a contributory factor. All 5 slides showed weak demonstration; in addition, two of the in-house sections (**H**) had non-specific staining in astrocytoma samples. Four of the five employed the Dako monoclonal, at 1:400 dilution (where entered); 3 used a Ventana CC1, and the UltraView kit; the other a Dako PT Link, and the EnVision FLEX kit. The other participant used the Dako polyclonal at 1:7000, without retrieval, with the Refine kit on a Bond III. The two Dako antibodies are the most widely used: Monoclonal 11/60 (18%) Polyclonal 26/60 (43%).

ACTH (**J&K**)

47 (78%) labs submitted slides stained with ACTH, which tallies well with the latest antibody repertoire levels: 80%. Nine different suppliers' markers were employed, of which six antibodies were used by just a single participant. 33 (70%) used the Dako monoclonal; six (13%) the Ventana polyclonal; and two (4%) the polyclonal from Cell Marque, which is also supplied through, and is essentially the same marker as, the Ventana 760-2708.
Six (10%) of the participants did not submit any slides for the 2nd antigen, but seven (12%) did request an alternative: CD34. *All* slides stained with CD34 (14) passed the assessment, there were no borderline scores either. The average CD34 score was 17/20.
For those participants using an anti ACTH marker, again, no slide was assessed as failed, but there were 11 borderline scores: 4/47 (9%) on the NEQAS pituitary sample (**J**) and 7/47 (15%) on in-house (**K**) control slides. Scores for ACTH (**J&K**) averaged 15/20.
Of the 4 NEQAS sub-optimal slides (**J**), three showed weak or incomplete demonstration of ACTH (Figs 8 & 11); the fourth had excessive background and non-specific staining. Two (6%) of the Dako (A02A3) users (n=33) were assessed borderline. The remaining two labs were the sole ThermoScientific and Biogenex.
For the 7 in-house (**K**) borderline slides, three (50%) were Ventana users; two (6%) stained with the Dako antibody; and both the single Millipore and ThermoScientific users (100%) were also assessed as having a borderline result.
Where a lab performed sub-optimally on either their NEQAS (**J**) or in-house (**K**) slides, there was usually only a slight improvement in the other slide. The only participant who scored borderline on both slides, used the ThermoScientific AH26 clone, at 1:800, without retrieval on a Bond Max (see Fig 11).

Selected Images showing Optimal and Sub-optimal Immunostaining

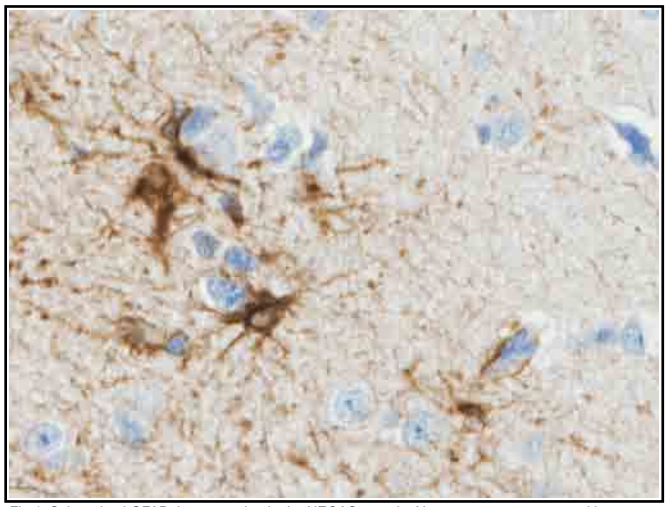


Fig 1. Sub-optimal GFAP demonstration in the NEQAS sample. Non-tumour areas are weakly stained, but overall the slide was adequately stained and passed the assessment. Dako polyclonal, 1:500, no pretreatment, on a Leica Bond Max, using Leica Bond Polymer Refine kit 2nd layer.

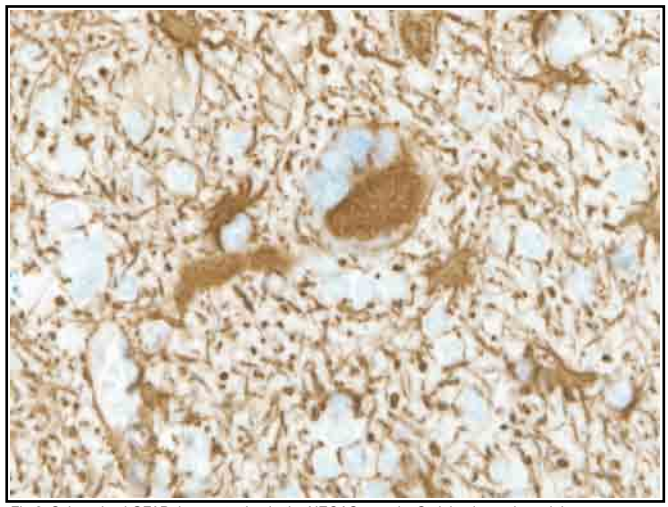


Fig 2. Sub-optimal GFAP demonstration in the NEQAS sample. Staining is weak, and the contrast is poor. This was assessed as borderline. Dako monoclonal (6F2), 1:400, 20 mins, with a high pH PT link RT, on the Dako Autostainer Plus Link, and a RTU Dako EnVision FLEX detection kit for 20 mins.

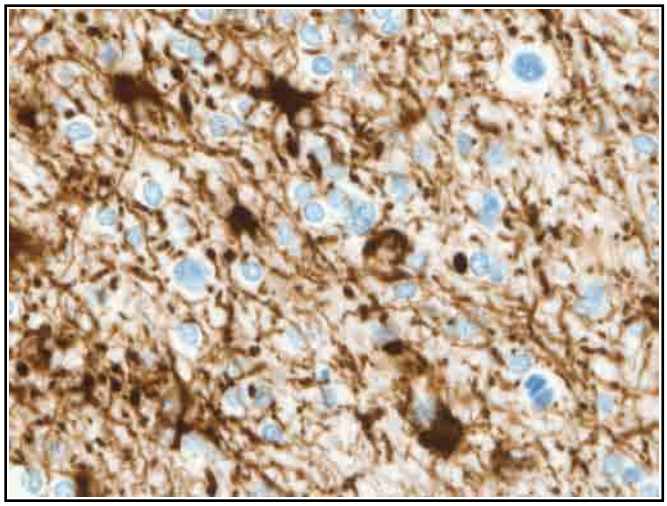


Fig 3. Excellent GFAP demonstration on the NEQAS tissue. Staining is crisp, counterstain is at the ideal intensity, and the slide is clean. Sigma monoclonal (GA5), 1:5000, 95 mins, with Ventana CC1 8mins, on the Ventana Benchmark XT, and Ventana UltraView Kit 2nd layer.

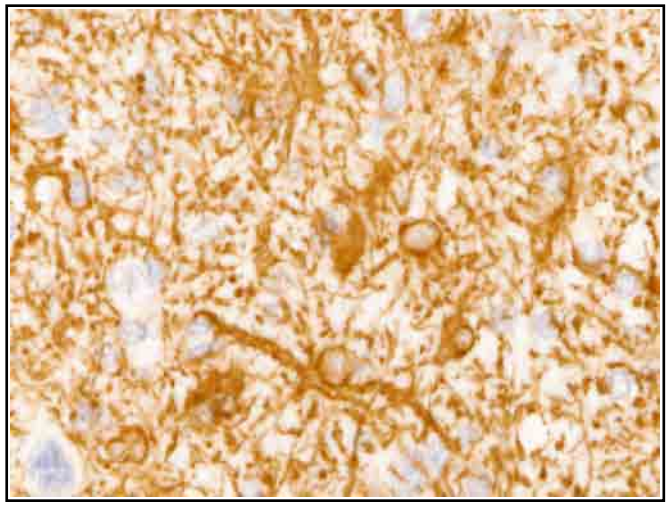


Fig 4. Sub-optimal GFAP demonstration in the NEQAS sample. Staining is weak with a pale brown colouration, low contrast and a pale counterstain. Dako monoclonal (6F2), no dilution given, Ventana CC1 64mins, on the Ventana Benchmark ULTRA, and Ventana UltraView Kit detection.

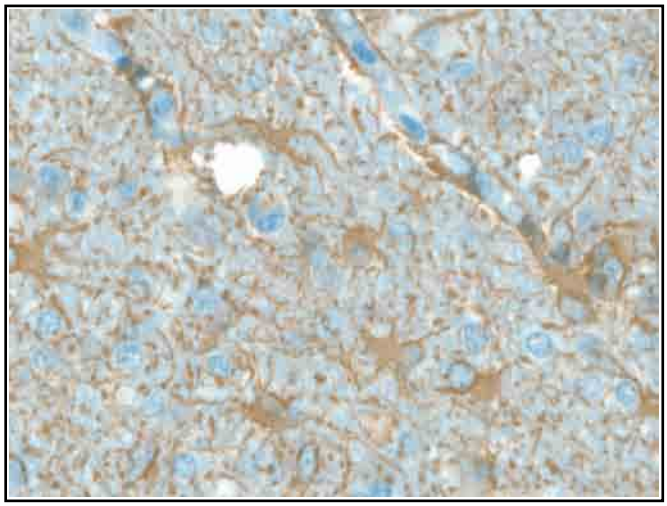


Fig 5. Sub-optimal GFAP demonstration in the NEQAS section. Staining is weak, and furthermore masked by the heavy counterstain. Assessed as borderline. Dako monoclonal (6F2), 1:400, 32 mins, with Ventana CC1 mild, on a Ventana Benchmark XT, and using the Ventana UltraView Kit.

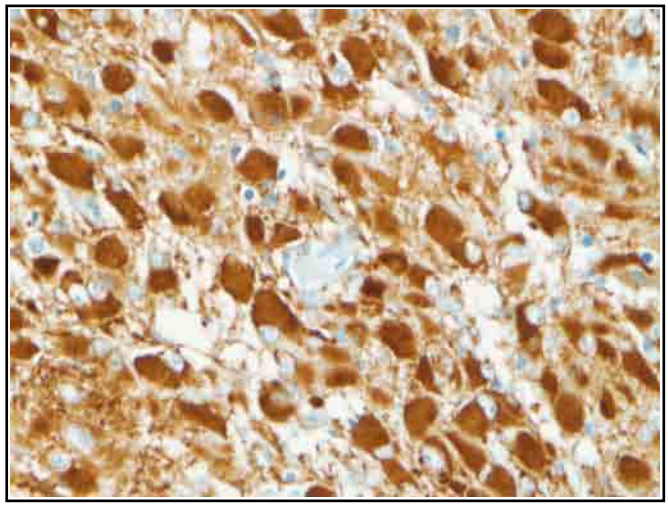


Fig 6. Nice example of GFAP on an in-house sample of glioblastoma. Tumour cells are nicely demonstrated, aided by a nice counterstain intensity. Dako polyclonal, 1:1000, 28 mins, Ventana CC1 mild for 4 mins, on a Ventana Benchmark ULTRA, and a RTU Ventana OptiView Kit 4 mins.

Selected Images showing Optimal and Sub-optimal Immunostaining

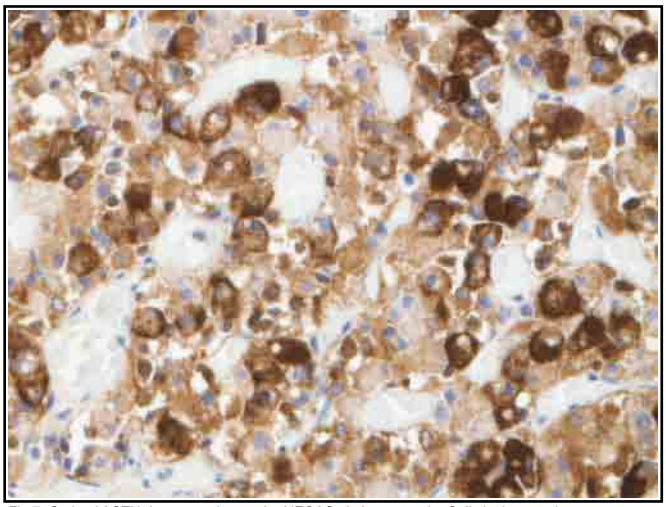


Fig 7. Optimal ACTH demonstration on the NEQAS pituitary sample. Cells in the anterior portion are selectively satined, contrast is good, and the background is clean. Dako monoclonal (02A3), 1:3000, 15 mins, no RT, on a Leica Bond-III, and RTU Leica Bond Polymer Refine kit, 8 mins.

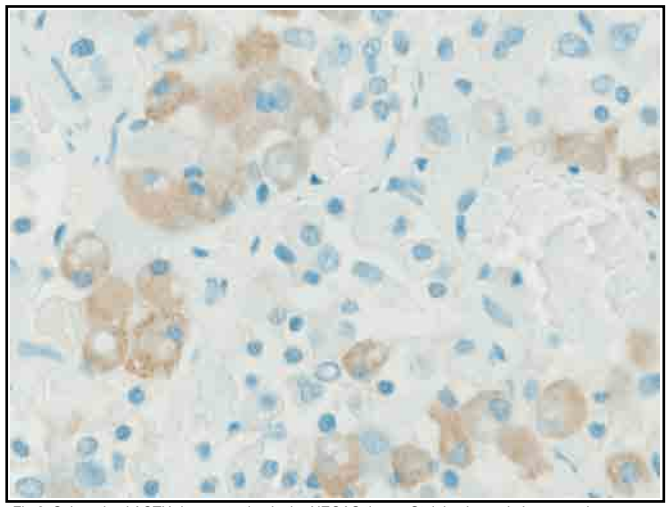


Fig 8. Sub-optimal ACTH demonstration in the NEQAS tissue. Staining is weak, but enough cells are positive to warrant a borderline score. Biogenex monoclonal (AH26), no dilution given, Ventana CC1 32 mins, on the Ventana Benchmark ULTRA, and Ventana UltraView Kit.

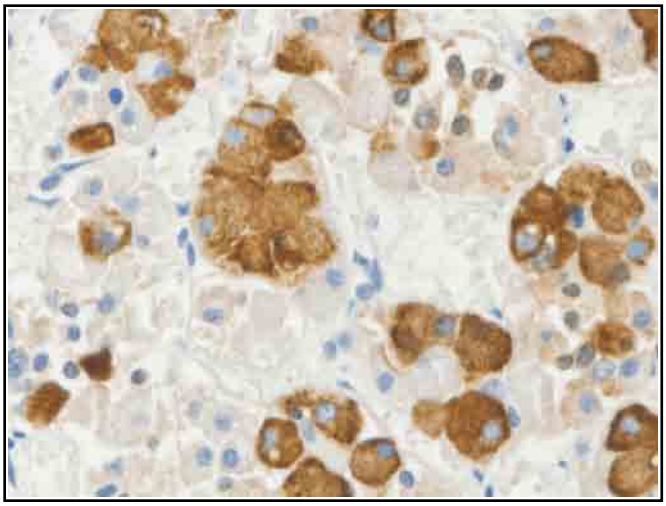


Fig 9. Optimal ACTH demonstration on the NEQAS pituitary slide. The staining was precise, and of the correct amount, with minimal background. Cell Marque 206A-74 antibody, 1:6000, 15 mins, no pretreatment, on a Leica Bond-III, and a Leica Bond Polymer Refine kit.

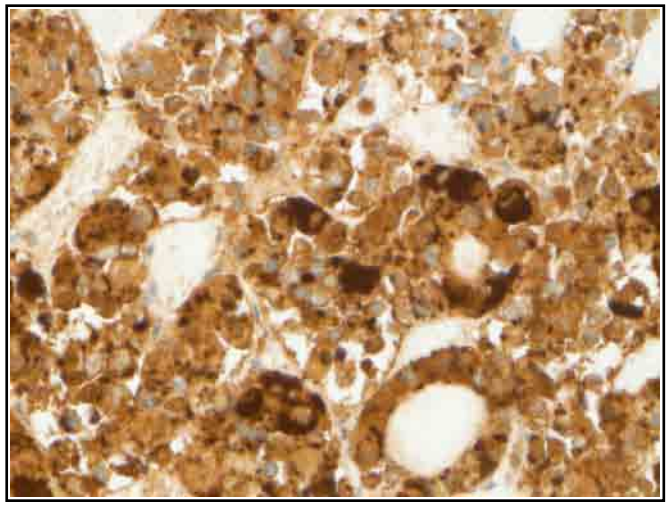


Fig 10. Sub-optimal ACTH in the NEQAS pituitary section. Staining is heavy and shows areas of non-specific demonstration, but just adequate for diagnostic purposes. Dako monoclonal (02A3), 1:75, pH9 Dako PTLink 20 mins, on the Dako Autostainer Link 48, and RTU Dako EnVision FLEX kit.

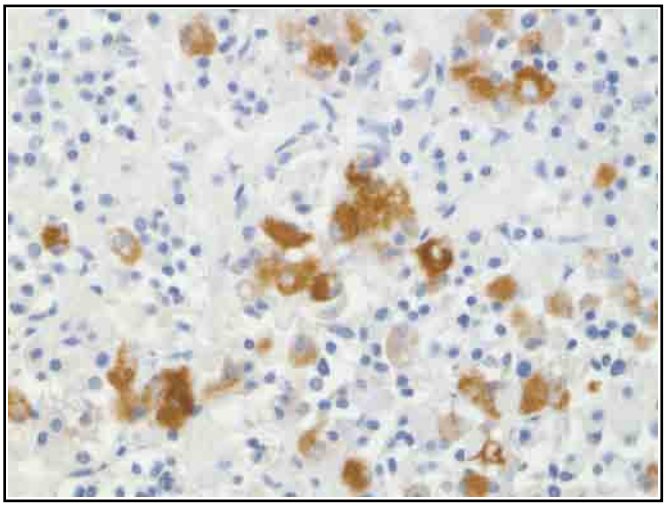


Fig 11. Sub-optimal ACTH demonstration in the NEQAS tissue. Staining is weak, and the counterstain a little heavy. Borderline assessment outcome. ThermoScientific monoclonal (AH26), 1:800, with no pretreatment, on a Leica Bond Max, and using the Leica Bond Polymer Refine kit.

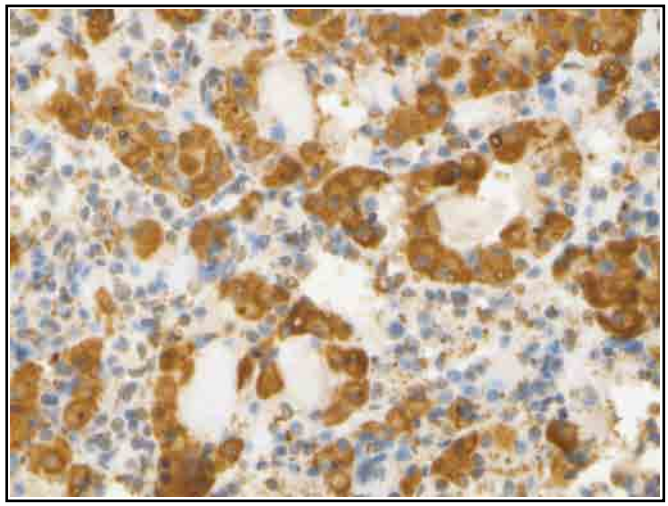
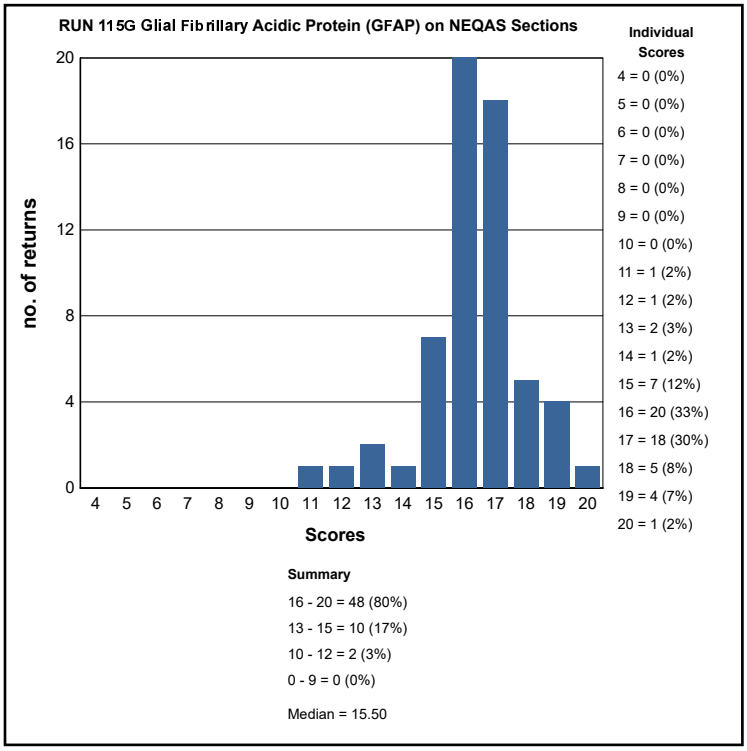
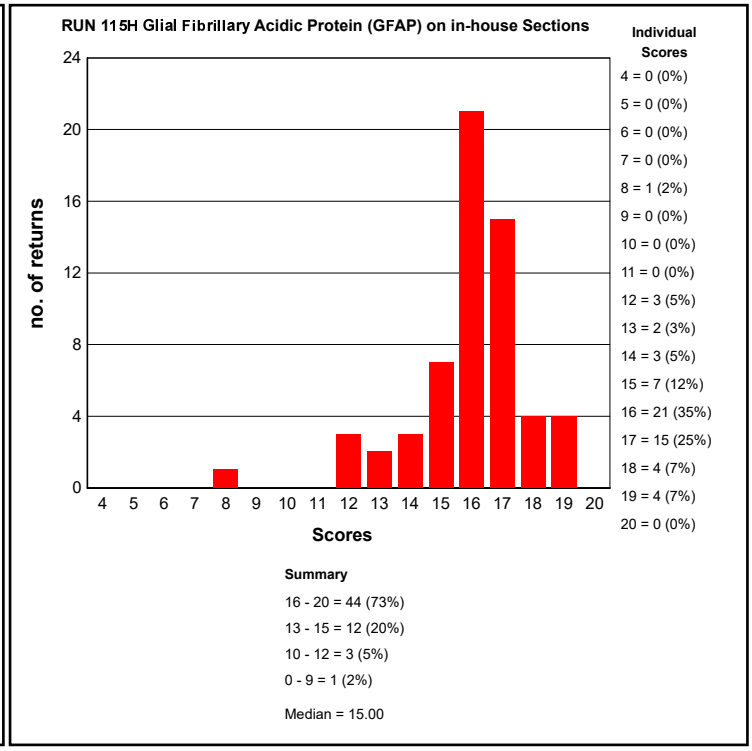
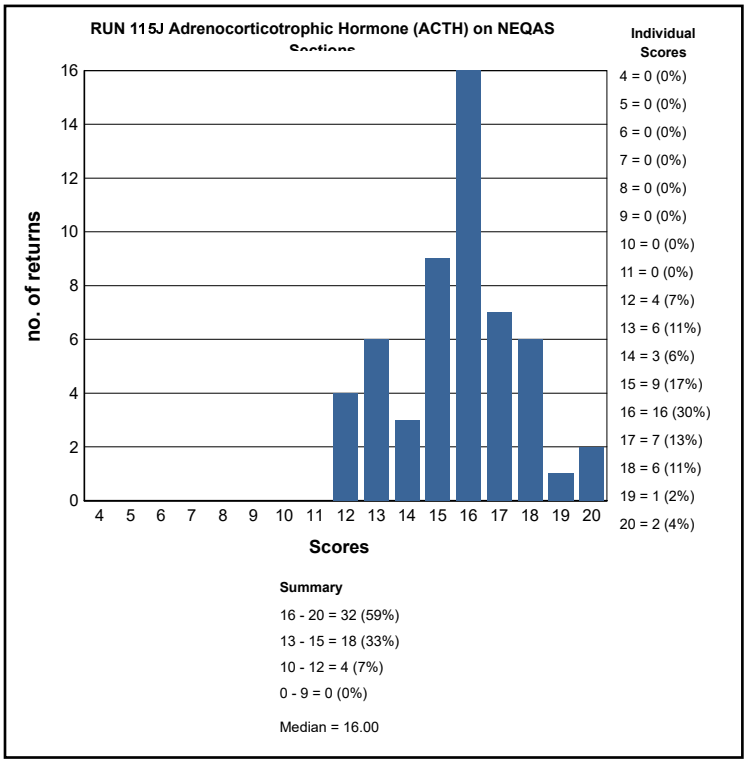


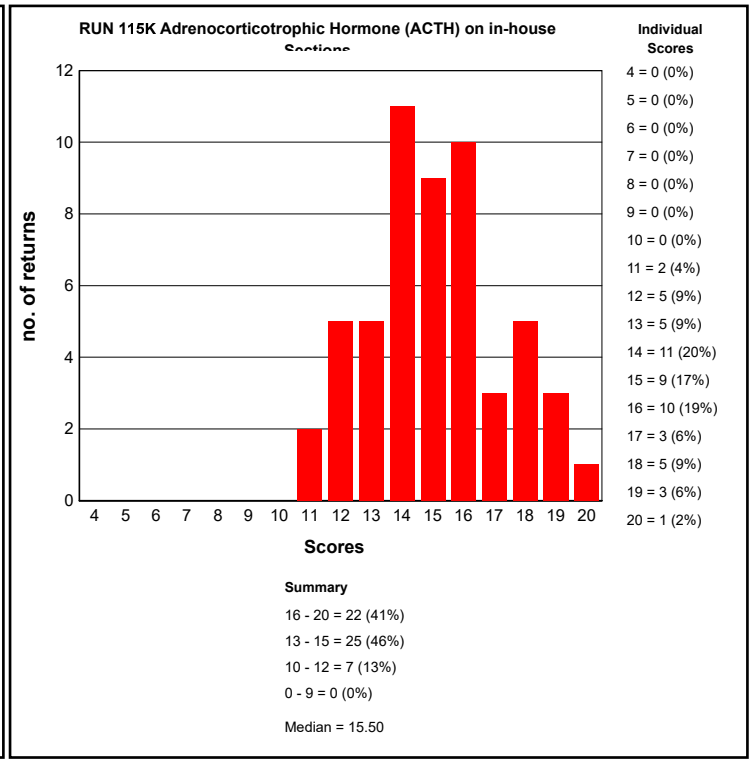
Fig 12. Nice example of ACTH on an in-house sample of pituitary adenoma. Staining is selective for the tumour, and the background relatively clean. Dako monoclonal (02A3), 1:3000, with Leica ER1 20 mins, on the Leica Bond-III, and with a Leica Bond Polymer Refine layer.

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: 115

Primary Antibody : Glial Fibrillary Acidic Protein (GFAP)

Antibody Details	N	%
Dako M0761 (6F2)	11	82
Dako Z0334 (R Poly)	26	100
Immunon 490740RB	1	100
Novocastra NCL-GFAP-GA5 (GA5)	2	100
Sigma G3895 (GA5)	2	100
Zymed/Invitrogen 08-1021 (ZCG29)	1	100
Dako IR524 (R Poly)	3	100
Novocastra PA0026 RTU (GA5)	4	100
Ventana 760-4345 (EP672Y)	6	100
Cell Marque (EP672Y) 258R	2	100
Dako Omnis GA524 (R Poly)	1	100

Neuropathology Run: 115

Primary Antibody : Adrenocorticotrophic Hormone (ACTH)

Antibody Details	N	%
Biogenex MU487-UC (Clone AH26)	1	0
Dako M3501 (Clone 02A3)	33	94
Novocastra NCL-ACTH (Clone 56)	1	100
ThermoScientific MS-452P (Clone AH26)	1	0
Ventana 760-2708 (Polyclonal)	6	100
Milipore CBL56 (Clone 56)	1	100
Neomarkers MS-452-PO (Clone AH26)	1	100
Cell Marque 206A-74	2	100
BioSB BSB-SB	1	100

Neuropathology Run: 115

Heat Mediated Retrieval	Adrenocorticotrophic Hormone (ACTH) N	Adrenocorticotrophic Hormone (ACTH) %	Glial Fibrillary Acidic Protein (GFAP) N	Glial Fibrillary Acidic Protein (GFAP) %
Dako Omnis	2	50	4	100
Dako PTLink	7	86	5	80
Leica ER1 10 mins	0	0	1	100
Leica ER1 20 mins	2	100	3	100
Leica ER1 30 mins	0	0	2	100
Leica ER2 10 mins	0	0	1	100
Leica ER2 20 mins	0	0	5	100
Microwave	0	0	2	100
None	21	95	13	100
Other	0	0	1	100
Ventana CC1 20mins	0	0	1	100
Ventana CC1 24mins	1	100	1	100
Ventana CC1 32mins	3	67	1	100
Ventana CC1 36mins	4	100	2	100
Ventana CC1 40mins	0	0	1	100
Ventana CC1 48mins	1	100	0	0
Ventana CC1 64mins	1	100	4	100
Ventana CC1 76mins	0	0	1	100
Ventana CC1 8mins	1	100	2	100
Ventana CC1 mild	4	100	5	80
Ventana CC1 standard	2	100	1	100
Ventana CC2 32mins	0	0	1	100
Water bath 95-98 OC	0	0	1	100

Neuropathology Run: 115

Enzyme Mediated Retrieval	Adrenocorticotrophic Hormone N	Adrenocorticotrophic Hormone %	Glial Fibrillary Acidic Protein (GFAP) N	Glial Fibrillary Acidic Protein (GFAP) %
AS PER KIT	1	0	0	0
Dako Proteinase K (S3020)	0	0	1	100
NOT APPLICABLE	23	96	27	96
Other	1	100	1	100
VBS Bond Enzyme 1	2	100	4	100
Ventana Protease 1 (760-2018)	0	0	5	100

Neuropathology Run: 115 Detection	Adrenocorticotrophic Hormone		Glial Fibrillary Acidic Protein (GFAP)	
	N	%	N	%
AS PER KIT	5	100	5	100
Dako EnVision FLEX (K8000/10)	5	80	4	75
Dako EnVision FLEX+ (K8002/12)	4	75	5	100
Dako Envision HRP/DAB (K5007)	2	100	1	100
Leica Bond Polymer Refine (DS9800)	15	93	17	100
Other	1	100	1	100
Vector Elite ABC Kit (PK-7200)	0	0	1	100
Ventana OptiView Kit (760-700)	5	100	8	100
Ventana UltraView Kit (760-500)	14	93	17	94

Neuropathology Run: 115 Chromogen	Adrenocorticotrophic Hormone (ACTH)		Glial Fibrillary Acidic Protein (GFAP)	
	N	%	N	%
AS PER KIT	9	100	8	100
DAKO DAB+	0	0	1	100
Dako DAB+ REAL Detection (K5001)	0	0	1	100
Dako EnVision Plus kits	2	100	2	100
Dako FLEX DAB	7	71	7	86
Dako REAL EnVision K5007 DAB	1	100	0	0
Leica Bond Polymer Refine kit (DS9800)	15	93	17	100
Other	3	100	3	100
Ventana DAB	2	100	2	100
Ventana Ultraview DAB	13	92	18	94

Neuropathology Run: 115 Automation	Adrenocorticotrophic Hormone (ACTH)		Glial Fibrillary Acidic Protein (GFAP)	
	N	%	N	%
Dako Autostainer Link 48	10	90	7	100
Dako Autostainer Plus Link	0	0	1	0
Dako Omnis	2	50	4	100
Leica Bond Max	5	80	6	100
Leica Bond-III	12	100	12	100
None (Manual)	2	100	2	100
Ventana Benchmark ULTRA	15	93	20	100
Ventana Benchmark XT	6	100	7	86

BEST METHODS - Gold Standard Antibody

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

Glial Fibrillary Acidic Protein (GFAP) - Method 1

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

Primary Antibody: Novocastra NCL-GFAP-GA5 (GA5) , 15 Mins, RT ºC Dilution 1: 200
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER1 30 mins, PH: 6
EAR:
Chromogen: Leica Bond Polymer Refine kit (DS9800), RT ºC., Time 1: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins Prediluted

Glial Fibrillary Acidic Protein (GFAP) - Method 2

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

Primary Antibody: Dako Z0334 (R Poly) Dilution 1: 1:2000
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER1 20 mins
EAR:
Chromogen: Leica Bond Polymer Refine kit (DS9800)
Detection: Leica Bond Polymer Refine (DS9800)

Glial Fibrillary Acidic Protein (GFAP) - Method 3

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

Primary Antibody: Dako Z0334 (R Poly) , 30 Mins, Room ºC Dilution 1: 10000
Automation: Dako Autostainer Link 48
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer, PH: 7.6
HMAR: Dako PTLink, Buffer: Dako EnVision retrieval sln High pH9, PH: 9
EAR: NOT APPLICABLE
Chromogen: Dako FLEX DAB, Room ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection: Dako EnVision FLEX+ (K8002/12) , 30 Mins, Room ºC Prediluted

Glial Fibrillary Acidic Protein (GFAP) - Method 4

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

Primary Antibody:	Dako M0761 (6F2) , 32 Mins, 37 ºC Dilution 1: 75
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

BEST METHODS - Secondary Antibody

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

Adrenocorticotrophic Hormone (ACTH) - Method 1

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

Primary Antibody:	Dako M3501 (Clone 02A3) , 15 Mins, RT ºC Dilution 1: 3000
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), RT ºC., Time 1: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, RT ºC Prediluted

Adrenocorticotrophic Hormone (ACTH) - Method 2

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

Primary Antibody:	Dako M3501 (Clone 02A3) , 32 Mins Dilution 1: 4000
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 mild
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

Adrenocorticotrophic Hormone (ACTH) - Method 3

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

Primary Antibody:	Dako M3501 (Clone 02A3) , 30 Mins, 23 ºC Dilution 1: 5000
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: Target retrieval solution High pH, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 23 ºC., Time 1: 10 Mins
Detection:	Other , 15 Mins, 23 ºC Prediluted

Adrenocorticotrophic Hormone (ACTH) - Method 4

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

Primary Antibody:	Novocastra NCL-ACTH (Clone 56) , 15 Mins, 20 ºC Dilution 1: 150
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	None
EAR:	VBS Bond Enzyme 1, 20 ºC. Digestion Time NEQAS: 5 Mins. In-House: 5 Mins
Chromogen:	Leica Bond Polymer Refine kit (DS9800), 20 ºC., Time 1: 8 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, 20 ºC Prediluted

Neil Bilbe and Irena Srebotnik Kirbis

	Gold Standard	Second Antibody
Antigens Assessed:	CK	Ki67
Sample circulated; cytospins and cell block sections:	Cell lines of melanoma, breast and cervical carcinoma, effusion with mesothelial cells, macrophages, RBCs.	Cell lines of melanoma, breast and cervical carcinoma, effusion with mesothelial cells, macrophages, RBCs.
Number of Registered Participants:	82 - Cell block 58 (71%), Cytospin 24 (29%)	
Number of Participants this Run	80 (98%)	

Introduction

Gold Standard: Cytokeratin

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

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

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

References
1. MJ Goddard et al. Comparison of commercially available cytokeratin antibodies in normal and neoplastic adult epithelial and non-epithelial tissues. J Clin Pathol 1991; 44:660-6632.
2. LJ Fowler & WA Lachar Application of immunocytochemistry to cytology. Archives of Pathology & Laboratory Medicine. 2008; 132(3): 373-38.
3. PA Fetsch & A Abati Immunocytochemistry in effusion cytology. Cancer Cytopathology. 2001; 93(5): 293-308.

Second Antigen: Ki67

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

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

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

References
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

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

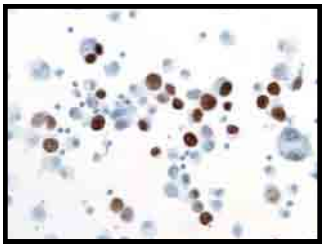
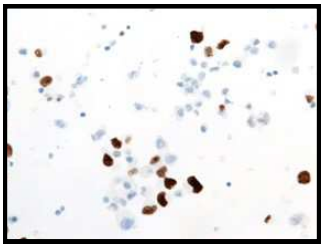
Assessment Summary:

There are currently 82 labs registered on the Cytopathology module. For Run 115 two labs did not submit any slides. Of the remaining 80, one participant returned their NEQAS slides (**R & T**) *unstained*. This left a total of 318 slides for the assessors to score. **R = 79, S = 80, T = 79, U = 80.**

The overall pass rate was 94% (299 slides), 5% borderline (16), and 1% failure rate (3). The average score for all slides was 16/20. See Table (3) for cell block v cytospin averages.

Examples of the immunocytochemical reactions on NEQAS slides prepared in a reference/supplier laboratory
Figs 1 & 2: Run 115 Ki67 samples - Cytospin (L) and Cell Block (R)

Run 115 Cytokeratin (R) samples see Run 114 Journal.
Assessment Outcomes:

The overall assessment outcomes are very similar to those for Run 114, but with a reduced level of borderline CK scores, down from 8% to 6%, (NEQAS = R), and 10% down to 3% (in-house = S). This brought the borderline levels for all slides

down from 7% to 5%. As for Run 114, only three slides failed the assessment (1%), all of which were Ki 67 slides (**T & U**).

CK (R & S)

No slides failed the assessment for this Run and the pass rate for the NEQAS slides (**R**) increased from 91% in Run 114 to 94%, mainly as a result of this reduced level of borderline scores, and absence of failed slides (one in Run 114). More impressive was the fact that there was a greater number of slides that scored 20/20, up from two (Run 114) to eight (Run 115), 4 NEQAS (**R**) slides, and 4 in-house (**S**), although interestingly, no participant scored 20/20 for both their NEQAS and in-house submissions. Given these findings it would be interesting to look at the protocols for the highest scoring submissions.

Of the 4 NEQAS (**R**) slides scoring 20/20, two were on cell blocks (Images Report Figs 3 A & B), and two were on cytospins (Images Report Fig 4), also see Fig 3 below:

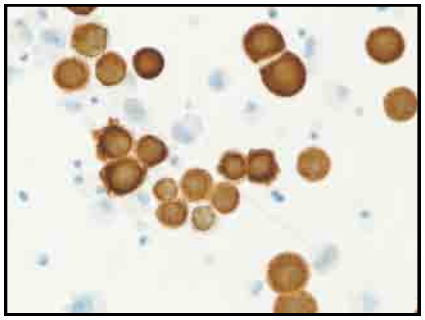


Fig 3: NEQAS Cytospin CK (**R**): scoring 20/20 for Run 115

Three of the slides had very similar protocols: Dako MNF116; 1:50, 1:250; 1:300 dilutions, no RT given; two on a Benchmark ULTRA, and one on the XT, but all used the UltraView kit. The other slide used the Becton Dickinson CAM 5.2 antibody at 1:50, no RT, but again in combination with the Ventana Benchmark ULTRA and UltraView detection kit.

For the in-house control slides (**S**), there was slightly more variance. Three used the Dako MNF116; diluted 1:100 and 1:200 (both on cell blocks) and 1:400 (smear), no retrieval; the two cell block slides again stained on the Benchmark ULTRA, one with the UltraView and one the OptiView kit. The smear was stained on a Leica Bond III with a Refine detection kit. The fourth slide was a FFPE section (user received a NEQAS cytospin!) stained using the Omnis prediluted AE1/ AE3, Omnis RT, and the Envision FLEX detection system.

Ki 67 (T & U)

In contrast to the Cytokeratin scores, there were three failed slides for the Ki 67 submissions. A single NEQAS (**T**) cytospin slide (Images Report Fig 11), and an in-house (**U**) smear from the same participant. The assessors found that the NEQAS slide (**T**) was both weakly stained and had non-specific staining, and the in-house (**U**) smear was too weakly stained for diagnostic purposes.

The third slide was an in-house (**U**) LBC sample from a lymph node. This again showed inappropriate and/or weak demonstration of Ki 67.

The number of slides scoring 20/20 totalled four, of which two were the NEQAS (cell block) and in-house (FFPE) from the *same* participant. The other two were a NEQAS (**T**) cytospin (Fig 4 below) and an in-house (**U**) cytospin control.

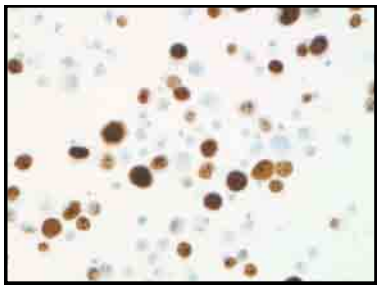


Fig 4: NEQAS Cytospin Ki 67 (**T**) scoring 20/20 for Run 115

Details of failed Ki 67 slides (3/159)

Both participants used the Dako MIB1 (employed by over 50% of participants) one at 1:100, the other at 1:150.; on a Leica Bond III and the Refine system; and the Benchmark ULTRA and UltraView kit respectively.

The participant who had submitted an in-house (**U**) LBC sample from a lymph node, stated that they had also used the Ventana CC1 retrieval method for 20 mins, although they did not use this on their NEQAS cytospin sample (**T**), for which they scored 16/20.

The lab which failed *both* their Ki 67 slides has not entered/ employed any retrieval details, on their NEQAS cytospin (**T**) and in-house (**U**) smear.

Details of the highest scoring Ki 67 slides (4/159)

One lab scored 20/20 for both their NEQAS cell block (**T**) and in-house (**U**) FFPE slides. This participant used the Ventana RTU Confirm Ki 67 monoclonal (clone 30-9), the second most popular antibody, used by around 25% of labs. They had stained on the BioGenex GenoMX 6000i platform (sole user), but again employed the Ventana OptiView Kit (760-700) detection system, a combination that obviously works well.

The other two slides were the NEQAS (**T**) cytospin (Fig 4 above) which used the Dako MIB1 clone, no RT, and the familiar Ventana Benchmark ULTRA, Ventana UltraView Kit (760-500) system.

The final slide was an in-house (**U**) cytospin sample stained again with the Dako MIB1 clone, at 1:200, on the Ventana Benchmark GX this time, but with the Ventana OptiView Kit (760-700).

(1) Summary Table - Scores All Slides:

Slide	Marker	Pass	Borderline	Fail
R (NEQAS)	CK (79)	94%	6%	0%
S (In-House)	CK (80)	98%	3%	0%
T (NEQAS)	Ki67 (79)	91%	8%	1%
U (In-House)	Ki67 (80)	94%	4%	3%
Total (Average)	318	94%	5%	1%

(2) Summary Table - NEQAS Slides Scoring 20:

Letter	Antigen	Type	Sample	Primary	Dilution 1:	Automation	Detection
R	CK	NEQAS	CB	Becton Dickinson 349205 (CAM5.2)	50	Ventana Benchmark ULTRA	UltraView kit
R	CK	NEQAS	CB	Dako M0821(MNF116)	300	Ventana Benchmark XT	UltraView kit
R	CK	NEQAS	CS	Dako M0821(MNF116)	50	Ventana Benchmark ULTRA	UltraView kit
R	CK	NEQAS	CS	Dako M0821(MNF116)	250	Ventana Benchmark ULTRA	UltraView kit
T	Ki67	NEQAS	CS	Dako 7240 (MIB-1)	Not given	Ventana Benchmark ULTRA	UltraView kit
T	Ki67	NEQAS	CB	Ventana RTU (30-9) 790-4286	Prediluted	BioGenex GenoMX 6000i	OptiView Kit

(3) Summary Table - Average Scores: Cell Block v Cytospin:

Letter	Antigen	Type	Sample	Average Score
R	CK	NEQAS	CB	16
R	CK	NEQAS	CS	16
T	Ki67	NEQAS	CB	16
T	Ki67	NEQAS	CS	15

Selected Images showing Optimal and Sub-optimal Immunostaining

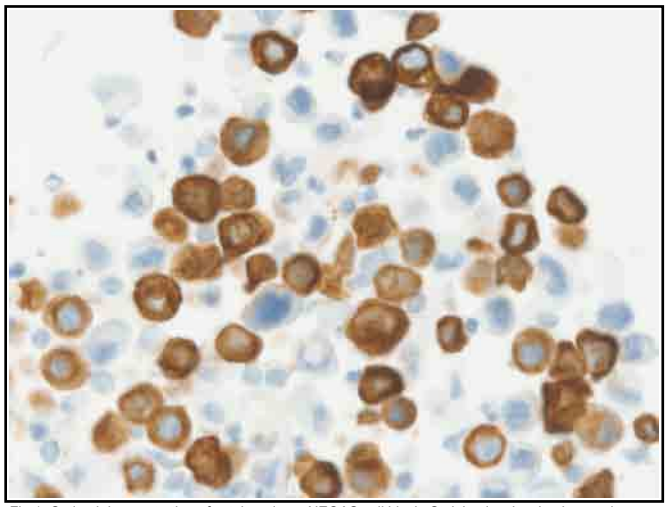


Fig 1. Optimal demonstration of cytokeratin on NEQAS cell block. Staining is crisp, background is clean, and the counterstain is of optimal intensity. Dako AE1/AE3, 1:200, 20 mins, with a high pH PT link for 20 mins, on a Dako Autostainer Link 48, using the Dako EnVision FLEX+ secondary layer 20 mins.

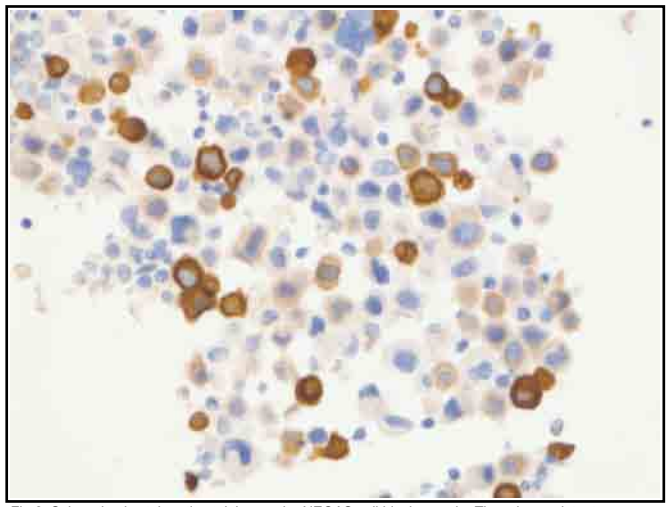


Fig 2. Sub-optimal cytokeratin staining on the NEQAS cell block sample. There is consistent, but weak non-specific staining of many cells (compare Fig 1). Adequate for diagnosis, assessed as a low pass. Dako AE1/AE3, 1:250, with Leica ER1 30 mins, on Leica Bond-III, and RTU Leica Bond Polymer Refine.

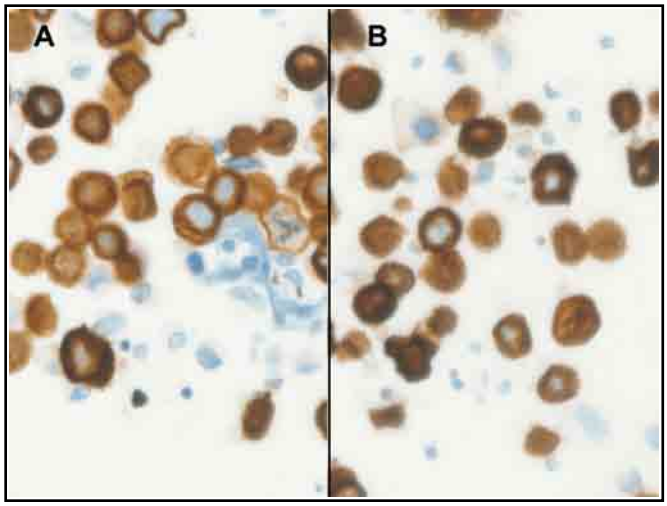


Fig 3. Optimal demonstration on cell block samples using (A) BD CAM 5.2, 1:50, no RT given, on a Ventana Benchmark ULTRA and Ventana UltraView Kit; (B) Dako MNF116, 1:300, again no RT details, on a Ventana Benchmark XT, and the Ventana UltraView Kit. Both slides are clean and precise.

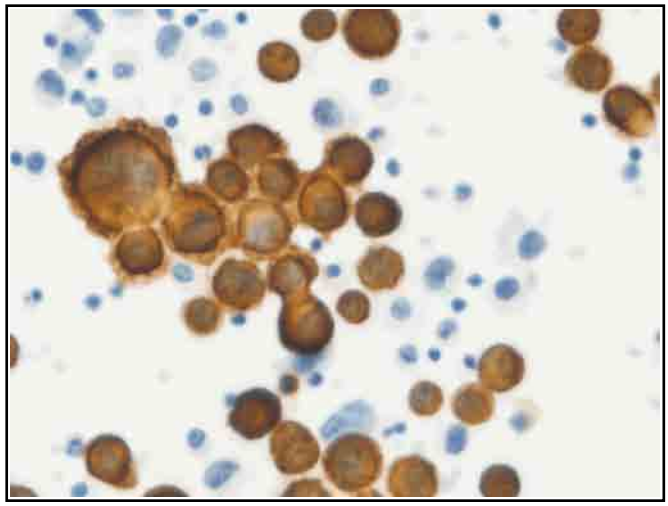


Fig 4. Optimal cytokeratin staining on a cytospin sample. Only epithelial cells are demonstrated. Dako MNF116, 1:50, 12 mins, no retrieval, employing the Ventana UltraView Kit, on a Ventana Benchmark ULTRA.

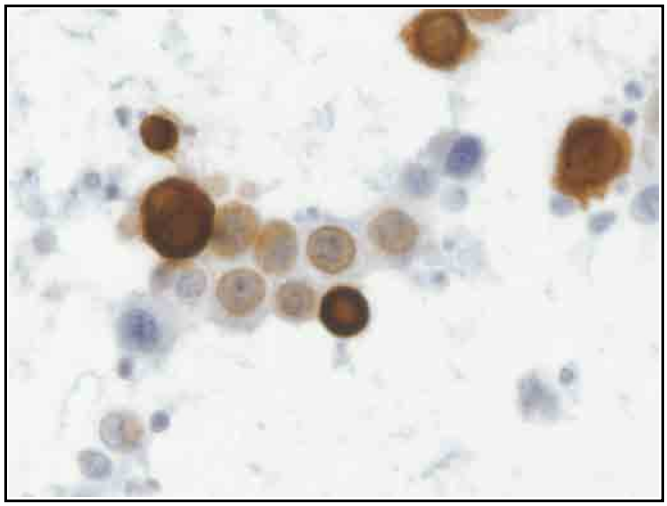


Fig 5. Sub-optimal cytokeratin staining on the NEQAS cytospin sample. There is both non-specific staining and a contaminant, resulting in a borderline result. Novocastra NCL-L-AE1/AE3, 1:250, 10 mins, with Leica ER2 10 mins, on a Leica Bond-III, with a RTU Leica Bond Polymer Refine, 8 mins.

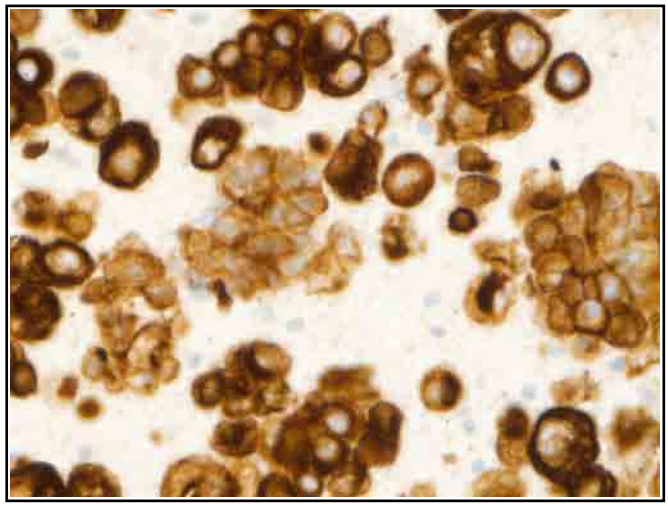


Fig 6. Excellent cytokeratin staining from an in-house cell block section of pleural effusion. There is little or no background staining of the matrix. Dako MNF116, 1:50, 30 mins, with a high pH Dako Omnis RT, on the Omnis platform. The secondary layer is the Dako EnVision FLEX kit for 20 mins.

Selected Images showing Optimal and Sub-optimal Immunostaining

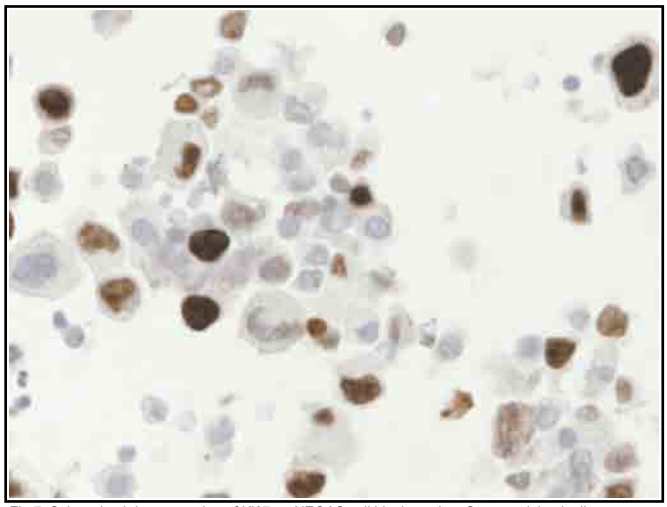


Fig 7. Sub-optimal demonstration of Ki67 on NEQAS cell block section. Some staining is dirty and muddy, and the haematoxylin appears not to have been sufficiently blued; although positive nuclear are present. Novocastra RTU (K2), 20 mins, with Leica ER2 10 mins, on Leica Bond-III, and Refine kit for 8 mins.

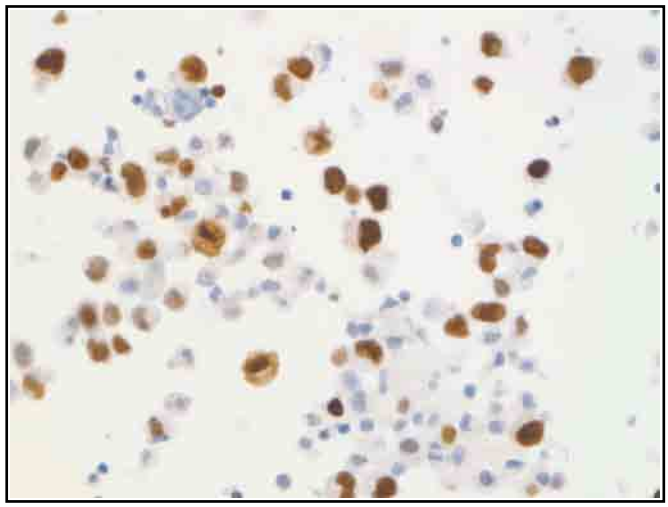


Fig 8. Sub-optimal demonstration of Ki67 on NEQAS cell block section; there is some non-specific staining and the background matrix has some colouration. Leica/Novocastra RTU (K2), 15 mins, Leica ER2 20 mins, Leica Bond-III, Leica Bond Polymer Refine kit. Similar to above protocol albeit slightly cleaner.

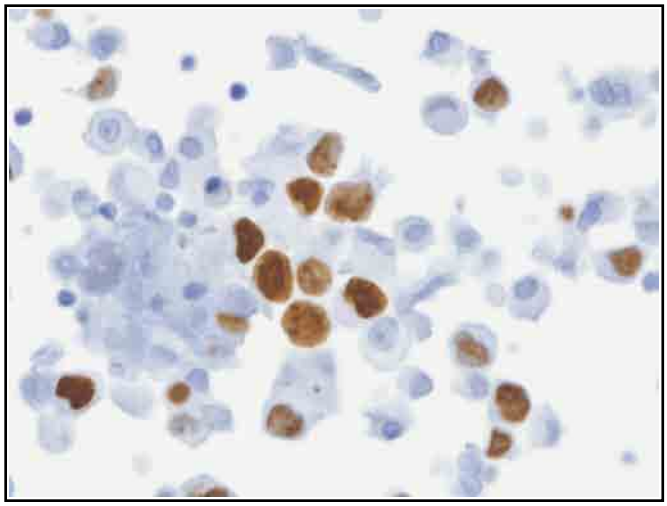


Fig 9. Sub-optimal demonstration of Ki67 on NEQAS cell block sample, the section shows morphological damage, causing cytoplasmic haematoxylin staining. Additionally, some cells are unstained. Dako FLEX RTU MIB-1, with the Dako PT Link, and the Dako Autostainer Link 48 platform.

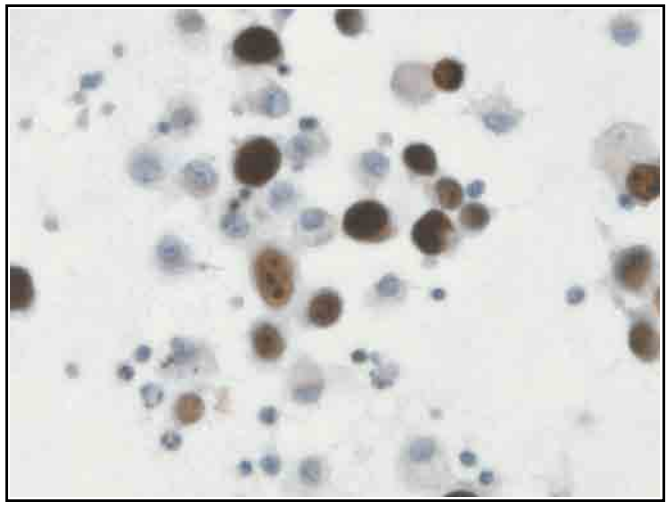


Fig 10. Sub-optimal demonstration of Ki67 on NEQAS cytospin slide. There is some non-specific staining, but this does not detract from its use for diagnostic purposes. Dako 7240 (MIB-1), 1:200, 60 mins, using a Pressure Cooker and EDTA, manual method with a RTU Dako REAL HRP/DAB kit.

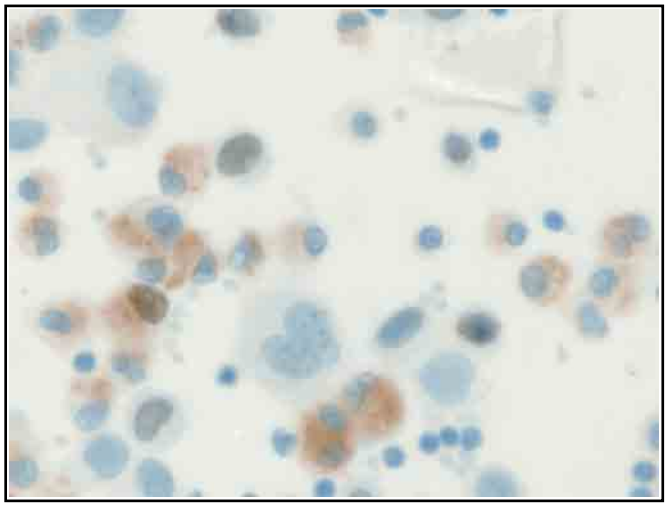


Fig 11. Sub-optimal demonstration of Ki67 on NEQAS cytospin slide. Staining is too weak and non-selective to be used with confidence, the slide failed the assessment. Dako 7240 (MIB-1), 1:100, 15 mins, no retrieval, on a Leica Bond-III, and with a RTU Leica Bond Polymer Refine kit for 8 mins.

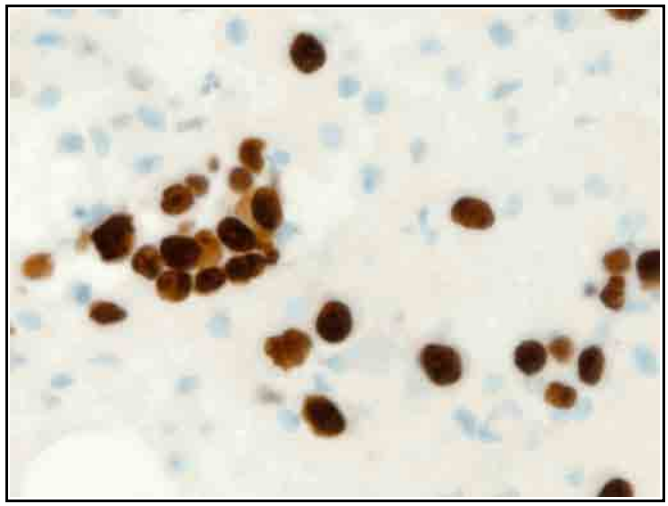
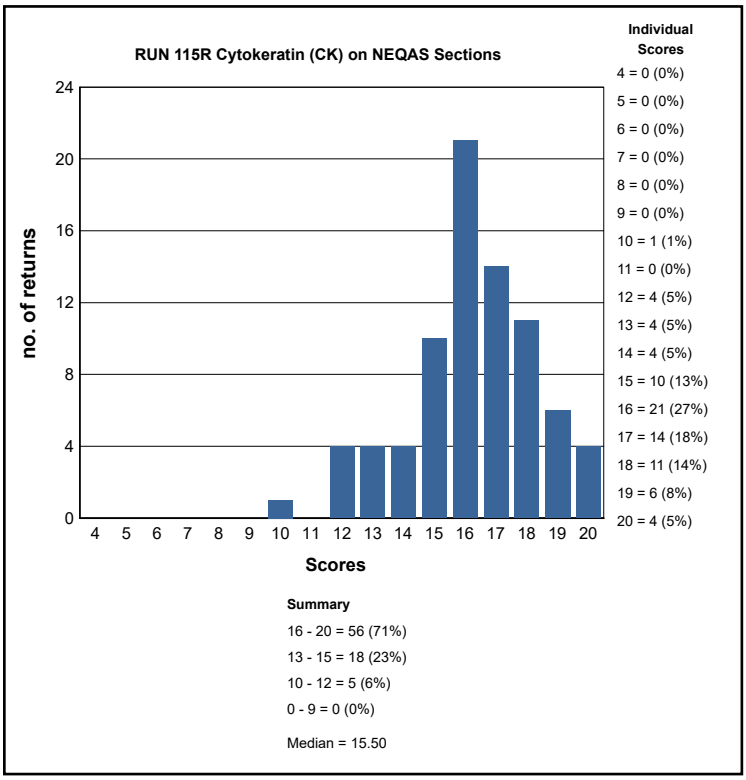
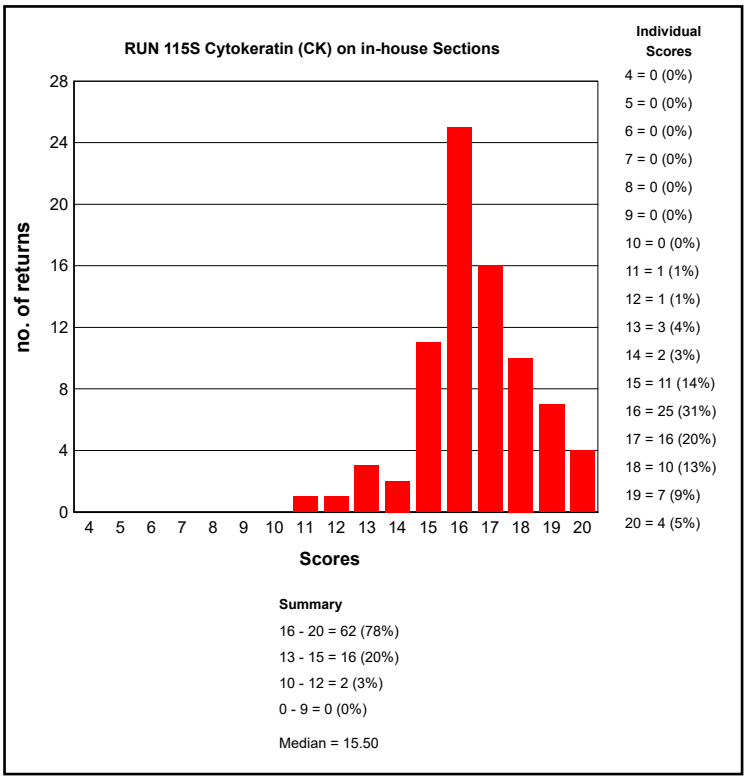
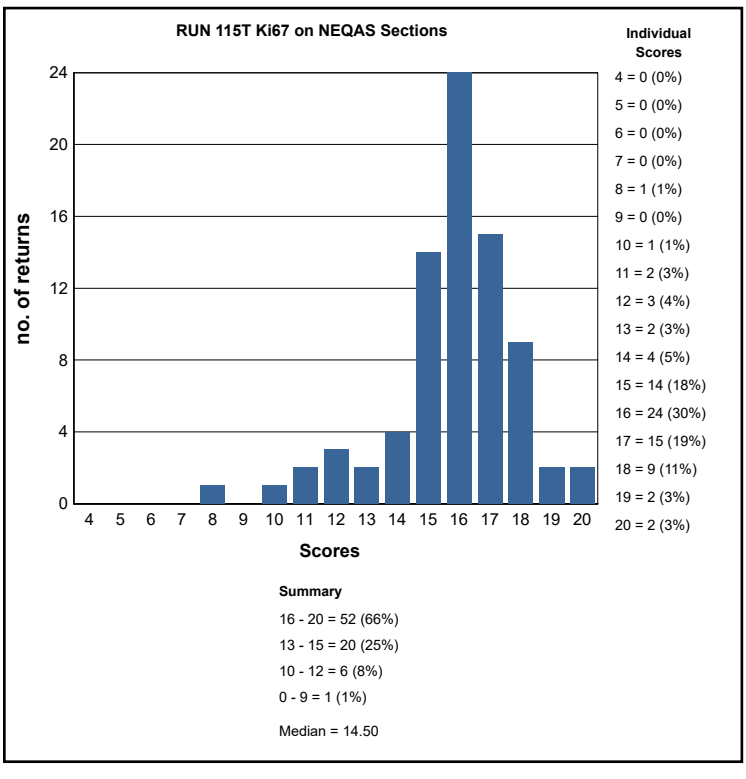


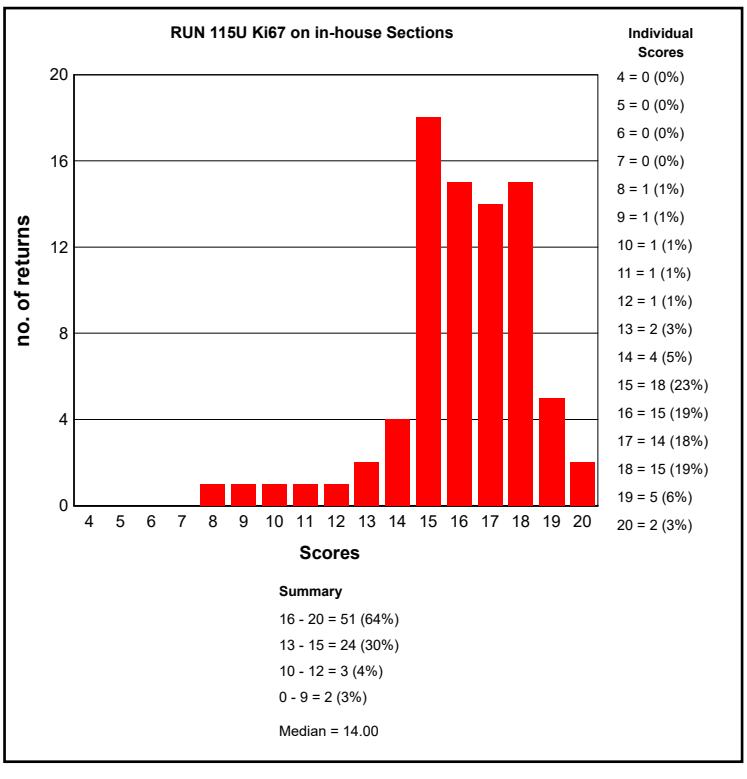
Fig 12. Nice example of Ki67 on an in-house cell block of pleural effusion. The staining is precise and the background and counterstain intensities are ideal. Dako 7240 (MIB-1), 1:50, 32 mins, with Ventana CC1 standard, Ventana Benchmark XT, and the UltraView Kit for 8 mins.

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: 115

Primary Antibody : Cytokeratin (CK)

Antibody Details	N	%
Becton Dickinson 349205 (CAM5.2)	3	100
BioGenex MU071-UC (clones AE1/AE3)	1	100
Dako M3515 (AE1/AE3)	18	83
Dako M0821(MNF116)	26	100
Leica/Novocastra RTU PA0909 (AE1/AE3)	1	100
Leica/Novocastra NCL-L-AE1/AE3	4	75
Ventana 760 2135 (AE1/AE3/PCK26)	3	100
Ventana 760 2595 AE1/AE3/PCK26	5	100
Other	6	100
Cell marque 307M-95 (CK7)	1	100
Biomedicals BMA-T-1302	1	100
Ventana CONFIRM 790-4373 (34BE12)	1	0
Dako FLEX RTU IR053 (AE1/AE3)	3	100
ImmunoBS MM-1012 (CK cocktail)	1	100
Ventana 790-4555 (CAM 5.2)	2	100
Leica/Novacastra NCL- L-CK5/6/8/18 (Multi 5D3/LP34	1	100
Dako Omnis FLEX GA053 (AE1/AE3)	2	100

Cytology Run: 115

Primary Antibody : Ki67

Antibody Details	N	%
Dako M7187 (Ki-67)	2	100
Dako 7240 (MIB-1)	41	85
Dako FLEX RTU IR626 (MIB-1)	4	100
Leica/Novocastra (MM1) NCL-Ki67-CE	1	100
Leica/Novocastra RTU (MM1) PA0118	1	100
Leica/Novocastra RTU (K2) PA0230	7	100
Ventana RTU (30-9) 790-4286	21	95
Other	2	100

Cytology Run: 115

Primary Antibody : Cytokeratin (CK)

Antigen Retrieval	N	%
YES	29	37
NO	50	63
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	29	
Not Specified	0	

Cytology Run: 115

Primary Antibody : Ki67

Antigen Retrieval	N	%
YES	30	38
NO	49	62
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	30	
Not Specified	0	

Cytology Run: 115

Heat Mediated Retrieval

Cytology Run: 115

Heat Mediated Retrieval

Cytology Run: 115

Enzyme Mediated Retrieval

Cytology Run: 115

Enzyme Mediated Retrieval

Cytology Run: 115

Detection	Cytokeratin (CK)		Ki67	
	N	%	N	%
AS PER KIT	4	75	7	100
BioGenex HRP (HK 519-06K)	1	100	0	0
Dako EnVision FLEX (K8000/10)	2	100	2	100
Dako EnVision FLEX+ (K8002/12)	7	100	6	100
Dako Envision HRP/DAB (K5007)	1	100	1	100
Dako Envision+ HRP mouse K4004/5/6/7	2	100	1	100
Dako REAL HRP/DAB (K5001)	0	0	1	100
Leica Bond Intense R Detection (DS9263)	0	0	1	100
Leica Bond Polymer AP Red Detection (DS9305)	1	100	0	0
Leica Bond Polymer Refine (DS9800)	21	86	19	84
Other	2	100	2	100
Ventana iView system (760-091)	2	100	2	50
Ventana OptiView (760-700) + Amp. (7/860-099)	2	100	1	100
Ventana OptiView Kit (760-700)	10	90	10	100
Ventana UltraView Kit (760-500)	22	100	23	96

Cytology Run: 115

Automation	Cytokeratin (CK)		Ki67	
	N	%	N	%
BioGenex GenoMX 6000i	1	100	1	100
Dako Autostainer Link 48	9	100	9	100
Dako Autostainer Plus Link	1	100	1	100
Dako Omnis	3	100	3	100
LabVision Autostainer	0	0	1	100
Leica Bond Max	7	86	5	80
Leica Bond-III	15	87	15	80
None (Manual)	1	100	1	100
Ventana Benchmark GX	3	67	4	100
Ventana Benchmark ULTRA	27	96	27	96
Ventana Benchmark XT	12	100	12	83

Cytology Run: 115

Chromogen	Cytokeratin (CK)		Ki67	
	N	%	N	%
AS PER KIT	7	100	10	90
BioGenex Liquid DAB (HK153-5K)	1	100	0	0
DAKO DAB+	2	100	2	100
Dako FLEX DAB	9	100	9	100
Dako REAL EnVision K5007 DAB	0	0	1	100
Leica Bond Polymer Refine kit (DS9800)	22	86	18	83
Other	7	100	6	100
Ventana DAB	8	75	7	86
Ventana iview	2	100	2	50
Ventana Ultraview DAB	21	100	24	96

BEST METHODS - Gold Standard Antibody

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

Cytokeratin (CK) - Method 1

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

Primary Antibody:	Dako M3515 (AE1/AE3) , 20 Mins, 23 ºC Dilution 1: 200
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: FLEX TRS HIGH PH
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 23 ºC., Time 1: 10 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 20 Mins, 23 ºC

Cytokeratin (CK) - Method 2

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

Primary Antibody:	Becton Dickinson 349205 (CAM5.2) , 32 Mins, RT ºC Dilution 1: 50
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	None
EAR:	Ventana Protease, RT ºC. Digestion Time NEQAS: 12 Mins. In-House: 12 Mins
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

Cytokeratin (CK) - Method 3

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

Primary Antibody:	Dako M0821(MNF116) , 12 Mins, 37 ºC Dilution 1: 50
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	None
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

Cytokeratin (CK) - Method 4

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

Primary Antibody:	Dako Omnis FLEX GA053 (AE1/AE3) , 12.5 Mins, RT ºC Prediluted
Automation:	Dako Omnis
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, RT ºC., Time 1: 5 Mins
Detection:	Dako EnVision FLEX (K8000/10) , 20 Mins, RT ºC Prediluted

BEST METHODS - Secondary Antibody

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

Ki67 - Method 1

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

Primary Antibody:	Dako 7240 (MIB-1)
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 2

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

Primary Antibody:	Ventana RTU (30-9) 790-4286
Automation:	BioGenex GenoMX 6000i
Method:	AS PER KIT
Main Buffer:	Optimax Wash Buffer
HMAR:	None
EAR:	
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700)

Ki67 - Method 3

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

Primary Antibody:	Dako 7240 (MIB-1)
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)

Ki67 - Method 4

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

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

The Alimentary Tract Module: GIST Run 115

Suzanne Parry

	First Antibody	Second Antibody
Antigens Assessed:	CD117	CD34
Tissue Sections circulated:	Normal appendix, GIST and Desmoid.	
Number of Registered Participants:	115	
Number of Participants this Run	111 (97%)	

Introduction

Gold Standard: CD117

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

Features of Optimal Immunostaining: **(See Figs 1, 2, 3,4 & 6)**

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

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

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

Second Antibody: CD34

CD34 is a transmembrane glycoprotein, which is expressed on immature haematopoietic stem/progenitor cells, capillary endothelial cells and embryonic fibroblasts. It can also be found in splenic marginal zones, dendritic interstitial cells around vessels, nerves, hair follicles, muscle cells and sweat glands in various tissues. CD34 labels capillaries in most tissues but may be absent in large veins and arteries, and is negative in the sinus endothelium of placenta and spleen. CD34 is an excellent indicator of vascular differentiation, regardless of the tumour grade. Prior to the discovery of CD117, CD34 positivity was the best available indicator for a GIST diagnosis, however, it is not a very specific marker for this use. Overall, about 60-70% of GISTs are positive for CD34, although this varies by tumour location: CD34 expression is highest in gastric GISTs (85%), but is only seen in about 50% of small intestinal GISTs (Hasegawa et al; Rudolph). Of the less common locations, CD34 is expressed in 100% of GISTs of the oesophagus, 65% colonic, 96 of rectal GISTs, and 65% of GISTs in non-GI locations, such as the mesentery and omentum (Miettinen et al). In summary, although GISTs often stain for CD34, this staining is not exclusive to GISTs, for example, competing diagnoses, such as schwannoma and solitary fibrous tumour, may also stain for CD34.

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

- Strong staining of the endothelial cells in the blood vessels and lymphatic vessels throughout the appendix
- Good localisation of CD34 to the interstitial cells of Cajal in the appendix
- A strong, distinct membranous reaction of virtually all the neoplastic cells of the gastrointestinal stromal tumour
- Minimal background staining

Features of Sub-optimal Immunostaining (See Figs 10 & 11)

· Weak or negative staining of the endothelial cells and other elements in the appendix
· Non-specific nuclear staining (possibly caused by over pre-treatment)
· Weak and/or patchy staining of the tumour cells of the GIST
· Excessive background / non specific staining
· Staining of the tumour cells in the desmoid tumour

Tissue Distribution and Assessment Procedure

Composite slides comprising of a GIST, appendix and desmoid tumour were distributed to all participants for them to stain with the requested antibodies CD117 and CD34 using their routine protocol. The circulated tissue was sequential sections from the same tissue block used for both the requested antibodies.
Assessment was carried out around a multi-header microscope with each slide being assessed by 4 independent assessors and a microscope lead. All 3 samples on the slide were viewed, and then a final overall score out of 5 was given by each individual assessor. These four scores were then combined to give an overall score out of 20.

Assessment Summary:

Results from the **CD117** assessment showed an overall acceptable pass rate of 84% lower than the previous run on the Neqas distributed material. The main reason for sub-optimal marks was due to very weak staining of the GIST or non-specific and background staining, predominantly in the desmoid tumour. Inappropriate antibody dilution factor and antigen retrieval were the main causes of these issues. The most popular CD117 antibody choice still remains the Dako polyclonal, used by 91 participants and showed an acceptable pass rate of 86% in this assessment run.
The **CD34** antibody was chosen as the secondary antibody for this current run 115 assessment. The acceptable pass rate was similar to that of the CD117 assessment. Only 1 lab failed the assessment. It was encouraging to see that most labs are using a composite control with a positive GIST tumour along with normal tissue.

NEQAS Pass Rates Run 114 & 115 CD117		
Run no	114	115
Acceptable	93% (N=102)	84%(N=83)
Borderline	5% (N=5)	14%(N=15)
Unacceptable	3% (N=3)	3% (N=3)

References

1. Cordless et al., Biology of Gastrointestinal Stromal Tumours. J Clin Oncol 2004, 22(18): 3813-3825.
2. Blay et al., Consensus meeting for the management of gastrointestinal stromal tumors. Report of the GIST Consensus Conference of 20-21 March 2004, under the auspices of ESMO. Ann Oncol 2005 6: 566-578.
3. Parfitt JR, Rodriguez-Justo M, Feakins R, Novelli MR (2008) Gastrointestinal Kaposi's sarcoma: CD117 expression and the potential for misdiagnosis as gastrointestinal stromal tumour. Histopathology 2008, 52: 816-23.
4. Novelli M, et al. DOG-1 and CD117 are the antibodies of choice in the diagnosis of gastrointestinal stromal tumours. Histopathology 2010, 57(2): 259-270.
6. Miettinen et al, Immunohistochemical spectrum of GISTs at different sites and their differential diagnosis with a reference to CD117 (KIT). Mod Pathol. 2000 Oct;13(10):1134-42.

Selected Images showing Optimal and Sub-optimal Immunostaining

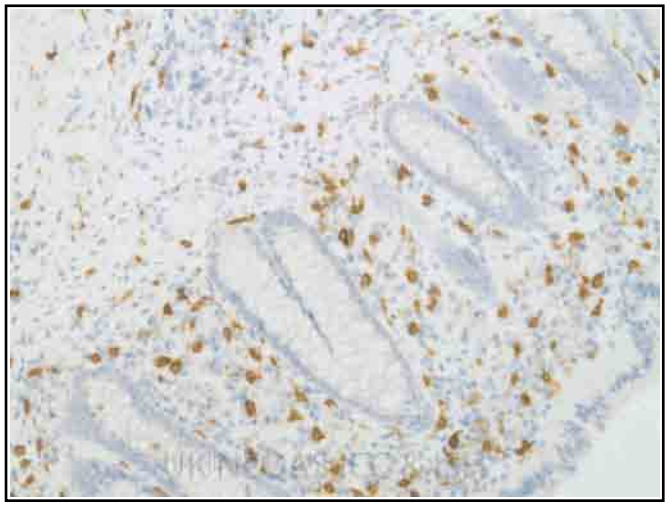


Fig 1. Good demonstration of CD117 in the UK NEQAS distributed appendix. The mast cells show distinct membranous staining, while the background remains clean. Stained with the Dako polyclonal antibody, 1:200 on a Leica Bond Max, ER2 retrieval for 20 minutess and Refine Detection.

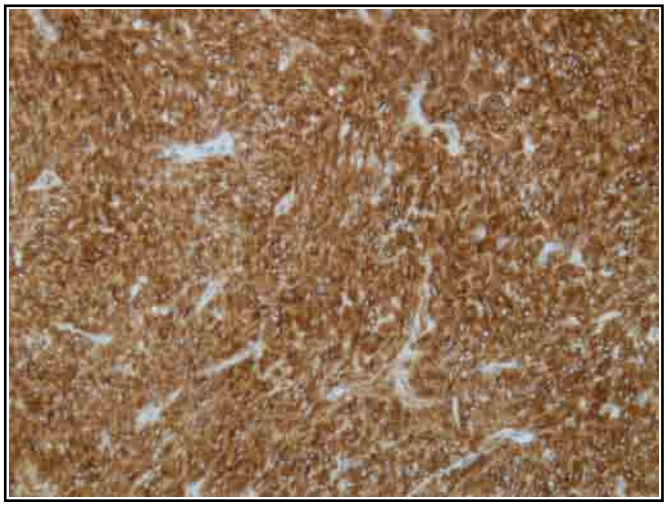


Fig 2. Good example of CD117 staining of the UK NEQAS distributed GIST, showing strong cytoplasmic and membranous staining in the tumour cells (same protocol as Fig 1).

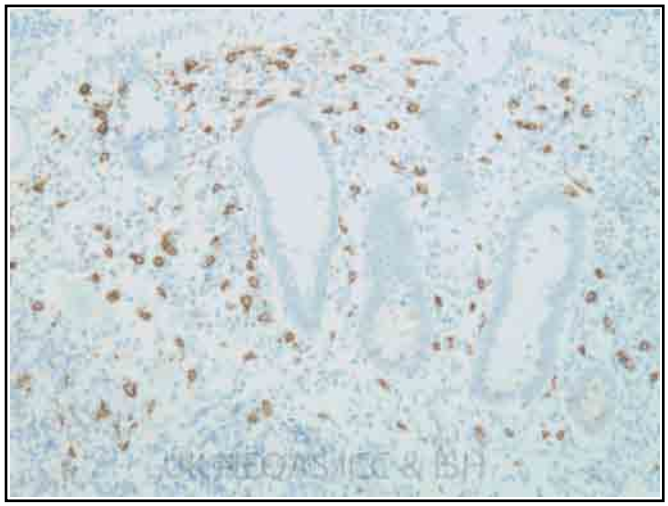


Fig 3. Optimal demonstration of CD117 in the UK NEQAS distributed appendix, showing strong crisp staining of the mast cells. Section stained with the Dako polyclonal antibody, 1:250, on the Dako autostainer and pretreatment in the PT link with high pH buffer solution.

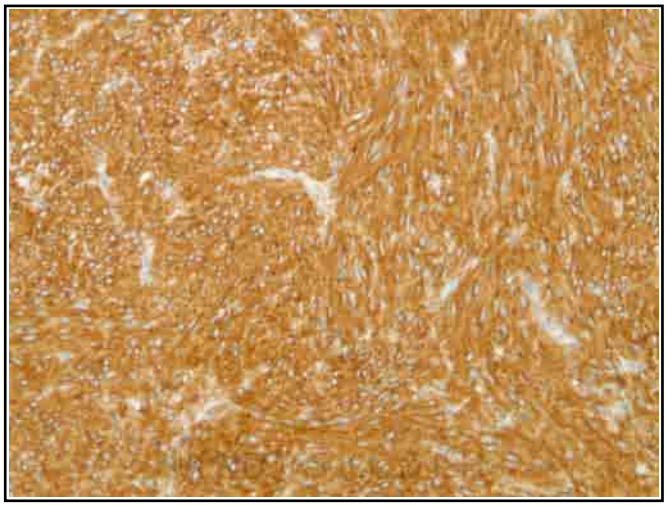


Fig 4. Another good example of optimal staining in the UK NEQAS GIST section: All the tumour cells show strong staining. The dako polyclonal antibody was used at a dilution of 1:300 on the Ventana Benchmark XT and CC1 standard antigen retrieval.

Fig 5. Poor desmontration of CD117 on the UK NEQAS distributed GIST section. Although most tumour cells are staining, the intesity is weak (compare to Figs 2 & 4). The appendix section on the same slide also showed very little staining of the mast cells. Stained with the Ventana 9.7 prediluted antibody on the Benchmark XT, CC1 retrieval for 64 minutes.

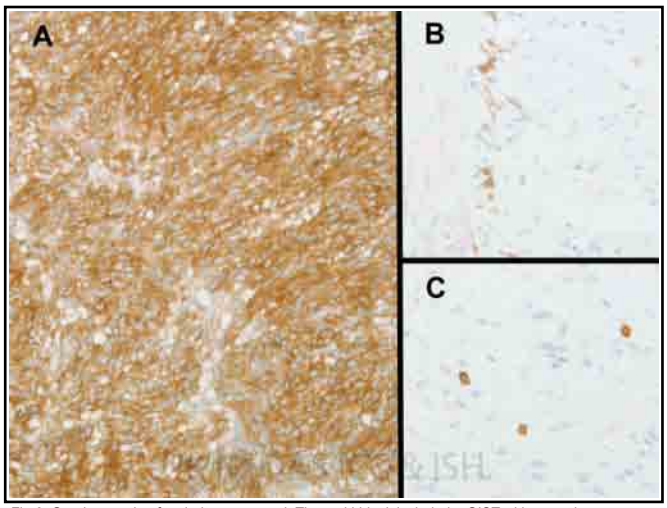


Fig 6. Good example of an in-house control. The multi-block included a GIST with normal epithelium and a demoid tumour. (A) shows strong staining of the GIST; (B) demonstrated the Cells of Cajhal; (C) mast cells staining in the desmoid section.

Selected Images showing Optimal and Sub-optimal Immunostaining

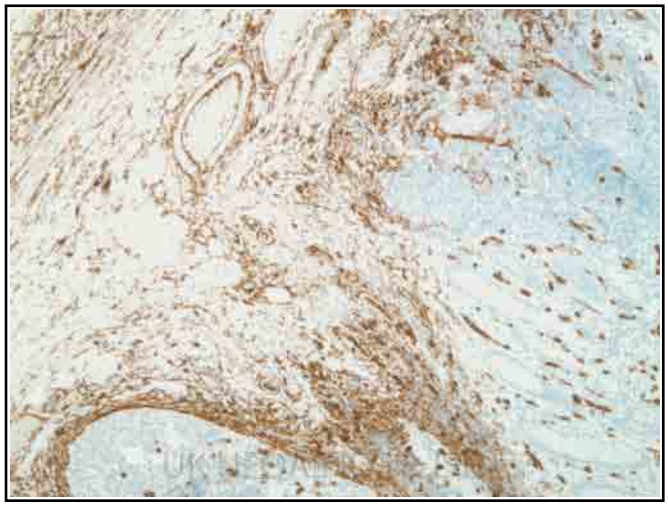


Fig 7. Optimal demonstration of CD34 in the UK NEQAS distributed appendix section. The example shows strong distinct staining of the vessels and endothelial cells while the background remains clean. Section stained with the Leica QBend 10 antibody, 1:100, on the Ventana ULTRA with Optiview detection.

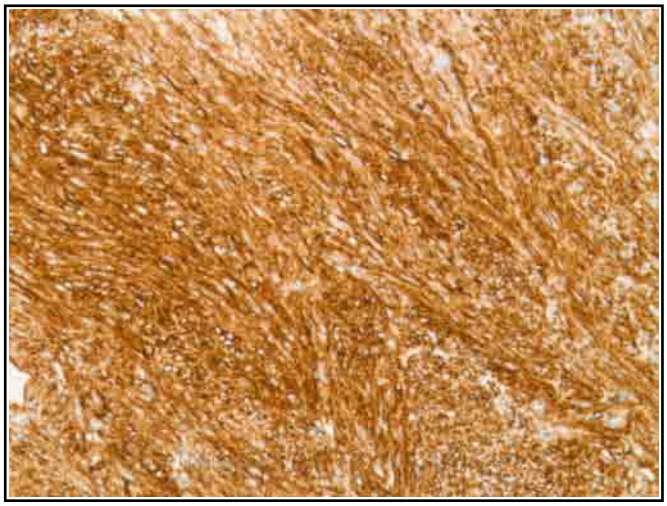


Fig 8. Good demonstration of CD34 in the UK NEQAS distributed GIST. The example shows intense, well localised membranous and cytoplasmic staining. Section stained with the Dako QBend 10 antibody, 1:50, on the Dako autostainer with pre-treatment in the PT Link, high pH buffer for 20 minutes.

Fig 9. Optimal staining in the UK NEQAS distributed desmoid section. The example shows strong staining in the endothelial cells and small vessels with minimal background (same protocol as Fig 8).

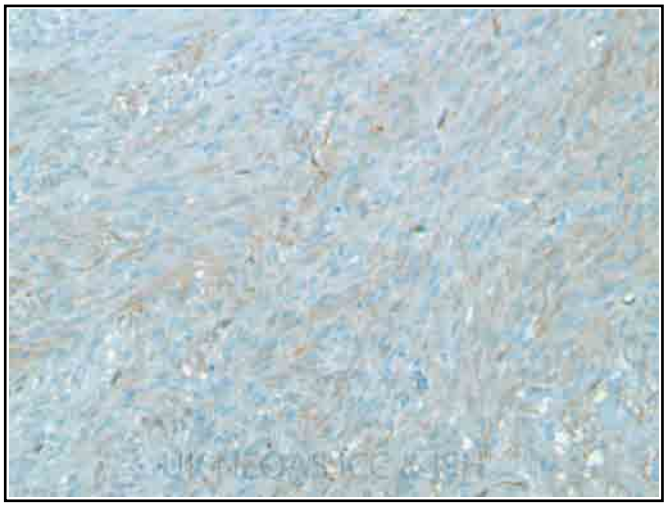


Fig 10. Sub-optimal demonstration of CD34 in the UK NEQAS GIST section. The staining is weak and patchy with some of the tumour cells not demonstrated (compare to Fig 8). A combination of insuffient antigen retrieval and sensitivity of the detection used may be the reason for the weaker staining: Leica RTU QBend 10 antibody on the Ventana Benchmark XT

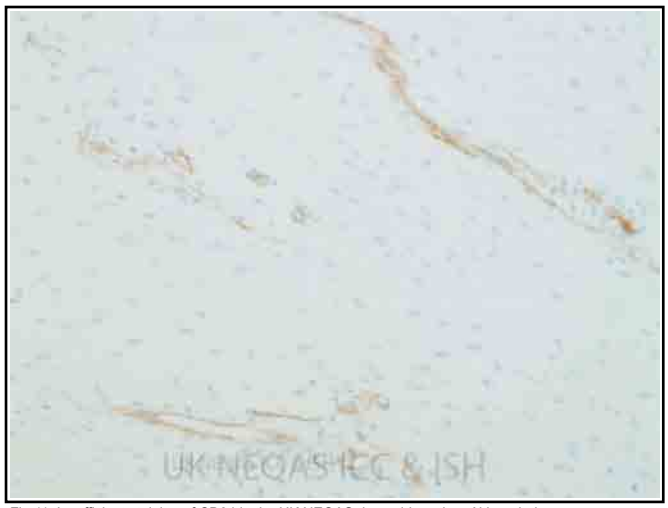


Fig 11. Insufficient staining of CD34 in the UK NEQAS desmoid section. Although the endothelial cells and vessels are demonstrated the staining is weak (compare to Fig 9). Same method as Fig 10

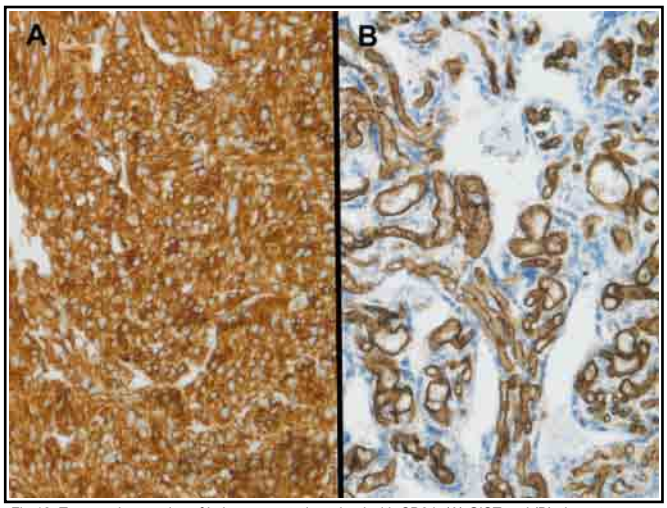
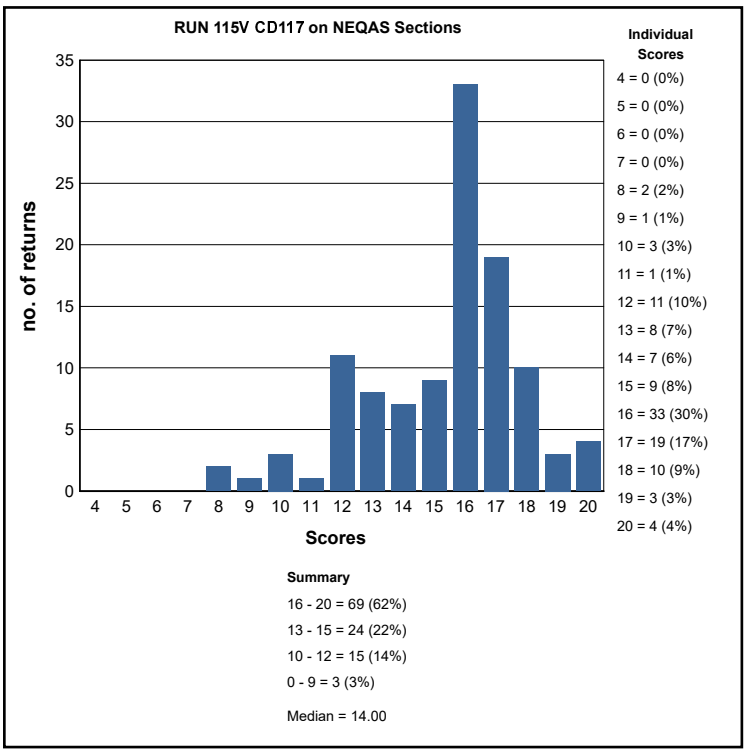
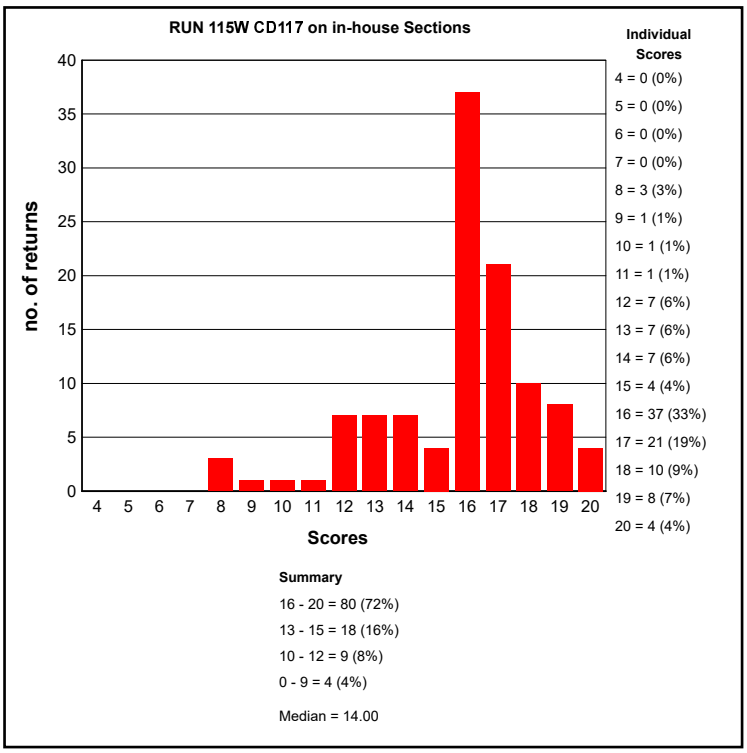
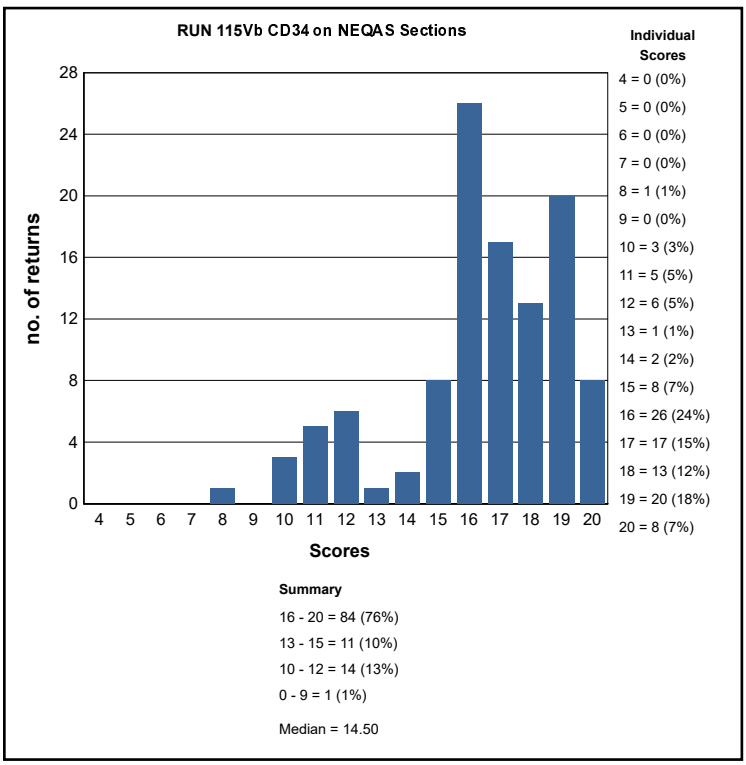


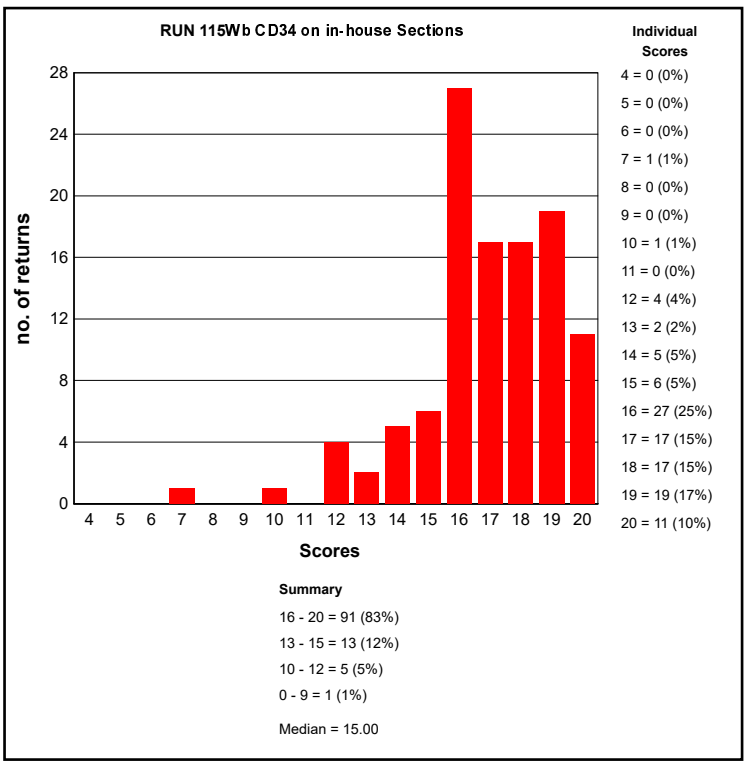
Fig 12. Two good examples of in house controls stained with CD34. (A) GIST and (B) placenta tissues; both showing strong crisp positive staining while the background remains clean. (A) stained with the Ventana antibody on the Benchmark XT; and (B) stained the Leica Qbend 10 antibody on the BondMax.

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: 115

Primary Antibody : CD117

Antibody Details	N	%
Dako A4502 (rb poly)	91	86
Leica/Novocastra NCL-CD117 (T595)	1	100
Ventana 790-2939 (rb poly)	1	0
Cell Marque 117R/S-xx (YR145)	4	100
Ventana 790-2951 (9.7)	10	70
Leica RTU (EP10) PA0007	3	100

Alimentary Tract Pathology Run: 115

Primary Antibody : CD34

Antibody Details	N	%
Dako IR/IS632 RTU (QBend10)	8	100
Dako M7165 (QBend10)	39	85
Leica NCL-END (QBend10)	14	93
Leica PA0212 (QBend10)	10	90
Leica NCL-E-ND (QBend10)	5	80
Leica RTU-END (QBend10)	3	67
Other	3	100
Serotec MCA 547 (QBend10)	1	0
Vector VP C345 (QBend10)	1	100
Ventana 790-2927 (QBend10)	24	83

Alimentary Tract Pathology Run: 115

Heat Mediated Retrieval	CD117 N	CD117 %	CD34 N	CD34 %
Dako Omnis	2	50	3	100
Dako PTLink	14	86	14	93
Leica ER1 10 mins	1	100	1	0
Leica ER1 20 mins	4	100	7	71
Leica ER1 30 mins	4	75	4	75
Leica ER2 10 mins	0	0	1	100
Leica ER2 20 mins	21	90	21	100
Leica ER2 30 mins	6	83	3	67
None	2	0	4	50
Pressure Cooker	1	100	1	100
Pressure Cooker in Microwave Oven	0	0	1	100
Ventana CC1 16mins	1	100	4	100
Ventana CC1 24mins	0	0	1	100
Ventana CC1 32mins	8	50	9	100
Ventana CC1 36mins	5	60	5	60
Ventana CC1 40mins	1	100	2	50
Ventana CC1 48mins	4	75	0	0
Ventana CC1 52mins	1	100	1	100
Ventana CC1 56mins	4	100	0	0
Ventana CC1 64mins	15	93	9	100
Ventana CC1 88mins	1	100	0	0
Ventana CC1 8mins	0	0	5	40
Ventana CC1 92mins	1	100	0	0
Ventana CC1 mild	6	100	7	71
Ventana CC1 standard	8	100	3	100

Alimentary Tract Pathology Run: 115

Enzyme Mediated Retrieval	CD117 N	CD117 %	CD34 N	CD34 %
AS PER KIT	0	0	4	100
NOT APPLICABLE	78	85	55	93
VBS Bond Enzyme 1	0	0	1	100
Ventana Protease 1 (760-2018)	0	0	1	100

Alimentary Tract Pathology Run: 115	CD117		CD34	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	9	100	9	78
BioGenex SS Polymer (QD 420-YIKE)	0	0	1	100
BioGenex SS Polymer (QD 430-XAKE)	1	100	0	0
Dako EnVision FLEX (K8000/10)	2	50	2	50
Dako EnVision FLEX+ (K8002/12)	8	75	7	100
Dako Envision HRP/DAB (K5007)	0	0	1	100
Dako Envision+ HRP mouse K4004/5/6/7	0	0	1	100
Dako Envision+ HRP rabbit K4008/9/10/11	1	100	0	0
Leica Bond Intense R Detection (DS9263)	1	100	0	0
Leica Bond Polymer Refine (DS9800)	34	88	35	91
Other	1	0	1	100
Ventana iView system (760-091)	1	100	1	100
Ventana OptiView (760-700) + Amp. (7/860-099)	1	100	1	100
Ventana OptiView Kit (760-700)	22	86	20	95
Ventana UltraView Kit (760-500)	28	82	28	71

Alimentary Tract Pathology Run: 115	CD117		CD34	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	1	100	1	100
Dako Autostainer Link 48	12	83	12	92
Dako Autostainer Plus Link	3	67	1	100
Dako Omnis	2	50	3	100
Leica Bond Max	11	73	11	73
Leica Bond-III	25	96	23	96
Ventana Benchmark GX	1	0	2	100
Ventana Benchmark ULTRA	35	86	36	81
Ventana Benchmark XT	20	85	14	79

Alimentary Tract Pathology Run: 115	CD117		CD34	
Chromogen	**N**	**%**	**N**	**%**
A. Menarini Liquid Stable DAB kit	1	100	0	0
AS PER KIT	18	83	18	94
BioGenex Liquid DAB (HK153-5K)	0	0	1	100
DAKO DAB+	1	0	1	100
Dako DAB+ REAL Detection (K5001)	0	0	1	100
Dako EnVision Plus kits	1	0	1	100
Dako FLEX DAB	10	80	8	88
Dako REAL EnVision K5007 DAB	0	0	1	100
Leica Bond Polymer Refine kit (DS9800)	32	88	33	85
NOT APPLICABLE	1	100	1	100
Other	6	83	5	100
Ventana DAB	9	100	8	100
Ventana iview	1	100	1	100
Ventana Ultraview DAB	30	83	29	69

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

Primary Antibody: Dako A4502 (rb poly) , 30 Mins, RT ºC Dilution 1: 1/200
Automation: Leica Bond Max
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 20 mins
EAR:
Chromogen: Leica Bond Polymer Refine kit (DS9800)
Detection: Leica Bond Polymer Refine (DS9800) , 15 Mins, RT ºC

CD117 - Method 2

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

Primary Antibody: Dako A4502 (rb poly) , 32 Mins, 37 ºC Dilution 1: 300
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)

CD117 - Method 3

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

Primary Antibody:	Dako A4502 (rb poly) Dilution 1: 250
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	AS PER KIT
HMAR:	Dako PTLink, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	AS PER KIT

CD117 - Method 4

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

Primary Antibody:	Ventana 790-2951 (9.7)
Automation:	Ventana Benchmark XT
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 32mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700)

BEST METHODS - Secondary Antibody

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

CD34 - Method 1

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

Primary Antibody:	Leica NCL-END (QBend10) , 15 Mins Dilution 1: 100
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

CD34 - Method 2

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

Primary Antibody:	Leica NCL-E-ND (QBend10) , 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:	
Chromogen:	Other, 36 ºC., Time 1: 8 Mins
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099) , 8 Mins, 36 ºC Prediluted

CD34 - Method 3

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

Primary Antibody:	Dako M7165 (QBend10) , 30 Mins, 20 ºC Dilution 1: 50
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer, PH: 7.6
HMAR:	Dako PTLink, Buffer: High PH TRS, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 20 ºC., Time 1: 10 Mins, Time 2: 10 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 15 Mins, 20 ºC Prediluted

CD34 - Method 4

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

Primary Antibody:	Ventana 790-2927 (QBend10) , 12 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 32mins, 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

Lynch Syndrome/HNPCC Run 115

Keith Miller and Suzanne Parry

	Gold Standard	Second Antibody
Antigens Assessed:	MSH2	MSH6
Tissue Sections circulated:	Positive and negative colonic tumours and normal appendix	
Number of Registered Participants:	91	
Number of Participants This Run:	82 (90%)	

General Introduction

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

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

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

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

Mismatch Repair Markers

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

Clinical Interpretation & Reporting Guidelines

We recommend reporting MMR IHC findings in tumours as either:

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

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

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

Clinical Reporting

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

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

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

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

Further Discussion on MMR proteins

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

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

Assessment Procedure:

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

Features of Optimal Immunostaining: (Figs 1, 3, 5, 7, 8, 10, &12)

Appendix: (Figs 1, 2 & 7)

Strong nuclear staining of the epithelium at the base and lower third-half of the crypts with fading of nuclear staining intensity in the middle and upper thirds of the crypts, to negative or weak staining of epithelial nuclei at the luminal surface.

- Strong staining of lymphoid follicles.

Tumour without loss of MMR protein: (Figs 5 ,10 & 12B)

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

Tumour with loss of MMR protein: (Figs 3, 8 & 12A)

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

Features of Sub-optimal Immunostaining: (Figs 2, 4, 6,9 & 11)

Appendix: (Figs 2)

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

Tumour without loss of MMR protein: (Figs 6 &11)

- 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 4 & 9)

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

Assessment Summary:

The pass rates for the **MSH2** assessment were higher than the previous time this antibody was assessed (Run 111), with 72% of labs achieving an acceptable pass, and a further 18% of participants receiving a borderline score (10-12/20), and therefore an overall pass of 90%. There was a fail rate of 10%, which was higher than the previous assessment for MSH2 (Run 111). However, similarly to previous runs, the main reason for a failed assessment was due to either weak staining or inappropriate non-specific staining. The Ventana G219-1129 clone was the most popular choice of antibody, and showed a pass rate of 78%. The Dako FE11 clone was also popular , used by 30 labs and showed a pass rate of 81%.

The **MSH6** assessment showed a higher pass rate to that of the previous run (111). 76% of participants received an acceptable pass, and a further 18% received a borderline pass. Similarly to the **MSH2** assessment, the fail rate was quite low with 5 laboratories (6%) receiving a score of under 10. The scores overall were higher than the last time **MSH2** was assessed. Again, weak staining was the main reason for failure or borderline scores (depending on the severity). The Ventana 44 clone was the most popular choice of MSH2 antibody used in this assessment by 27 labs, and showed a pass rate of 81%. The Dako EP49 clone was also commonly used, and showed an overall pass rate of 80%.

In-House Control Tissue Recommendations

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

References

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

Selected Images showing Optimal and Sub-optimal Immunostaining

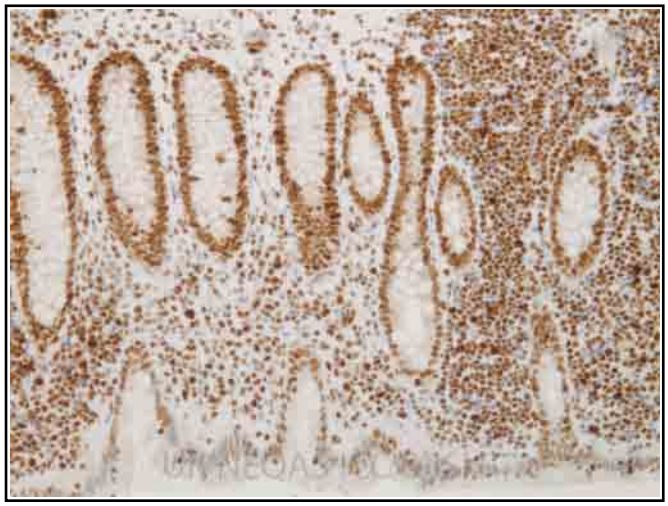


Fig 1. Optimal demonstration of MSH 2 in the UK NEQAS distributed appendix, showing strong staining of the basal and lower half of the epithelial crypts, fading towards the luminal surface. The Ventana (G219-1129) pre-dilute antibody was used on the Ventana Benchmark XT, pre-treatment CC1 for 32 mins with the optiview kit.

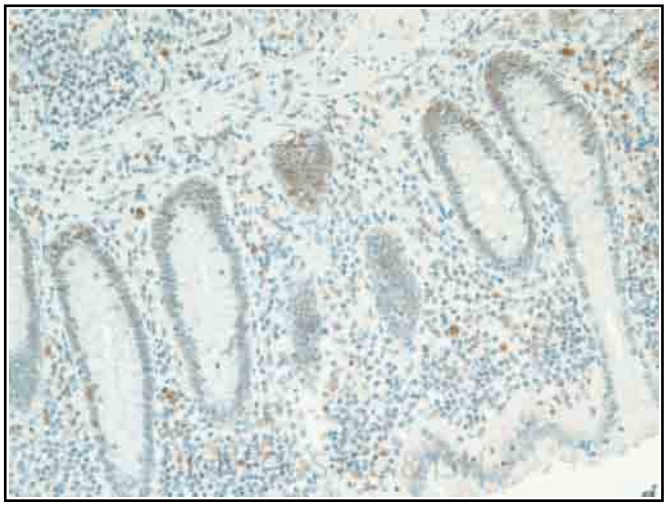


Fig 2.Sub-optimal weak MSH2 staining in the UK NEQAS distributed appendix. Section was stained using the Invitrogen (FE11) primary antibody, dilution 1:100 on the Leica Bond III with a pre-treatment of ER1 20 mins.

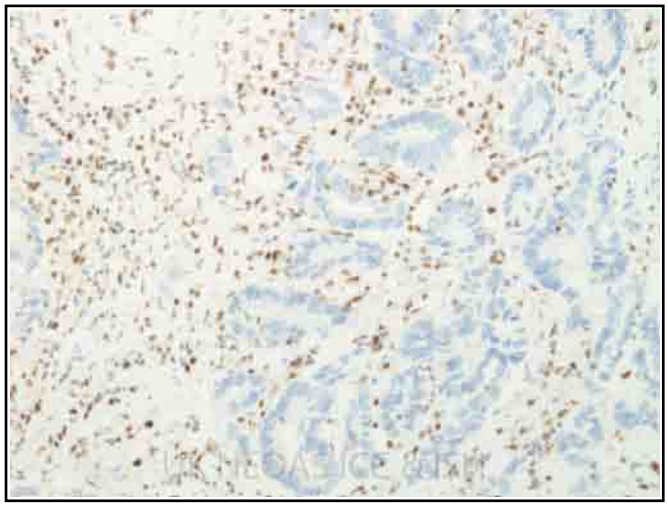


Fig 3. A good example of MSH2 in the UK NEQAS distributed negative tumour. The tumour is negative, whilst the intra-tumoral lymphoid and stromal cells are stained. The epitomics (9AC-0211) antibody was used at a dilution 1:800 with a pre-treatment of ER2 20 mins on the Leica Bond Max.

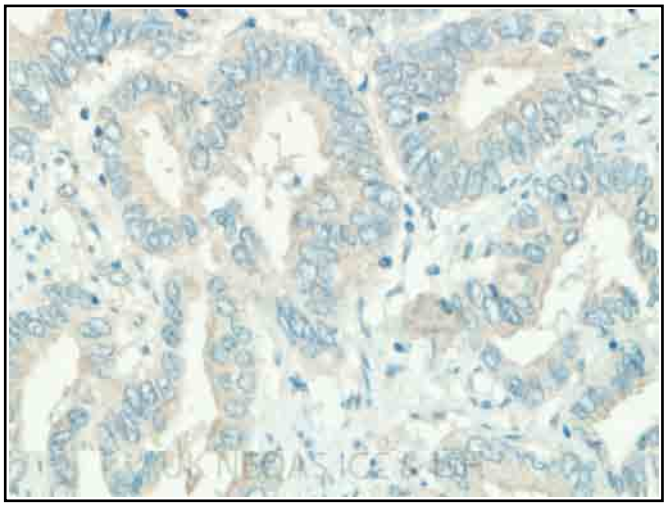


Fig 4. Poor MSH 2 demonstration, none of the expected lymphoid or stromal cells is staining and excessive cytoplasmic staining in the tumour cells. Same protocol is used as in (Fig 2).

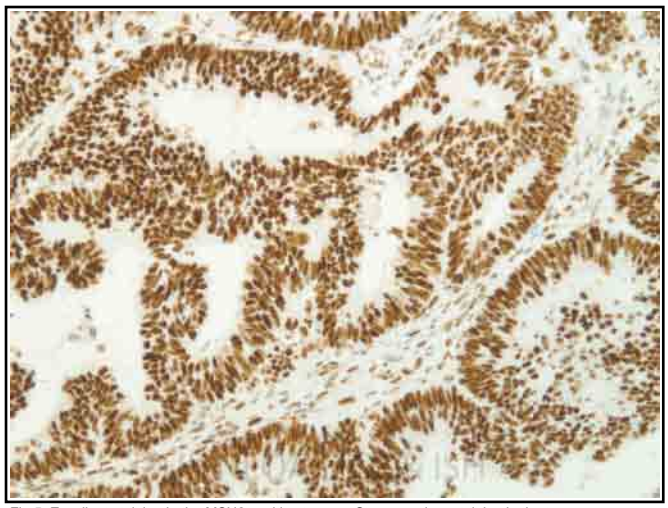


Fig 5. Excellent staining in the MSH2 positive tumour. Strong nuclear staining in the tumour cells as well as the intra-tumoral lymphoid and stromal cells. The protocol used was with the Dako Flex RTU antibody on the DAKO auto-stainer with the PT link with a High pH buffer.

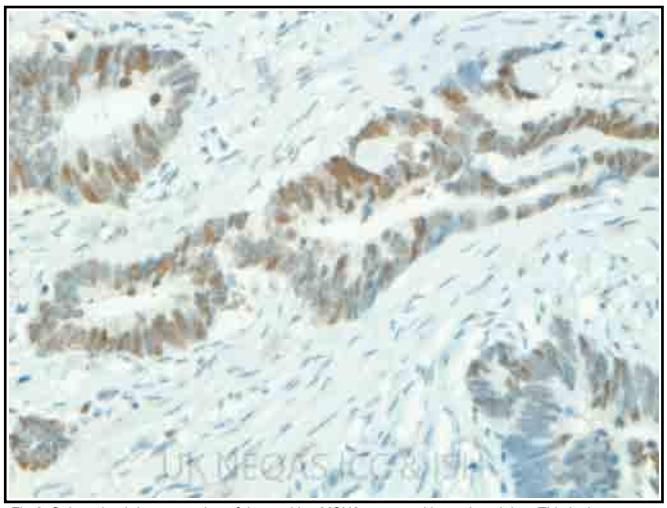


Fig 6. Sub-optimal demonstration of the positive MSH2 tumour with weak staining. This is the same protocol as used in Fig 2 and Fig 4. The reason for weaker staining may be due to the protocol using a low pH buffer instead of a high pH buffer.

Selected Images showing Optimal and Sub-optimal Immunostaining

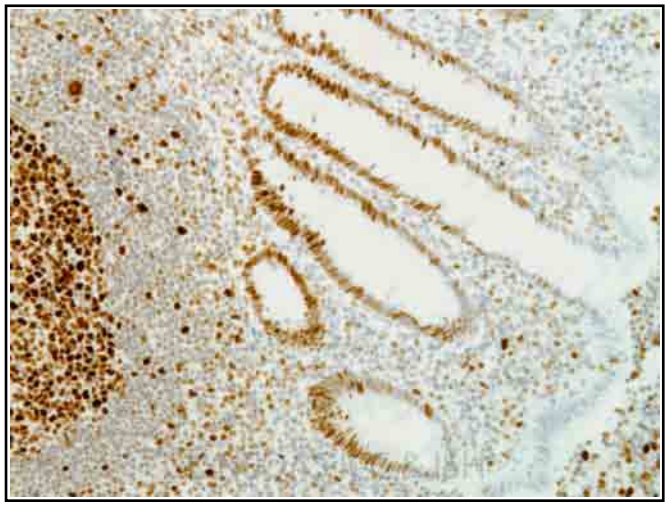


Fig 7. Optimal demonstration of MSH6 in the UK NEQAS distributed appendix, showing strong staining of the basal and lower half of the epithelial crypts, fading towards the luminal surface. The method used was on the DAKO Omnis with the DAKO RTU (EP49) primary antibody.

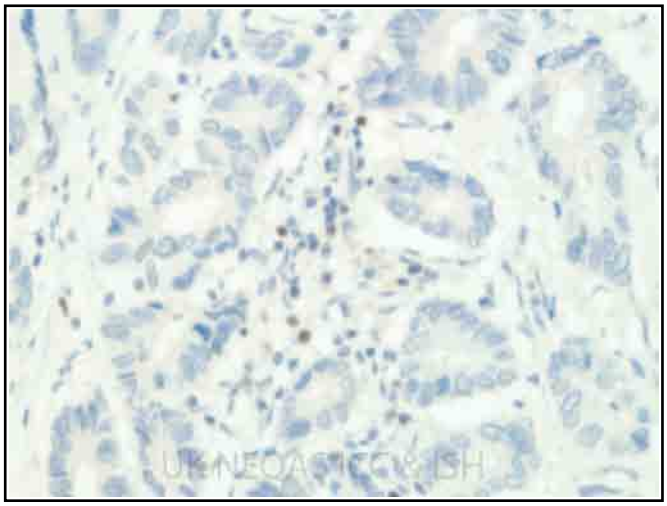


Fig 8. Acceptable staining in the MSH6 negative tumour. Slight blushing in the cytoplasm of the tumour cells. Section was stained with DAKO (EP49) 1:50 dilution with pre-treatment ER2 for 20 mins on the Leica Bond III.

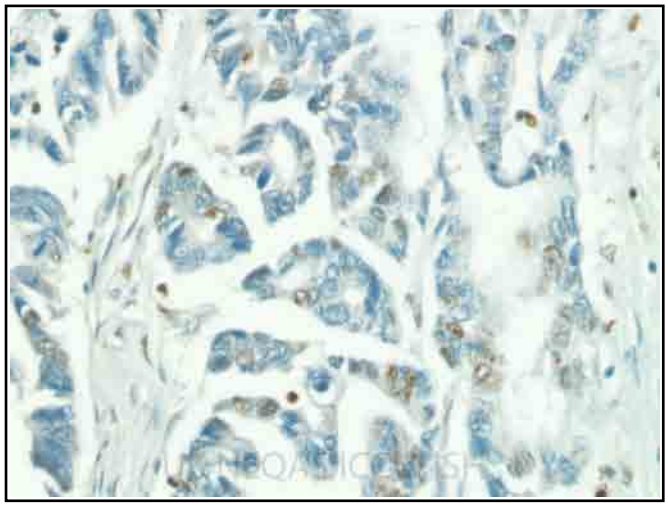


Fig 9. Unacceptable staining in the MSH6 negative tumour. False positive nuclear staining in the negative tumour. This laboratory also had overstaining in the appendix and positive tumour as well as the negative tumour. The section was stained on the DAKO auto-stainer with the PT link using the DAKO (EP49) at a 1:50 dilution.

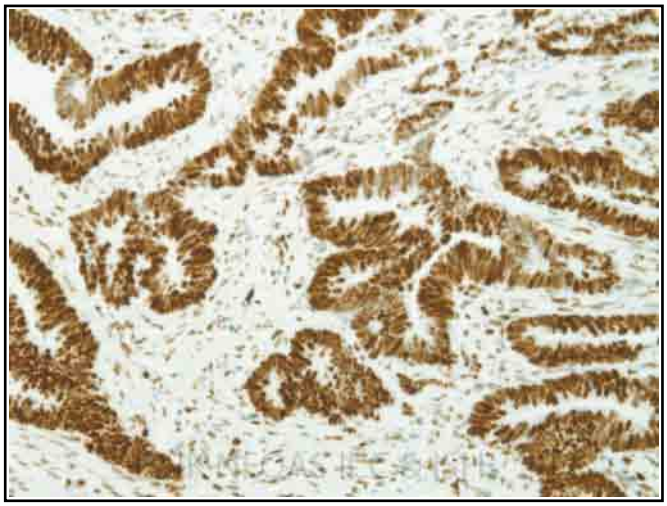


Fig 10. Good demonstration in the MSH 6 positive tumour, strong staining in the tumour and the stromal cells. The primary antibody used was the Abcam (EPR3945) on the Ventana Benchmark XT using CC1 64 mins as the pre-treatment.

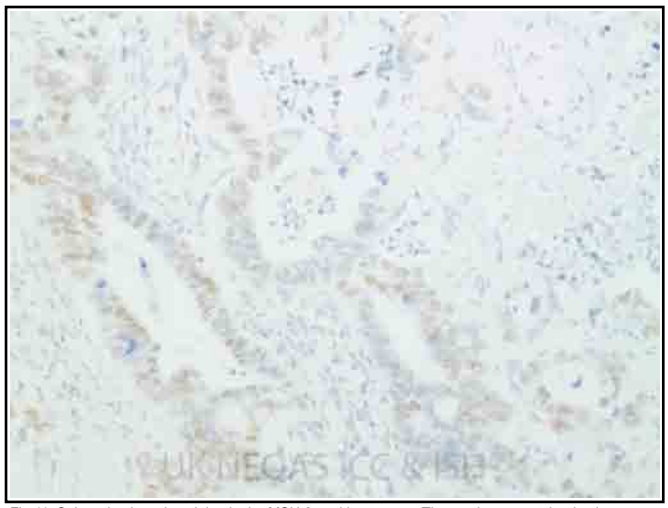


Fig 11. Sub-optimal weak staining in the MSH 6 positive tumour. The section was stained using the Cell Marque RTU antibody (287R-27/28) on the Leica Bond Max using pre-treatment ER2 for 30 mins.

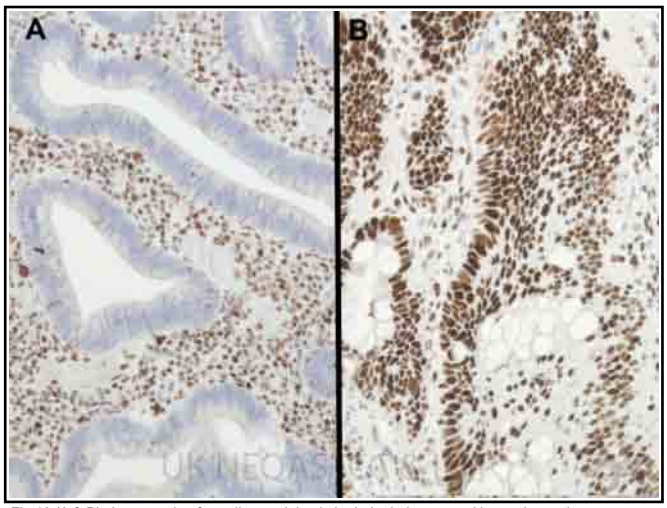
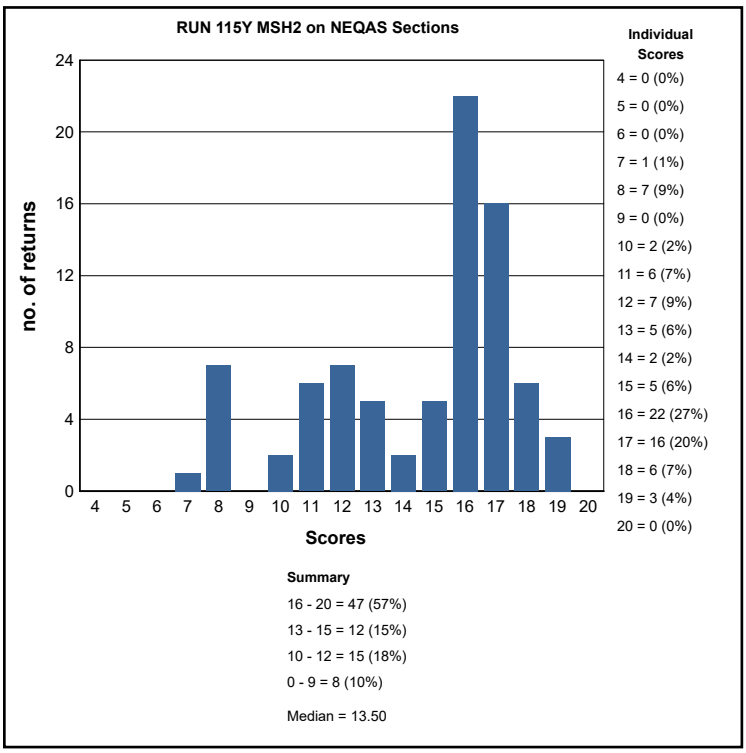
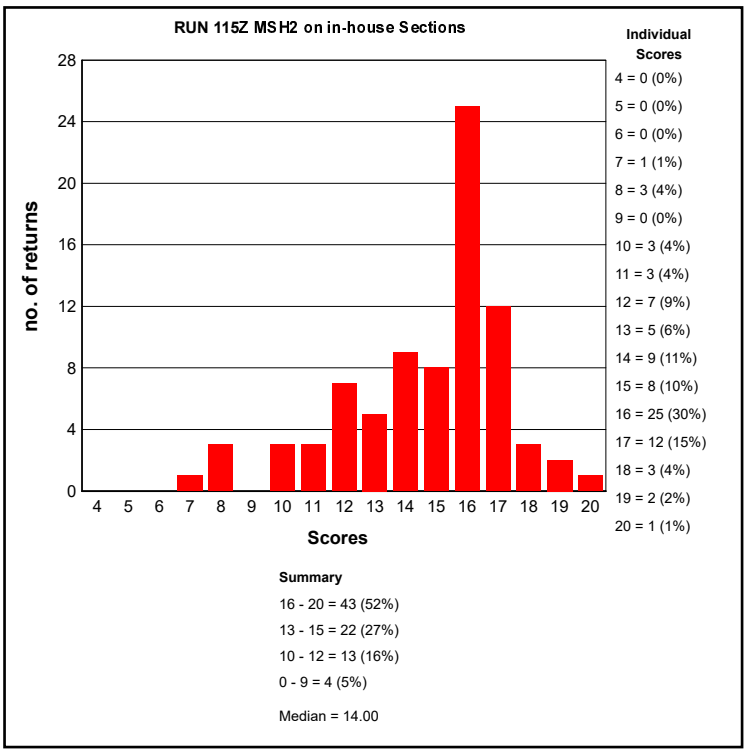
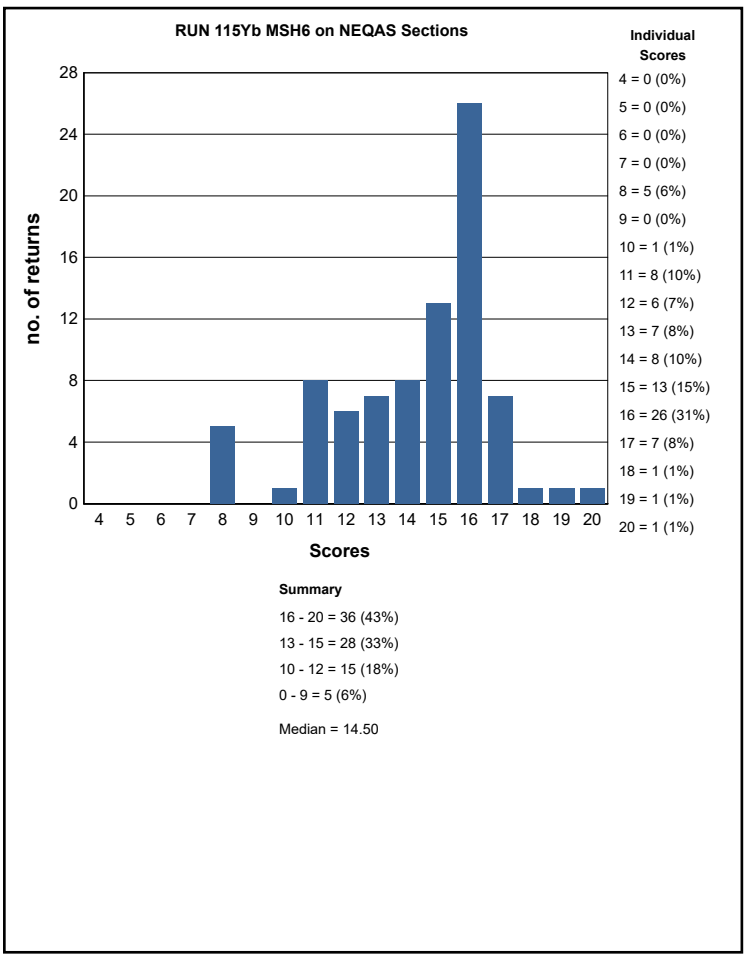


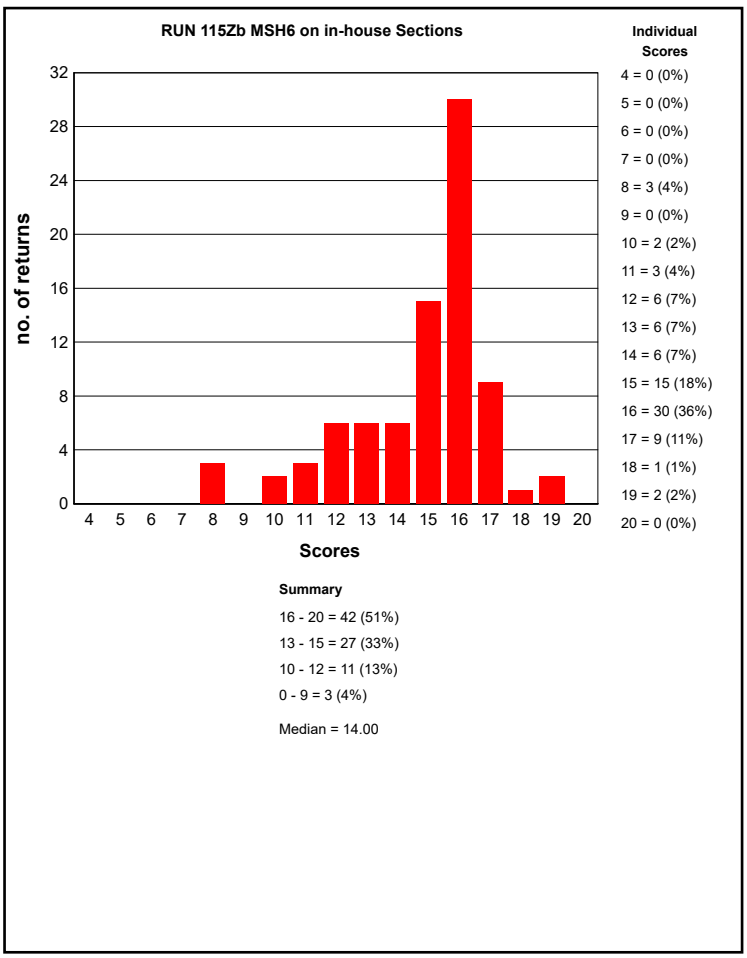
Fig 12 (A & B). An example of excellent staining in both the in-house positive and negative tumours. The method used was the DAKO (EP49) primary antibody 1:50. Pre-treatment used was ER2 for 30 mins on the Leica Bond III.

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: 115

Primary Antibody : MSH2

Antibody Details	N	%
Other	4	25
BD Bio/Pharmingen 556349 (G219-1129)	4	50
Leica/Novocastra NCL-MSH2 (25D12)	4	50
Invitrogen 33-7900 (FE11)	1	0
Ventana 760-4265 (G219-1129)	32	78
Cell Marque CMAx/Cx (G219-1129)	4	75
Calbiochem NA27/T (FE11)	1	0
Leica/Novocastra Bond RTU PA0048 (25D12)	1	100
Dako FLEX RTU IR085 (FE11)	16	75
Dako M3639 (FE11)	14	86
Epitomics AC-0211 RED2	1	100

Primary Antibody : MSH6

Antibody Details	N	%
Other	1	100
Abcam AB92471 (EPR3945)	2	100
Leica/Novocastra NCL-L-MSH6 (PU29)	1	0
Ventana CONFIRM 790-4455 (44)	27	81
BD T. Labs/BioSci/Pharmingen 610919 (44/MSH6)	3	33
BD T. Labs/BioSci/Pharmingen 610918 (44)	2	0
Cell Marque 287R-24/25/26 (SP93)	2	100
Cell Marque 287R-27/28 RTU (SP93)	1	0
Epitomics AC-0047 (EP49)	3	100
Dako FLEX RTU IR086 (EP49)	19	89
Dako M3646 (EP49)	23	70

HNPCC Run: 115

Heat Mediated Retrieval	MSH2 N	MSH2 %	MSH6 N	MSH6 %
Dako Omnis	2	100	3	100
Dako PTLink	10	90	9	89
Leica ER1 20 mins	2	0	1	0
Leica ER1 30 mins	0	0	3	67
Leica ER2 20 mins	11	73	7	71
Leica ER2 30 mins	8	75	11	64
Leica ER2 40 mins	5	60	3	100
Microwave	1	0	1	0
Ventana CC1 24mins	3	0	0	0
Ventana CC1 32mins	9	56	4	100
Ventana CC1 36mins	0	0	1	0
Ventana CC1 40mins	2	100	4	75
Ventana CC1 48mins	2	100	5	80
Ventana CC1 52mins	1	100	0	0
Ventana CC1 56mins	2	100	2	50
Ventana CC1 64mins	16	75	12	58
Ventana CC1 72mins	2	100	3	100
Ventana CC1 88mins	1	100	2	100
Ventana CC1 92mins	2	50	4	100
Ventana CC1 mild	1	100	0	0
Ventana CC1 standard	1	100	4	75
Ventana CC2 64mins	0	0	1	100
Ventana CC2 92mins	0	0	2	100
Water bath 95-98 OC	1	0	1	100

HNPCC Run: 115

Enzyme Mediated Retrieval	MSH2 N	MSH2 %	MSH6 N	MSH6 %
AS PER KIT	5	80	5	60
NOT APPLICABLE	47	68	48	75

HNPCC Run: 115	MSH2		MSH6	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	10	80	8	75
Dako EnVision FLEX (K8000/10)	1	100	2	100
Dako EnVision FLEX+ (K8002/12)	6	100	6	100
Dako Envision HRP/DAB (K5007)	1	0	1	100
Leica Bond Polymer Refine (DS9800)	24	67	24	71
Other	0	0	1	100
Ventana OptiView (760-700) + Amp. (7/860-099)	5	60	5	80
Ventana OptiView Kit (760-700)	28	79	26	85
Ventana UltraView Kit (760-500)	6	33	8	38

HNPCC Run: 115	MSH2		MSH6	
Automation	**N**	**%**	**N**	**%**
Dako Autostainer Link 48	7	100	7	100
Dako Autostainer plus	1	100	1	0
Dako Autostainer Plus Link	2	50	1	100
Dako Omnis	2	100	3	100
Leica Bond Max	9	33	8	50
Leica Bond-III	17	82	17	76
None (Manual)	2	0	2	50
Ventana Benchmark GX	2	100	2	100
Ventana Benchmark ULTRA	31	71	34	79
Ventana Benchmark XT	10	70	9	67

HNPCC Run: 115	MSH2		MSH6	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	20	80	22	82
Dako EnVision Plus kits	1	100	1	100
Dako FLEX DAB	7	100	8	100
Dako REAL EnVision K5007 DAB	2	0	2	50
Leica Bond Polymer Refine kit (DS9800)	25	68	24	71
Other	4	100	4	75
Ventana DAB	15	60	12	83
Ventana Ultraview DAB	9	56	11	55

BEST METHODS - Gold Standard Antibody

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

MSH2 - Method 1

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

Primary Antibody: Epitomics AC-0211 RED2 , 60 Mins, AMB ºC Dilution 1: 800
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 2: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 15 Mins, AMB ºC

MSH2 - Method 2

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

Primary Antibody: Ventana 760-4265 (G219-1129) , 16 Mins, 37 ºC Prediluted
Automation: Ventana Benchmark XT
Method: Ventana Optiview
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 32mins
EAR:
Chromogen: AS PER KIT, 37 ºC., Time 1: 8 Mins
Detection: Ventana OptiView Kit (760-700) , 8 Mins, 37 ºC Prediluted

MSH2 - Method 3

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

Primary Antibody:	Dako FLEX RTU IR085 (FE11)
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	AS PER KIT
HMAR:	Dako PTLink
EAR:	
Chromogen:	AS PER KIT
Detection:	AS PER KIT

MSH2 - Method 4

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

Primary Antibody:	Leica/Novocastra Bond RTU PA0048 (25D12)
Automation:	Leica Bond-III
Method:	Other
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 30 mins
EAR:	AS PER KIT
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	AS PER KIT

BEST METHODS - Secondary Antibody

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

MSH6 - Method 1

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

Primary Antibody:	Dako M3646 (EP49) , 20 Mins Dilution 1: 50
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 30 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800), Time 1: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins

MSH6 - Method 2

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

Primary Antibody:	Abcam AB92471 (EPR3945)
Automation:	Ventana Benchmark XT
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana OptiView Kit (760-700)

MSH6 - Method 3

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

Primary Antibody:	Dako FLEX RTU IR086 (EP49) , 25 Mins, RT ºC Prediluted
Automation:	Dako Omnis
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis
EAR:	
Chromogen:	Dako FLEX DAB, RT ºC., Time 1: 5 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 20 Mins, RT ºC Prediluted

MSH6 - Method 4

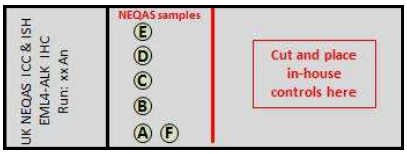
Participant scored 18/20 (UK NEQAS Slide) and 16/20 (In House slide) using this method.

Primary Antibody:	Dako FLEX RTU IR086 (EP49) , 20 Mins, 18 ºC Prediluted
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer, PH: 7
HMAR:	Dako PTLink, Buffer: Target Retrieval Solution (High pH), PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 18 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Other , 15 Mins, 18 ºC Prediluted

Suzanne Parry

Antibody Assessed	ALK
Samples Circulated	Composite slide (see table below)
Number Registered Participants/Submitted	66/56 (85%)

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



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

Introduction

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

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

Assessment Criteria

Same slide NEQAS & In-house controls

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

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

Interpretation criteria incorporating staining intensity

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

Assessment scoring guidelines

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

Table 2: Assessment interpretation

Results & Discussion

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

Distributed NEQAS Sample Results

There was a continued increase in number of participants from the previous run, with an 80% (N=45) acceptable pass rate for the NEQAS section. No laboratories received a borderline pass and 11 laboratories (20%) failed the assessment. This was mainly due to weak or very weak staining compared to the expected level of staining this was mainly due to laboratories not following the recommended staining protocols or inadequate antigen retrieval. The most popular antibody of choice was the Ventana/Roche (D5F3) with 44 participants using this antibody with a 91% pass rate. The Cell Signalling Technology (D5F3) was also used by 3 participants achieving a 100% pass rate.

Features of Optimal Staining. (Figs 1, 3, 5, 7, 8,10 &12A)

- Moderate to strong cytoplasmic staining of the ganglion cells in the appendix (Fig 1A&B)
- Moderate to strong granular staining of the positive tumour sample (Fig 5)
- Moderate to strong granular staining of the positive tumour cell line samples . The ALK negative cells within the mixed cell line should remain unstained.
- No staining in the negative tumour sample (Fig 7)
- No background or inappropriately localised staining

.

Features of Sub-optimal Staining (Figs 2, 6, 9 &11)

- False negative or absence of ALK staining where tumour cells should be staining positive (fig 9A)
- Non-specific / Excessive Tyramide staining (Fig 4)
- Absence of staining in appendix

In-house Control Results

Of the 56 participants taking part in this assessment, 35 (63%) achieved an acceptable result. 15 (27%) participants obtained a borderline pass result and 6 participants failed with the in-house sections. This was mainly due to the use of an inappropriate control for the lung cancer setting.

Lymphoma control

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

ALK IHC Control Recommendation

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

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

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

- Commercially available control material such as cell lines or xenografts, showing at least positive and negative ALK IHC expression may also be an acceptable alternative.
- In all cases the control material should initially be validated using FISH.

- It is also recommended that control material should be cut and placed alongside the clinical sample being tested.

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13. Fiona H. Blackhall, Solange Peters, Lukas Bubendorf et al., (2014) Prevalence and Clinical Outcomes for Patients With ALK-Positive Resected Stage I to III Adenocarcinoma: Results From the European Thoracic Oncology Platform Lungscape Project. J.Clin. Oncol 32 (25): 2780 - 2788.
14. Georg Hutarew, Cornelia Hauser-Kronberger, Felix Strasser et al., (2014) Immunohistochemistry as a screening tool for ALK rearrangement in NSCLC: evaluation of five different ALK antibody clones and ALK FISH. histopahology 65:398-407.

Acknowledgements

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

Selected Images showing Optimal and Sub-optimal Immunostaining

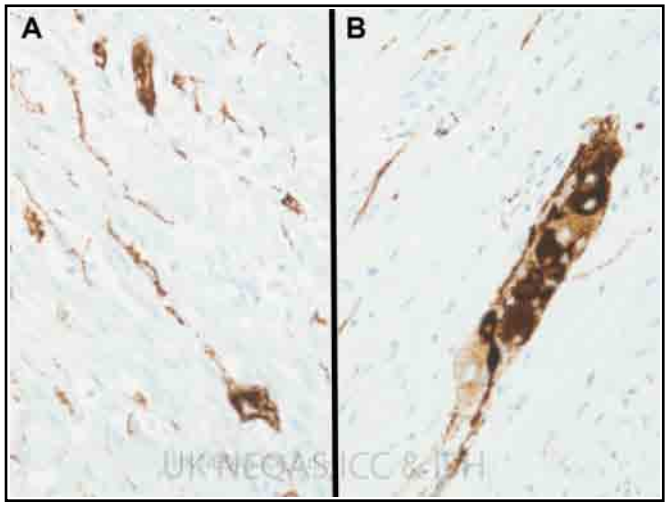


Fig 1. Two good examples of ALK in the UK NEQAS distributed appendix sample A. Both sections show the expected strong staining of ganglion cells and axons. (A) stained with the Ventana D5F3 assay and recommended protocol. (B) stained with the Cell Signaling D5F3 antibody on the Ventana Benchmark XT with OptiView detection.

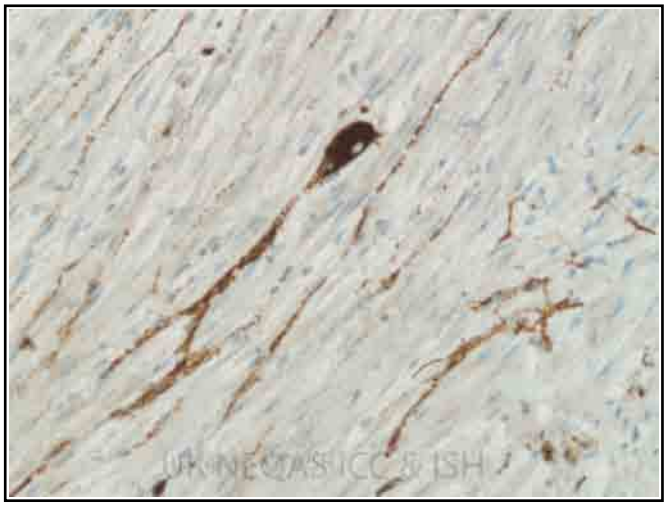


Fig 2. Sub-optimal demonstration of ALK on the UK NEQAS distributed appendix sample A. Although the ganglion and axons are staining as expected, the section also shows non-specific background staining and teramide deposit. Stained with the Ventana D5F3 assay on the ULTRA stainer.

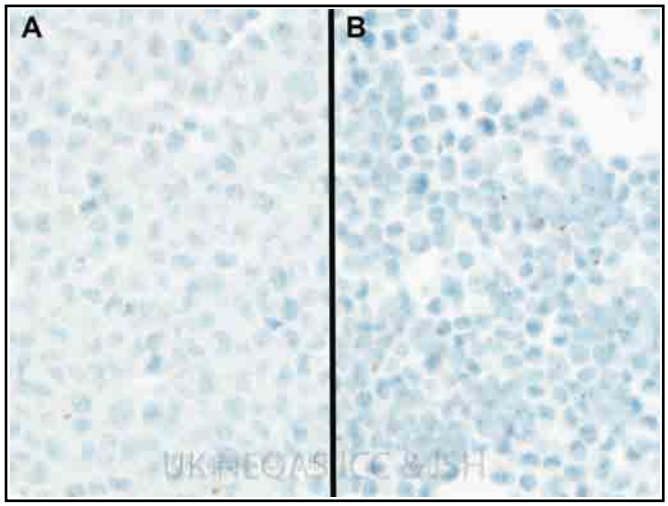


Fig 3. Optimal demonstration of ALK in the UK NEQAS distributed negative cell line sample B. As expected, both (A) and (B) show no staining. (A) stained with the Ventana D5F3 and (B) with the Cell Signaling antibody on the Ventana Benchmark.

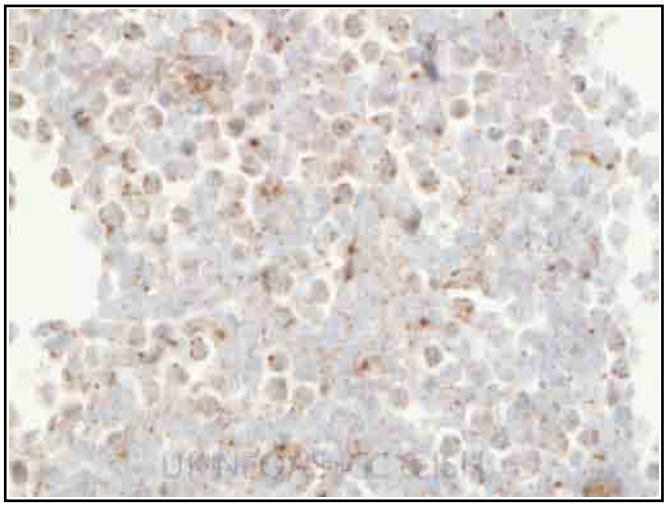


Fig 4. Sub-optimal staining with ALK on the UK NEQAS distributed negative cell line sample B, showing excessive background and teramide deposit. Stained with the Ventana D5F3 assay on the Benchmark GX.

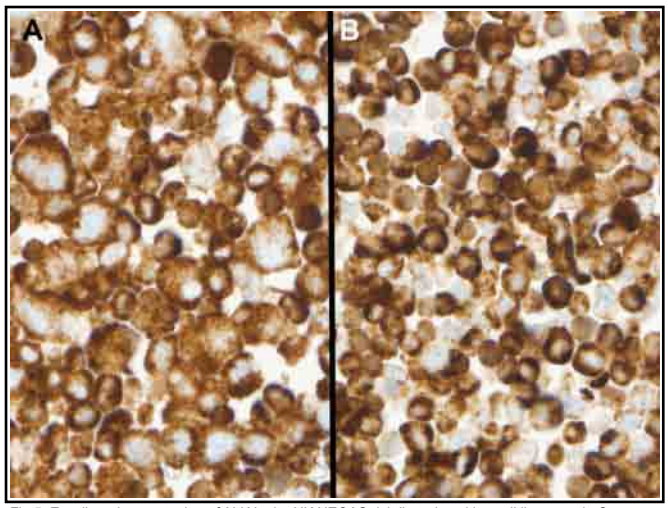


Fig 5. Excellent demonstration of ALK in the UK NEQAS dsitributed positive cell line sample C. Both sections show strong membranous and cytoplasmic staining of the neoplastic cells. (A) stained with the Ventana D5F3 assay with the recommended protocol. (B) stained with the Cell Signaling D5F3 antibody on the Ventana Benchmark XT with OptiView detection.

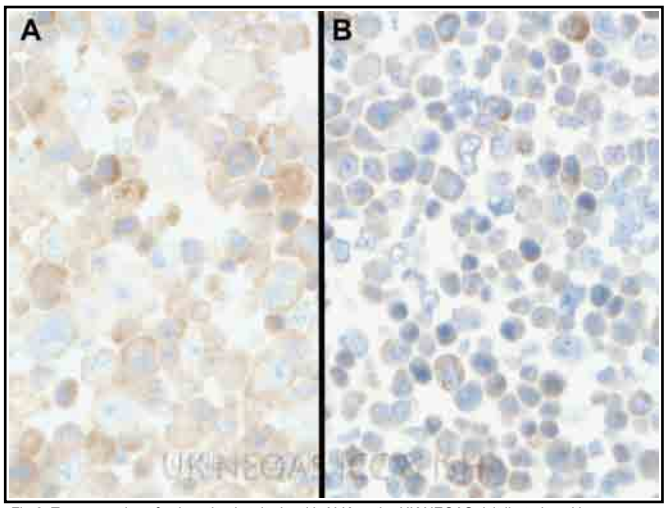


Fig 6. Two examples of sub-optimal stainnig with ALK on the UK NEQAS dsitributed positive cell line sample C. Both sections show weaker staining than expected. (A) stained with the Dako ALK1 antibody, 1:20, on the autostainer with prereatment in the PT link. This antibody is not recommended for use in the lung setting. (B) stained with the Novocastra 5A4 antibody,

Selected Images showing Optimal and Sub-optimal Immunostaining

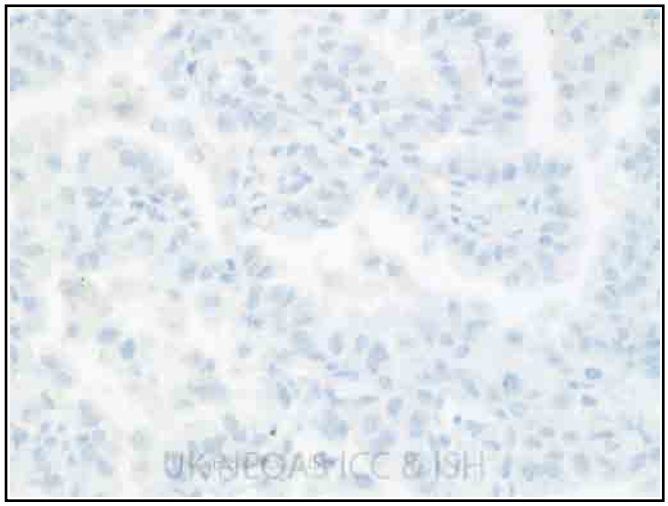


Fig 7. Acceptable ALK IHC result in the UK NEQAS distributed negative tumour sample D. Stained using the Ventana D5F3 assay with the recommended protocol with OptiView detection kit and an amplification step.

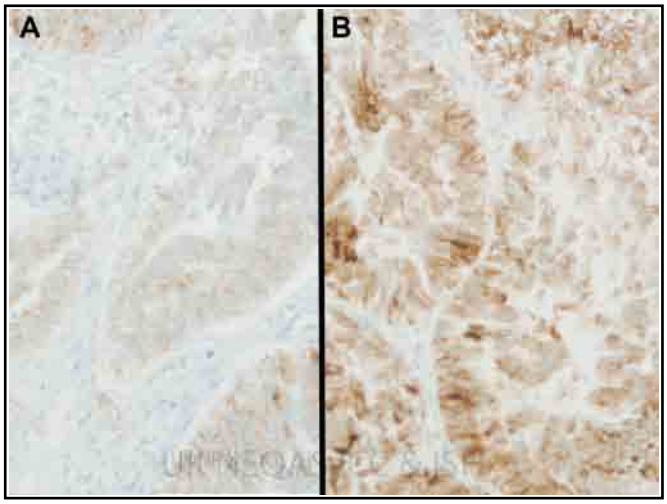


Fig 8. Expected level of staining in the UK NEQAS distributed positive tumour sample E. Due to the heterogenety of the sample, the tissue used in Block 1 (Example A) showed weaker staining than that seen in Block 2 (Example B). Both tissue blocks were created from the same tumour and were tested positive by FISH.

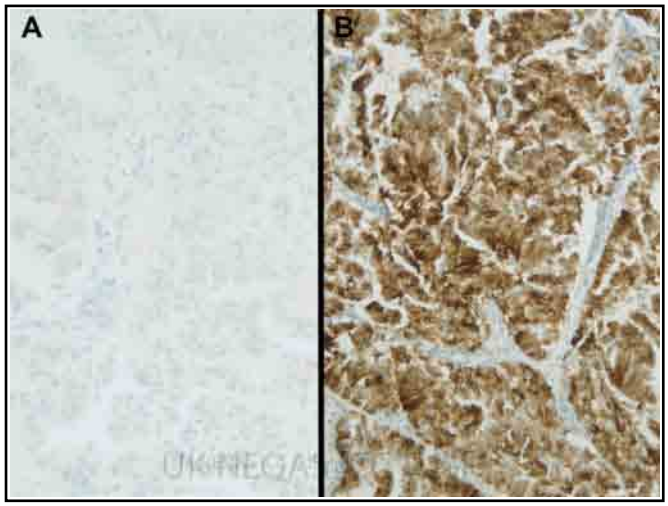


Fig 9. Two examples of poor ALK staining in the UK NEQAS dsitributed positive tumour sample E (compare to Fig 8). (A) shows very weak/false negative staining, which is most likely caused by insufficient antigen retrieval and detection. This lab is using a pretreatment of CC1 on the Ventana for 32 minutes. CC1 extended is the recommended protocol.

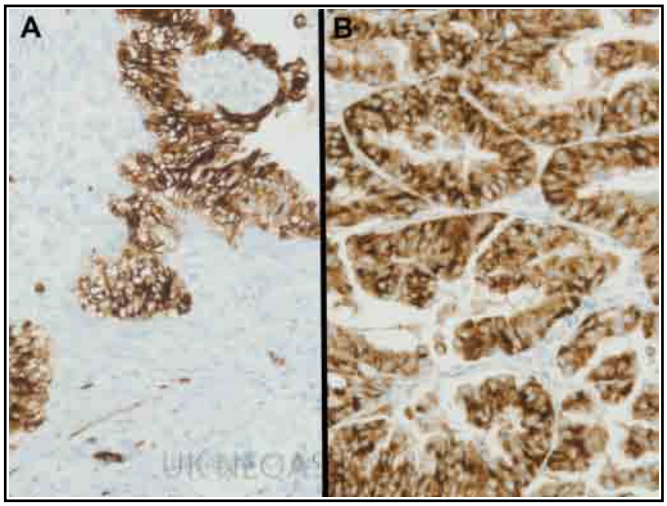


Fig 10. Two examples showing the optimal level of ALK staining on the UK NEQAS distributed tumour sample F. Although the examples are from different tissue blocks used in the assessment, both samples show strong membranous and cytoplasmic staining as expected. Both sections stained on the Ventana using the D5F3 clone.

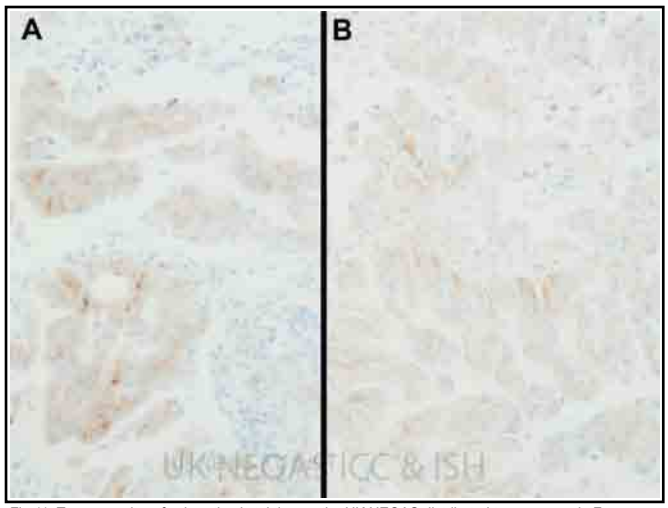


Fig 11. Two examples of sub-optimal staining on the UK NEQAS distributed tumour sample F (compare to Fig 10). The staining is much weaker than expected. (A) stained using the same protocol as Fig 9A, and (B) was stained with the Abcam (5A4) antibody, 1:50, on the Ventan ULTRA with the UltraView detection kit.

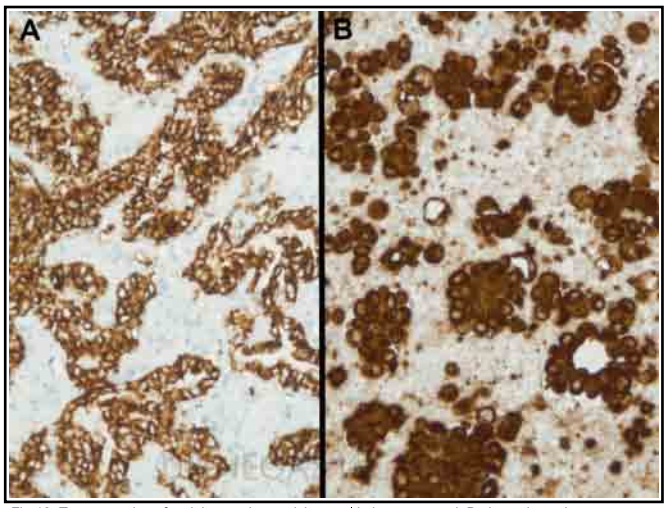
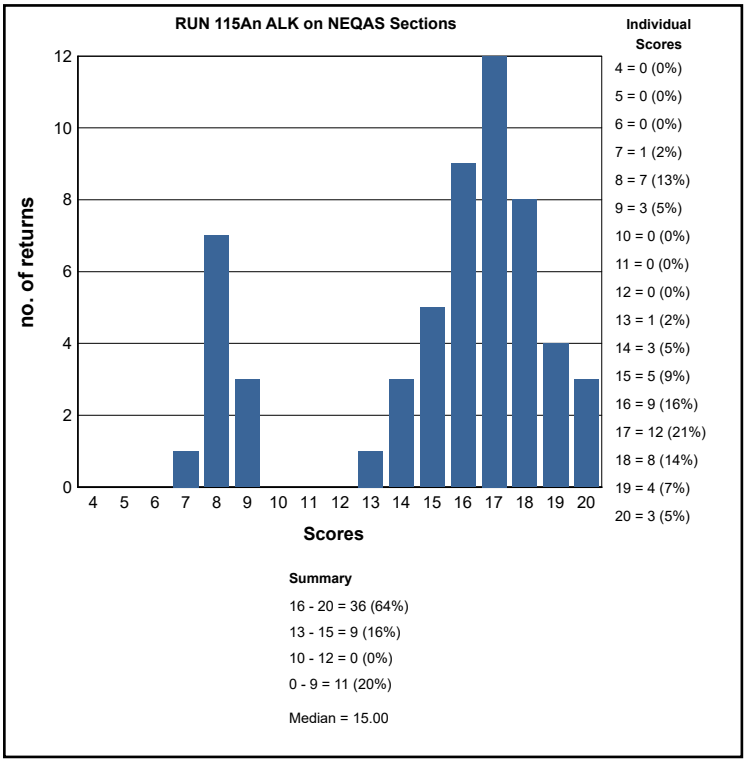
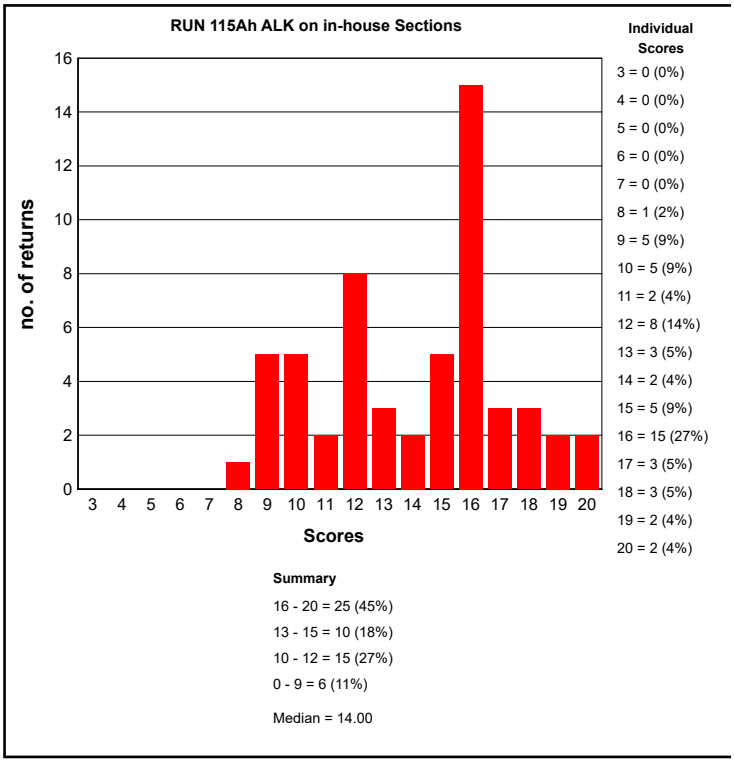


Fig 12. Two examples of staining on the particiapants' in house control. Both sections show good strong membranous staining of the tumour cells. However, section (B) also shows excessive background and teramide deposit.

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: 115

Primary Antibody	N	%
Abcam (5A4)	1	0
Cell Sign. Tech. (D5F3)	3	100
Dako M7195 (ALK1)	1	0
Novocastra NCL-ALK (5A4)	6	33
Ventana/Roche (D5F3)	44	91

ALK NSCLC Run: 115

Automation	N	%
Dako Autostainer Link 48	3	33
Leica Bond Max	1	0
Ventana Benchmark GX	4	75
Ventana Benchmark ULTRA	17	82
Ventana Benchmark XT	30	90

ALK NSCLC Run: 115

Heat Mediated Retrieval	N	%
Dako PTLink	2	50
Leica ER2 20 mins	1	0
None	1	0
Other	2	100
Ventana CC1 32mins	1	0
Ventana CC1 48mins	1	0
Ventana CC1 64mins	1	100
Ventana CC1 88mins	1	100
Ventana CC1 92mins	35	91
Ventana CC1 extended	5	80
Ventana CC1 standard	4	75
Ventana CC2 92mins	1	100

ALK NSCLC Run: 115

Detection	N	%
AS PER KIT	1	0
Dako EnVision FLEX (K8000/10)	1	100
Leica Bond Polymer Refine (DS9800)	1	0
Other	1	0
Ventana OptiView (760-700) + Amp. (7/860-099)	29	90
Ventana OptiView Kit (760-700)	20	90
Ventana UltraView Kit (760-500)	1	0

ALK NSCLC Run: 115		
Enzyme Retrieval	**N**	**%**
AS PER KIT	2	50
NOT APPLICABLE	26	92
Ventana Protease	2	50

ALK NSCLC Run: 115		
Chromogen	**N**	**%**
AS PER KIT	19	84
Dako EnVision Plus kits	1	100
Dako FLEX DAB	1	0
Leica Bond Polymer Refine kit (DS9800)	1	0
Ventana DAB	30	90
Ventana Ultraview DAB	3	33

BEST METHODS

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

ALK - Method 1

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

Primary Antibody:	Ventana/Roche (D5F3) Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	
Main Buffer:	
HMAR:	Ventana CC1 extended
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 20/20 (In House slide) using this method.

Primary Antibody:	Ventana/Roche (D5F3) , 12 Mins Prediluted
Automation:	Ventana Benchmark XT
Method:	
Main Buffer:	
HMAR:	Ventana CC1 92mins
EAR:	
Chromogen:	AS PER KIT
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099) , 12 Mins

ALK - Method 3

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

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

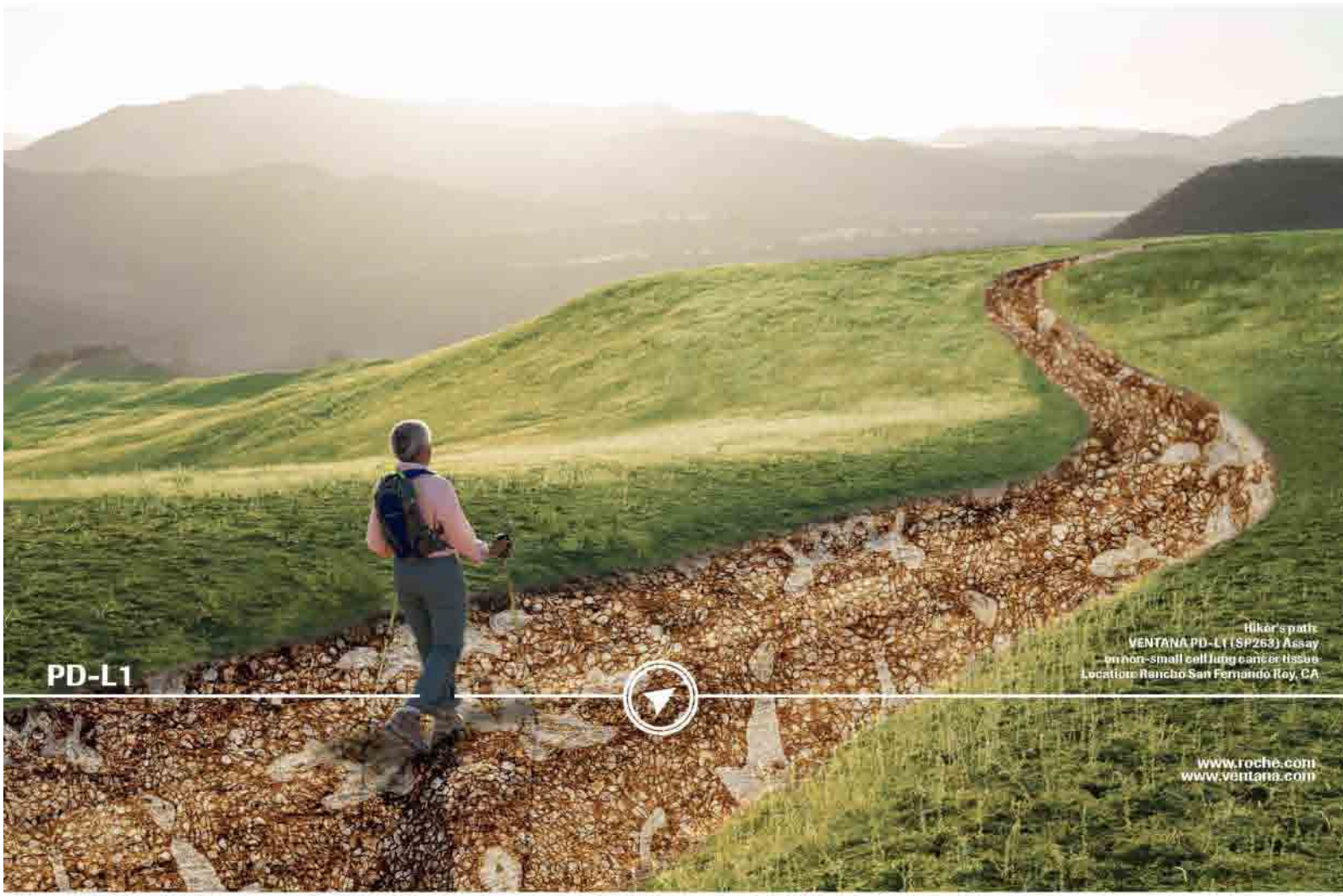
Guiding immunotherapy

VENTANA PD-L1 (SP263) Assay

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

Empowering pathologists to answer PD-L1 questions by

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



For healthcare professional use only.

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

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

The HER2 ISH Module - Interpretive Run 44

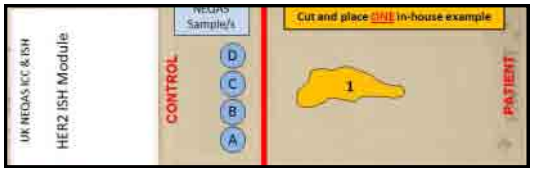
Suzanne Parry and Dawn Wilkinson

Gene Assessed:	HER2
Sections Circulated:	Composite slide consisting of 4 breast carcinoma sections (see table below)
Number of Registered Participants:	172
Number of Participants This Run	158 (92%)

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

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

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



Introduction

Breast cancer HER2 status should be determined in all newly diagnosed and recurrent breast cancers. In-situ hybridization (ISH), using either fluorescent (FISH)[4] or brightfield chromogenic methods[5] are now recognised techniques to determine the amplification status of breast cancers in either 2+ IHC equivocal cases or as an upfront method.
Although single HER2 probe methods are available, such as Ventana Silver ISH (SISH)[3], the dual probe (ratio) based techniques are the method of choice, with chromogenic assays now used as abundantly as **FISH**
For UK laboratories who are accredited to carry out diagnostic HER2 ISH testing, participation in an external quality assessment (such as the UK NEQAS ICC & ISH) is a requirement, and therefore poor performance is monitored on a regular basis to enhance confidence in HER2 ISH testing. It should also be emphasised that the scheme remains very much open to non-UK laboratories as an aid to monitoring and improving performance.

Recommendations

There are several recent recommendations regarding HER2 ISH testing including those by CAP (USA) and UK[6,7]. It is advisable that these guidelines are followed and processes of introducing and quality controlling clinical HER2 ISH testing are properly validated prior to their introduction into the laboratory.

Updated Assessment Procedure

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

Assessment Results

All laboratories except one laboratory used a dual probe ratio method. The laboratory that did use a single copy method achieved excellent results. 71% of participants achieved acceptable results. 20% received a borderline pass and 9% failed this run. The most popular brightfield method was the Ventana DDISH with 50 laboratories using this technique. The most popular FISH method was with the Pathvysion Vysis Kit. With 44 laboratories using this technique. The most popular FISH kit achieved a 77% acceptable pass rate compared to the most popular brightfield kit which achieved a 64% pass rate.

The UK laboratories achieved a higher overall acceptable pass rate of 91% (38 laboratories) compared to all participants with a total overall acceptable pass rate of 71% (101 laboratories). UK laboratories had a 2% fail rate (1 laboratory) compared to all participants (13 laboratories) which was higher with a 9% fail rate overall.

The ISH results have improved from the previous run with an acceptable pass rate of 71% compared to Run 43 of 50% and fewer borderline passes 20% compared to previous run of 31%.

Frequency Histograms

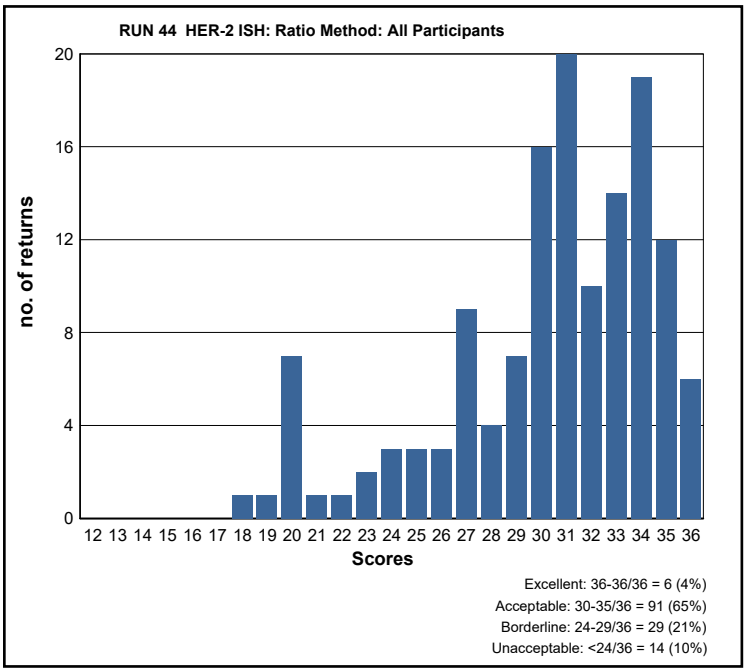
Frequency histograms of the assessment results are also provided showing the distribution of HER2 copy and Cen17 counts and ratio calculations. Participants are encouraged to use the provided frequency histograms to gauge their performance and variability in counts for each of the distributed samples.

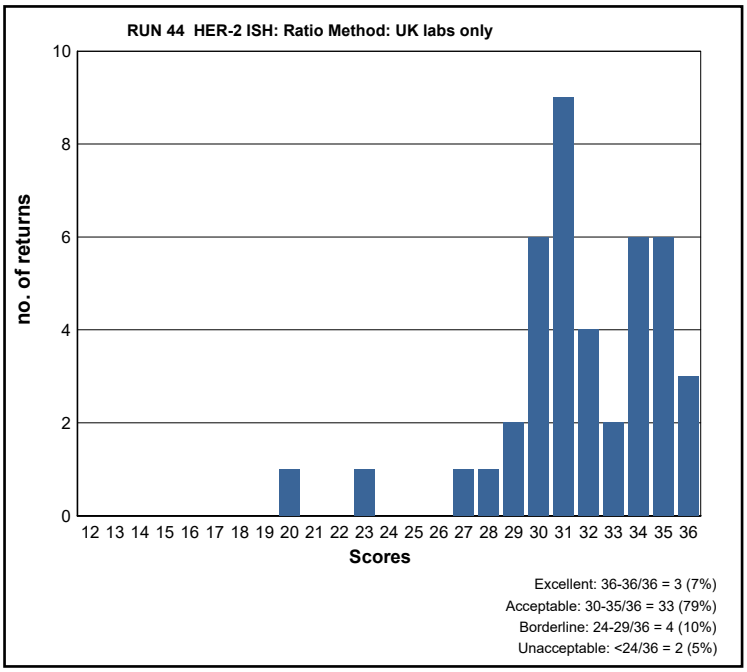
HER2 ISH Method and Probe Enumeration

The table on the next page (Table 1) shows the various ISH methods employed along with mean HER2, Cen17 counts and ratio calculations.

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

GRAPHICAL REPRESENTATION OF PASS RATES (ratio methods only)





METHODS USED and PASS RATES

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

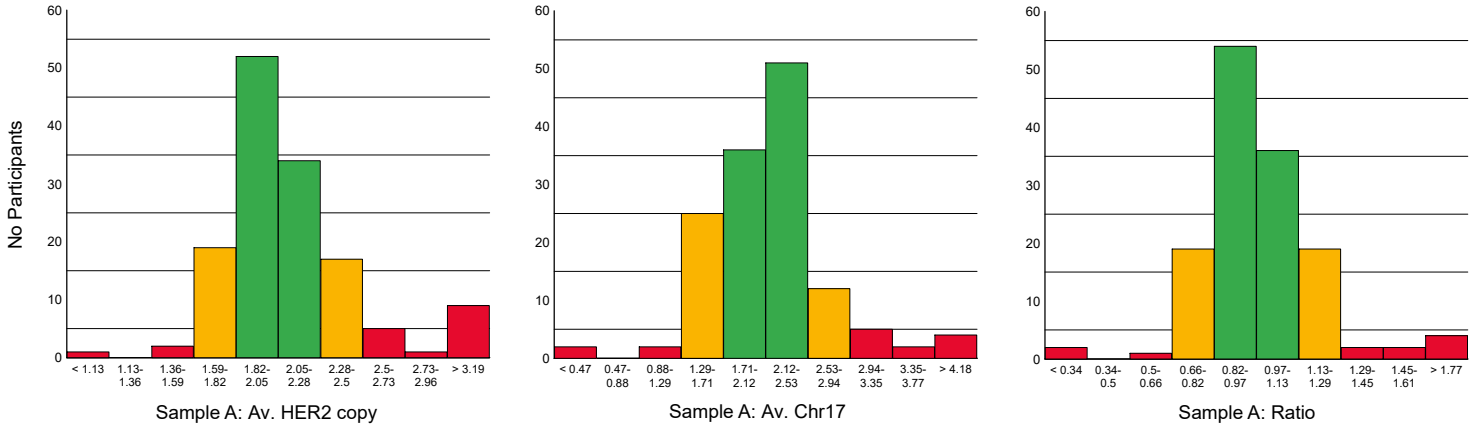
Ratio Method	N	% Pass (score ≥ 30/36)
Ratio: Dako IQFISH pharmDX	11	64%
Ratio: Dako Pharm Dx	2	100%
Ratio: In house FISH	2	50%
Ratio: Kreatech Probes	4	75%
Ratio: Leica HER2 FISH TA9217	5	100%
Ratio: Other - CISH	1	100%
Ratio: Other - FISH	5	80%
Ratio: Pathvysion Vysis Kit	43	74%
Ratio: Ventana BDISH 800-098/505	4	75%
Ratio: Ventana DDISH (780/800-4422)	50	60%
Ratio: Ventana Inform Silver ISH	4	75%
Ratio: Zytovision ZytoDot 2C	1	0%
Ratio: Zytovision ZytoLight	7	86%

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

FREQUENCY HISTOGRAMS

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

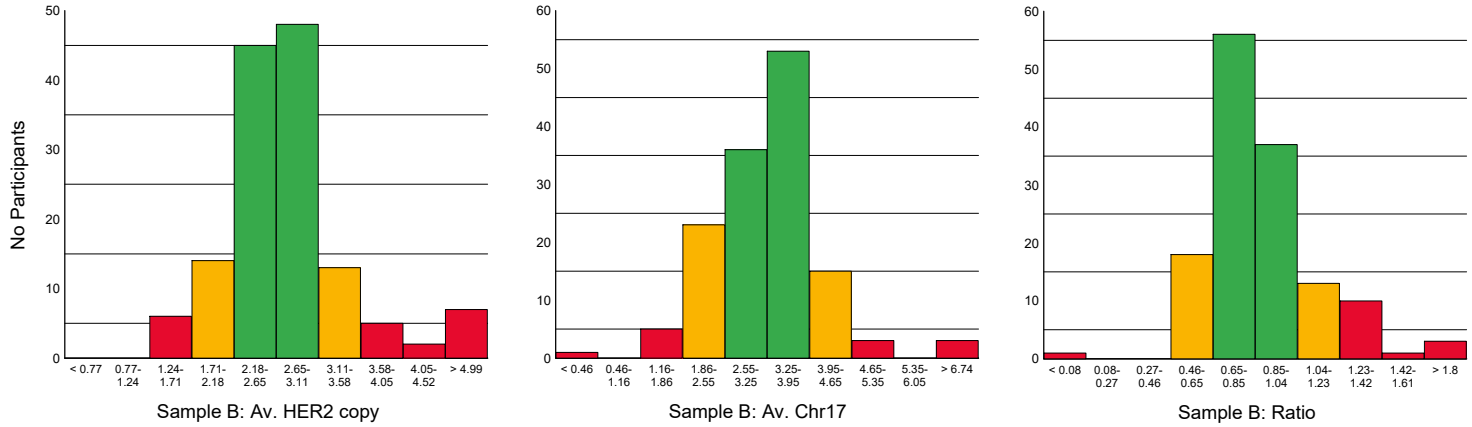
Green = mean +/- 1 Stdev Amber = mean +/- 2 Stdev Red = mean ≥ +/- 3 stdev



Sample A: Av. HER2 copy

Sample A: Av. Chr17

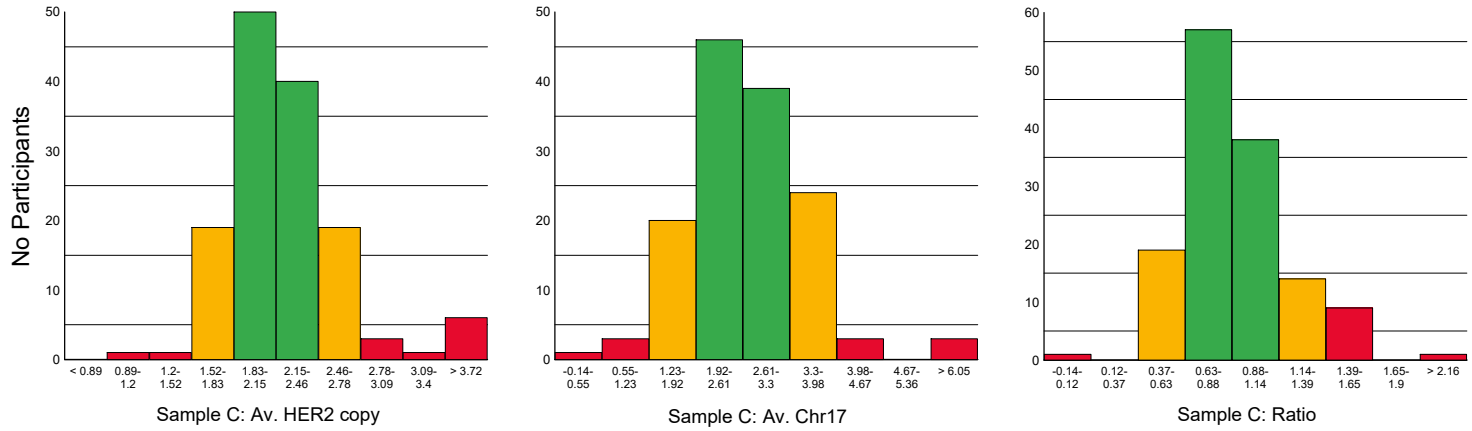
Sample A: Ratio



Sample B: Av. HER2 copy

Sample B: Av. Chr17

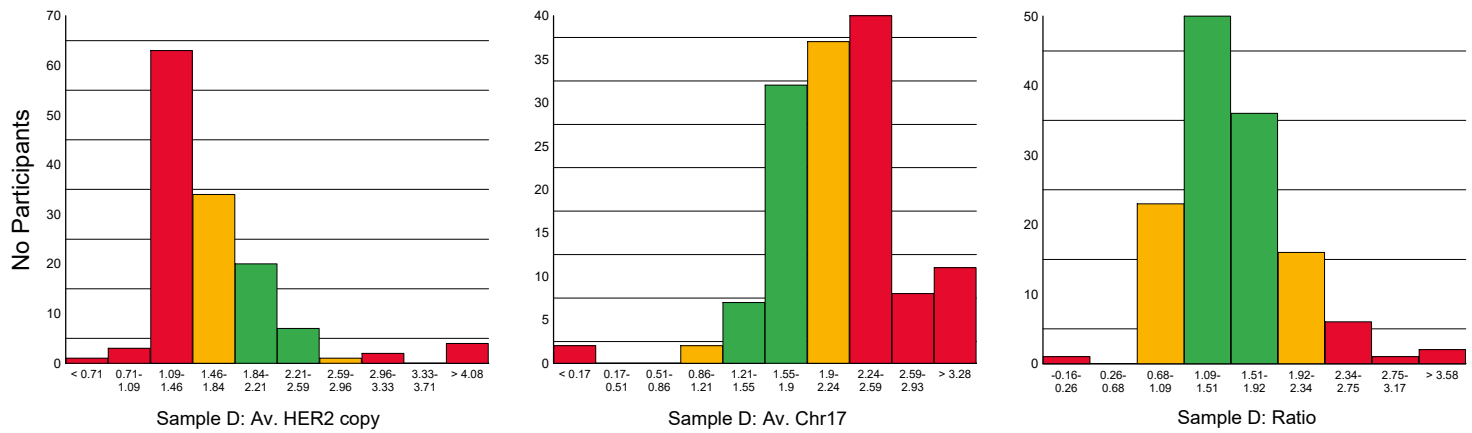
Sample B: Ratio



Sample C: Av. HER2 copy

Sample C: Av. Chr17

Sample C: Ratio



Sample D: Av. HER2 copy

Sample D: Av. Chr17

Sample D: Ratio

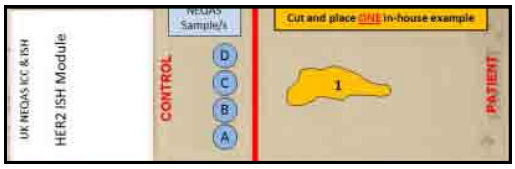
The HER2 ISH Module - Technical Run 44

Suzanne Parry and Dawn Wilkinson

Gene Assessed:	HER2
Sections circulated:	Composite slide consisting of 4 breast carcinoma sections (see table below)
Number of Registered Participants:	172
Number of Participants Taking Part this Run	134 (78%) (75 Fluorescent and 59 Chromogenic)

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

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

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

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

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

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

Participants are also required to provide an **in-house** example. Ideally, the section should be placed on the same slide as the Neqas material, however, the scheme will accept separate in house controls.

Results Summary

CISH Results

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

The overall results showed a decline in acceptable pass rates compared to the previous run (Run 43); only 36% of labs achieved an acceptable pass on the UK Neqas material, compared to 48% on the previous run. The failures were mostly due to weak or no CEN17 signals, which is similar to what we have been seeing in previous assessment runs over the last year. The borderline passes were mainly marked down due to weak, but still readable signals, again mostly due to weak Cen17 signals. Some slides showed dust-like silver chromogen signals, but unless obscuring the specific HER2 signals the slide was deemed still readable and did not fail, but was given a borderline pass. These observational results refer mainly to the Ventana BDISH or DDISH methods, which were used by most labs (86%) who submitted brightfield ISH slides for this technical assessment.

The Dako DuoCISH method was used by a few labs, but the data has only collected the results from 1 lab as the other participants using this kit did not submit their methodology details. However, these slides were recognised as being stained with the Dako DuoCISH kit by the assessment team as the staining is very distinct: The HER2 signals are red and the Cen17 signals are blue. The cytoplasm also has a very red blush which is inherent of this method. Data from the 1 lab shown in the report graph indicate that this lab achieved an acceptable level of staining. The Zytovision ZytoDot 2C method was also used by 1 lab, and this achieved a borderline pass. Marks were lost due to weak signals in some of the cores. The Zytovision ZytoDot 2C assay is another CISH methodology recognised by it's green HER2 signals and red Cen 17 signals.

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

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

FISH Results

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

Overall the FISH results showed slightly lower rates than the previous assessment (Run 43): 60% of labs received an acceptable pass, and a further 9% achieved a borderline. The current run showed a fail rate of 31%, which was similar to that of the CISH assessment. The main reason for unacceptable results was due to weak or no signals. We cannot be sure if this fading or total loss of signal may be due to slides not being sealed or stored correctly. However, UK Neqas does provide recommendations for this to try and prevent any loss of signal (see later paragraph on Recommendations for Returning FISH slides).

The Pathvysion Vysis kit still remains the most popular FISH method; used by 39 labs for this assessment and showed an acceptable pass rate of 49%. A further 8% of labs using the Vysis kit received a borderline score, and 44% of Vysis users failed the assessment. The Dako IQFISH and the Leica FISH kits were the next popular choices of kits. The Dako showed very good acceptable pass rates of 92% and the Leica showed an acceptable pass rate of 60%. A variety of other kits were used, including the Dako Pharm Dx, Kreatech Probes and the Zytovision ZytoLight. The numbers of users of these kits was comparatively low.

Validating ISH

It is important to emphasise that when introducing ISH into the laboratory or when changing methodology, e.g. from FISH to CISH, the new technique must be fully validated and staff must undergo extra training to gain expertise with the new method employed.
The UK NEQAS data indicates that there is a definite move towards the use of the Ventana DDISH method and laboratories introducing either this method or another ISH method should make sure that:

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

Recommendations for Returning FISH Slides for NEQAS Assessments

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

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

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

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

Selected Images showing Optimal and Sub-optimal Immunostaining

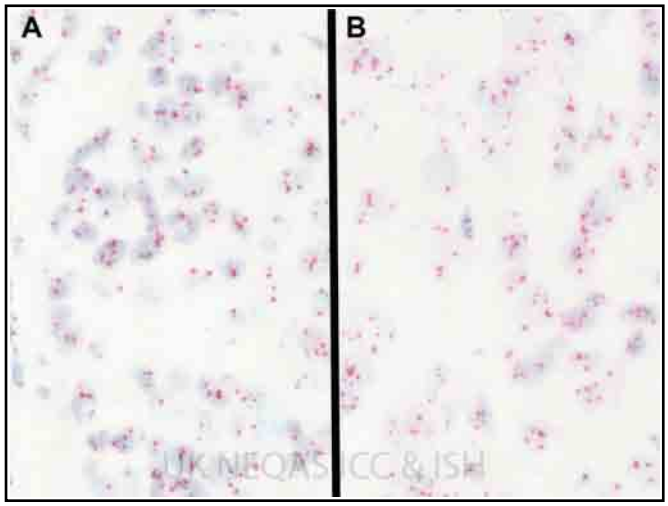


Fig 1. Acceptable Ventana DDISH in the UK NEQAS distributed non-amplified samples A and B. Both examples show distinct HER2 signals (black) and Chr17 signals (red) at the expected signal level.

Fig 2. Acceptable Ventana DDISH in the UK NEQAS non-amplified distributed sample C, showing strong HER2 signals (black) and Chr17 signals (red) and the expected average copy numbers per cell.

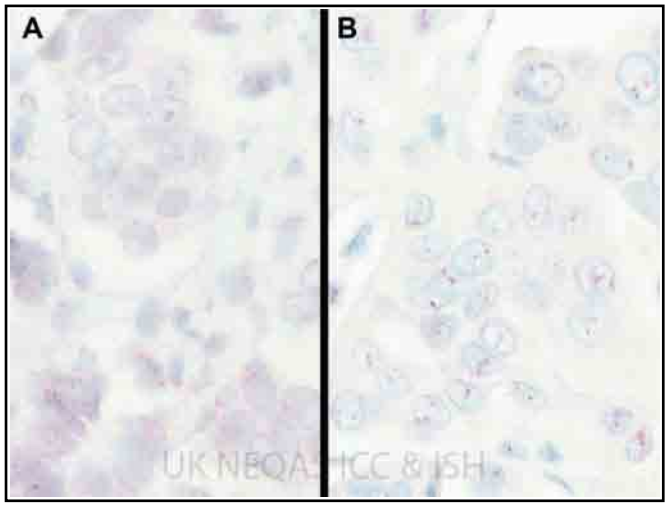


Fig 3. Two examples showing unacceptable Ventana DDISH staining in the UK NEQAS non-amplified samples: A shows excessive leaching of Chr17 and no HER2 signals, and B shows weak and patchy staining of both the Chr17 and HER2 signals.

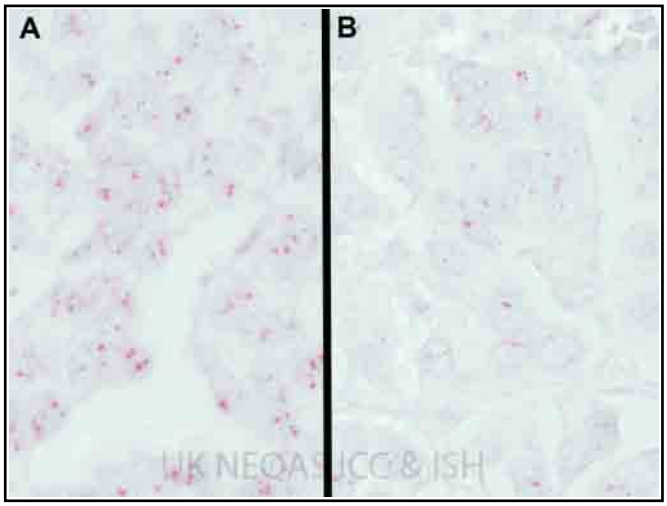


Fig 4. Unacceptable Ventana DDISH staining in the UK NEQAS non-amplified samples (B and C): Both cores show little HER2 signals and morphology damage, most likely caused by excessive pretreatment.

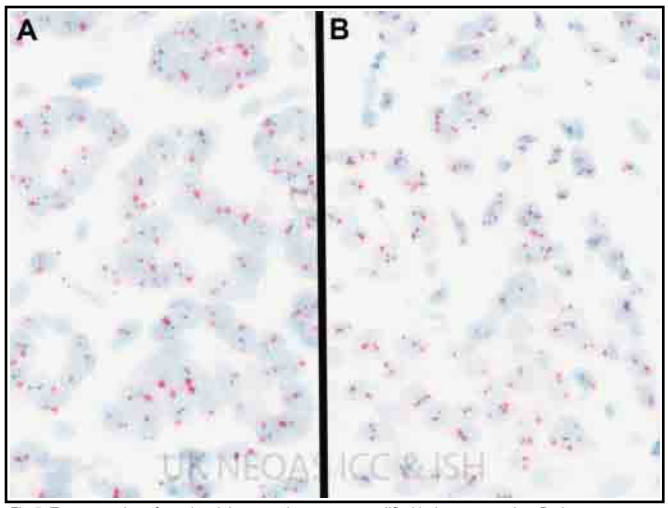


Fig 5. Two examples of good staining seen in two non-amplified in-house samples. Both stained with the Ventana DDISH assay. Fig 6. Two poor in-house examples: (A) is an amplified case, but shows excessive leaching of the Chr17 probe. While (B) shows excessive

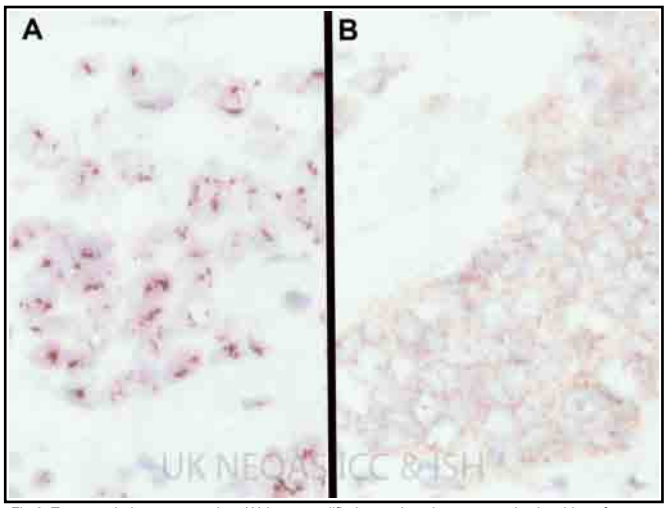
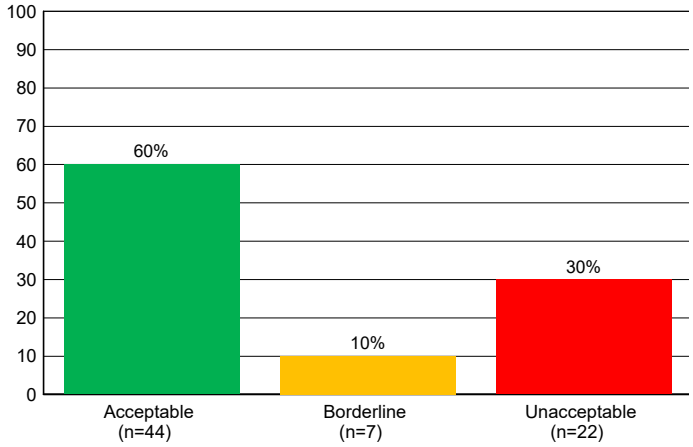


Fig 6. Two poor in-house examples: (A) is an amplified case, but shows excessive leaching of the Chr17 probe. While (B) shows excessive non-specific silver deposit. Both stained using the Ventana DDISH assay.

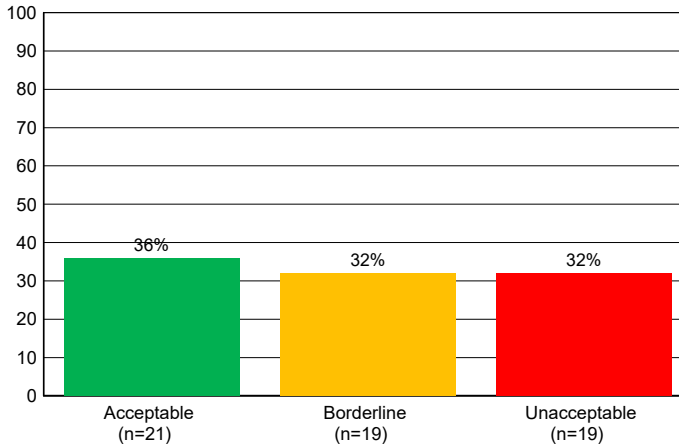
Technical ISH: Pass Rates and Methods

Overall Pass Rates

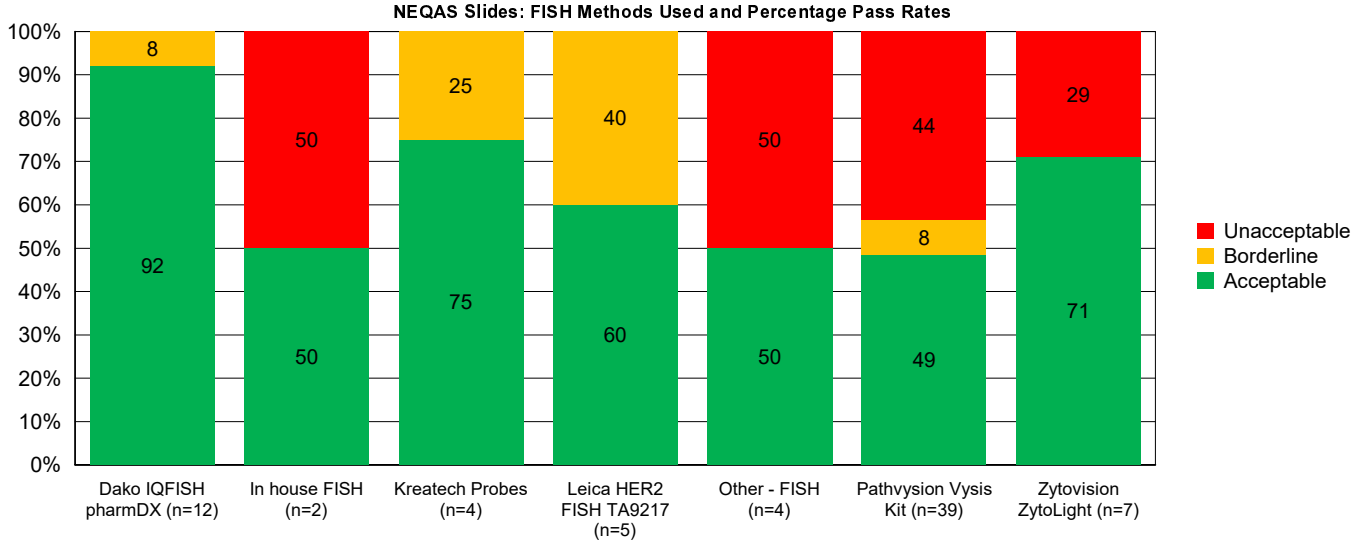
FISH NEQAS slide (n=73)



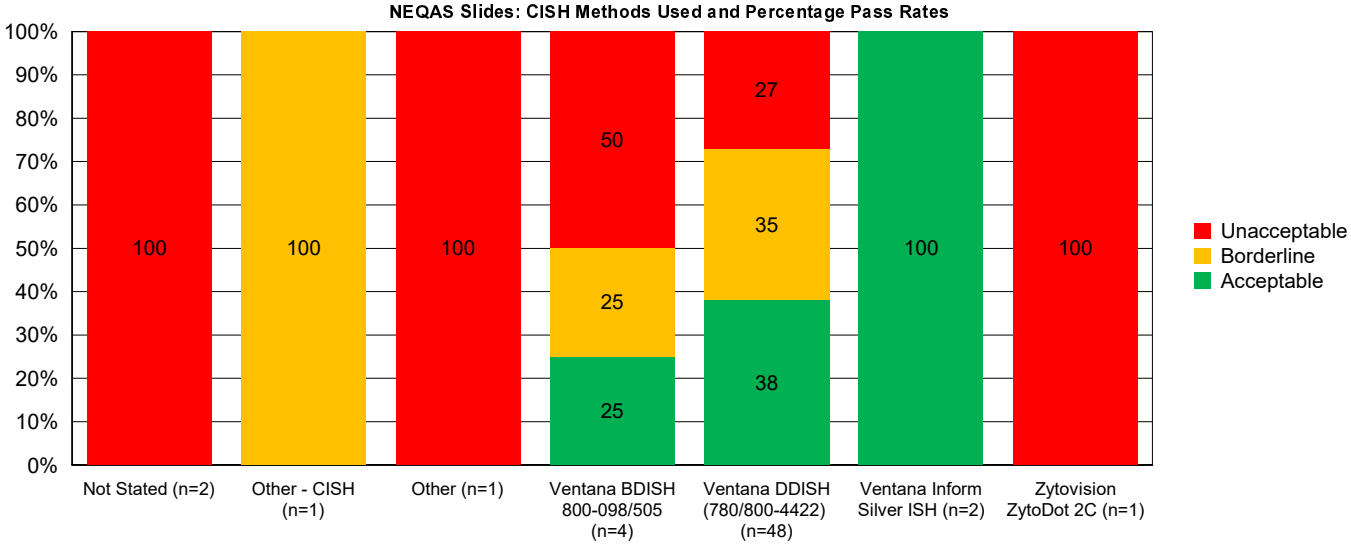
CISH NEQAS Slide (n=59)



NEQAS Slides: FISH Methods Used and Percentage Pass Rates



NEQAS Slides: CISH Methods Used and Percentage Pass Rates



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