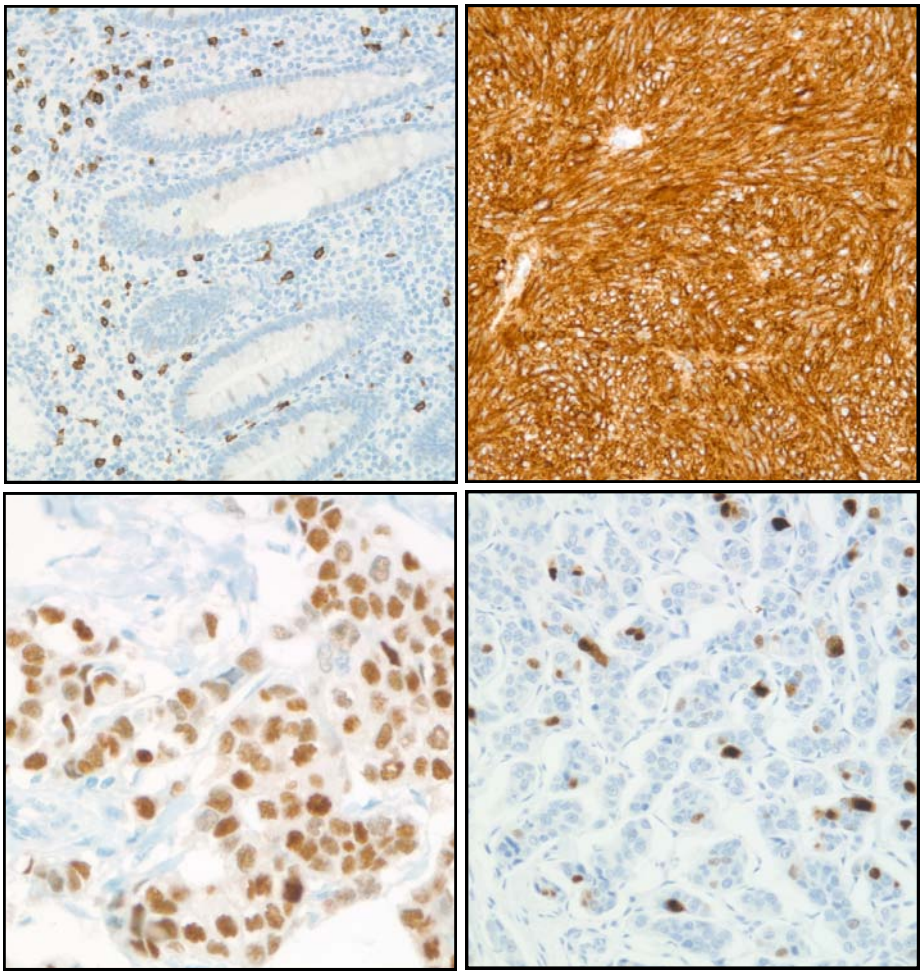
Immunocytochemistry

Improving Immunocytochemistry for Over 25 Years

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

Assessment Dates: 7th January — 22nd January 2016



Cover Photo: Taken from the Alimentary Tract and Hormone Receptor Modules:

Top Left: Optimal CD117 staining of mast cells on the NEQAS appendix sample
Top Right: Excellent DOG-1 demonstration on the NEQAS GIST sample
Bottom Left: Optimal PR staining on the NEQAS high-expressing breast tumour sample
Bottom Right: Good PR staining on the NEQAS low-expressing breast tumour sample

Immunocytochemistry Modules

In situ Hybridisation Modules

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

Accreditation Update:

We are pleased to announce that following our UKAS visit in February we are now fully accredited to ISO: 17043

7833

See the website for further details: www.uknegasiccish.org

UK NEQAS
Immunocytochemistry & In-Situ Hybridisation

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General Information

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

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ASSESSORS

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Panel for Lung Cancer

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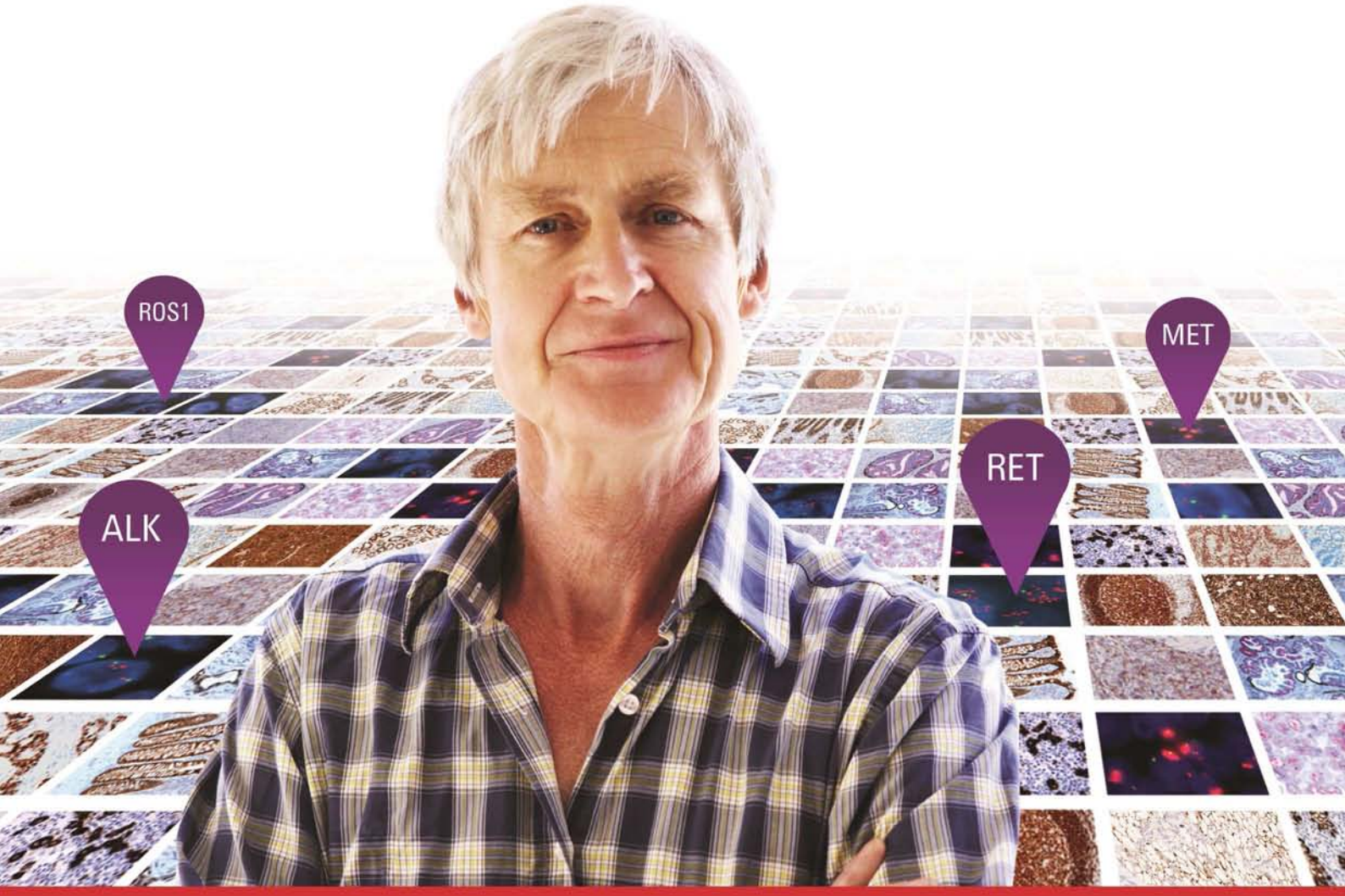


Agilent Technologies

General Pathology Module Run 112

Julie Williams and Suzanne Parry

	Gold Standard	Second Antibody
Antigens Assessed:	Desmin	Ber-EP4
Tissue Sections circulated:	Normal Appendix & Leiomyosarcoma	Normal Appendix, Lung Carcinoma & Mesothelioma
Number of Registered Participants:	330	
Number of Participants this Run	316 (96%)	

Introduction

Gold Standard: Desmin

Desmin is a 53KDa cytoplasmic intermediate filament protein that is characteristically found in all three types of muscle cells (smooth muscle, cardiac and skeletal muscle). The protein is composed of an N-terminal head-piece and a C-terminal tail-piece. It forms cytoskeletal networks across the muscle fibre at the plasma nuclear membrane border, with particular localisation at the sub-plasmalemmal region and the Z-band. Desmin's main diagnostic use is in the demonstration of rhabdomyosarcomas, leiomyomas, leiomyosarcomas (Chang et al., Pollock et al.) and other tumours with myoid differentiation, although it is important to use Desmin within a panel of antibodies.

Features of Optimal Immunostaining:
Appendix: (Figs1 & 2)
- Strong staining of the smooth muscle layers of the muscularis propria and around the vessels in the submucosa
- Minimal background staining

Leiomyosarcoma: (Fig 4)
- Strong cytoplasmic staining of the tumour cells
- Minimal background staining

Features of Suboptimal Immunostaining: (Figs 3 & 5)
- Weak, uneven or no staining
- Non-specific staining of cell types not expected to stain, e.g. lymphocytes and epithelial cells (often due to excessive antigen retrieval)
- Excessive background staining (particularly in the connective tissue)

References:
1. T Chang et al. Immunocytochemical study of small round cell tumours in routinely processed specimens. Arch Pathol lab Med 1989; 113:1343-8
2. L Pollock et al. Desmin expression in rhabdomyosarcoma: influence of the desmin clone and immunocytochemical method. J. Clin pathol 1195;48:535-8

Second Antigen: BER-EP4

Ber-EP4 is an epithelial specific glycoprotein antigen, located on the cell surface (mostly basolaterally) and in the cytoplasm of virtually all epithelial cells. Exceptions to this are most squamous epithelial and myoepithelial cells, adult hepatocytes and parietal cells in gastric glands. The antibody is also not positive in spleen, brain, connective tissue, bone marrow, smooth and striated muscle, heart and endothelia. Ber-EP4 is useful as a differential marker in the diagnosis of adenocarcinoma versus malignant mesothelioma (Sheibani et al.). The antibody is also useful in differentiating between basal and squamous cell carcinomas of the skin (Beer et al.), and the detection of micro metastases in patients with oesophageal carcinoma (Hosch et al.).

Features of Optimal Immunostaining:
Appendix (Fig 7)
- Strong cytoplasmic and basolateral of the epithelial cells.
- No background staining

Lung Carcinoma: (Fig 9)
- Moderate to strong cytoplasmic and membranous staining in all of the tumour cells
- Minimal background staining

Sub-optimal Immunostaining: (Figs 8, 9, 11 & 12)
- Weak, uneven or no staining
- Diffuse staining
- Non-specific staining of cell types not expected to stain
- Excessive background staining
- Non-specific inappropriate staining in the mesothelioma tissue (Fig 12)

References:
1. Sheibani K, et al. Ber-EP4 antibody as a discriminant in the differential diagnosis of malignant mesothelioma versus adenocarcinoma. Am J Surg Pathol 1191;15:779-84.
2. Beer et al. Ber-EP4 and epithelial membrane antigen aid distinction of basal cell, squamous cell and basosquamous carcinomas of the skin. Histopathology 2000;37:218-23.
3. Hosch et al. Malignant potential and cytogenic characteristics of occult disseminated tumour cells in esophageal cancer. Cancer Res 2000;60:6836-40.

Assessment Summary:

Desmin was the chosen gold standard marker for this assessment run. The pass rates were slightly lower than the last time this was assessed, with 77% of labs achieving an acceptable pass, compared to 92% in run 110. A further 15% received a borderline pass (scores of 10-12/20) and 25 labs (8%) failed the assessment. The main reason for a borderline or failed result was due to very little or no staining of the desmin filaments in the appendix and/or the leiomyosarcoma tumour. Many of these labs are not using antigen retrieval, which would account for little or no staining. Other labs used a variety of antigen retrieval methods, with most labs opting for the appropriate heat mediated protocol recommended by the suppliers of their particular antibody. The Dako D33 clone was the most popular choice of antibody, used by 54% of participating labs, and showed a pass rate of 82%.This antibody worked well on all the commercially available staining platforms. The in–house controls showed a higher acceptable pass rate of 95% compared to that on the Neqas samples.

The second antibody assessed for this run was **Ber-EP4.** This showed a pass rate of 84%, with a further 12% receiving a borderline pass. Again, the reason for failure was due to very weak staining caused by insufficient pre-treatment. The assessors noted that several slides stained with the OptiView detection kit on the Ventana Benchmark machines showed a very granular pattern of staining, and this was thought to be inherently due to the detection kit. Many labs are using enzyme pre-treatment, which produced good results on all of the staining platforms with the recommended conditions according to the commercial datasheets.. The Dako Ber-EP4 was the most popular antibody, used by 58% of participating labs, and showed an acceptable pass rate of 84%. The in house controls produced slightly higher pass rates compared to the Neqas samples with 94% achieving an acceptable pass. Appendix and colon/bowel were the most commonly used in-house control tissues. These tissues are appropriate to demonstrate the epithelial cell staining.

Selected Images showing Optimal and Sub-optimal Immunostaining

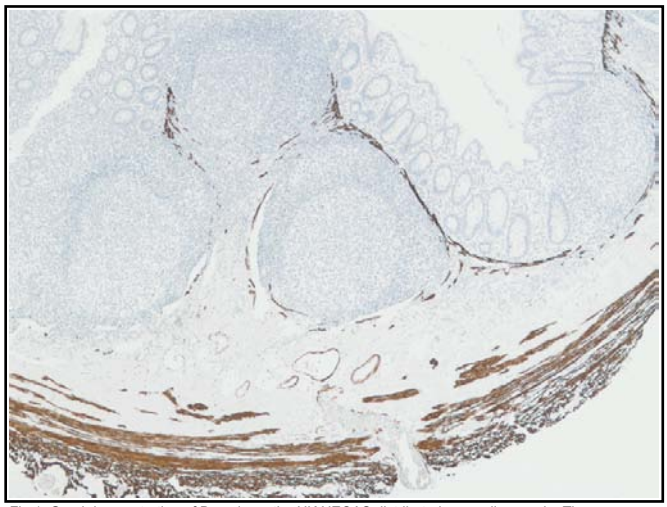


Fig 1. Good demonstration of Desmin on the UK NEQAS distributed appendix sample. The section shows strong staining in the smooth muscle layers of the muscularis propria and around the vessels in the submucosa. Section stained with the Ventana DER11 pre-diluted antibody on the Benchmark XT with no pre-treatment.

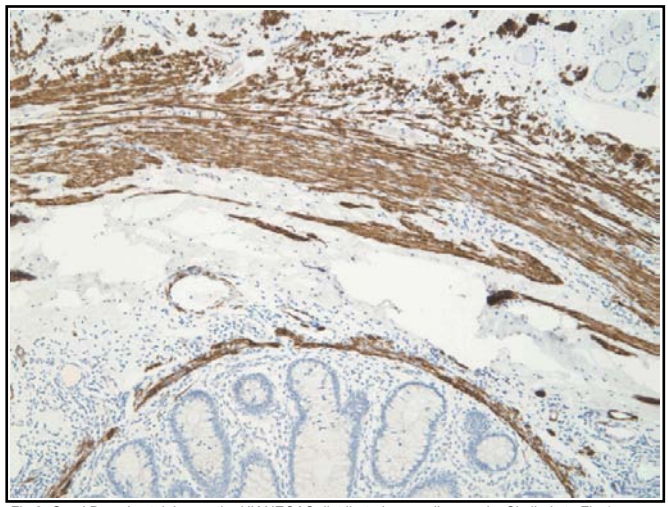


Fig 2. Good Desmin staining on the UK NEQAS distributed appendix sample. Similarly to Fig 1, the staining is strong in the smooth muscle layers and vessels of the submucosa. Section stained using the Leica RTU DER11 antibody on the Leica BondMax and ER2 antigen retrieval buffer for 20 minutes.

Fig 3. Suboptimal demonstration of Desmin on the UK NEQAS appendix: Although the expected smooth muscle and vessels are staining, there is also excessive background staining, making it more difficult to read the finer vessel staining. Oddly, the staining method used is virtually identical to that used in Fig 2, with the only difference being that a lyophilised DER11

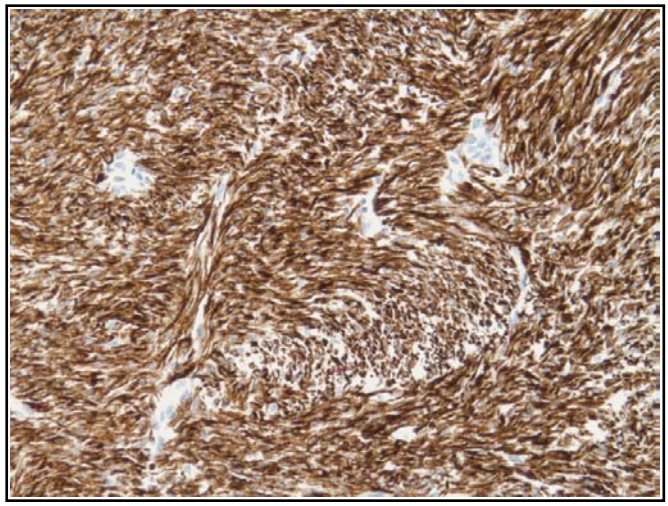


Fig 4. Good example of Desmin staining on the UK NEQAS distributed leiomyosarcoma, showing strong cytoplasmic staining in all the tumour cells (same methodology as Fig 2).

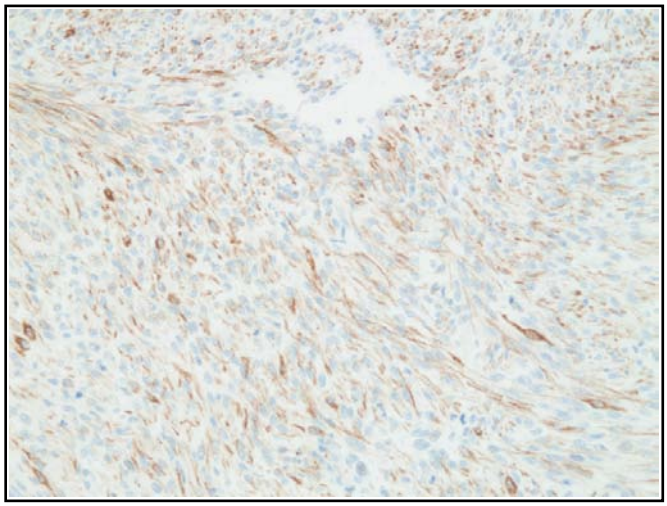


Fig 5. Poor demonstration of Desmin in the UK NEQAS distributed leiomyosarcoma tumour: The staining is patchy and many of the tumour cells expected to stain are not demonstrated (compare to Fig 4). This section was stained using the Dako D33 antibody, 1:200, on the Autostainer with pre-treatment in the PT link using high pH buffer for 20 minutes.

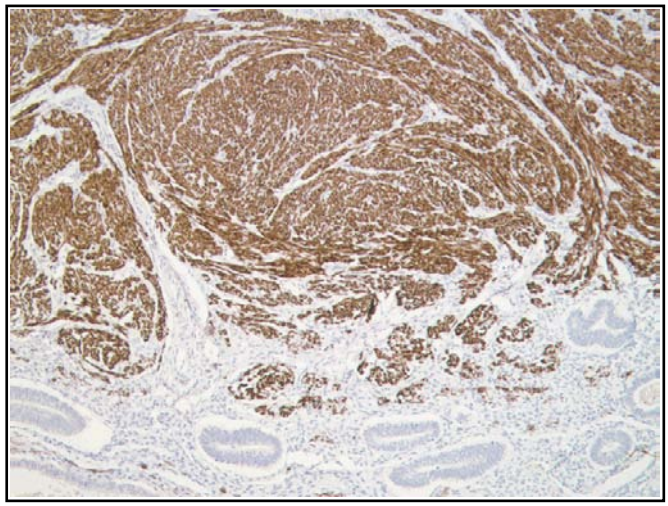


Fig 6. Good example of an in house uterus control stained with Desmin. The staining is strong and distinct, while the background remains clean (same methodology as Figs 2&4).

Selected Images showing Optimal and Sub-optimal Immunostaining

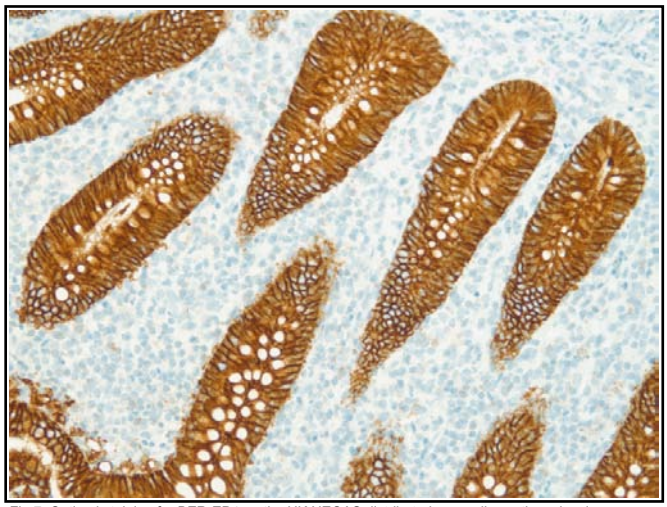


Fig 7. Optimal staining for BER-EP4 on the UK NEQAS distributed appendix section, showing strong cytoplasmic and basolateral staining of the epithelial cells. Section stained with the Ventana pre-diluted antibody on the Benchmark XT, CC1 antigen retrieval for 32 minutes and Optiview detection kit.

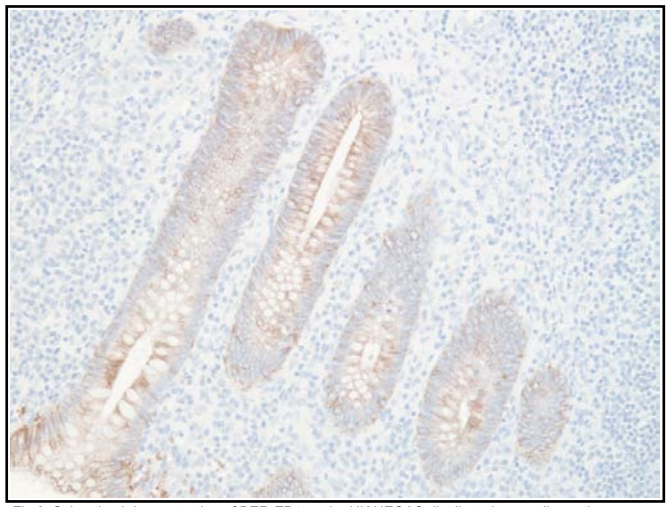


Fig 8. Suboptimal demonstration of BER-EP4 on the UK NEQAS distributed appendix section (compare to Fig 7). The staining is weak, with several cells not staining at all. Stained using the Dako antibody, 1:50, on the Ventana Benchmark XT and no pre-treatment. Insufficient antigen retrieval is the likely cause of the weak staining.

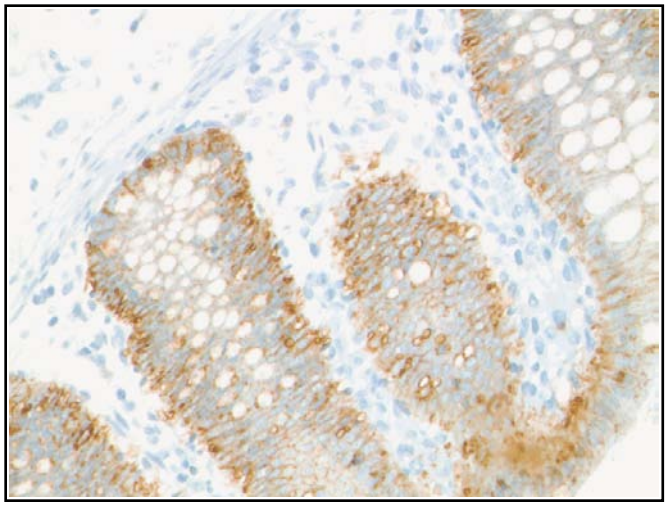


Fig 9. Borderline level of BER-EP4 staining on the UK NEQAS distributed appendix section. The staining is diffuse and is also very granular. This granular pattern was seen in several slides stained using the Ventana detection system. Stained with the ThermoScientific antibody, 1:30, Optiview detection on the Ventana ULTRA and CC1 antigen retrieval for 32 minutes.

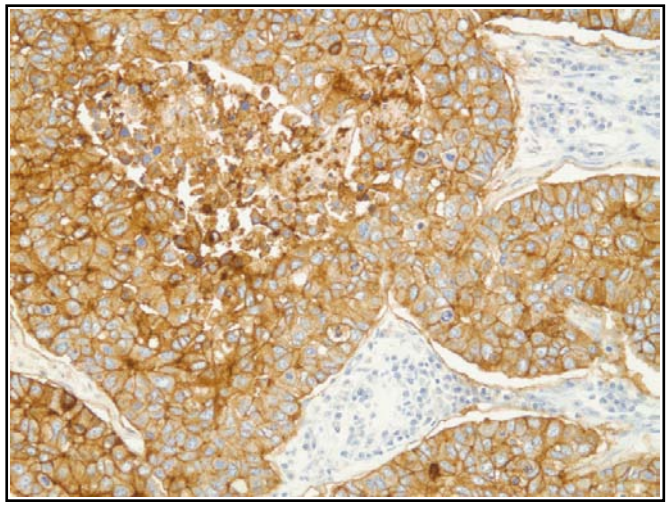


Fig 10. Optimal staining of the UK NEQAS lung carcinoma tissue. The section shows strong cytoplasmic and membranous staining in all of the tumour cells. Stained using the Dako antibody, 1:200, on the Leica BondMax, with ER1 antigen retrieval for 10 minutes.

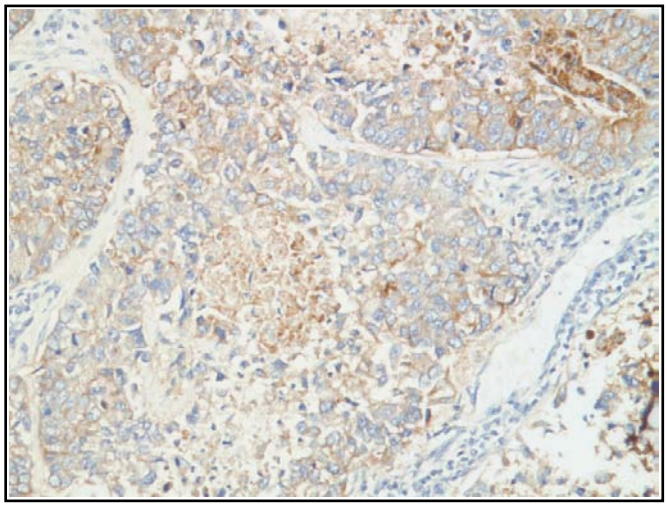


Fig 11. Sub-optimal staining of BER-EP4 in the UK NEQAS distributed lung carcinoma (compare to Fig 10). Staining of the tumour cells is very weak with several cells expected to stain not demonstrated. This is probably caused by the lack of pre-treatment. Stained with the Dako antibody 1:200, on the Leica BondMax and no antigen retrieval.

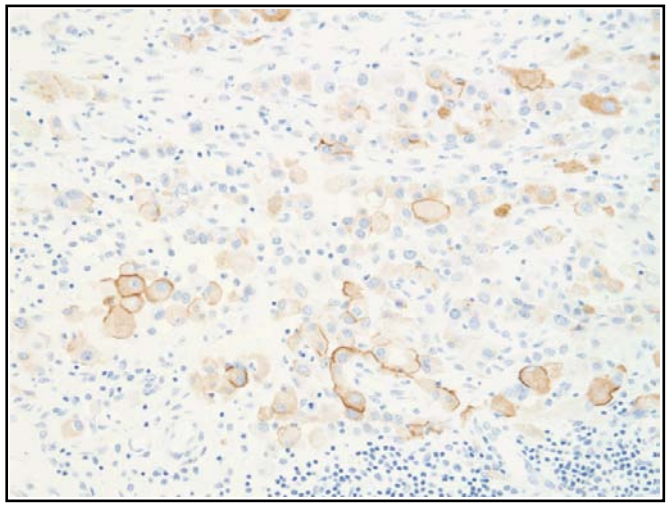
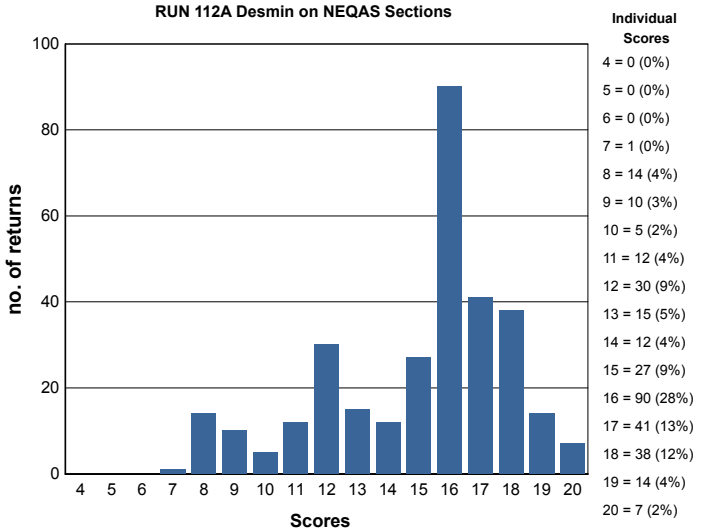
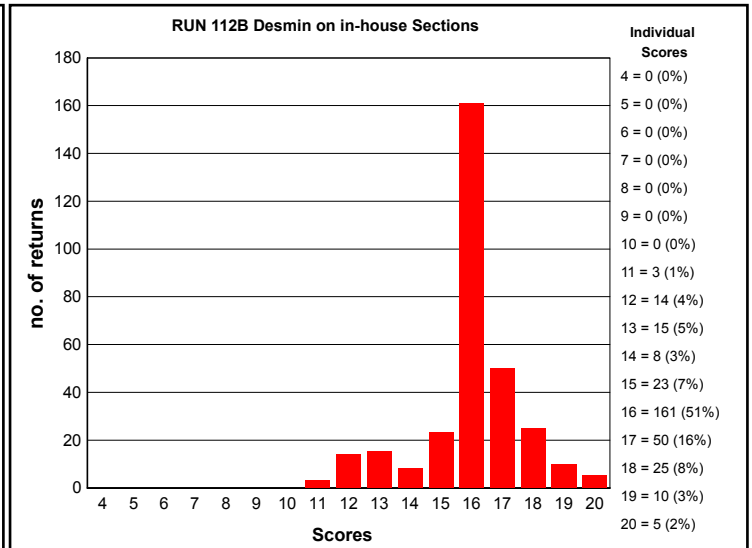
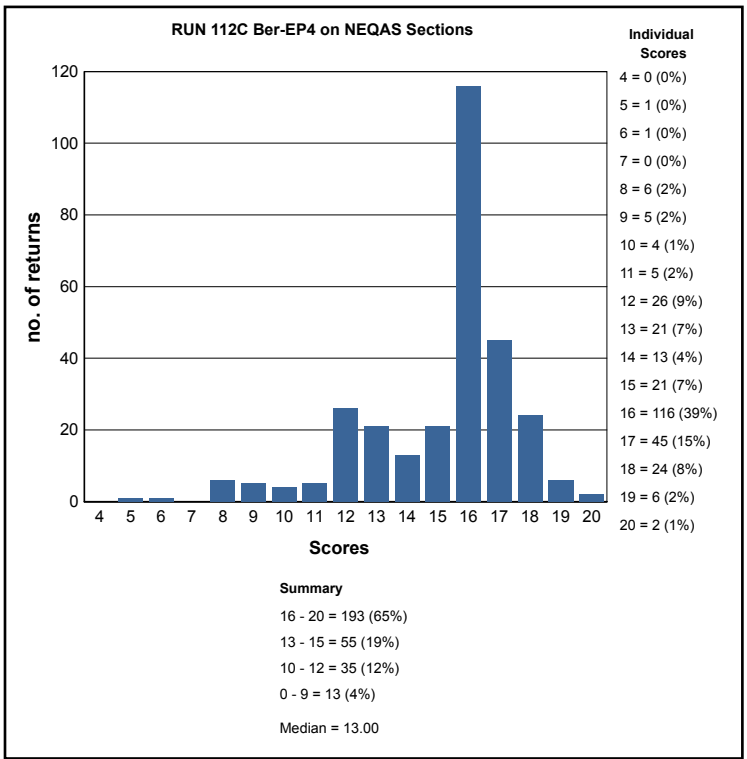


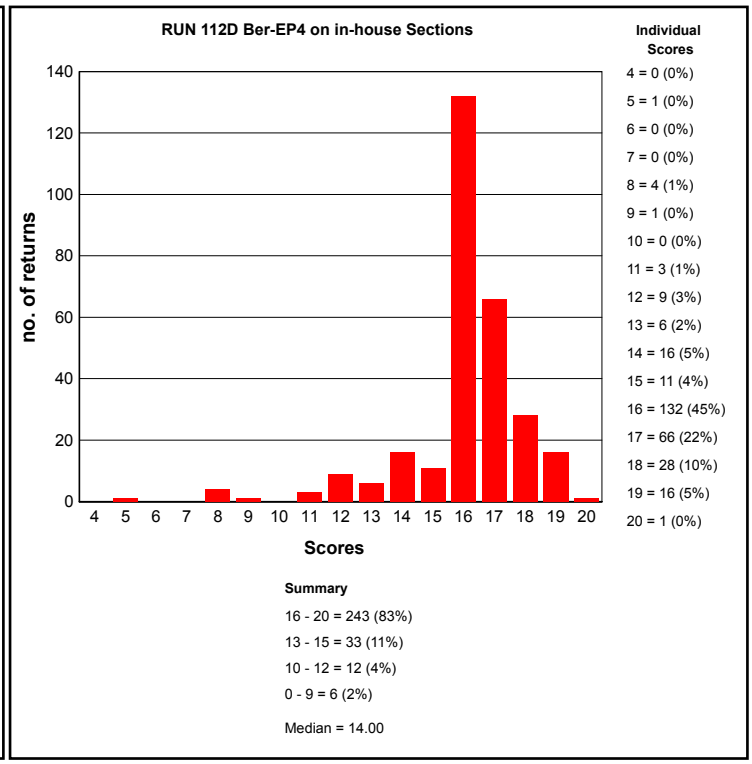
Fig 12. Sub-optimal demonstration of BER-EP4 in the UK NEQAS distributed mesothelioma. This section should be negative for BER-EP4, however, the example shows non-specific inappropriate staining. The Dako antibody was used at a 1:200 on the Leica BondMax with no pre-treatment.

GRAPHICAL REPRESENTATION OF PASS RATES









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

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

General Pathology Run: 112

Primary Antibody : Desmin

Antibody Details	N	%
Cell Marque 243M (D33)	5	40
Dako IR606 RTU Flex Link (D33)	15	80
Dako IS606 RTU Flex Plus (D33)	5	100
Dako M0760 (D33)	152	66
Dako M724 (DER11)	3	33
Leica PA0032 RTU (DER11)	21	95
Leica/Novocastra NCL-DES (DER11)	32	88
Leica/Novocastra NCL-L-DES (DER11)	17	94
Other	7	57
Ventana 760 2513 (DER11)	54	96

General Pathology Run: 112

Primary Antibody : Ber-EP4

Antibody Details	N	%
Abcam Ber-EP4 ab7504	1	0
Biocare (IntelliPath) Ber-EP4 SKU:107	1	100
Cell Marque Ber-EP4 248M-94/95/96/97/98	7	57
Dako Ber-EP4 M0804	183	84
Dako RTU Auto Link Ber-EP4 IR637	14	93
Dako RTU Auto Plus Ber-EP4 IS637	6	67
Dako RTU Omnis FLEX Ber-EP4 GA637	6	100
Thermo Sci/Neomarkers BerEP4 MS-1898	3	33
Ventana Ber-EP4 760-43-83	41	93

General Pathology Run: 112

Heat Mediated Retrieval	Ber-EP4 N	Ber-EP4 %	Desmin N	Desmin %
Biocare Decloaking Chamber	0	0	2	100
Dako Omnis	7	100	5	20
Dako Pascal	0	0	1	100
Dako PTLink	31	94	44	77
Lab vision PT Module	2	100	5	60
Leica ER1 10 mins	6	83	3	100
Leica ER1 20 mins	18	94	11	73
Leica ER1 30 mins	0	0	5	20
Leica ER1 40 mins	1	100	1	100
Leica ER2 10 mins	2	100	4	100
Leica ER2 20 mins	5	60	52	85
Leica ER2 30 mins	1	0	14	100
Leica ER2 40 mins	0	0	2	100
Microwave	2	0	4	50
None	48	81	33	88
Other	1	100	2	50
Pressure Cooker	3	33	9	89
Steamer	1	100	2	0
Ventana CC1 16mins	4	100	3	100
Ventana CC1 20mins	2	50	0	0
Ventana CC1 24mins	3	100	2	50
Ventana CC1 32mins	12	83	18	72
Ventana CC1 36mins	9	100	6	100
Ventana CC1 40mins	1	100	3	67
Ventana CC1 48mins	0	0	2	100
Ventana CC1 52mins	0	0	2	100
Ventana CC1 56mins	1	100	3	100
Ventana CC1 64mins	3	67	15	80
Ventana CC1 88mins	0	0	1	100
Ventana CC1 8mins	10	90	5	80
Ventana CC1 extended	0	0	2	100
Ventana CC1 mild	13	92	13	38
Ventana CC1 standard	5	100	22	68
Ventana CC2 56mins	0	0	1	100
Ventana CC2 mild	1	100	0	0
Water bath 68 OC	1	100	1	100
Water bath 95-98 OC	0	0	1	0

General Pathology Run: 112

Enzyme Mediated Retrieval	Ber-EP4 N	Ber-EP4 %	Desmin N	Desmin %
AS PER KIT	5	60	4	50
BioGenex Protease	1	100	0	0
Dako Proteinase K (S3004)	1	100	0	0
Dako Proteinase K (S3020)	3	67	1	100
Enzyme digestion + HIER	2	100	0	0
NOT APPLICABLE	56	86	115	79
Other	12	100	0	0
Roche Pronase (165921)	1	100	0	0
SIGMA Proteinase 24	1	100	0	0
Trypsin	1	0	0	0
VBS Bond Enzyme 1	34	82	5	100
VBS Bond Enzyme 2	1	100	1	100
Ventana Protease	6	100	5	80
Ventana Protease 1 (760-2018)	59	78	34	94

General Pathology Run: 112	Ber-EP4		Desmin	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	24	75	21	71
BioGenex SS Polymer (QD 420-YIKE)	1	100	0	0
Dako EnVision FLEX (K8000/10)	5	80	6	50
Dako EnVision FLEX+ (K8002/12)	25	96	32	75
Dako Envision HRP/DAB (K5007)	5	60	6	83
Dako Envision+ HRP mouse K4004/5/6/7	3	67	2	50
Dako rb-a-mo Ig (E0354)	1	0	1	100
LabVision UltraVision ONE Polymer (TL-012/5-HDJ/T	1	100	1	0
Leica Bond Polymer Define (DS9713)	2	50	3	100
Leica Bond Polymer Refine (DS9800)	63	86	88	84
MenaPath X-Cell Plus (MP-XCP)	0	0	4	100
None	1	100	1	100
NOT APPLICABLE	0	0	1	100
Novocastra Novolink PDS (RE7-140/150/280/290-K)	1	0	2	0
Other	9	78	7	43
Ventana iView system (760-091)	5	60	6	67
Ventana OptiView (760-700) + Amp. (7/860-099)	1	100	1	100
Ventana OptiView Kit (760-700)	39	95	42	88
Ventana UltraView Kit (760-500)	56	84	79	77

General Pathology Run: 112	Ber-EP4		Desmin	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	0	0	1	100
BioGenex Optimax	1	100	0	0
Dako Autostainer	3	67	5	60
Dako Autostainer Link 48	31	94	38	76
Dako Autostainer plus	1	0	3	100
Dako Autostainer Plus Link	3	100	4	75
Dako Omnis	7	100	5	20
LabVision Autostainer	3	100	4	25
Leica Bond Max	29	86	45	84
Leica Bond X	1	0	0	0
Leica Bond-III	51	86	52	83
Menarini - Intellipath FLX	1	100	4	100
None (Manual)	5	20	7	57
Other	1	100	1	100
Shandon Sequenza	2	100	4	75
Ventana Benchmark GX	4	100	4	100
Ventana Benchmark ULTRA	74	84	78	78
Ventana Benchmark XT	45	80	56	75

General Pathology Run: 112	Ber-EP4		Desmin	
Chromogen	**N**	**%**	**N**	**%**
A. Menarini Liquid Stable DAB kit	1	100	0	0
AS PER KIT	39	90	29	76
BioGenex Liquid DAB (HK153-5K)	1	100	0	0
Dako DAB K3468	0	0	1	0
Dako DAB Liquid (K3465)	0	0	1	100
DAKO DAB+	1	0	2	0
Dako DAB+ Liquid (K3468)	3	33	3	33
Dako EnVision Plus kits	4	100	4	100
Dako FLEX DAB	27	93	37	70
Dako REAL EnVision K5007 DAB	7	71	7	71
LabVision DAB	1	100	0	0
Leica Bond Polymer Refine kit (DS9800)	68	85	90	83
menapath xcell kit DAB (MP-860)	0	0	4	100
NOT APPLICABLE	1	100	1	100
Other	11	91	14	86
Sigma DAB (D5637)	1	0	1	0
Sigma DAB (D5905)	1	0	1	100
Ventana DAB	23	91	28	89
Ventana iview	6	67	6	67
Ventana Ultraview DAB	70	80	82	73

BEST METHODS - Gold Standard Antibody

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

Desmin - Method 1

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

Primary Antibody: Leica PA0032 RTU (DER11) , 30 Mins Prediluted

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), 20 ºC., Time 1: 5 Mins, Time 2: 5 Mins

Detection: Leica Bond Polymer Refine (DS9800) , 20 Mins, 20 ºC Prediluted

Desmin - Method 2

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

Primary Antibody:	Ventana 760 2513 (DER11) , 24 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 32mins, Buffer: CC1
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

Desmin - Method 3

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

Primary Antibody:	Ventana 760 2513 (DER11) , 12 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	None
EAR:	Ventana Protease 1 (760-2018), 36 ºC. Digestion Time NEQAS: 12 Mins. In-House: 12 Mins
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700)

Desmin - Method 4

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

Primary Antibody:	Dako IS606 RTU Flex Plus (D33) , 32 Mins, Room Temp ºC
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)

BEST METHODS - Secondary Antibody

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

Ber-EP4 - Method 1

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

Primary Antibody:	Dako RTU Omnis FLEX Ber-EP4 GA637 , 20 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) , 32 ºC

Ber-EP4 - Method 2

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

Primary Antibody:	Dako Ber-EP4 M0804 , 15 Mins, 21 ºC
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	
EAR:	VBS Bond Enzyme 1 Digestion Time NEQAS: 10 Mins. In-House: 10 Mins
Chromogen:	AS PER KIT
Detection:	, 8 Mins, 21 ºC

Ber-EP4 - Method 3

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

Primary Antibody:	Dako RTU Auto Link Ber-EP4 IR637 , 20 Mins, 21 ºC Prediluted
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	AS PER KIT
HMAR:	Dako PTLink, Buffer: Dako low pH TRS
EAR:	
Chromogen:	AS PER KIT, 21 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	, 20 Mins, 21 ºC Prediluted

Ber-EP4 - Method 4

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

Primary Antibody:	Ventana Ber-EP4 760-43-83 , 28 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	None
EAR:	Ventana Protease 1 (760-2018), 36 ºC. Digestion Time NEQAS: 4 Mins. In-House: 4 Mins
Chromogen:	AS PER KIT, 36 ºC., Time 1: 8 Mins, Time 2: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 36 ºC Prediluted

Breast Hormonal Receptor Module Run 112

Merdol Ibrahim and Suzanne Parry

Antigen Assessed:	Progesterone Receptor (PgR)
Tissue Sections circulated:	Composite slide consisting of 3 breast carcinomas with different levels of receptor expression. Most slides also included normal tonsil.
Number of Registered Participants:	291
Number of Participants This Run	287 (99%)

Circulated Tissue
The table below shows the staining characteristics of the tissue sections circulated during Run 112. This composed of three infiltrating ductal carcinomas (IDCs) with differing levels of receptor expression along with normal breast and a section of tonsil. The staining of the breast tumours were characterised using the Novocastra/Leica 16 (A), Ventana 1E2 (A&B), and Dako PgR636 (A&B) antibody clones.

Sections	% Positivity	Expected Staining Intensity	Allred / Quick Score
A. IDC	>95%	High	8
B. IDC	30%	Moderate to High	6 (the expression level varied depending on the serial section received)
C. IDC	0%	Negative	0
D. Normal Breast	Approximately 10% in normal gland	Moderate to high	0 (Negative)
E. Tonsil	≤ 2%	Weak to Medium	0 (Negative)

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

General Guideline Used in The Assessment of slides:

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

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

In-House Tissue Recommendations:

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

The required composite control should consist of the following samples:

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

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

Introduction

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

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

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

Choice of Tissue for Assessments

This assessment consisted of invasive breast tumours (samples A-C) and also normal breast and tonsil (samples D & E). The tonsil was used as a further negative control and to gauge the specificity of the test. Unfortunately some of the Neqas distributed samples may not have contained a tonsil section.

NEQAS tissue samples were tested by staining every 50th section from the relevant tissue blocks using the Leica 1A6 clone. This made sure that any heterogeneity in the tissue samples did not result in a participant being penalised. Furthermore, samples were also tested by the relevant commercial companies to further verify the expected level of staining and included Leica (clone 1A6), Dako (clone PgR 636) and Ventana (clone 1E2). It should be noted that the submitted 1E2 clone showed nuclear staining in the germinal centres of the tonsil section in less than 2% of cells.

Assessment Results

Features of Optimal Immunostaining (Figs 1-6)

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

Features of Sub-optimal Immunostaining (Figs 7-10)

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

NEQAS Slide Results

The overall pass rate on the NEQAS sections was slightly higher than the previous PR assessment (Run 107): 88% received an acceptable pass (scores of ≥13-20/20), compared to 83% at Run 107. A further 8% of labs received a borderline score of 10-12/20. 10 labs (4%) failed the assessment. As with previous assessment PR runs, the majority of the PR antibodies, irrespective of which isoform used (A and/or B), produced the expected staining results.

The most popular antibody used in this assessment run was the Ventana 1E2 clone, used by 91 participants and showed an acceptable pass rate of 83%. It was also encouraging to see that the 1E2 clone did not exhibit the high rates of discordant staining observed in previous UK NEQAS assessments, with only one participant slide showing 'false-positive staining' (Fig 10). The second most popular antibody was the Leica/Novocastra PgR 16A clone, used by 66 participants and showed a pass rate of 97%.

In-House Tissue Results

94% of participants also submitted their in-house controls for assessment. These showed an acceptable pass rate of 80%, and a further 19% of labs received a borderline pass. 1% of participants failed the assessment on their in-house submitted slide. The reasons for a failed result, include inappropriate control with no tumour, excessive cytoplasmic staining, excessive pre-treatment, inappropriate non-specific staining, and poor tissue morphology. Several labs that received a borderline pass had not submitted the required composite control including a high, mid/low and a negative expressing tumour, and therefore received a maximum score of 3/5 from each assessor.

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 PR; 2) re-tested and validated staining of every 50th section from the relevant tissue blocks using the Leica 16 (A) clone; 3) re-tested and validated samples by the relevant commercial companies to further verify the expected level of staining. Such companies include the Novocastra/Leica (clone 16 A), Dako (clone PgR 636 A&B) and the Ventana (clone 1E2) antibodies.

- 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-low-mid (Allred 3-6) and a negative (Allred 0) PR-expressing tumour, Participants are given a maximum score of 12/20. The presence of normal staining glands is also encouraged.

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

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.

Assessment Summary

The main observations for sub-optimal results on the NEQAS samples was either due to weaker staining than expected in the tumour sections or due to inappropriate non-specific staining, such as staining of the fibroblasts, plasma cells or the negative PR-expressing tumour.

Similarly to the last two previous assessments, a few laboratories are experiencing issues with adhesion of their in-house tissue. This has been noted since the scheme has asked labs to cut and place their own in-house control sections together on the same slides as the UK NEQAS sections. The in-house sections are therefore stained together on the same slide and at the same time as the NEQAS sections. Any participant that experienced issues with adherence was asked to send in a separate in-house control slide. Despite a few labs having adhesion problems, this method of assessing has proven to be very helpful at assessment, enabling the assessors to review both sections at the same time, and it was noted that most laboratories received a similar score for both their in-house and NEQAS samples. A few labs did receive lower scores on the NEQAS tissue, and this was mostly due to weak and lower expression of the mid-expressing tumour. This may have been caused if the slides were not stained soon after receipt, and therefore the expression level of PR may have been reduced due to loss of antigenicity. UK

NEQAS advises that sides are stained as soon as possible receipt to the laboratory. Another factor to take into consideration is that the sensitivity of the lab's assay may be acceptable for their own local in-house tissue sections, which they have validated and verified on their tissue, however it can indicate that the sensitivity of the assay may not be optimal. A protocol should be in place that will stain effectively on both the NEQAS samples and the labs own samples.

UK NEQAS ICC and ISH would like to reassure participants that we have validated our one slide approach and this will be continually monitoring to ensure our samples are of appropriate quality.

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1. Jennet M. Harvey, Gary M. Clark, C. Kent Osborne, and D. Craig Allred (1999) Estrogen Receptor Status by Immunohistochemistry Is Superior to the Ligand-Binding Assay for Predicting Response to Adjuvant Endocrine Therapy in Breast Cancer J Clin Oncol 17:1474-1481.
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8. Davies C, Godwin J, Gray R et al., (2011) Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. The Lancet 378: 771-784.

Acknowledgments

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

Selected Images showing Optimal and Sub-optimal Immunostaining

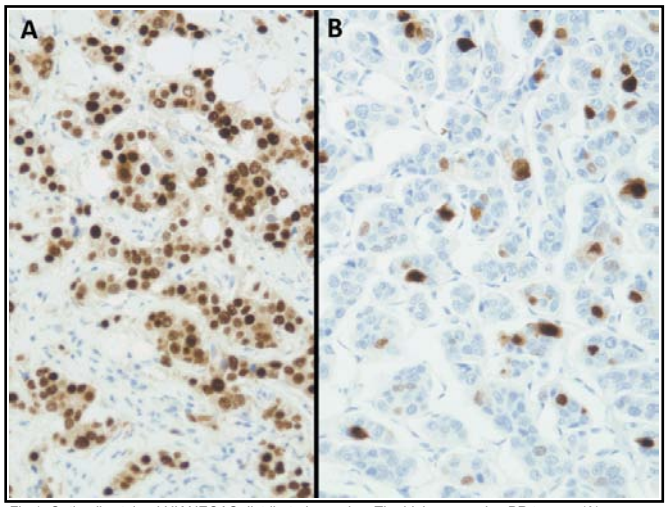


Fig 1. Optimally stained UK NEQAS distributed samples: The high expressing PR tumour (A) shows intense staining in over 95% of neoplastic cells, while the low-mid-expressing tumour (B) shows varying intensity of positive staining in approximately 30% of neoplastic cells. Stained with the Leica 16 (A) antibody, 1:100, on the Leica Bond III with antigen retrieval for 30 minutes

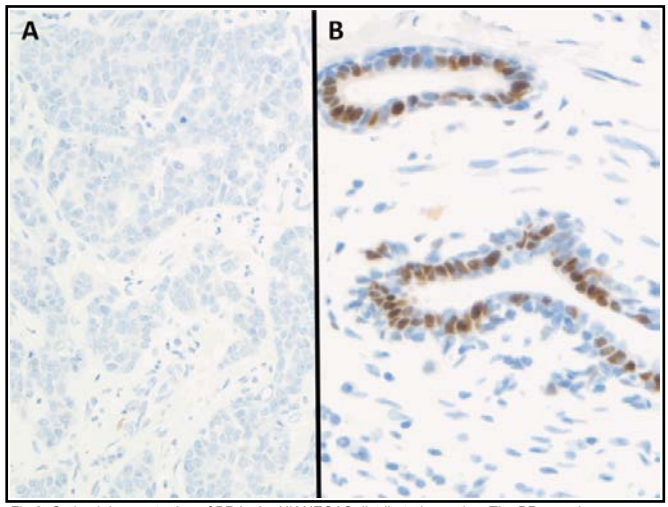


Fig 2. Optimal demonstration of PR in the UK NEQAS distributed samples. The PR negative tumour (A) remains unstained, and the normal glands show the expected percentage of nuclei staining positive for PR. (Same protocol as Fig 1).

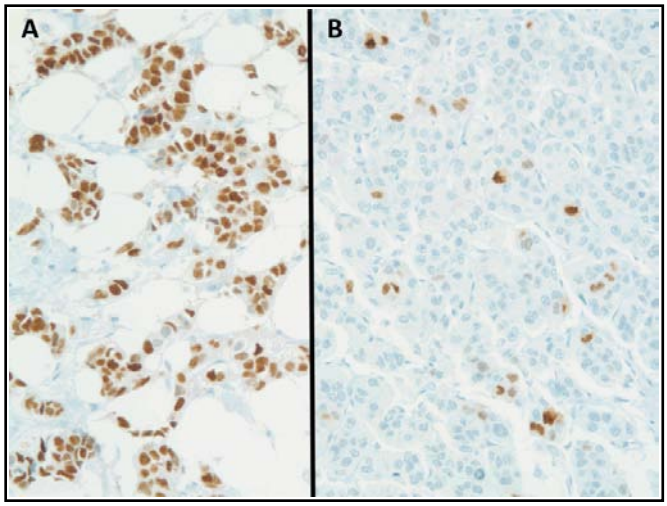


Fig 3. Optimal demonstration of PR in the UK NEQAS high (A) and low-mid (B)-expressing tumours, showing the expected level of staining in both samples. Stained with the Ventana pre-diluted 1E2 (A&B) antibody on the Benchmark XT with CC1 standard antigen retrieval.

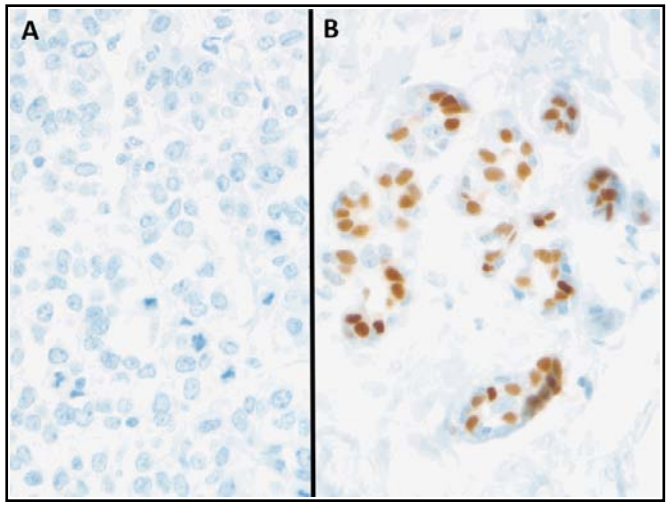


Fig 4. Optimal level of expression in the UK NEQAS distributed samples stained with the Ventana 1E2 (A&B) clone. As expected, the PR negative tumour (A) remains unstained, and the normal glands (section B) show the expected percentage of positive staining (Same protocol as Fig 3).

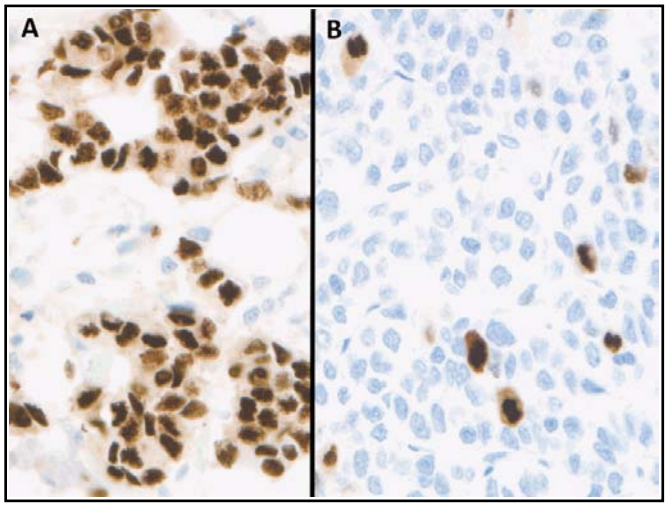


Fig 5. Optimally stained UK NEQAS distributed samples: The high expressing PR tumour (A) shows intense staining in over 95% of neoplastic cells, while the low-mid-expressing tumour (B) shows staining in less than 30% of tumour cells. Stained with the Dako RTU PgR636 (A&B) antibody, on the Autostainer with pre-treatment in the PT link for 20 minutes in high pH buffer.

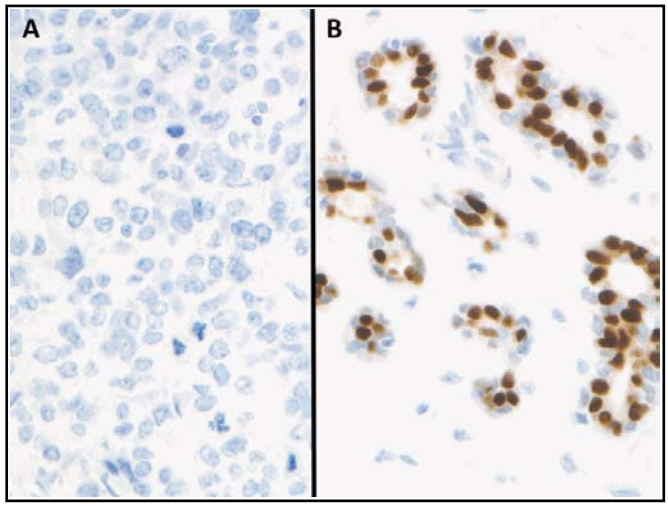


Fig 6. . Optimal demonstration of PR in the UK NEQAS distributed samples. The PR negative tumour (A) remains unstained, and the normal glands show the expected percentage of nuclei staining positive for PR. (Same protocol as Fig 5).

Selected Images showing Optimal and Sub-optimal Immunostaining

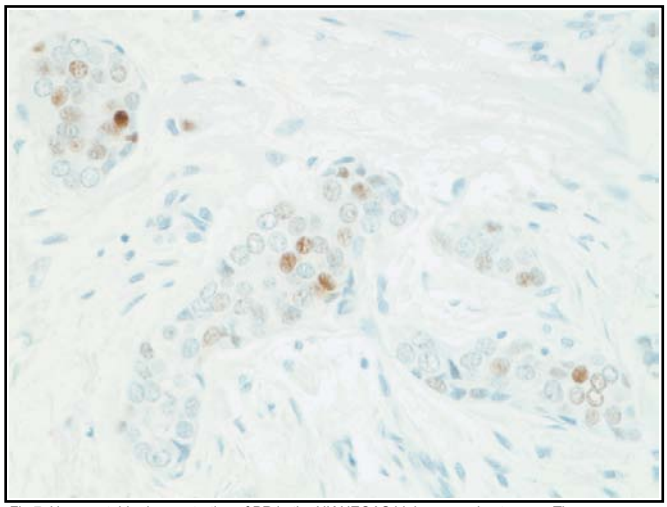


Fig 7. Unacceptable demonstration of PR in the UK NEQAS high-expressing tumour. The staining is very weak with much lower percentage of tumour cells staining than is expected, and therefore more representative of a mid-expressor (compare to Figs 1A, 3A & 5A). This is most likely caused by insufficient antigen retrieval. Stained with the Leica PgR 16 (A) antibody,

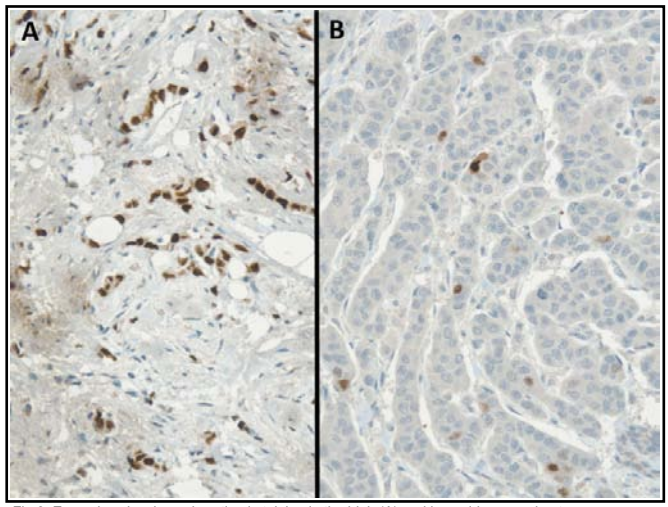


Fig 8. Examples showing sub-optimal staining in the high (A) and low-mid-expressing tumours (B). Both sections show excessive background staining. This is most likely caused by excessive antigen retrieval. Stained with the Dako PgR 363 (A&B) antibody, 1:60, on the Ventana Benchmark XT with CC1 extended antigen retrieval.

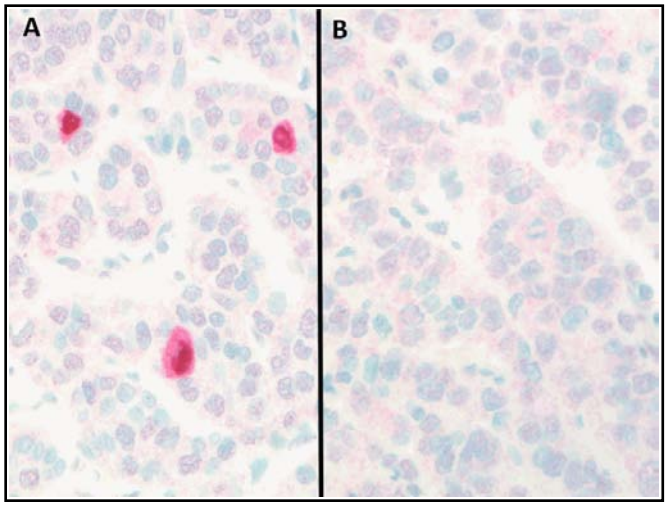


Fig 9. Sub-optimal demonstration on the low-mid (A) and negative expressing (B) tumours. Although the expression is as expected, the staining is diffuse and leaching into the cytoplasm of the cells. Stained with the Leica PgR 16 (A) antibody, 1:200, on the Leica BondMax, ER2 retrieval for 20 minutes and Refine Red detection kit.

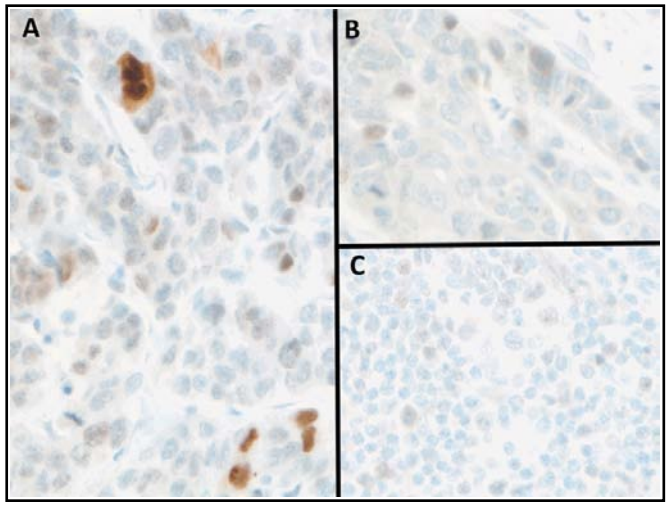


Fig 10. Unacceptable demonstration of PR on the UK NEQAS low-mid (A) and negative tumours (B), and the normal tonsil (C): All sections show non-specific nuclear staining. The negative tumour appears to show false positive staining, and the tonsil is staining more lymphocytes than expected. Stained with the Ventana 1E2 (A&B) antibody on the ULTA with

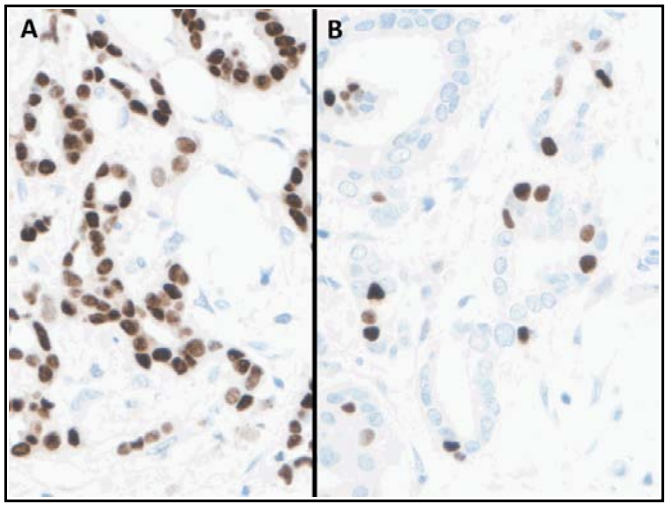


Fig 11. Good examples of in house controls for PR, showing high (A) and mid- expressing tumours (B). Stained with the Leica PgR 16 (A) antibody on the Bond III with ER1 retrieval for 30 minutes.

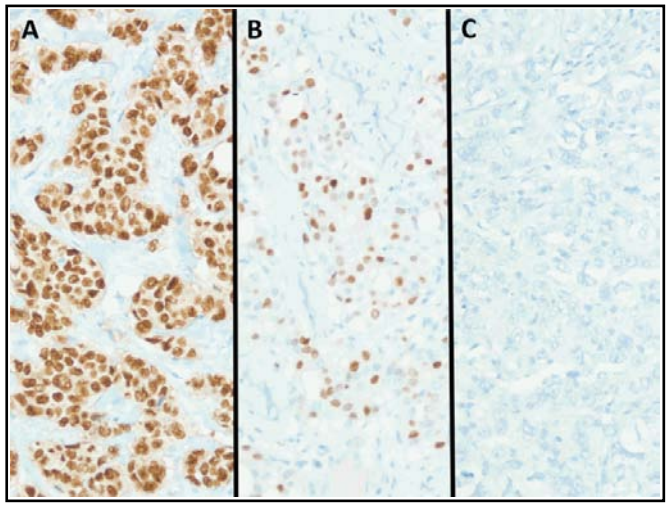
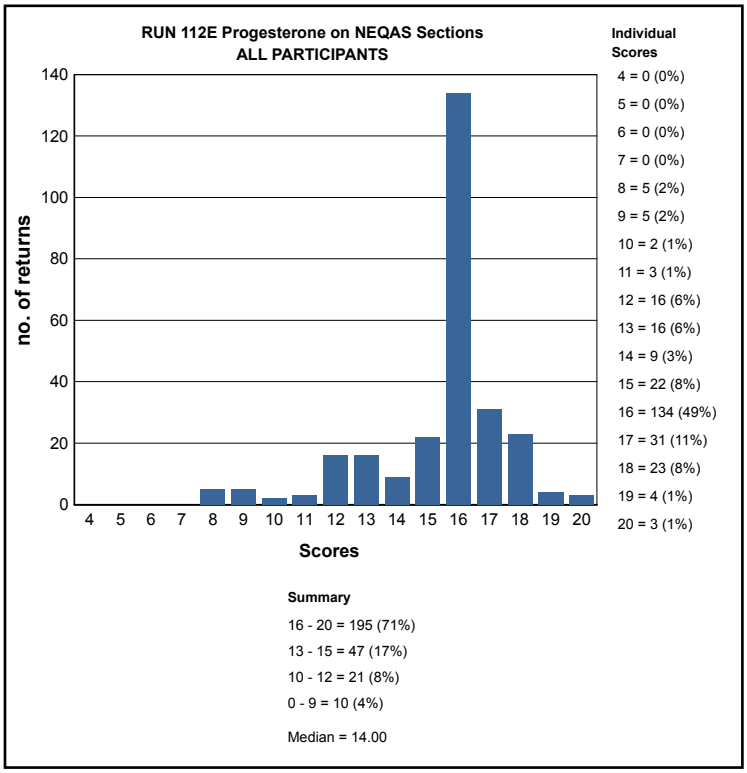
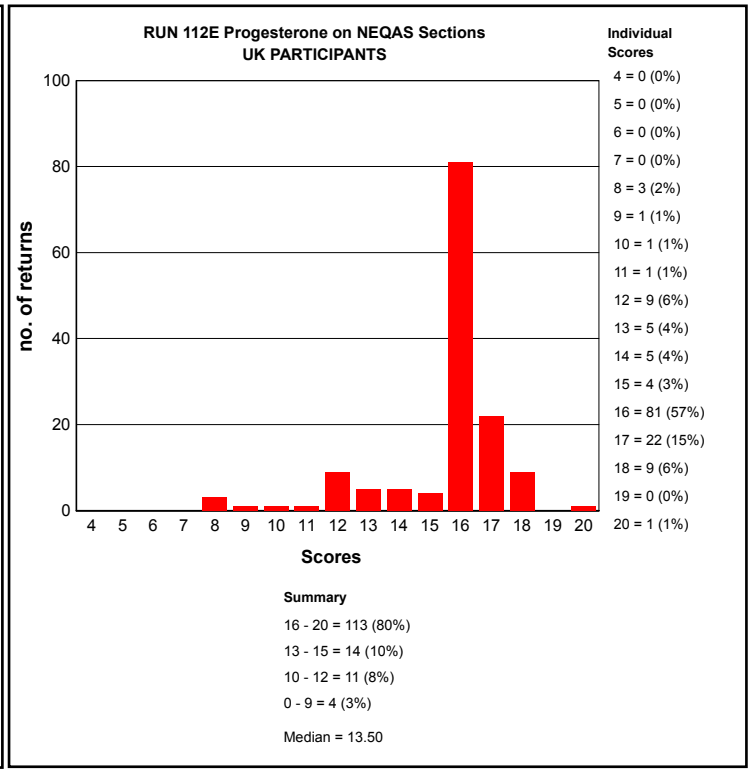
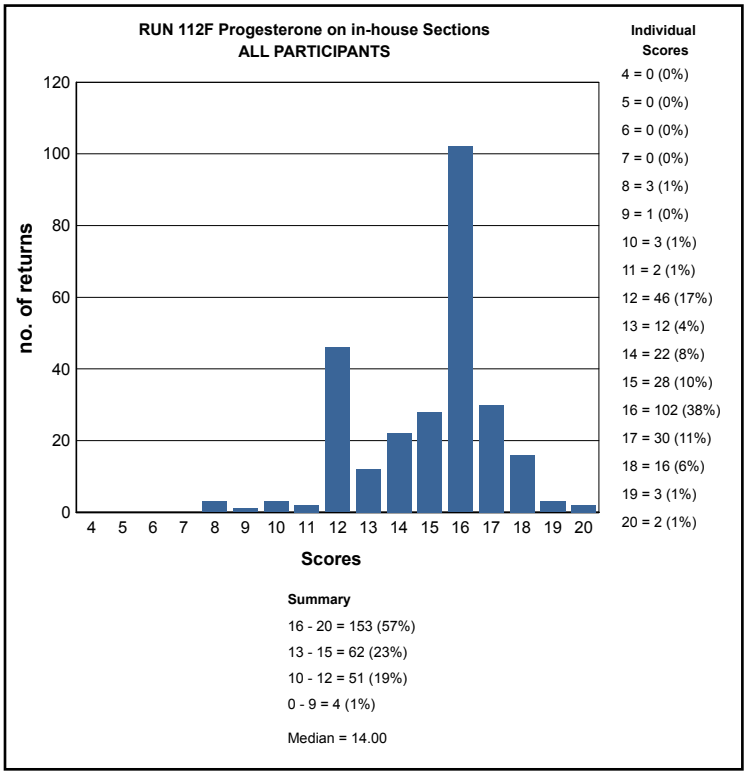


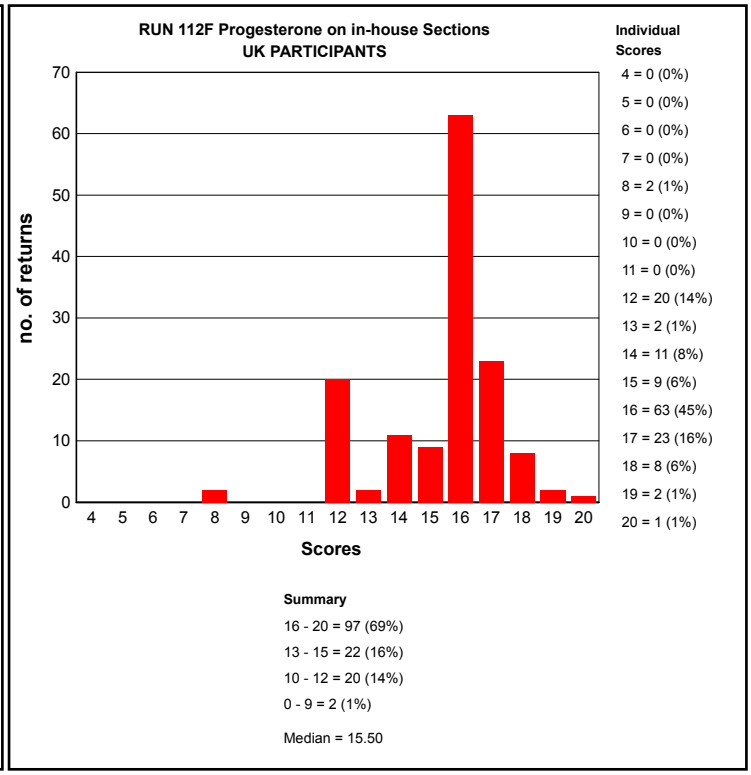
Fig 12. Good example of an in house multi-block control for PR. The section contains high, mid, and negative expressing tumours (A-C respectively). A control containing tumours of known differing expression levels is important to gauge the sensitivity of the assay. Stained with the Ventana 1E2 (A&B) antibody on the ULTRA with CC1 standard 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: 112

Primary Antibody : Progesterone

Antibody Details	N	%
Cell Marque 323R-16 (A)	1	0
Dako IR068 (PgR 636) (A&B)	16	100
Dako IS068 (PgR 636) (A&B)	2	100
Dako K1904 (PgR 1294 (b))	1	100
Dako M3569 (PgR 636) (A&B)	39	87
Dako N1630 RTU (PgR 636) (A&B)	1	100
NeoMarkers RM-9102-S (SP2) (A&B)	1	100
Novocastra NCL-L-PGR-312 (16) (A)	29	97
Novocastra NCL-L-PGR-AB (16+SAN27) (A&B)	10	100
Novocastra NCL-L-PGR/2 (1A6) (A&B)	2	100
Novocastra NCL-PGR (1A6) (A&B)	2	50
Novocastra NCL-PGR-312 (16) (A)	25	92
Novocastra NCL-PGR-AB (16+SAN27)	2	100
Novocastra PA0312 (16) (A)	9	100
Novocastra RTU-PGR-312 (16) (A)	3	100
Novocastra RTU-PGR-AB (16+SAN27) (A&B)	1	100
Other	15	87
Ventana 760 2816 PgR (16) (A)	1	100
Ventana 790-2223 (1E2) (A&B)	63	79
Ventana 790-4296 (1E2) (A&B)	28	86
Ventana 790-4324 (SP2) (A&B)	3	100

Breast Steroid Hormone Receptor Run: 112

Automation

	Progesterone N	Progesterone %
Dako Autostainer	1	0
Dako Autostainer Link 48	33	97
Dako Autostainer plus	1	100
Dako Autostainer Plus Link	3	100
Dako Omnis	5	100
LabVision Autostainer	4	100
Leica Bond Max	30	93
Leica Bond-III	43	98
Menarini - Intellipath FLX	2	100
None (Manual)	5	80
Other	1	0
Shandon Sequenza	3	100
Ventana Benchmark GX	8	88
Ventana Benchmark ULTRA	75	84
Ventana Benchmark XT	53	79

Breast Steroid Hormone Receptor Run: 112

Heat Mediated Retrieval

	Progesterone N	Progesterone %
Biocare Decloaking Chamber	1	100
Dako Omnis	5	100
Dako Pascal	1	100
Dako PTLink	34	97
Lab vision PT Module	3	100
Leica ER1 20 mins	12	92
Leica ER1 30 mins	19	100
Leica ER1 40 mins	2	100
Leica ER2 10 mins	3	100
Leica ER2 20 mins	29	93
Leica ER2 30 mins	7	100
Leica ER2 40 mins	1	100
Microwave	2	100
Other	1	100
Pressure Cooker	8	50
Pressure Cooker in Microwave Oven	1	100
Ventana CC1 16mins	2	50
Ventana CC1 20mins	1	100
Ventana CC1 24mins	1	100
Ventana CC1 32mins	6	83
Ventana CC1 36mins	13	77
Ventana CC1 40mins	2	100
Ventana CC1 48mins	2	100
Ventana CC1 52mins	4	100
Ventana CC1 56mins	1	100
Ventana CC1 64mins	31	87
Ventana CC1 72mins	1	100
Ventana CC1 76mins	1	100
Ventana CC1 8mins	2	50
Ventana CC1 92mins	2	100
Ventana CC1 extended	5	60
Ventana CC1 mild	16	81
Ventana CC1 standard	38	82
Ventana CC2 32mins	1	100
Ventana CC2 36mins	1	100
Ventana CC2 64mins	1	100
Ventana CC2 standard	2	50
Water bath 95-98 OC	3	100

Breast Steroid Hormone Receptor Run: 112

Enzyme Mediated Retrieval

	Progesterone N	Progesterone %
AS PER KIT	7	86
NOT APPLICABLE	158	89
Ventana Protease 1 (760-2018)	2	50

Breast Steroid Hormone Receptor Run: 112	Progesterone	
Detection	**N**	**%**
AS PER KIT	9	100
Biocare polymer (M4U534)	1	100
Dako EnVision FLEX (K8000/10)	8	88
Dako EnVision FLEX+ (K8002/12)	24	100
Dako Envision HRP/DAB (K5007)	5	60
Dako Envision+ HRP mouse K4004/5/6/7	2	100
Dako rb-a-mo Ig (E0354)	1	100
Dako REAL HRP/DAB (K5001)	1	100
LabVision UltraVision LP HRP (TL 125 HLJ)	1	100
Leica Bond Polymer Refine (DS9800)	68	97
MenaPath X-Cell Plus (MP-XCP)	1	100
None	3	67
NOT APPLICABLE	1	100
Novocastra Novolink PDS (RE7-140/150/280/290-K)	1	100
Other	4	75
Ventana iView system (760-091)	5	100
Ventana OptiView Kit (760-700)	17	94
Ventana UltraView Kit (760-500)	112	79

Breast Steroid Hormone Receptor Run: 112	Progesterone	
Chromogen	**N**	**%**
AS PER KIT	21	90
Dako DAB K3468	1	100
DAKO DAB+	2	100
Dako EnVision Plus kits	2	100
Dako FLEX DAB	31	97
Dako REAL EnVision K5007 DAB	6	50
Dako REAL K5001 DAB	1	100
Leica Bond Polymer Refine kit (DS9800)	65	98
menapath xcell kit DAB (MP-860)	1	100
Other	9	89
Sigma DAB (D5905)	1	100
Ventana DAB	8	100
Ventana Enhanced Alk. Phos. Red Detection Kit	1	100
Ventana iview	4	100
Ventana Ultraview DAB	114	80

BEST METHODS

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

Progesterone - Method 1

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

Primary Antibody: Novocastra NCL-L-PGR-312 (16) (A) , 20 Mins Dilution 1: 200
Automation: Dako Autostainer Link 48
Method: Dako FLEX kit
Main Buffer: PBS + Tween
HMAR: Dako PTLink
EAR: NOT APPLICABLE
Chromogen: Dako FLEX DAB
Detection: Dako EnVision FLEX (K8000/10)

Progesterone - Method 2

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

Primary Antibody: Ventana 790-4296 (1E2) (A&B) , 20 Mins, 42 ºC Prediluted
Automation: Ventana Benchmark ULTRA
Method: Ventana UltraView DAB
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 64mins
EAR:
Chromogen: Ventana Ultraview DAB
Detection: Ventana UltraView Kit (760-500)

Progesterone - Method 3

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

Primary Antibody: Dako IR068 (PgR 636) (A&B) , 20 Mins, RT ºC Prediluted
Automation: Dako Autostainer Link 48
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako PTLink, Buffer: Dako TRS High
EAR:
Chromogen: Dako FLEX DAB, RT ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection: Dako EnVision FLEX+ (K8002/12) , 20 Mins, RT ºC Prediluted

Progesterone - Method 4

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

Primary Antibody:	Novocastra NCL-PGR-312 (16) (A) , 15 Mins, rt ºC Dilution 1: 100
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), Time 2: 12 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, rt ºC

Breast HER2 IHC Module Run 112

Suzanne Parry and Keith Miller

Antigen Assessed:	HER2
Sections Circulated:	Composite slide consisting of 4 breast carcinoma cell lines (see table below)
Number of Registered Participants:	350
Number of Participants this Run	311 (89%)

Specific Guidelines Used in The Assessment of Slides:

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

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

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

Validation of Distributed UK NEQAS ICC & ISH Cell Lines

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

Updated Assessment and Scoring Procedure

UK NEQAS Specific Membrane Scoring Algorithm:

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

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

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

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

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

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

Introduction

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

Recommended HER2 testing guidelines include the ASCO/ CAP guidelines by Wolff et al. (2007), the UK guidelines by Walker et al., (2008), the ASCO/CAP guidelines by Wolff et al., 2013., and the updated UK guidelines by Rakha et al., (2015). These publications provide invaluable guidelines covering interpretation, tissue fixation and antibody validation. The articles by Walker et. al. and Rakha 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.

The UK NEQAS HER2 IHC module assesses the technical quality of staining achieved by laboratories and provides feedback to laboratories to help improve the analytical phase of patient diagnosis. Participants who continue to have difficulty in producing acceptable staining results should be aware that the members of staff at UK NEQAS ICC are always willing to assist any laboratory struggling to meet the required standard of HER2 immunostaining, and would be happy to receive requests for assistance via e-mail in the first instance (see front of journal or the UK NEQAS ICC & ISH website for contact details).

In-House Control Tissue Recommendations

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

Assessment Summary:

Pass rates for the assessment are shown in the subsequent summary graphs. As with most recent assessment runs the Ventana 4B5 system still remains the most popular choice of HER2 antibody. This was used by 57% of participants in the current run and showed an overall acceptable pass rate of 75%. 7% (N=23) of labs are using the Dako HercepTest, and 6% (N=19) of labs are using the Leica Oracle kit. 25% of participants used lab-devised methods in this assessment run, using a variety of antibodies, pre-treatment methods and platforms. These labs showed an acceptable pass rate of 27%. All data is based on the methodology information submitted by participants, but unfortunately not all methodology was provided by all participants.

Important: Any laboratory using the Ventana OptiView detection kit for HER2 testing should be aware that they are doing so 'off label usage' as this detection system has not been validated for 4B5 HER2 testing.

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 Recommendations 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

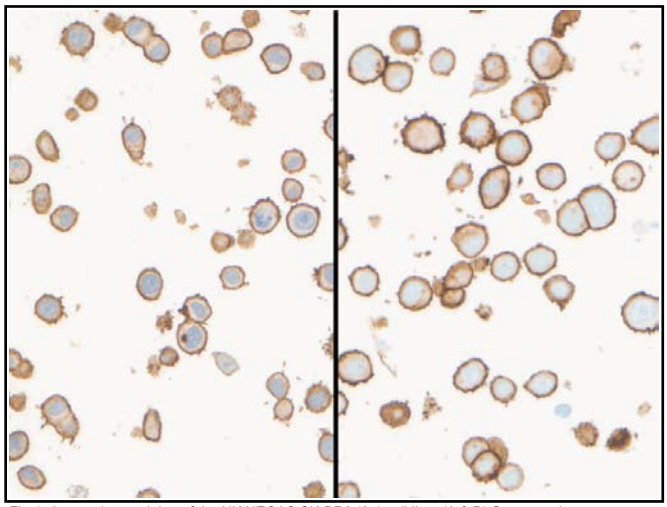


Fig 1. Appropriate staining of the UK NEQAS SK-BR3 (3+) cell line. (A & B) Strong and complete circumferential membrane. Stained using (A) the Leica Oracle kit, on a Bond III as per recommendations and (B) Ventana Pathway 4B5, CC1 retrieval on the Benchmark ULTRA with UltraView detection.

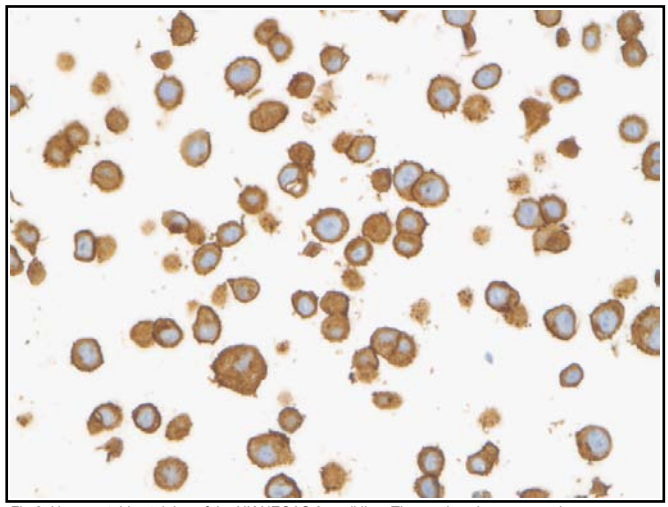


Fig 2. Unacceptable staining of the UK NEQAS 3+ cell line. The section shows excessive cytoplasmic staining, making it difficult to read the membranes. The staining was carried out using a lab-devised method with the Dako polyclonal antibody and antigen retrieval in the pressure cooker.

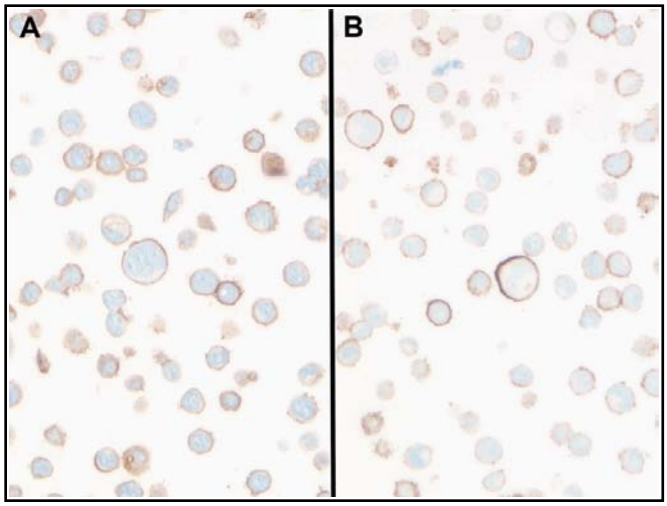


Fig 3. Two examples showing the expected level of staining of the UK NEQAS MDA-MB-453 (2+) cell line. (A & B) Majority of cells show weak to moderate complete membrane staining. Stained using (A) the Dako HercepTest on the autostainer with pre-treatment in the PT link. (B) stained with the Ventana Pathway 4B5 (same method as in Fig 1B).

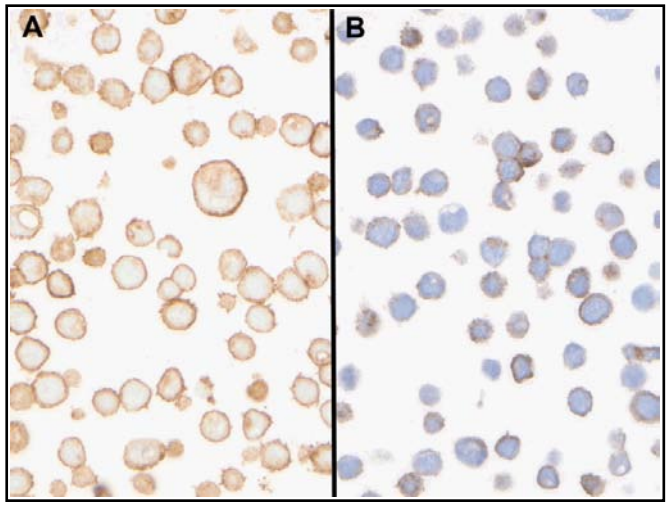


Fig 4. Unacceptable staining of the UK NEQAS 2+ distributed cell lines. (A) shows excessive membrane staining, which is more representative of 3+ staining. (B) shows morphology damage and excessive counterstain.

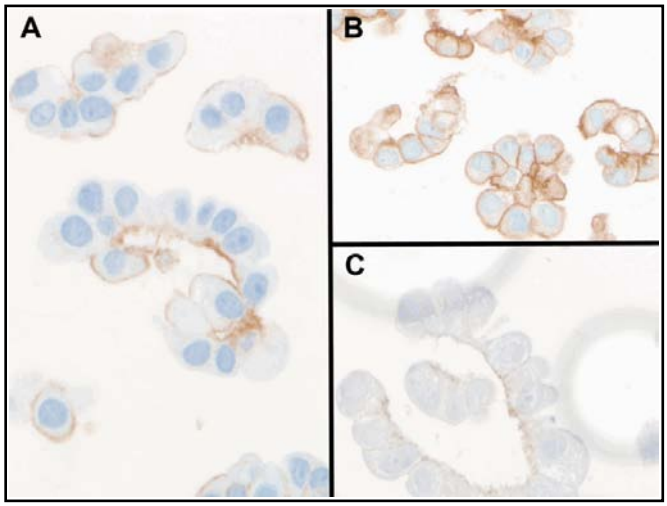


Fig 5. Staining of the UK NEQAS MDA-MB-175 (1+) cell line: (A) shows the expected level of staining, which is partial membranous. In (B) the staining is too high and more representative of 2+staining. In (C) any possible staining is masked by the grey counterstain. This section also had water droplets covering most of the slide, making some areas of the slide difficult to read.

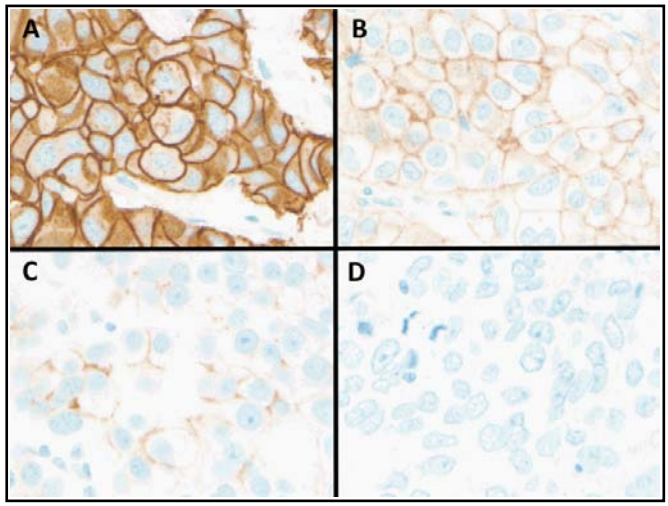
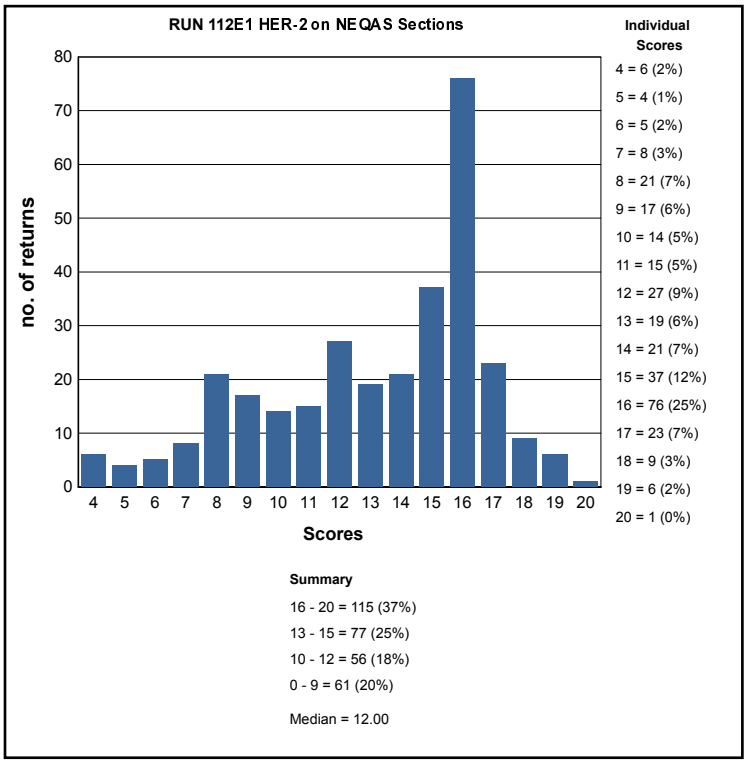
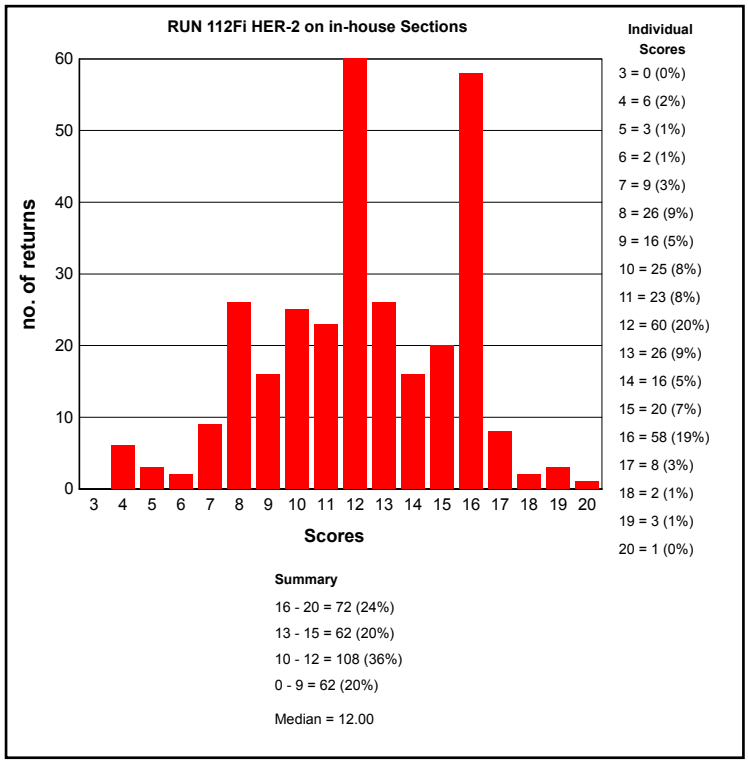


Fig 6. Good in house breast controls optimally stained with HER2. (A) 3+ (B) 2+ (C) 1+ and (D) negative. Section stained with the Ventana 4B5 on the Benchmark ULTRA with CC1 retrieval for 36mins and Ultraview detection.

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: 112

Primary Antibody	N	%
Biocare CME 342 A,B (EP1045Y)	4	25
BioGenex (EP1045Y) rb mono	4	50
Biogenex AM134-5M (CB11)	3	0
Cell Marque 237R (SP3)	5	40
Cell Marque CMA 601 (CB11)	2	0
Dako A0485 C-erB-2 (poly)	30	40
Dako HercepTest K5204 (poly)	4	25
Dako HercepTest K5205 (poly)	1	0
Dako HercepTest K5207 (poly)	4	100
Dako Link HercepTest SK001 (poly)	14	79
Labvision / Neomarkers RM-9103 (SP3)	7	0
Leica Oracle HER2 Bond IHC (CB11)	19	79
Novocastra NCL-L-CB11 (CB11)	10	20
Novocastra NCL-L-CBE356 (10A7)	1	100
Novocastra RTU-CB11 (CB11)	1	0
Other	10	20
Ventana Confirm 790-4493 (4B5)	39	67
Ventana Pathway 790-100 (4B5)	11	82
Ventana Pathway 790-2991 (4B5)	125	76

Breast HER2 ICC Run: 112

Automation	N	%
BioGenex GenoMX 6000i	4	0
Dako Autostainer	4	25
Dako Autostainer Link 48	26	54
Dako Autostainer plus	4	0
Dako Autostainer Plus Link	4	100
Dako Omnis	1	0
LabVision Autostainer	2	0
Leica Bond Max	22	45
Leica Bond-III	18	83
Menarini - Intellipath FLX	1	0
None (Manual)	25	28
Other	5	20
Shandon Sequenza	1	0
Ventana Benchmark GX	13	54
Ventana Benchmark ULTRA	82	77
Ventana Benchmark XT	83	72

Breast HER2 ICC Run: 112

Heat Mediated Retrieval	N	%
Biocare Decloaking Chamber	8	25
Dako Omnis	1	0
Dako Pascal	1	0
Dako PTLink	32	53
Lab vision PT Module	1	0
Leica ER1 10 mins	5	80
Leica ER1 20 mins	8	38
Leica ER1 25 mins	17	76
Leica ER1 30 mins	3	0
Leica ER1 40 mins	1	100
Leica ER2 10 mins	1	0
Leica ER2 20 mins	2	100
Microwave	9	11
None	6	50
Other	4	75
Pressure Cooker	7	0
Steamer	1	0
Ventana CC1 16mins	3	33
Ventana CC1 20mins	5	20
Ventana CC1 24mins	2	50
Ventana CC1 32mins	11	36
Ventana CC1 36mins	37	84
Ventana CC1 40mins	2	50
Ventana CC1 48mins	1	0
Ventana CC1 52mins	3	67
Ventana CC1 56mins	5	100
Ventana CC1 64mins	6	83
Ventana CC1 76mins	2	100
Ventana CC1 8mins	1	100
Ventana CC1 mild	74	70
Ventana CC1 standard	13	92
Ventana CC2 16mins	1	100
Ventana CC2 36mins	2	100
Ventana CC2 standard	1	100
Water bath 95-98 OC	14	43

Breast HER2 ICC Run: 112

Detection	N	%
AS PER KIT	31	65
Biocare polymer (M4U534)	1	100
Biocare SLAB (STU HRP 700H,L10)	2	50
BioGenex HRP (HK 519-06K)	2	0
BioGenex SS Polymer (QD 420-YIKE)	1	0
BioGenex SS Polymer (QD 430-XAKE)	1	0
Dako HerCep Test (K5204)	1	0
Dako EnVision FLEX (K8000/10)	11	27
Dako EnVision FLEX+ (K8002/12)	8	25
Dako Envision HRP/DAB (K5007)	9	33
Dako Envision+ HRP rabbit K4008/9/10/11	1	0
Dako HerCep Test Autor (K5207)	2	100
Dako HerCep Test Autor (SK001)	7	86
Dako rb-a-mo Ig (E0354)	1	0
LabVision UltraVision ONE Polymer (TL-012/5-HDJ/T	1	0
Leica Bond Polymer Define (DS9713)	1	100
Leica Bond Polymer Refine (DS9800)	23	52
None	2	50
Other	11	9
Ventana iView system (760-091)	6	100
Ventana OptiView Kit (760-700)	12	50
Ventana UltraView Kit (760-500)	149	74

Breast HER2 ICC Run: 112

Enzyme Retrieval	N	%
AS PER KIT	17	65
Enzyme digestion + HIER	1	0
NOT APPLICABLE	149	63
Other	2	0
Ventana Protease	1	100
Ventana Protease 1 (760-2018)	1	100

Breast HER2 ICC Run: 112

Chromogen	N	%
A. Menarini Liquid Stable DAB kit	1	0
AS PER KIT	49	73
BioGenex DAB (QD430)	1	0
BioGenex Liquid DAB (HK153-5K)	2	0
BioGenex liquid DBA (HK-124-7K)	1	0
DAKO DAB+	1	100
Dako DAB+ Liquid (K3468)	3	0
Dako DAB+ REAL Detection (K5001)	2	0
Dako EnVision Plus kits	3	33
Dako FLEX DAB	21	43
Dako REAL EnVision K5007 DAB	8	38
LabVision DAB	1	0
Leica Bond Polymer Refine kit (DS9800)	27	56
Other	21	24
Sigma DAB (D5905)	1	0
Ventana DAB	7	86
Ventana iview	4	100
Ventana Ultraview DAB	142	73

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

Primary Antibody:	Leica Oracle HER2 Bond IHC (CB11) , 30 Mins, 22 ºC Prediluted
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590), PH: 7.6
HMAR:	Leica ER1 25 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), 22 ºC., Time 1: 10 Mins
Detection:	AS PER KIT , 10 Mins, 22 ºC Prediluted

HER-2 - Method 2

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

Primary Antibody:	Dako Link HercepTest SK001 (poly)
Automation:	Dako Autostainer Plus Link
Method:	AS PER KIT
Main Buffer:	AS PER KIT
HMAR:	Dako PTLink
EAR:	AS PER KIT
Chromogen:	AS PER KIT
Detection:	AS PER KIT

HER-2 - Method 3

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

Primary Antibody:	Ventana Pathway 790-2991 (4B5) , 12 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 36mins
EAR:	
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

ISH Best Practices

A Tissue/Slides

1 Pre-analytics:
Verify sample collection conditions (6-48 hours in NBF)

2 Water Quality:
Ensure water quality is as per CAP guidelines

3 Pre-processing/Sectioning:
- Ensure tissue fits into cassette
- Cut 4 um thick sections

4 Slides:
- Use Superfrost Plus slides or equivalent charged
- Do not 'double dip' slides

B Pre-run

1 Labels:
Centre label with no overhang

2 Dispensers:
- inspect all tips and remove plugs
- prime liquid meniscus to end of dispenser

3 Slide Placement:
Placed towards operator

4 Protocol:
- Ensure protocol optimisation
- Start with package insert protocol

5 Instrument Priming:
Prime instrument at least once before run0

C Post-run

1 Post-Processing Slides:
- Wash slides in soapy DI water
- If using red detection, dry slides by baking at 60°C for 60 min
- Dip in clean xylene (< 30 sec), then permanently mount

2 Bulk Preparation:
Rinse with DI water

3 Decontaminate Carboys:
Decontaminate 20 L bulk carboys every quarter per operator's manual

4 Instrument:
- Decontaminate instrument per operator's manual quarterly
- Level 1/Preventative Maintenance
- Ensure L1/PM are performed according to recommended schedule

For online training on our Dual ISH Assay, please visit **www.her2dualish.com**

For a full description of ISH Best practices, please contact your local Product Specialist.

www.roche.com **www.ventana.com**

Gastric HER2 ICC Module Run 112

Merdol Ibrahim and Suzanne Parry

Antigen Assessed:	**HER2 for IHC**
The NEQAS distributed sections consisted of a composite slide containing 4 surgical /resections of intestinal gastric carcinoma:	The expected expression levels of each tissue sample were: **A.** 0 **B**. 0 or 1+ depending on the serial section received **C**. 2+ **D**. 3+
Number of Registered Participants	78
Number of Participants this Run	65 (83%)

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

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

Validation of Distributed Samples

IHC Validation of Distributed Samples

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

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

Table 2: HER2 IHC staining and & ISH results

Section labelled from left to right at slide label end	Staining pattern with IHC	HER2 status by ISH
A	**0**	Non-Amplified
B	**0 or 1+**	Non-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

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 who are HER2 positive (IHC 3+ and IHC 2+/ISH+) have been shown to benefit from Herceptin (Trastuzumab) therapy and increased overall survival rate. 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 IHC 2+/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– 4)

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

Features Of Sub-optimal or Unacceptable Staining: (Fig 5)

- 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

Pass Rates

The pass rates for the UK NEQAS distributed samples were considerably lower than the previous assessment (Run 111), with 62% of labs receiving an acceptable pass, compared to 82% in the previous run (111). A further 20% of labs received a borderline pass (scores of 10-12/20), and 10 labs (15%) failed the assessment. The borderline results were mostly given due to weaker staining than expected, which was mostly seen on tissue core C. This sample should have been a 2+ expressing tumour, but for some labs the staining was nearing to a 1+ expression. A few labs also showed over staining, and core C was appearing towards a 3+. Very weak staining was the most common reason why some labs failed the assessment (scores of ≤9/20), with several labs showing no staining at all in the 2+ (core C) tumour section, and the 3+ (core D) appearing to be more representative of a 2+ - expressing tumour. For some of these labs, it was obvious that an incorrect antigen retrieval time or incubation with the antibody time was the reason for either over-staining or weak staining. For example, Ventana recommends specific antigen retrieval methods, and times and temperature of antibody incubation depending on the detection kit used and whether the staining is carried out on the Benchmark XT or the ULTRA platform. It is therefore very important for labs to follow the correct recommended protocol according to the automated platform and detection kit they are using.

95% (N=62) of labs that participated in the assessment also submitted their in-house controls. The overall pass rate was higher than that seen on the Neqas samples, with 50% receiving an acceptable pass, and 34% receiving a borderline score. Many of the labs that received a borderline pass lost marks because they did not include a 2+-expressing tumour as part of their control: UK Neqas requires labs to submit a composite control consisting of 3+, 2+ and 1+/0 expressing gastric/breast control tumour samples. Labs are given a maximum score of 12/20, i.e. 3/5 from each assessor if they do not provide the required composite control. Several other labs were marked down for poor tissue quality or fixation, and 10 labs (16%) failed the assessment, and this was mainly due to poor tissue quality.

Methodologies

Most 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 and showed an acceptable pass rate of 49% on the UK NEQAS distributed section. All 6 labs that are using the standardised Dako HercepTest received acceptable passes. The Leica Oracle kit was used by 1 lab, but unfortunately the user failed the assessment due to weak and a lower expression HER2 expression than expected. The sections also showed excessive cytoplasmic staining.

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 acceptable for labs to submit an in-house control which might also be heterogeneous in nature, for example, with areas of both 3+ and 2+ membrane expression. If this control has been used, participants must clearly indicate the areas where the assessment should be carried out.

Additional Comments:

- The Ventana 4B5 is known to stain intestinal metaplasia (not illustrated) and participants are not penalised when this staining is observed.
- Any laboratory using the Ventana OptiView detection kit for HER2 testing should be aware that they are doing so 'off label usage' as this detection system has not been validated for 4B5 HER2 testing.

References:

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

Acknowledgments

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

Selected Images showing Optimal and Sub-optimal Immunostaining

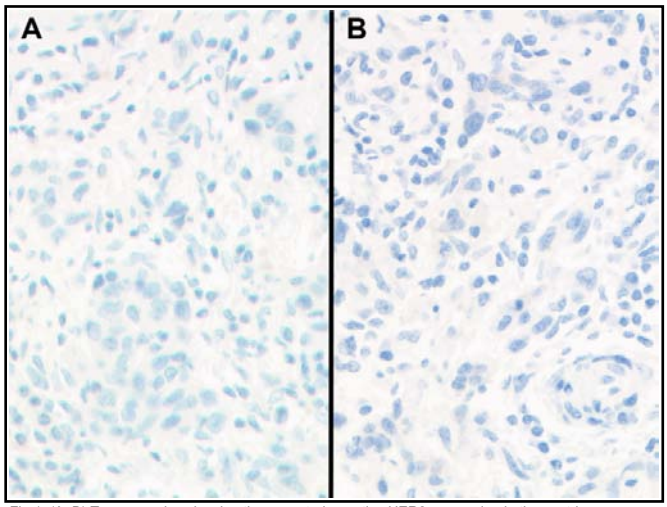


Fig 1. (A, B) Two examples showing the expected negative HER2 expression in the gastric tumour sample 'A'. Section (A) stained with the Ventana 4B5 Pathway on the Benchmark ULTRA, CC1 36 minutes. (B) stained with the Dako HercepTest with antigen retrieval in the PT link.

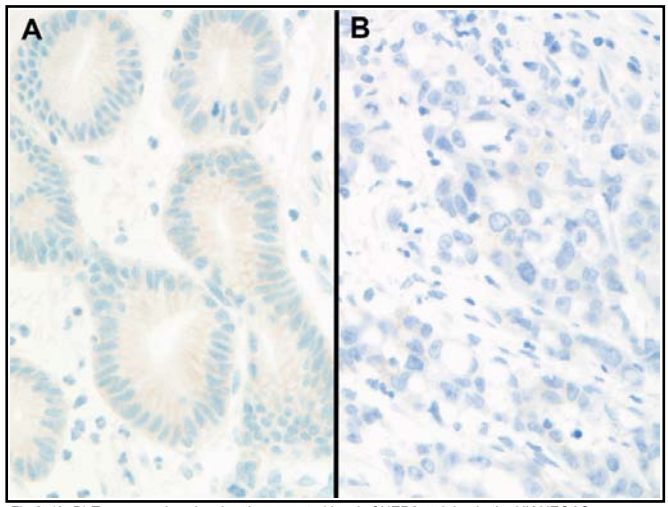


Fig 2. (A, B) Two examples showing the expected level of HER2 staining in the UK NEQAS distributed sample B. The expression level varied from a 1+ to 0 expression depending on the block and serial section received. (A) stained with the Ventana 4B5 Pathway shows 1+ expression, which is incomplete membrane staining, and (B) stained with the Dako HercepTest

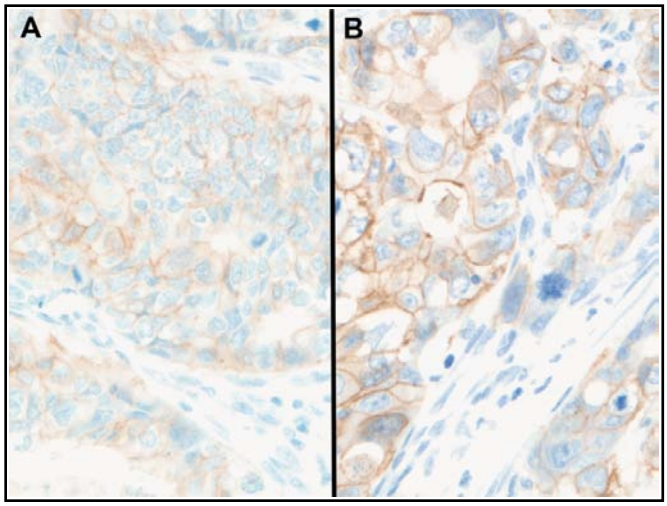


Fig 3. (A, B) Two good examples of HER2 2+ staining on the UK NEQAS distributed gastric sample 'C'. Both show complete membrane staining, but lower expression than expected in a 3+.(A) Stained on a Ventana ULTRA with the 4B5 pathway and CC1 retrieval for 36 minutes, and (B) stained with the Dako HercepTest with Dako PT link retrieval.

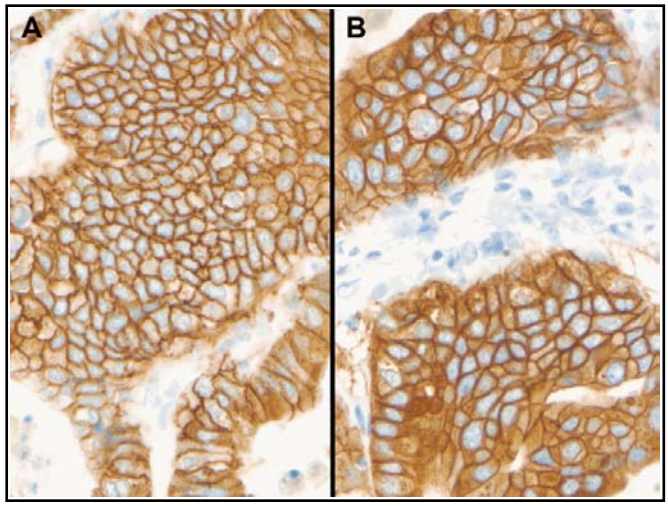


Fig 4. (A, B) Two good examples of HER2 3+ from the NEQAS distributed gastric sample 'D', both showing intense complete membrane staining. (A) Stained on a Ventana Benchmark ULTA with the 4B5 Pathway, retrieval for 36 minutes in CC1 buffer. (B) stained with the Dako Link HercepTest asnd retrieval in the PT link.

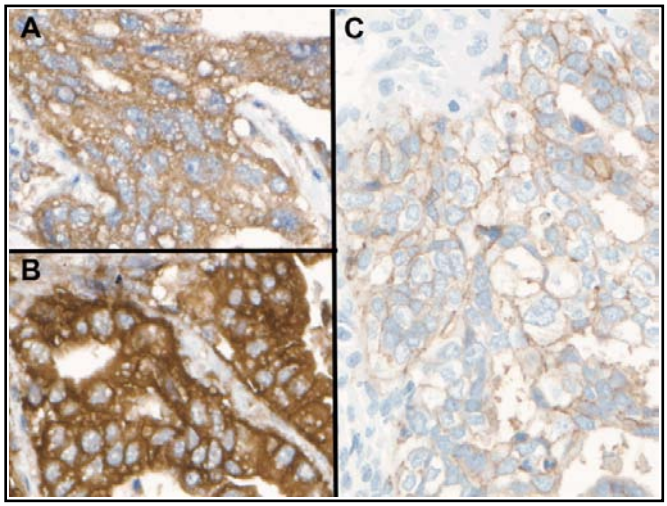


Fig 5. Unacceptable staining in the UK NEQAS samples. (A) and (B) show excessive staining, and cytoplasmic staining with morphology damage in the samples 'C' and 'D' respectfully. This was stained using a lab-devised method. The example in (C) should show HER2 3+expression, but the staining is weak and more representative of a 2+.

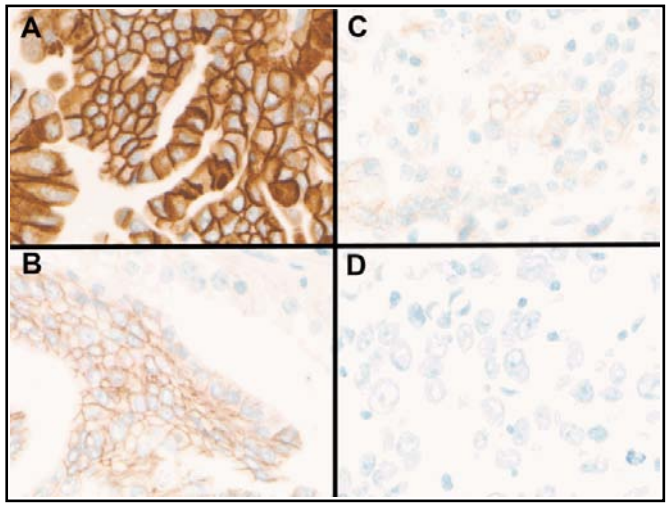
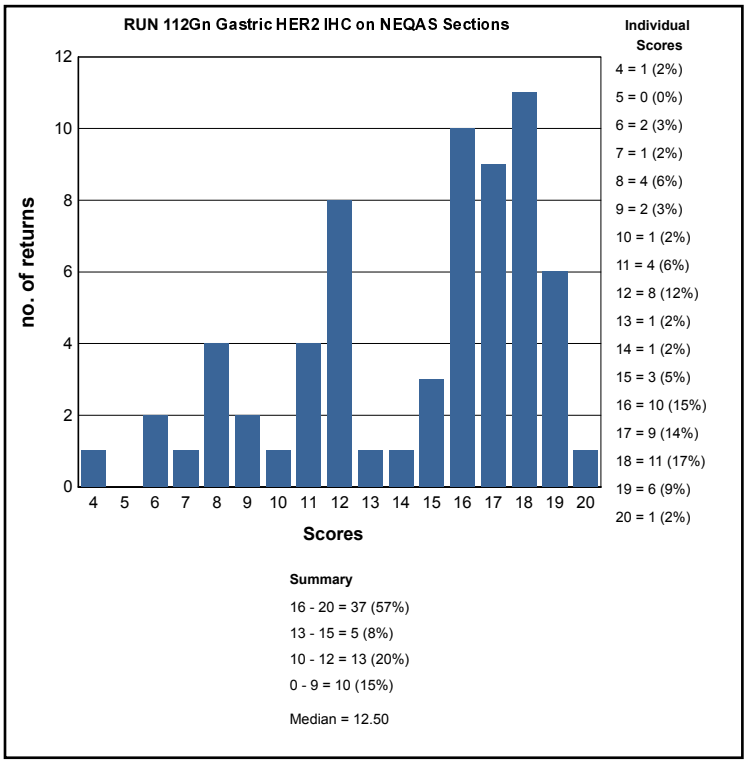
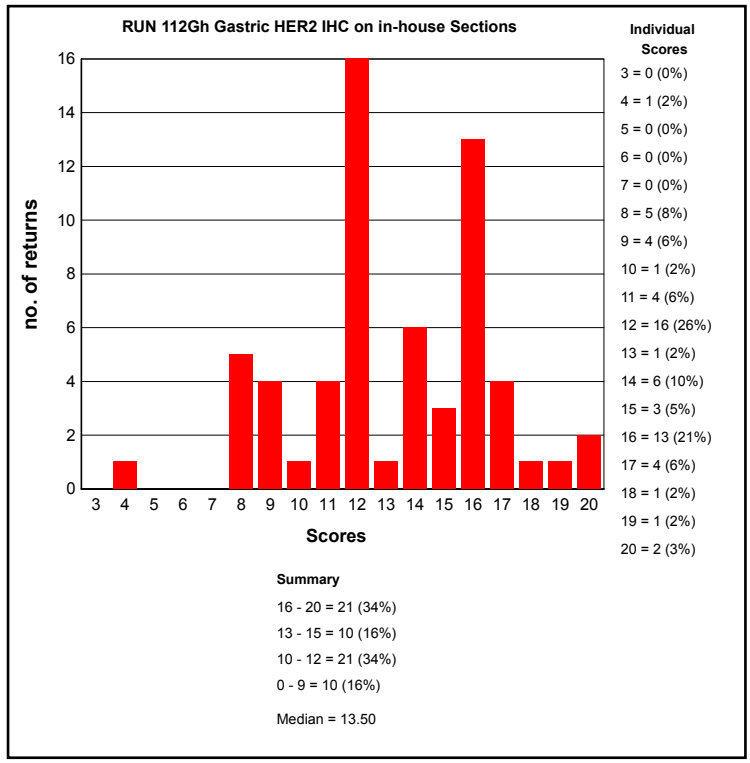


Fig 6. Good example and HER2 staining of an in-house control. The multi-block contains (A) 3+, (B) 2+, (C) 1+ and (D) negative expressing gastric tumours.

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: 112

Primary Antibody	N	%
Dako Link HercepTest SK001 (poly)	6	100
Leica Oracle HER2 Bond IHC (CB11)	1	0
Ventana Confirm 790-4493 (4B5)	15	40
Ventana Confirm 790-4493 (4B5)	1	0
Ventana Confirm 790/800-2996 (4B5)	1	100
Ventana Pathway 790-100 (4B5)	4	75
Ventana Pathway 790-2991 (4B5)	1	0
Ventana Pathway 790-2991 (4B5)	34	76

Gastric HER2 ICC Run: 112

Automation	N	%
Dako Autostainer Link 48	4	100
Dako Autostainer Plus Link	2	100
Leica Bond Max	1	0
Ventana Benchmark GX	3	67
Ventana Benchmark ULTRA	24	71
Ventana Benchmark XT	29	59

Gastric HER2 ICC Run: 112

Heat Mediated Retrieval	N	%
Dako PTLink	5	100
Leica ER1 25 mins	1	0
Ventana CC1 16mins	1	0
Ventana CC1 20mins	1	0
Ventana CC1 24mins	3	33
Ventana CC1 32mins	5	60
Ventana CC1 36mins	10	80
Ventana CC1 40mins	1	100
Ventana CC1 48mins	1	100
Ventana CC1 56mins	1	100
Ventana CC1 64mins	4	100
Ventana CC1 mild	24	63
Ventana CC1 standard	4	25
Ventana CC2 36mins	1	100

Gastric HER2 ICC Run: 112

Detection	N	%
AS PER KIT	6	67
Dako HerCep Test Autor (SK001)	3	100
Leica Bond Polymer Refine (DS9800)	1	100
Ventana iView system (760-091)	3	67
Ventana OptiView Kit (760-700)	4	50
Ventana UltraView Kit (760-500)	45	64

Gastric HER2 ICC Run: 112

Enzyme Retrieval	N	%
AS PER KIT	4	25
NOT APPLICABLE	26	69

Gastric HER2 ICC Run: 112

Chromogen	N	%
AS PER KIT	11	73
DAKO DAB+	1	100
Dako FLEX DAB	2	100
Other	1	100
Ventana DAB	4	75
Ventana iview	1	0
Ventana Ultraview DAB	43	63

BEST METHODS

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

Gastric HER2 IHC - Method 1

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

Primary Antibody:	Ventana Pathway 790-2991 (4B5) Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 36mins
EAR:	
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

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) , 16 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 mild
EAR:	
Chromogen:	Ventana Ultraview DAB, 37 ºC., Time 1: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC Prediluted

Gastric HER2 IHC - Method 3

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

Primary Antibody:	Dako Link HercepTest SK001 (poly)
Automation:	Dako Autostainer Plus Link
Method:	AS PER KIT
Main Buffer:	AS PER KIT
HMAR:	Dako PTLink
EAR:	AS PER KIT
Chromogen:	AS PER KIT
Detection:	AS PER KIT

Lymphoma Module Run 112

David Blythe and Suzanne Parry

	Gold Standard	Second Antibody
Antigens Assessed:	Cyclin D1	CD30
Tissue Sections circulated:	Reactive Tonsil and Mantle Cell Lymphoma	Hodgkin's Lymphoma
Number of Registered Participants:	223	
Number of Participants this Run	207 (93%)	

Introduction

Gold Standard: Cyclin D1

Cyclin D1 is a 36KDa protein encoded by the CCND1 (bcl-1) gene. It is part of the cyclin family, and functions as a regulator of Cyclin-dependent kinases (CDKs) CDK4 and CDK6., whose activity is required for cell cycle G1/S transition. Mutations, amplification and overexpression of the cyclin D1 gene alters cell cycle progression, and are seen frequently in a variety of tumours, including breast carcinomas, head and neck squamous cell carcinoma and oesophageal cancers. Cyclin D1 is also expressed in some lymphoid neoplasms, and immunohistochemical staining of Cyclin D1 is particularly useful to diagnose mantle cell lymphoma (MCL) and to distinguish this from chronic lymphocytic leukaemia, small lymphocytic lymphomas and other non-Hodgkin lymphomas (Chan et al., Swerdlow et al.). This diagnosis is a very important one to make due to the tumour's unresponsiveness to treatment and it's prognosis.

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

- Moderate to strong staining in the squamous epithelium of the tonsil.
- Nuclear staining in the majority of tumour cells of the MCL.
- Characteristically the staining will feature a mixture of strongly, moderately and weakly stained cells.
- Minimal background staining.

Features of Sub-optimal Immunostaining (Figs 3 & 5):

- Very weak staining of the tonsil squamous epithelial cells or in the tumour nuclei of the MCL.
- Uneven staining.
- Diffuse or predominantly cytoplasmic staining.
- Non-specific or background staining.

References:

1. Chan JKC. Expert opinion: Immunostaining for cyclin D1 and the diagnosis of mantle cell lymphoma: is there a reliable method? Histopathol1999; 34:7-20.
2. Swerdlow SH, Williams ME. From centrocytic to mantle cell lymphoma: A clinicopathologic and molecular review or 3 decades. Hum pathol 2002; 33:7-20.

Second Antigen: CD30

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

Features of Optimal Immunostaining (Figs 7 & 11):

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

Features of Sub-optimal Immunostaining (Figs 8, 9 & 10):

- Weak, uneven or negative staining of the HRS.
- Excessive background staining, or non-specific staining of cell types or components not expected to stain.
- Tissue destruction due to harsh heat induced antigen retrieval.

References

1. de Bruin PC, et al. CD30 expression in normal and neoplastic lymphoid tissue: biological aspects and clinical implications (review). Leukaemia 1995;9: 1620-7.

Assessment Summary:

The pass rates for the **Cyclin D1** assessment were slightly higher than the previous run (111), with 77% of labs achieving an acceptable pass, and a further 16% receiving a borderline pass. In parallel, fewer labs failed the assessment: Failure rate of 6% (N=13). The reason for failure for all of these labs was due to very weak staining, which was mostly noticeable on the UK Neqas MCL tissue, but also seen on the Neqas tonsil. The borderline scores were also mostly given to the sections which showed weak staining, but were still considered diagnostic. The Labvision (Thermo Sci) SP4 antibody still remains the most popular choice, and this showed an acceptable pass rate of 72% in the current assessment. The Ventana rabbit monoclonal antibody SP4-R was also very popular, used by 42 participants and showed an acceptable pass rate of 98%. As noted on previous Cyclin D1 assessments runs, the Leica P2D11F11 clone overall showed weaker staining irrespective of which commercial platform or antigen retrieval method used. The participant's in-house controls for Cyclin D1 showed similar pass rates to that of the UK Neqas material, and it was good to see that many labs are now using a multi block of both tonsil and a MCL for their in-house control. Again, very weak staining was the reason for the failed results on the in-house material.

The second chosen antibody for the Run 112 lymphoma assessment was **CD30**. This showed even better pass rates, with 82% of labs achieving an acceptable pass, and a further 6% received a borderline mark. Only 3 labs (1%) failed the assessment, and this was again due to very weak staining on the UK Neqas distributed samples. A range of suppliers, platforms and protocols were used, but the most popular choice of antibody was the Dako BER-H2 clone; used by 107 labs and showed an acceptable pass rate of 96%. Almost all labs submitted a lymph node with Hodgkin's disease for their in-house control material. Few labs submitted tonsil instead. The pass rates for the in-house was similar to that on the Neqas material, with only 6 labs failing the assessment, which was due to weak staining or non-specific staining.

Selected Images showing Optimal and Sub-optimal Immunostaining

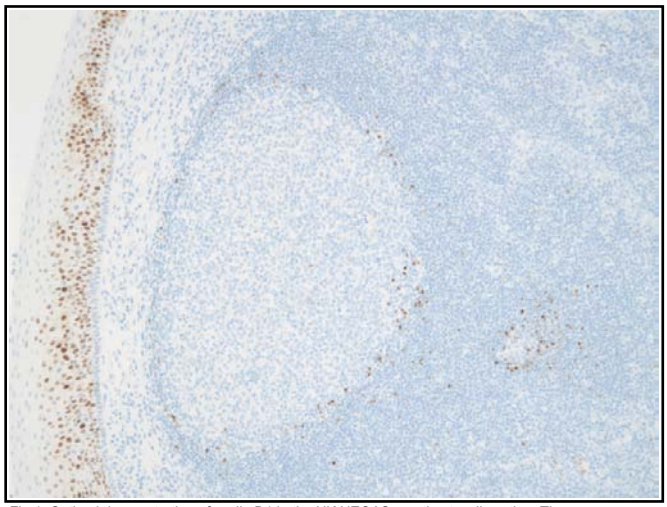


Fig 1. Optimal demonstration of cyclin D1 in the UK NEQAS reactive tonsil section. The squamous epithelial cells show moderate to strong nuclear staining and there are a few endothelial cells staining in the germinal centre. Section stained with the Dako RTU EP12 antibody on the Autostainer with pre-treatment in the PT link.

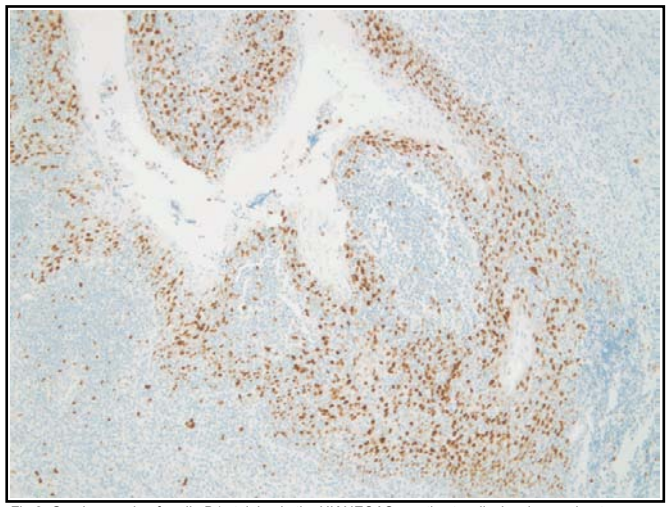


Fig 2. Good example of cyclin D1 staining in the UK NEQAS reactive tonsil, showing moderate to strong nuclear staining in the squamous epithelial cells and a few endothelial cells in the germinal centre. Stained using the LabVision SP4 antibody on the Ventana ULTRA with CC1 antigen retrieval for 48 minutes.

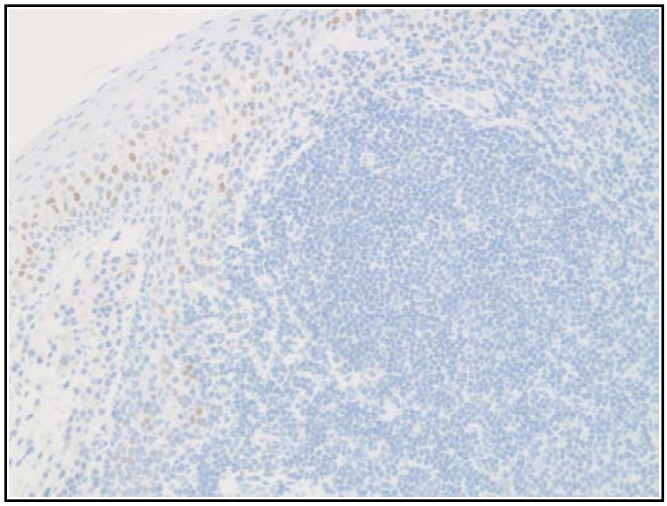


Fig 3. Suboptimal demonstration of cyclin D1 in the UK NEQAS distributed tonsil (compare to Figs 1&2). The staining is weak with fewer cells demonstrated than expected. Stained using the Thermo Scientific DC5-6 antibody (no dilution provided), on the Leica BondMax with ER2 antigen retrieval for 30 minutes.

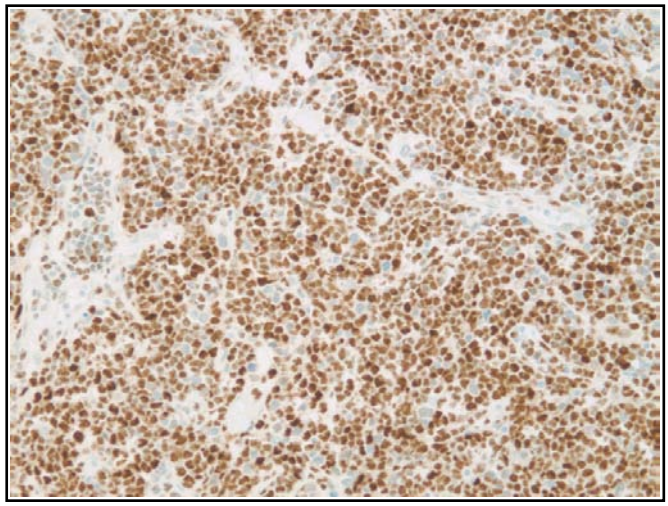


Fig 4. Optimal demonstration of cyclin D1 on the UK NEQAS distributed mantle cell lymphoma (MCL): Virtually all of the tumour cells show strong nuclear staining. (Same protocol as Fig 2).

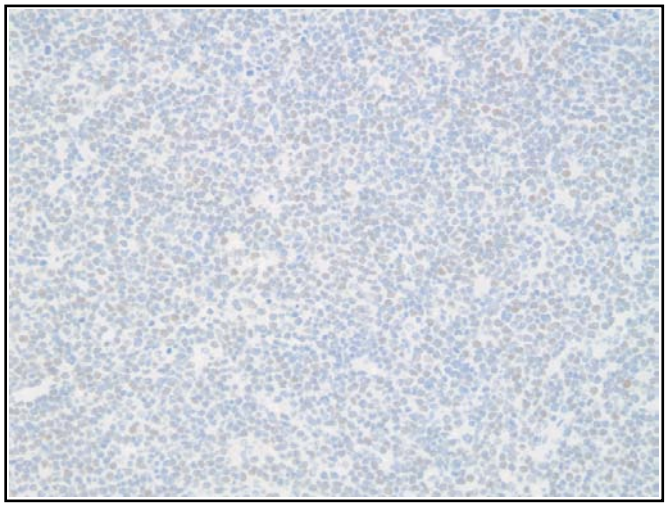


Fig 5. Unacceptable demonstration of cyclin D1 on the UK NEQAS distributed mantle cell lymphoma (compare to Fig 4). Not only is the staining very weak, but many cells expected to be positive are not staining. (Same protocol as Fig 3).

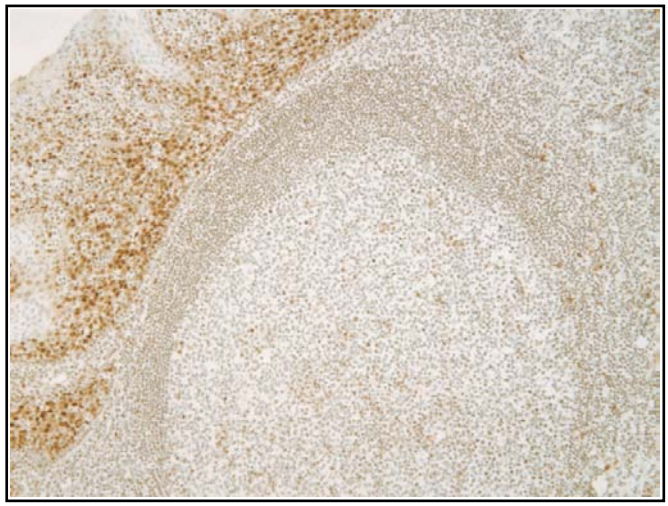


Fig 6. Poor demonstration of cyclin D1 on an in-house tonsil section. The section shows non-specific and background staining. Staining was carried out with the Ventana SP4-R pre-diluted antibody on the Benchmark ULTRA with CC1 retrieval for 36 minutes.

Selected Images showing Optimal and Sub-optimal Immunostaining

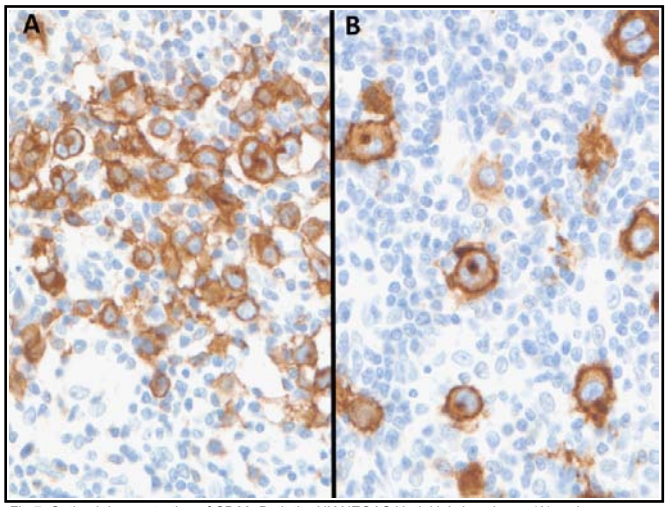


Fig 7. Optimal demonstration of CD30: Both the UK NEQAS Hodgkin's lymphoma (A) and participant's in house Hodgkin's control (B) show strong membrane and paranuclear staining of the Hodgkin's cells. Stained with the Dako Ber-H2 antibody, 1:50, using the Dako PT Link with 20 minutes in low pH buffer on the Autostainer and FLEX detection kit.

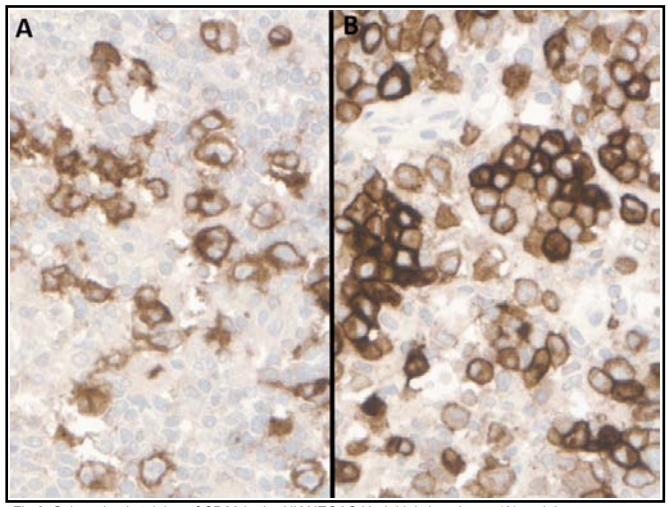


Fig 8. Sub-optimal staining of CD30 in the UK NEQAS Hodgkin's lymphoma (A) and the participant's in house Hodgkin's control (B): Although both sections show the expected staining of Hodgkin's cells, there is also non-specific background staining. Stained with the Dako Ber-H2 antibody (no dilution provided), on the Leica Bond III, and antigen retrieval with ER1

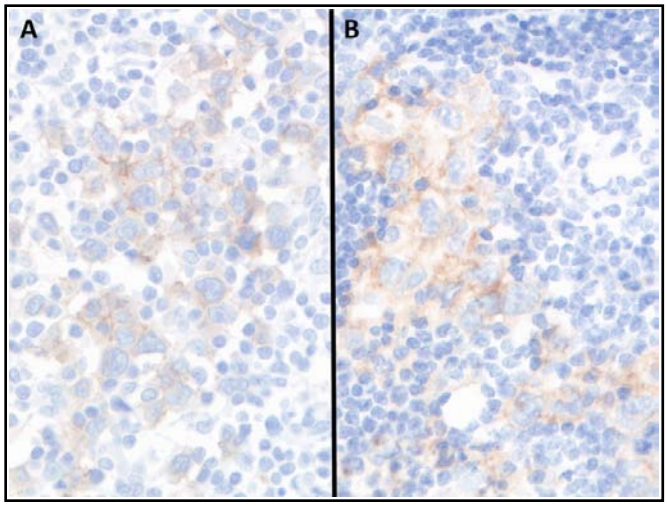


Fig 9. Poor demonstration of CD30 in the UK NEQAS Hodgkin's lymphoma (A) and the participant's in house Hodgkin's control (B): Both sections show weak and diffuse staining. Sections stained with the Ventana BER-H2 pre-diluted antibody, on the Benchmark XT with 56 minutes of antigen retrieval in CC1 buffer.

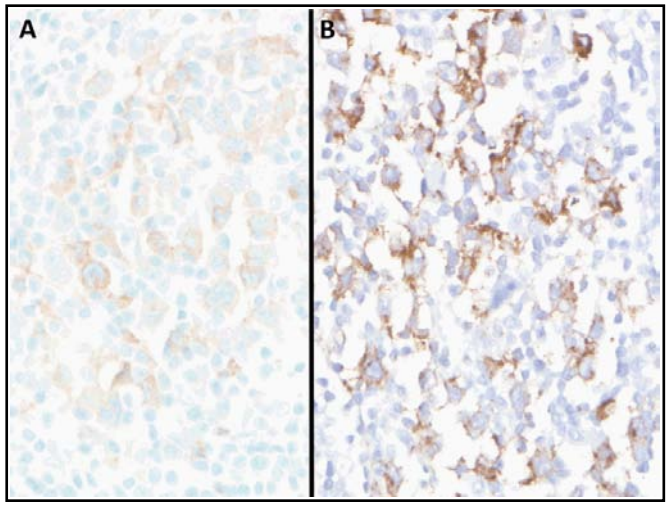


Fig 10. Two examples of poor staining on the UK NEQAS Hodgkin's lymphoma section: (A) shows weak staining, while (B) appears to be over pre- treated. (A) stained with the Dako Ber-H2 antibody, 1:400, on the Ventana XT, CC1 44 minutes. (B) stained with the Ventana Ber-H2 prediluted antibody on the Ventana ULTRA, CC1 for 32 minutes and Optiview detection

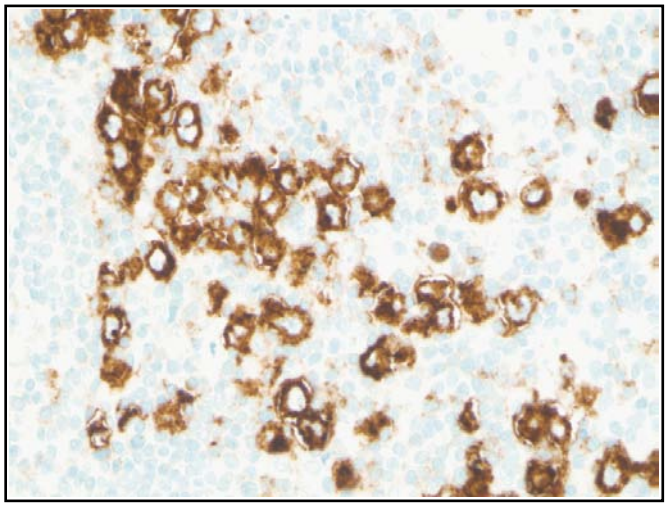


Fig 11. Acceptable level of CD30 staining in the UK NEQAS distributed tumour section. The staining is strong and clearly demonstrates the Hodgkin's cells, however, there is a slight granular pattern. Stained with the Ventana prediluted antibody on the Benchmark ULTRA, with CC1 pre-treatment for 64 minutes.

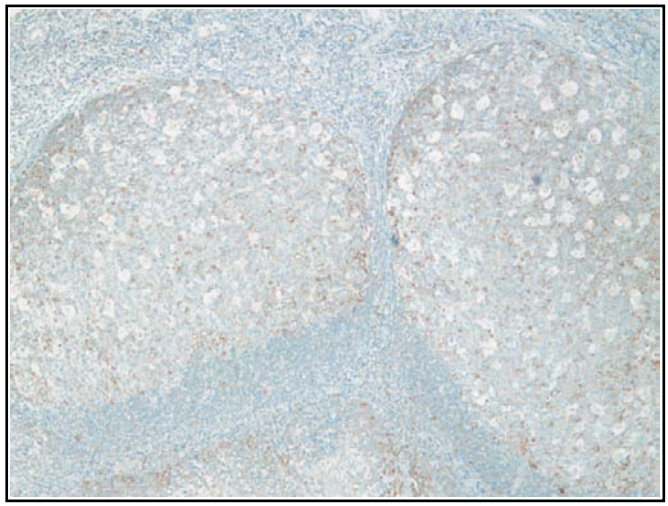
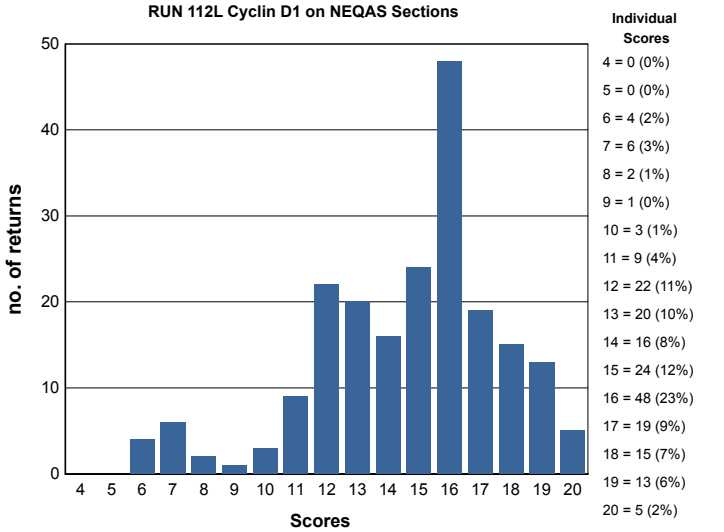
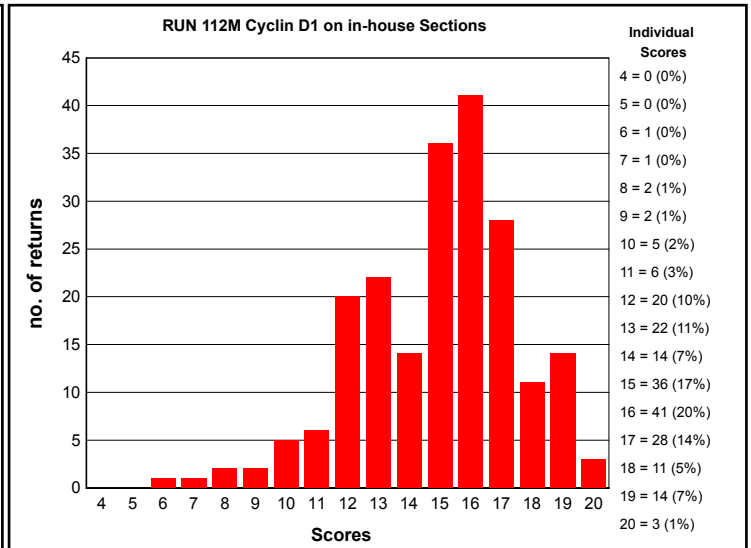
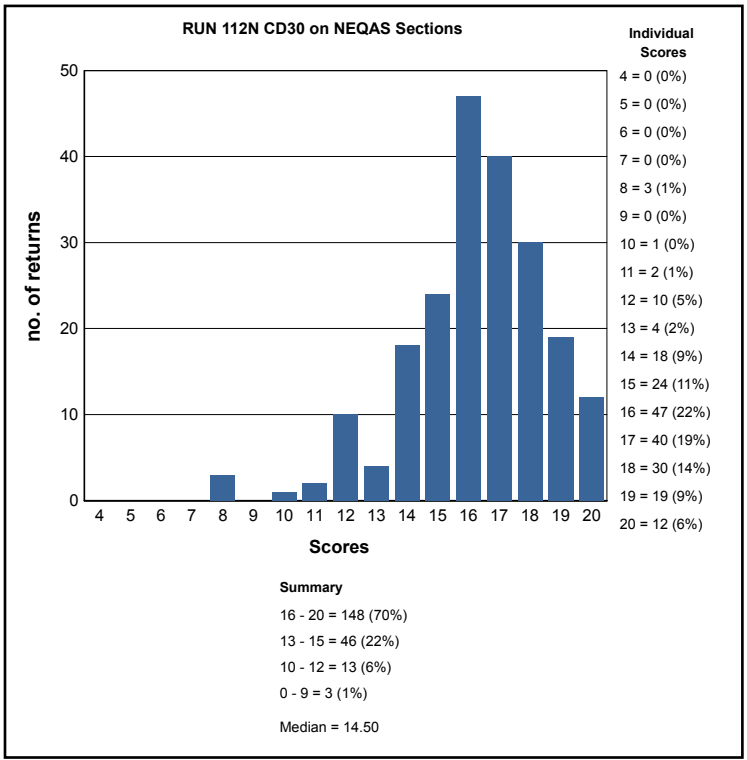


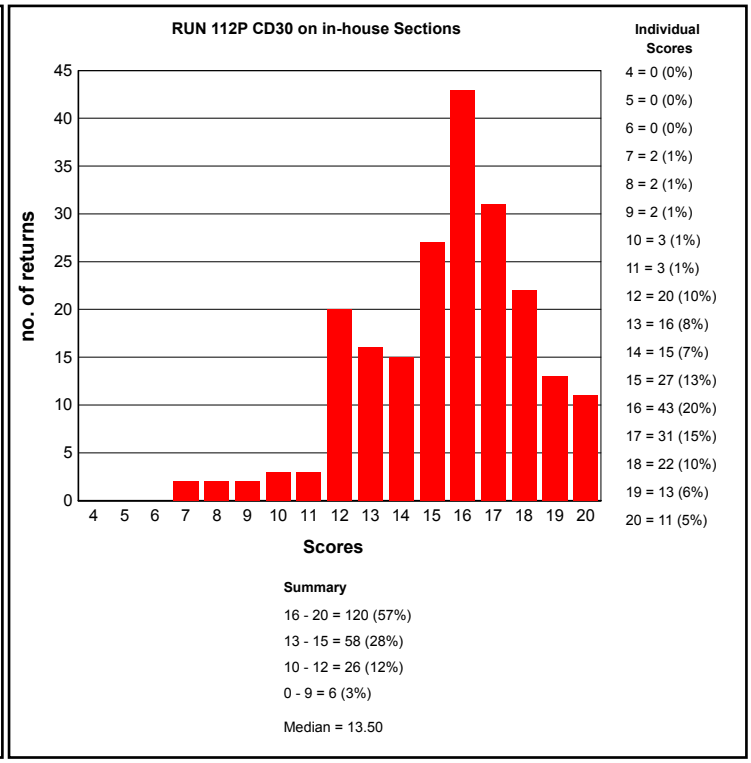
Fig 12. Unacceptable non-specific staining of lymphocytes in the germinal centres of an in house tonsil control. This is most likely caused by the antibody being used at too high a concentration. The Dako Ber-H2 antibody was used at 1:10 dilution on the Ventana Benchmark XT, CC1 mild pre-treatment.

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: 112

Primary Antibody : Cyclin D1

Antibody Details	N	%
AbCam SP4 16663	2	0
Cell Marque (RBT14) CRC011	1	100
Cell Marque (SP4) 241-R	9	67
Dako (DCS-6) M7155	1	0
Dako (EP12) M3642	21	86
Dako (SP4) M3635	2	50
Dako FLEX RTU (SP4) IR152	2	100
Dako RTU (EP12) IR083/IS083	16	94
Lab Vision/Thermo Sci MS 210P (DCS-6)	1	0
LabVision/Thermo Sci (SP4) RM-9104	60	72
Leica/Novo (P2D11F11) NCL-Cyclin D1-GM-CE	8	38
Leica/Novo RTU (P2D11F11) NCL-RTU-CyclinD1	1	0
Menapath (SP4) MP-307	2	100
NeoMarkers/Thermo Sci (EPR2241IHC) RM-2113	8	75
Neomarkers/Thermo Sci MS 210 PO (DC5-6)	2	0
Other	18	67
Vector rbm VP-RM03 (SP4)	2	100
Ventana (SP4-R) 790-4508	42	98
Ventana rbm 760-4282 (SP4)	5	100

Lymphoma Run: 112

Primary Antibody : CD30

Antibody Details	N	%
Cell Marque 130M (Ber-H2)	8	88
Dako IR/IS602 (Ber-H2)	16	94
Dako M0751 (Ber-H2)	90	94
Dako N1558 (Ber-H2)	1	100
labvn/Thermo MS-361-S (BER-H2)	1	100
Leica/Novocastra Bond RTU PA0153 (1G12)	4	100
Leica/Novocastra Bond RTU PA0790 (JCM182)	17	100
Leica/Novocastra NCL-CD30 (1G12)	4	75
Leica/Novocastra NCL-CD30-365 (15B3)	3	100
Leica/Novocastra NCL-L-CD30 (1G12)	6	67
Leica/Novocastra NCL-L-CD30-591 (JCM182)	9	100
Leica/Novocastra RTU-CD30 (1G12)	6	83
Other	16	94
Ventana 790 2926 (Ber-H2)	23	87

Lymphoma Run: 112

Heat Mediated Retrieval	CD30 N	CD30 %	Cyclin D1 N	Cyclin D1 %
_Leica Bond III ER1	1	100	0	0
_Leica BondMax ER1	1	0	0	0
_Leica BondMax ER2	4	75	0	0
_Ventana Benk CC1 (Mild)	1	100	0	0
_Ventana Benk XT CC1 (Mild)	1	100	0	0
_Ventana Benk XT CC1 (Standard)	1	100	0	0
Biocare Decloaking Chamber	1	100	0	0
Dako Omnis	4	100	5	80
Dako Pascal	1	100	1	100
Dako PTLink	27	100	23	100
Lab vision PT Module	2	100	2	50
Leica ER1 10 mins	1	0	0	0
Leica ER1 20 mins	12	100	0	0
Leica ER1 30 mins	6	83	2	0
Leica ER1 40 mins	0	0	1	0
Leica ER2 10 mins	3	100	1	0
Leica ER2 20 mins	23	96	29	59
Leica ER2 30 mins	12	92	26	46
Leica ER2 40 mins	4	100	10	50
Microwave	2	100	2	50
None	0	0	1	100
Pressure Cooker	1	100	2	100
Pressure Cooker in Microwave Oven	0	0	1	0
Steamer	0	0	1	0
Ventana CC1 16mins	2	100	0	0
Ventana CC1 20mins	0	0	1	100
Ventana CC1 24mins	1	0	2	100
Ventana CC1 32mins	8	88	12	100
Ventana CC1 36mins	1	100	1	100
Ventana CC1 40mins	0	0	6	100
Ventana CC1 44mins	2	50	0	0
Ventana CC1 48mins	4	100	4	100
Ventana CC1 52mins	1	100	0	0
Ventana CC1 56mins	4	75	4	100
Ventana CC1 64mins	23	96	23	96
Ventana CC1 76mins	2	50	2	100
Ventana CC1 80mins	2	100	0	0
Ventana CC1 88mins	1	100	0	0
Ventana CC1 92mins	3	100	2	50
Ventana CC1 extended	3	33	6	83
Ventana CC1 mild	6	100	9	67
Ventana CC1 standard	17	88	22	95
Ventana CC2 32mins	1	100	0	0
Ventana CC2 56mins	1	100	0	0
Ventana CC2 64mins	0	0	1	100
Ventana CC2 92mins	0	0	1	100
Water bath 95-98 OC	0	0	1	100

Lymphoma Run: 112

Enzyme Mediated Retrieval	CD30 N	CD30 %	Cyclin D1 N	Cyclin D1 %
AS PER KIT	5	100	1	100
NOT APPLICABLE	99	91	104	73
Ventana Protease	0	0	1	100

Lymphoma Run: 112	CD30		Cyclin D1	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	11	100	13	85
BioGenex SS Polymer (QD 420-YIKE)	0	0	1	100
Dako EnVision FLEX (K8000/10)	3	100	5	80
Dako EnVision FLEX+ (K8002/12)	21	100	14	100
Dako Envision HRP/DAB (K5007)	2	100	3	67
Dako Envision+ HRP mouse K4004/5/6/7	2	100	0	0
Dako Envision+ HRP rabbit K4008/9/10/11	0	0	2	100
Dako rb-a-mo Ig (E0354)	1	100	0	0
LabVision UltraVision ONE Polymer (TL-012/5-HDJ/T	1	100	0	0
Leica Bond Polymer Define (DS9713)	0	0	1	0
Leica Bond Polymer Refine (DS9800)	72	90	62	48
MenaPath X-Cell Plus (MP-XCP)	1	100	0	0
None	1	100	1	100
NOT APPLICABLE	0	0	2	50
Other	5	100	8	63
Ventana iView system (760-091)	4	75	4	100
Ventana OptiView (760-700) + Amp. (7/860-099)	3	100	1	100
Ventana OptiView Kit (760-700)	31	94	38	100
Ventana UltraView Kit (760-500)	45	87	50	86

Lymphoma Run: 112	CD30		Cyclin D1	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	0	0	1	100
Dako Autostainer Link 48	24	100	21	100
Dako Autostainer plus	2	100	2	50
Dako Autostainer Plus Link	4	100	3	100
Dako Omnis	4	100	5	80
LabVision Autostainer	3	67	3	33
Leica Bond Max	38	84	29	41
Leica Bond-III	39	97	41	54
Menarini - Intellipath FLX	1	100	0	0
None (Manual)	3	100	2	100
Shandon Sequenza	1	100	1	0
Ventana Benchmark GX	2	100	2	100
Ventana Benchmark ULTRA	56	95	63	94
Ventana Benchmark XT	29	79	33	88

Lymphoma Run: 112	CD30		Cyclin D1	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	26	88	22	86
BioGenex Liquid DAB (HK153-5K)	0	0	1	100
DAKO DAB+	2	100	2	100
Dako EnVision Plus kits	1	100	4	100
Dako FLEX DAB	21	100	18	94
Dako REAL EnVision K5007 DAB	2	100	3	67
LabVision DAB	1	100	0	0
Leica Bond Polymer Refine kit (DS9800)	67	94	61	46
menapath xcell kit DAB (MP-860)	1	100	0	0
NOT APPLICABLE	1	100	3	67
Other	13	92	15	80
Sigma DAB (D4168)	1	100	0	0
Sigma DAB (D5905)	0	0	1	100
Ventana DAB	15	87	22	91
Ventana iview	4	75	1	100
Ventana Ultraview DAB	51	88	54	91

BEST METHODS - Gold Standard Antibody

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

Cyclin D1 - Method 1

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

Primary Antibody: Dako RTU (EP12) IR083/IS083 , 20 Mins, 20 ºC Prediluted
Automation: Dako Autostainer Link 48
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako PTLink
EAR: NOT APPLICABLE
Chromogen: Dako EnVision Plus kits
Detection: AS PER KIT

Cyclin D1 - Method 2

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

Primary Antibody: LabVision/Thermo Sci (SP4) RM-9104 , 30 Mins, 20 ºC Dilution 1: 50
Automation: Dako Autostainer Plus Link
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

Cyclin D1 - Method 3

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

Primary Antibody:	Ventana (SP4-R) 790-4508 , 16 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 40mins
EAR:	
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700)

Cyclin D1 - Method 4

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

Primary Antibody:	LabVision/Thermo Sci (SP4) RM-9104 , 40 Mins, 36 ºC
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 48mins
EAR:	Ventana Protease
Chromogen:	Ventana DAB
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099) , 4 Mins

BEST METHODS - Secondary Antibody

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

CD30 - Method 1

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

Primary Antibody:	Leica/Novocastra NCL-CD30-365 (15B3) Dilution 1: 40
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

CD30 - Method 2

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

Primary Antibody:	Leica/Novocastra NCL-L-CD30 (1G12) , 32 Mins, RT ºC Dilution 1: 30
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins, Buffer: Ventana reaction buffer
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB, RT ºC., Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, RT ºC

CD30 - Method 3

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

Primary Antibody:	Dako M0751 (Ber-H2) , 40 Mins, 21 ºC Dilution 1: 50
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: HIGH PH TARGET RETRIEVAL SOLUTION, PH: 9
EAR:	
Chromogen:	Dako FLEX DAB, 21 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 15 Mins, 21 ºC Prediluted

CD30 - Method 4

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

Primary Antibody:	Cell Marque 130M (Ber-H2) , 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, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT, 37 ºC., Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 37 ºC Prediluted

Neil Bilbe

	Gold Standard	Second Antibody
Antigens Assessed:	Synaptophysin	CK
Tissue Sections circulated:	GBM/Oligo and cerebellum	Metastatic breast carcinoma
Number of Registered Participants:	64	
Number of Participants this Run	60 (94%)	

Introduction

Gold Standard: Synaptophysin

Synaptophysin is a 38kDa integral synaptic vesicle glycoprotein with four transmembrane domains. It is present in neuroendocrine cells and in virtually all neurons in the brain and spinal cord involved in synaptic transmission. Synaptophysin is also detected in the epithelium of the choroid plexus, adrenal cortical cells, goblet cells and Paneth cells (Wiedenmann et al). The antibody is a useful marker of a wide spectrum of neuroendocrine tumours, including neuroblastomas, ganglio-neuroblastomas, ganglioneuromas, gangliogliomas, pheochromocytomas, and paragangliomas (Gould et al). Neuroendocrine neoplasms of epithelial type are also detected, including pancreatic islet-cell neoplasms, medullary thyroid carcinomas, pituitary and parathyroid adenomas, bronchopulmonary and gastrointestinal tract carcinoids. Synatposhysin also stains positive for adrenal cortical adenomas.

Features of Optimal Immunostaining:

- Intense, punctate staining around the neuron perikarya.
- Intense staining in normal cerebellum.
- Clean background.

Features of Sub-optimal Immunostaining:

- Weak or uneven staining.
- Poor localisation of antigen
- Excessive background staining, or non-specific staining of cell types or components not expected to stain.
- Tissue destruction due to harsh heat induced antigen retrieval.

References:

1. Wiedenmann B, et al. Synaptophysin: A marker protein for neuroendocrine cells and neoplasms. Proc Natl Acad Sci 1986; 83:3500-4.
2. Gould VE et al. Synaptophysin expression in neuroendocrine neoplasms as determined by immunocytochemistry. Am J Pathol 1987; 126:243-57.
3. Kwon SE, Chapman ER. Synaptophysin regulates the kinetics of synaptic vesicle endocytosis in central neurons. Neuron 2011:70:847-85.

Second Antigen: Cytokeratin

Cytokeratins are a family of water-soluble proteins with molecular weights between 40-70 kD that form the cytoskeleton of epithelial cells. At least 19 different cytokeratins have been identified and can be divided into two subfamilies. Subfamily A comprises relatively acidic cytokeratins with a pH under 5.5 whereas members of subfamily B have a relatively basic pH of 6 or over.

Positive staining occurs in the cytoplasm of squamous and columnar epithelium of the cervix, colon, oesophagus, skin, small intestine, stomach and tonsil. Other tissues that stained included glandular tissue (mammary, parathyroid, prostate sweat and thyroid), astrocyte, white matter of the cerebellum, glial filaments of the cerebrum, distal tubule and Bowman's capsule of the kidney, bile duct, pneumocytes, bronchi, mesothelium, interlobular duct of the pancreas, anterior pituitary cell, interlobular duct and acinar cells of the salivary gland, reticular cells and Hassall's bodies of the thymus, and endometrium and smooth muscle of the uterus. Negative staining was noted for adrenal, bone marrow, heart, pericardium, peripheral nerve, skeletal muscle, spleen and testis.

All epithelial neoplasms stain positively, transitional cell carcinomas are weakly stained. It is also used for the differential diagnosis of diffuse malignant mesothelioma of the sarcomatoid (spindle-cell) type cases, from other types of spindle cell neoplasms. Cytokeratins are usually negative for melanoma, lymphoma, neurofibroma, and sarcoma.

Features of Optimal Immunostaining

- Specific staining in the tumour cells, and other epithelia
- Clean background with no non-specific staining
- Good contrast with counterstain

Features of Suboptimal Immunostaining

- Weak or uneven staining of the metastatic tumour
- Diffuse, poorly-localised staining.
- Excessive background or non-specific specific staining
- Weak or overtly heavy counterstain

References:

1. Chu P, Wu E, Weiss LM. Cytokeratin 7 and cytokeratin 20 expression in epithelial neoplasms: A survey of 435 cases. Mod Pathol. 2000;13:962–72
2. R Moll et al. Cytokeratin 20 in human carcinomas. A new histodiagnostic marker detected by monoclonal antibodies. Am J Pathol 1992; 140:427-47.
3. R Moll. Cytokeratins in the histological diagnosis of malignant tumours. Int J Biol Markers. 1994; 9(2): 63-9.
4. A Kende et al. Expression of cytokeratins 7 and 20 in carcinomas of the gastrointestinal tract. Histopathology 2003; 42 (2):137-140.
5. Tseng SCG, Jarvinen MJ, Nelson WG, Huang JW, Woodcock-Mitchell J, Sun TTI. Correlation of specific keratins with different types of epithelial differentiation: Monoclonal antibody studies. Cell 1982;30(2):361-72
6. Eichner R, Bonitz P, Sun T-T. Classification of epidermal keratins according to their immunoreactivity, isoelectric point and mode of expression. J Cell Biol 984;98(4):1388-96

Assessment Summary:

There were four non-submissions (6%) out of the 64 registered labs. One participant did not return any of the two in-house controls (**H & K**) leaving a total of 238 sections for assessment. The majority of participants had placed their in-house control on the NEQAS slide as required, but for the Synaptophysin (3) and the Cytokeratin (2) these were sent in separately (4%), primarily due to section adhesion. There also appeared to be less evidence of section lifting, which was a problem when the single slide assessment on HistoBond® slides was introduced (runs 110 and 111).

The overall quality was excellent, with only 3 (1%) slides (all Synaptophysin) failing the assessment, 13 were assessed as borderline (5%) and the remaining 222 (93%), scoring ≥ 13/20. Of these 222 slides, twenty scored a maximum 20/20 (8%): **G**=3, **H**=2, **J**=8, and **K**=7.

Of the 16 sub-optimal sections, 14 were stained with Synaptophysin, with two in-house Cytokeratin (**K**) sections also being assessed as borderline. This was reflected in the average scores of **15.5** for Synaptophysin (**G** & **H**) and **17.1** for Cytokeratin (**J** & **K**). Two of the three failed Synaptophysin sections were the NEQAS (**G**) and in-house (**H**) slides from the same laboratory (no methodology received) and the other was on a NEQAS section that employed a Ventana Confirm RTU antibody, SP11 clone, with CC1 antigen retrieval, on a Benchmark ULTRA. All three sections though had weak staining. No protocols were received for either of the two labs who scored borderline for their in-house Cytokeratin (**K**) sections.

Noticeable features of the assessment were the low intensity of Synaptophysin staining in many of the cerebellar samples in the Gold slides. (**G**); whereas the main feature of the Cytokeratin assessments was the performance of the clones MNF116 and AE1/AE3. It was noticeable that overall the MNF116 clone produced a much cleaner result than the AE1/AE3 stained slides (see figs 7-11).

Summary Table:

	Pass	Bord	Fail	No of slides		Pass	Bord	Fail
G/Synap	48	10	2	60	G/Synap	80%	17%	3%
H/Synap	57	1	1	59	H/Synap	97%	2%	2%
J/CK	60	0	0	60	J/CK	100%	0%	0%
K/CK	57	2	0	59	K/CK	97%	3%	0%
Total	222	13	3	238	Average	93%	5%	1%

Selected Images showing Optimal and Sub-optimal Immunostaining

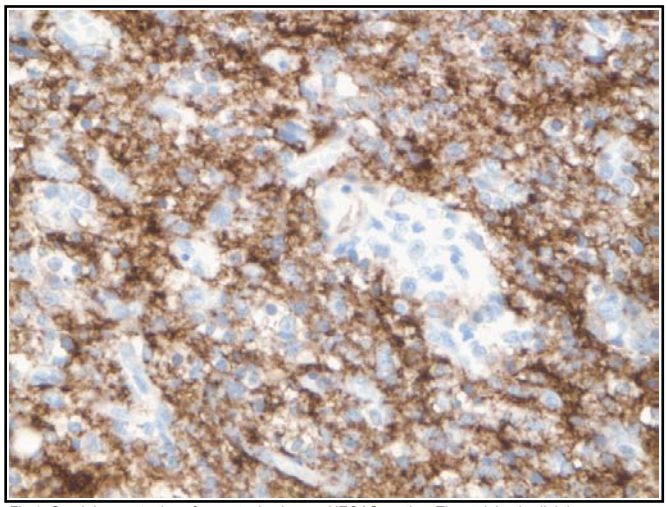


Fig 1. Good demonstration of synaptophysin on a NEQAS section. The staining is slightly weak, and there is a hint of background, but otherwise adequate. Novocastra NCL-L-SYNAP-299 (27G12), no dilution or times given. Leica ER1 20 mins, Leica Bond-III, and a Leica Bond Polymer Refine kit.

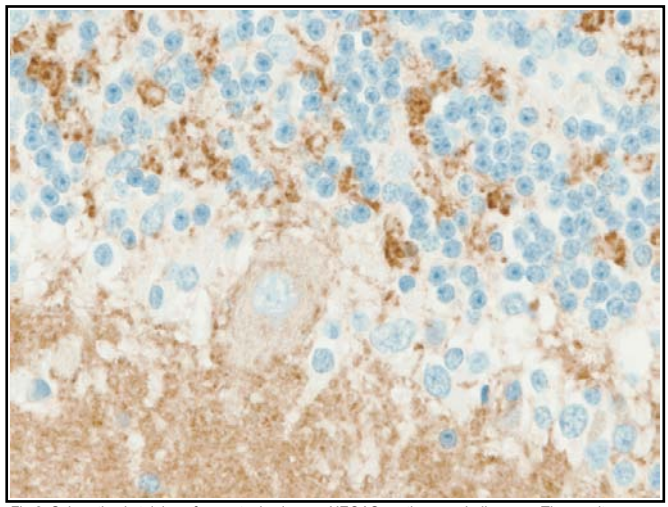


Fig 2. Sub-optimal staining of synaptophysin on a NEQAS section, cerebellar area. The result is weak, with a borderline assessment outcome. Ventana CONFIRM 790-4407 (SP-11), Ventana CC1 52 mins, Ventana Benchmark ULTRA, with the Ventana UltraView Kit (760-500).

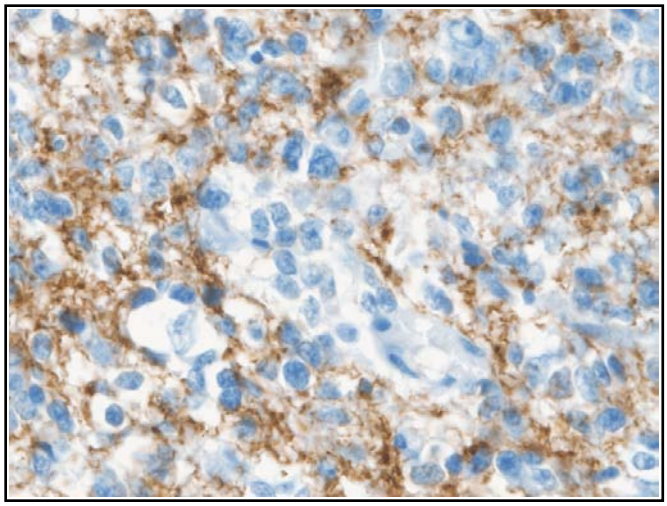


Fig 3. Sub-optimal staining of synaptophysin. The tumour in the NEQAS section is weakly demonstrated (compare fig 5) and the counterstain slightly heavy, although still adequate for diagnostic purposes. Novocastra NCL-SYNAP-299, 1:50, 32 mins, with the Ventana CC1 standard, on a Ventana Benchmark XT and the Ventana UltraView Kit.

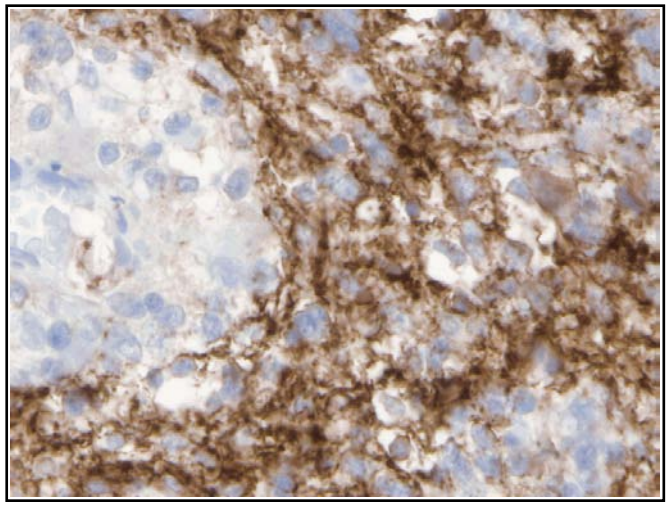


Fig 4. Good synaptophysin demonstration of the tumour in the NEQAS section, but the background is just a little dirty for it to be assessed as optimal. Dako monoclonal, 1:100, 15 mins, with Leica ER2 20 mins, on a Leica Bond Max, and the Leica Bond Polymer Refine kit.

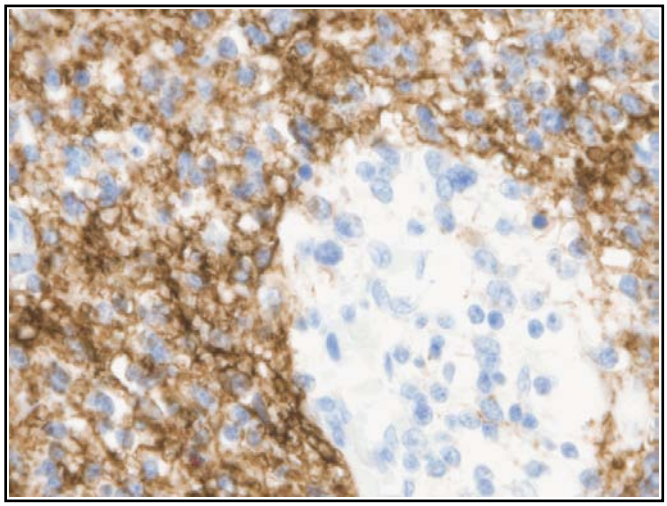


Fig 5. Nice synaptophysin demonstration in the NEQAS section. All of the tumour is demonstrated, there is no background staining, and the counterstain intensity is ideal. Dako monoclonal, 1:100, 15 mins, with Leica ER1 20 mins, on the Leica Bond-III, using a RTU Bond Polymer Refine kit for 8 mins.

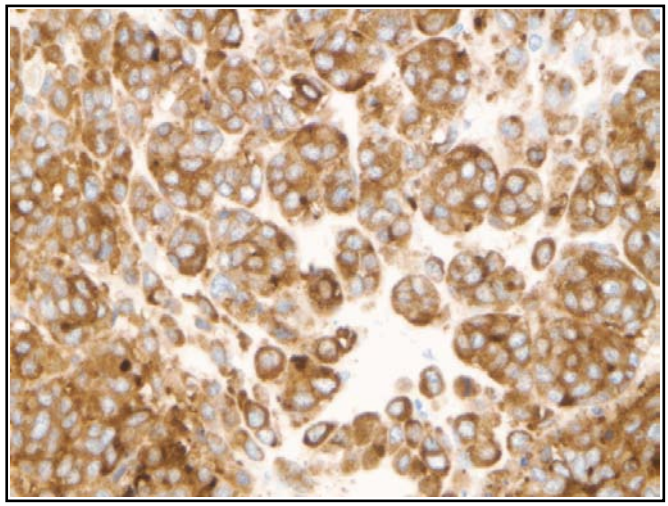


Fig 6. Excellent synaptophysin staining on an in-house section from a case of metastatic neuroendocrine tumour in the ovary. Rosettes of tumour are nicely demonstrated against a clean background. Novocastra NCL-L-SYNAP-299, 1:10, 15 mins, with Leica ER1 20 mins, on a Leica Bond-III, with a Leica Bond Polymer Refine kit for 8 mins.

Selected Images showing Optimal and Sub-optimal Immunostaining

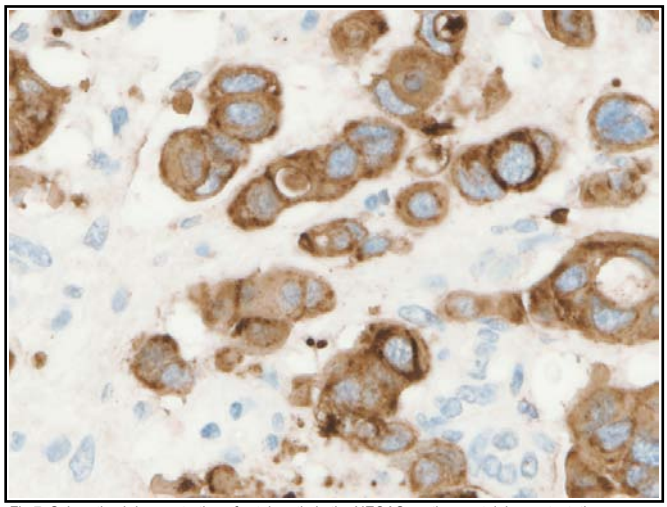


Fig 7. Sub-optimal demonstration of cytokeratin in the NEQAS section containing metastatic breast disease. Staining is weak, and there is some background. The slide was still more than adequate for diagnosis. Dako Clone AE1/AE3, with Leica ER1 30 mins, on a Leica Bond-III, and a Leica Bond Polymer Refine kit.

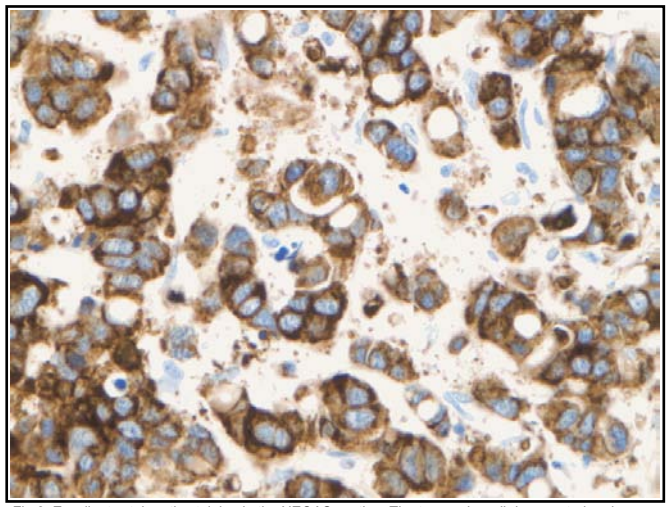


Fig 8. Excellent cytokeratin staining in the NEQAS section. The tumour is well demarcated and the background clean (compare fig 10). Dako Clone MNF116, 1:500, with Leica ER1 10 mins, on a Shandon Sequenza, Leica Bond Polymer Refine.

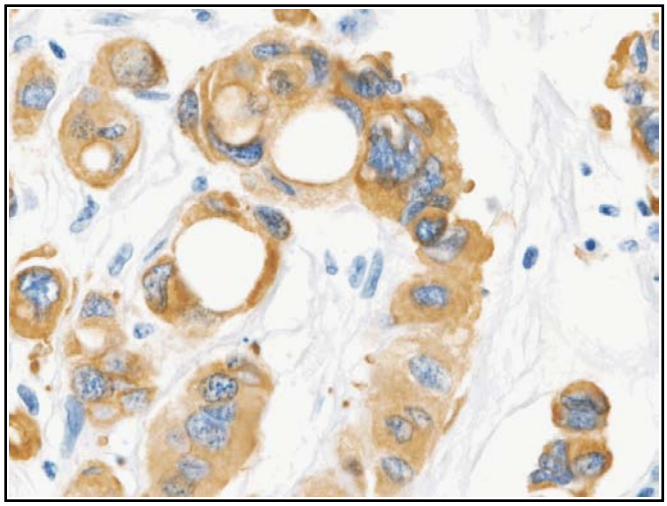


Fig 9. Sub-optimal cytokeratin staining on the NEQAS section. Although demonstrated, the tumour is only weakly positive. The assessors considered the intensity of staining just adequate for diagnostic purposes. Novocastra NCL-AE1/AE3, 1:300, 28 mins, no retrieval given, on a Ventana Benchmark XT,with a Ventana UltraView Kit.

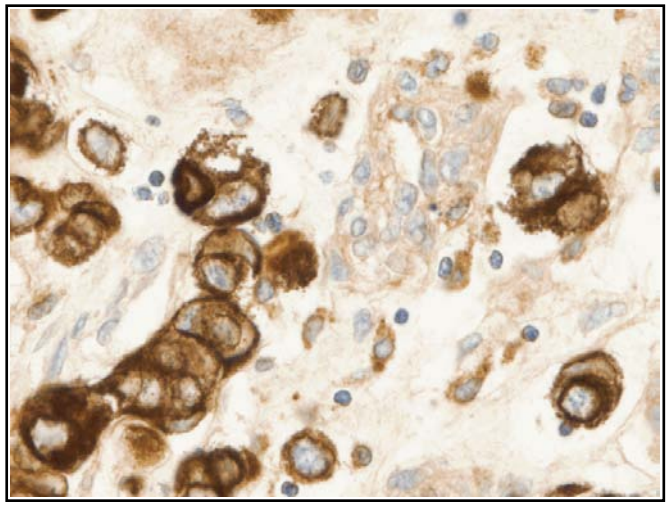


Fig 10. Sub-optimal cytokeratin staining on the NEQAS tissue. The slide is dirty, and there is evidence of morphological damage to the tumour cells. This was assessed as a low pass. RTU Leica AE1/AE3, with no retrieval, on a Leica Bond Max, and with the Leica Bond Polymer Refine kit.

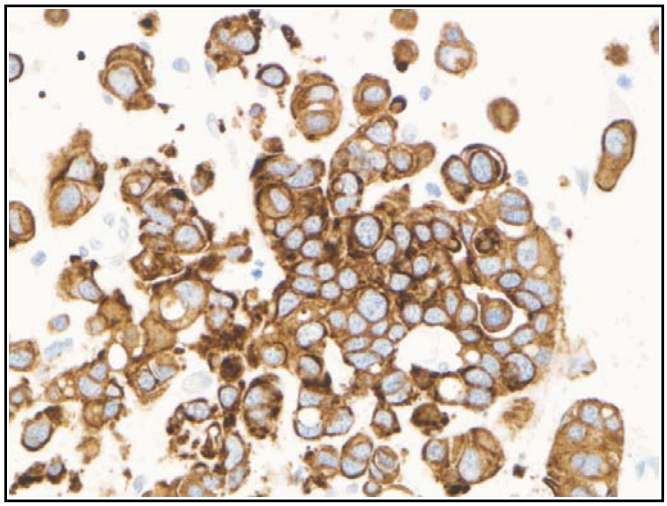


Fig 11. Optimal demonstration of cytokeratin on the NEQAS slide. The tumour is nicely and consistently stained against a clear background. Dako MNF116, 1:400, with Leica ER2 20 mins, on the Leica Bond-III, using the RTU Leica Bond Polymer Refine kit for 8 mins.

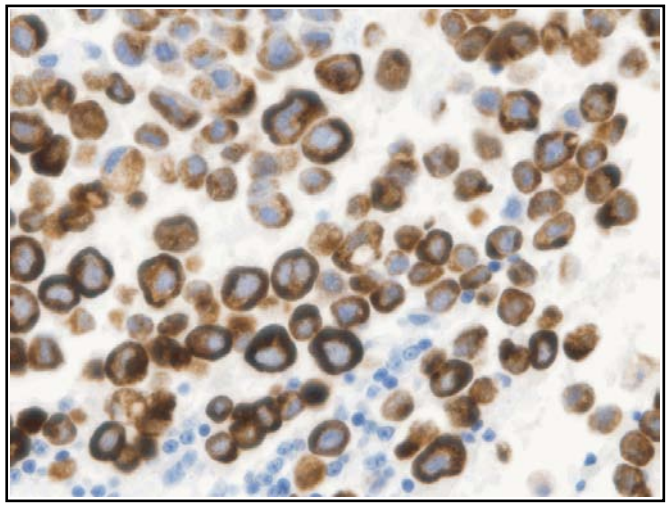
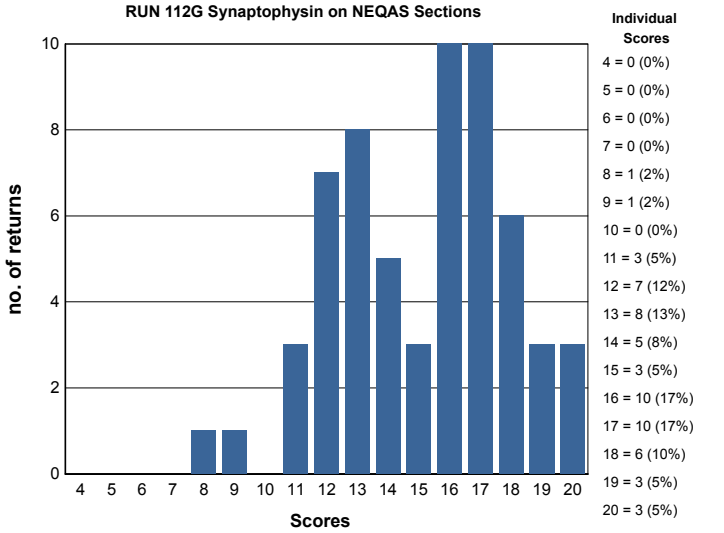
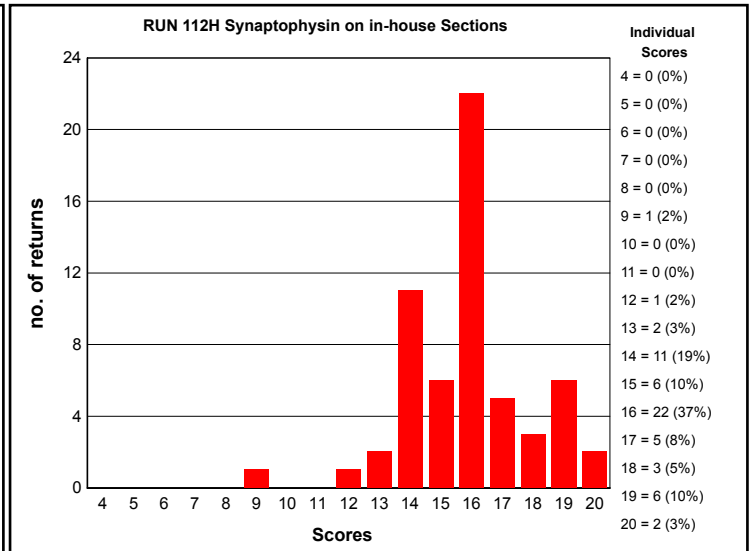
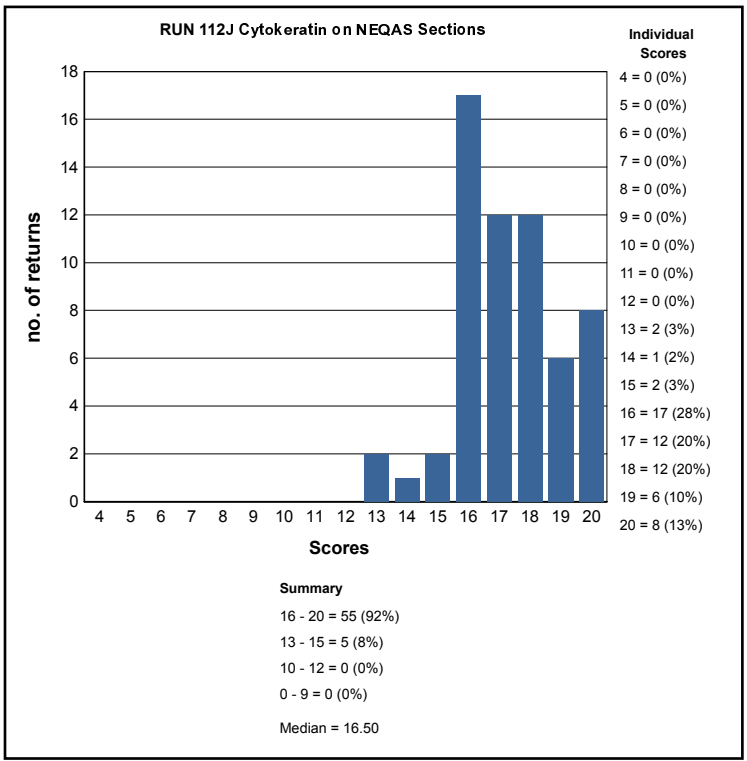


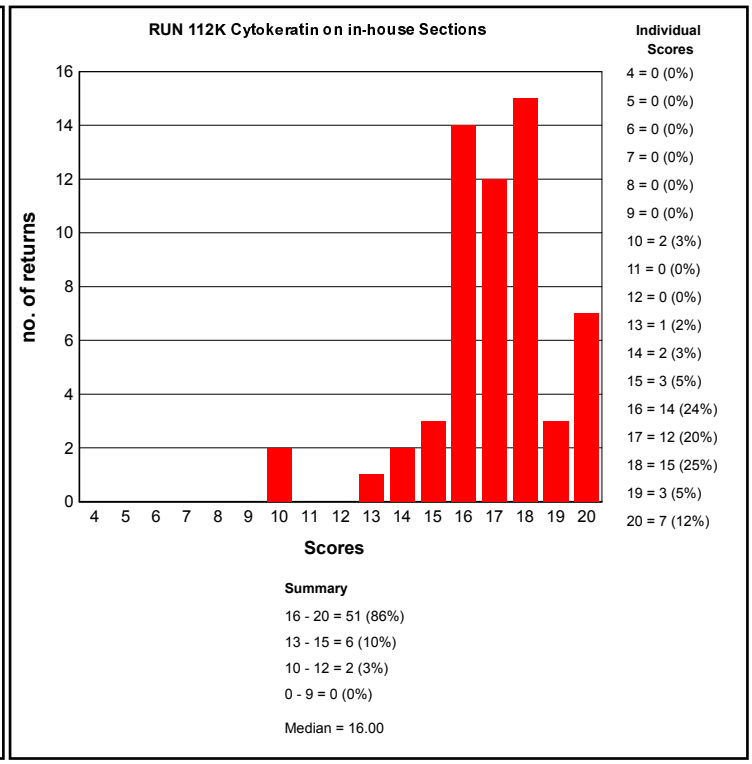
Fig 12. Excellent example of cytokeratin staining on an in-house section of metastatic breast carcinoma. Tumour cells are beautifully demonstrated, and the counterstain is of ideal intensity. Dako MNF116, 1:200, 15 mins, no retrieval, on a Leica Bond-III, and a Leica Bond Polymer Refine kit.

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: 112

Primary Antibody : Synaptophysin

Antibody Details	N	%
BioGenex AM 6 (clone SNP)	2	100
Biogenex MU 363 UC	1	100
DAKO FLEX IR776 (SY38)	1	100
Dako M0776 (clone SY38)	5	60
NeoMarkers SP11	1	0
Novocastra Bond RTU PA0299 (rb poly)	2	100
Novocastra NCL-L-SYNAP-299 (27G12)	10	80
Novocastra NCL-SYNAP-299 (27G12)	9	89
Other	16	100
Ventana 760 2668 (rb poly)	1	0
Ventana 760-4595 (MRQ-40)	3	33
Ventana CONFIRM 790-4407 (SP-11)	8	75

Neuropathology Run: 112

Primary Antibody : Cytokeratin

Antibody Details	N	%
Becton Dickinson 349205 (CAM 5.2)	4	100
DakoCytomation M0821 (Clone MNF116)	13	100
DakoCytomation M3515 (Clone AE1/AE3)	12	100
Novocastra NCL-AE1/AE3	4	100
Other	19	100
Sigma C2562 pan-ck	1	100
Ventana 760 2595 (Clone AE1/AE3)	3	100

Neuropathology Run: 112

Heat Mediated Retrieval	Cytokeratin N	Cytokeratin %	Synaptophysin N	Synaptophysin %
_Leica BondMax ER2	1	100	0	0
Biocare Decloaking Chamber	1	100	1	100
Dako Omnis	2	100	2	100
Dako PTLink	8	100	10	90
Leica ER1 10 mins	1	100	0	0
Leica ER1 20 mins	2	100	3	100
Leica ER1 30 mins	1	100	4	100
Leica ER1 40 mins	0	0	1	100
Leica ER2 10 mins	1	100	0	0
Leica ER2 20 mins	5	100	9	78
Leica ER2 30 mins	1	100	2	50
Microwave	1	100	1	100
None	7	100	1	100
Other	0	0	1	100
Ventana CC1 16mins	1	100	0	0
Ventana CC1 24mins	0	0	2	50
Ventana CC1 32mins	3	100	2	50
Ventana CC1 36mins	2	100	0	0
Ventana CC1 40mins	1	100	1	100
Ventana CC1 48mins	1	100	0	0
Ventana CC1 52mins	0	0	2	50
Ventana CC1 56mins	0	0	1	100
Ventana CC1 64mins	1	100	7	86
Ventana CC1 mild	2	100	2	0
Ventana CC1 standard	3	100	7	86

Neuropathology Run: 112

Enzyme Mediated Retrieval	Cytokeratin N	Cytokeratin %	Synaptophysin N	Synaptophysin %
AS PER KIT	1	100	0	0
BioGenex Protease	1	100	0	0
Dako Proteinase K (S3020)	1	100	0	0
MP BioMedicals Trypsin 150213	1	100	0	0
NOT APPLICABLE	20	100	37	81
Other	2	100	1	100
Sigma Pepsin (P7000)	1	100	0	0
VBS Bond Enzyme 1	6	100	0	0
Ventana Protease	1	100	0	0
Ventana Protease 1 (760-2018)	8	100	0	0

Neuropathology Run: 112	Cytokeratin		Synaptophysin	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	5	100	8	88
Biocare polymer (M4U534)	1	100	1	100
Dako EnVision FLEX (K8000/10)	0	0	2	100
Dako EnVision FLEX+ (K8002/12)	6	100	4	100
Dako Envision HRP/DAB (K5007)	2	100	2	100
Leica Bond Polymer Refine (DS9800)	16	100	18	83
None	1	100	1	100
NOT APPLICABLE	1	100	0	0
Other	2	100	1	100
Ventana OptiView Kit (760-700)	5	100	8	75
Ventana UltraView Kit (760-500)	14	100	14	64
Vision BioSystems Refine (DS9800)	1	100	0	0

Neuropathology Run: 112	Cytokeratin		Synaptophysin	
Automation	**N**	**%**	**N**	**%**
BioGenex Optimax	1	100	0	0
Dako Autostainer Link 48	7	100	10	90
Dako Autostainer plus	1	100	0	0
Dako Autostainer Plus Link	1	100	0	0
Dako Omnis	2	100	2	100
Leica Bond Max	6	100	6	67
Leica Bond X	1	100	0	0
Leica Bond-III	11	100	14	93
Menarini - Intellipath FLX	1	100	1	100
None (Manual)	1	100	1	100
Shandon Sequenza	2	100	1	100
Ventana Benchmark ULTRA	14	100	16	69
Ventana Benchmark XT	8	100	8	75

Neuropathology Run: 112	Cytokeratin		Synaptophysin	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	10	100	12	83
Dako DAB+ REAL Detection (K5001)	1	100	0	0
Dako EnVision Plus kits	3	100	1	100
Dako FLEX DAB	4	100	6	100
Dako REAL EnVision K5007 DAB	0	0	2	100
Dako REAL K5001 DAB	1	100	0	0
Leica Bond Polymer Refine kit (DS9800)	18	100	18	83
Other	2	100	3	100
Ventana DAB	1	100	2	100
Ventana Ultraview DAB	16	100	15	60

BEST METHODS - Gold Standard Antibody

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

Synaptophysin - Method 1

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

Primary Antibody: Novocastra NCL-L-SYNAP-299 (27G12) , 15 Mins, RT ºC Dilution 1: 100
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER1 30 mins, PH: 6
EAR:
Chromogen: Leica Bond Polymer Refine kit (DS9800), RT ºC., Time 1: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins, RT ºC Prediluted

Synaptophysin - Method 2

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

Primary Antibody: Dako M0776 (clone SY38) , 20 Mins Dilution 1: 50
Automation: Dako Omnis
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako Omnis, Buffer: Dako low pH TRS
EAR:
Chromogen: Dako FLEX DAB, Time 1: 5 Mins, Time 2: 5 Mins
Detection: Other , 10 Mins Prediluted

Synaptophysin - Method 3

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

Primary Antibody:	Biogenex MU 363 UC , 24 Mins, 22 ºC Dilution 1: 50
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 30 mins, Buffer: ER2
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), 22 ºC., Time 1: 9 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, 22 ºC Prediluted

Synaptophysin - Method 4

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

Primary Antibody:	Novocastra NCL-SYNAP-299 (27G12) , 24 Mins, 36 ºC Dilution 1: 75
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	AS PER KIT

BEST METHODS - Secondary Antibody

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

Cytokeratin - Method 1

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

Primary Antibody:	DakoCytomation M0821 (Clone MNF116) , 15 Mins, 25 ºC Dilution 1: 200
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Optimax Wash Buffer
HMAR:	None
EAR:	VBS Bond Enzyme 1, 37 ºC. Digestion Time NEQAS: 10 Mins. In-House: 10 Mins
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, 25 ºC

Cytokeratin - Method 2

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

Primary Antibody:	DakoCytomation M0821 (Clone MNF116) , 28 Mins, 37 ºC Dilution 1: 100
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	
EAR:	Ventana Protease 1 (760-2018), 37 ºC. Digestion Time NEQAS: 12 Mins. In-House: 12 Mins
Chromogen:	Ventana Ultraview DAB, 37 ºC., Time 1: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC Prediluted

Cytokeratin - Method 3

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

Primary Antibody:	Novocastra NCL-AE1/AE3 , 30 Mins, 25 ºC Dilution 1: 100
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	
EAR:	Dako Proteinase K (S3020)
Chromogen:	Ventana Ultraview DAB
Detection:	

Cytokeratin - Method 4

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

Primary Antibody:	Ventana 760 2595 (Clone AE1/AE3) , 8 Mins, 36 ºC
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 36mins
EAR:	Ventana Protease, 36 ºC. Digestion Time NEQAS: 8 Mins. In-House: 8 Mins
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

Neil Bilbe and Irena Srebotnik Kirbis

	Gold Standard	Second Antibody
Antigens Assessed:	S100	CK 5/6
Sample circulated; cytospins and cell block sections:	Human melanoma and human carcinoma, cell lines, plus an effusion with mesothelial cells, macrophages and RBCs.	Human melanoma and human carcinoma, cell lines, plus an effusion with mesothelial cells, macrophages and RBCs.
Number of Registered Participants:	84 - Cell block 59 (70%), Cytospin 25 (30%)	
Number of Participants this Run	81(96%)	

Introduction

Gold Standard: Melanoma markers

S100 is a multigene family of low molecular weight proteins and is demonstrated in some Langerhans' cells and melanocytes of the skin, interdigitating reticulum cells in lymph nodes, medullary epithelial reticular cells in the thymus, chondrocytes in cartilaginous tissue, adipocytes in some, but not other biopsies, myoepithelial cells in salivary glands and breast, folliculostellate cells of the pituitary gland, and Schwann cells and glial cells of nervous tissue. Weak labelling is found in epithelial cells of the mammary and sweat glands. It stains the majority of malignant melanomas.

The following rarely or never express S100: adenocarcinomas of the alimentary tract, lung, and prostate, transitional cell carcinoma, malignant mesothelioma, fibromatosis, fibrohistiocytic tumours, smooth muscle tumours, malignant lymphomas and germinal cell tumours.

In some tumours, the S-100 protein positivity is restricted to so-called sustentacular cells: phaeochromocytoma/ paraganglioma (particularly when benign), and medullary thyroid carcinoma. S-100 positive dendritic cells are particularly numerous in sclerosing variant of papillary carcinoma.

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. Liu K, Dodge R, Glasgow BJ, Layfield LJ. Fine-needle aspiration: Comparison of smear, cytospin, and cell block preparations in diagnostic and cost effectiveness. Diagn Cytopathol. 1998;19:70–4.
2. Dalquen P, Sauter G, Epper R, Kleiber B, Feichter G, Gudat F. Immunocytochemistry in diagnostic cytology. Recent Results Cancer Res. 1993;133:47–80
3. Leung SW, Bedard YC. Immunocytochemical staining on ThinPrep processed smears. Mod Pathol 1996; 9(3): 304–6.
4. Beaty M, Fetsch PA, Wilder AM, Marincola FM, Abati A. Effusion cytology of malignant melanoma. Cancer (Cancer Cytopathol) 1997; 81(1): 57–63.
5. Erdag, G, Chowdhuri, SR, Fetsch, P, Erickson, D, Hughes, MS & Filie, AC 2013, 'Kba.62 and S100 protein expression in cytologic samples of metastatic malignant melanoma' *Diagnostic Cytopathology*, vol 41, no. 10, pp. 847-851.

Second Antigen: CK 5/6

Cytokeratins are alpha-type fibrous polypeptides with a diameter of 7-11 nm. They are important components of the cytoskeleton in almost all epithelial cells as well as in some non-epithelial cell types. Cytokeratins are, generally, held to be the most ubiquitous markers of epithelial differentiation, and, so far, 20 distinct types numbered by Moll (4, 5) have been revealed. The CK 5 is a high molecular weight, basic type of cytokeratin, with a molecular mass of 58 kDa, expressed in the basal, the intermediate and the superficial cell layers of stratified epithelia as well as in transitional epithelia, complex epithelia, and in mesothelial cells and mesothelioma. CK 5 has not, with few exceptions, been found in simple epithelia and in non-epithelial cells. CK 6 is also a high molecular weight, basic type of cytokeratin, with a molecular mass of 56 kDa, expressed by proliferating squamous epithelium often paired with CK 16 (48 kDa) (4, 5).

Cytokeratin 5/6 is intended for use in immunocytochemistry. Antibodies to cytokeratin 5/6 (CK 5/6) have been found valuable for the distinction between low differentiated squamous cell carcinoma and adenocarcinoma. Furthermore, the expression of CK5 by mesothelium makes this antibody valuable in the differentiation between epithelioid mesothelioma and lung carcinoma (1) when used together with other antibodies against mesothelioma markers, such as calretinin and thrombomodulin (2). Anti-CK 5/6 has also been found useful in the differential diagnosis of atypical proliferations of the breast (3). Differential identification is aided by the results from a panel of antibodies.

Features of Optimal Immunostaining:

- Strong staining in the cytoplasm
- Clean background with no staining of the melanoma cells within the cytological effusion.
- Adequate counterstain.

Features of Sub-optimal immunostaining:

- Weak, uneven, or less staining than expected .
- Diffuse staining in the membranes.
- High background or non-specific staining of cell types not expected to stain, including the melanoma cells within the sample
- Excessive or very weak counterstain

References

1. Clover J, Oates J, Edwards C. Anti-cytokeratin 5/6: a positive marker for epithelioid mesothelioma. Histopathology 1997;31:140-3.
2. Cury PM, Butcher DN, Fisher C, Corrin B, Nicholson AG. Value of the mesothelium-associated antibodies thrombomodulin, cytokeratin 5/6, calretinin, and CD44H in distinguishing epithelioid pleural mesothelioma from adenocarcinoma metastatic to the pleura. Mod Pathol 2000;13:107-12.
3. Otterbach F, Bankfalvi A, Bergner S, Decker T, Krech R, Boecker W. Cytokeratin 5/6 immunohistochemistry assists the differential diagnosis of atypical proliferations of the breast. Histopathology 2000;37:232-40.
4. Moll R, Franke WW, Schiller DL. The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumors and cultured cells. Cell 1982;31:11-24.
5. Moll R, Löwe A, Laufer J, Franke WW. Cytokeratin 20 in human carcinomas. A new histodiagnostic marker detected by monoclonal antibodies. Am J Pathol 1992;140:427-47.

References (cell blocks in cytology)

1. Nithyananda A. Nathan; Cell Block Cytology; Improved Preparation and Its Efficacy in Diagnostic Cytology Am J Clin Pathol 2000;114:599-606

Assessment Summary:

At the start of the run there were 84 registered participants; 80 labs had submitted at the time of assessment and another submitted at a later date. This resulted in a total of 316 slides assessed and used in the analyses.

The Gold antigen requested for this run was **S100**, rather than either; HMB-45, Melan-A, *or* S100 as for the previous two runs (110, 111). The purpose of this was to assess the laboratories' ability to employ S100 to demonstrate melanoma cells.

The scheme has seen, and reported, that out of the three antibodies usually permitted for 'melanoma markers', S100 has generally performed less well, or less consistently than the other two markers, particularly with samples prepared into cell blocks (see Journal writes ups).

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

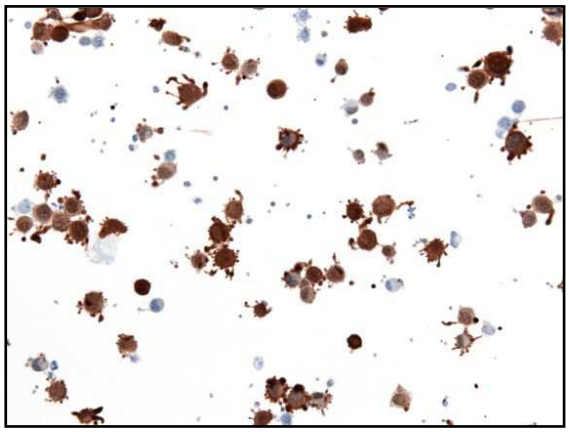


Fig 1: S100 on cytospin prepared from sample 112 R

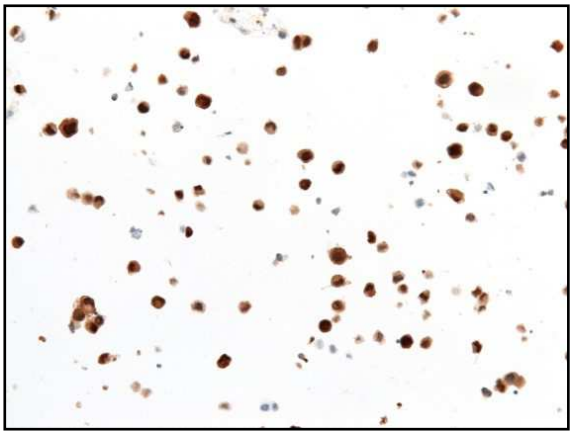


Fig 2: S100 on cell block prepared from sample 112 R

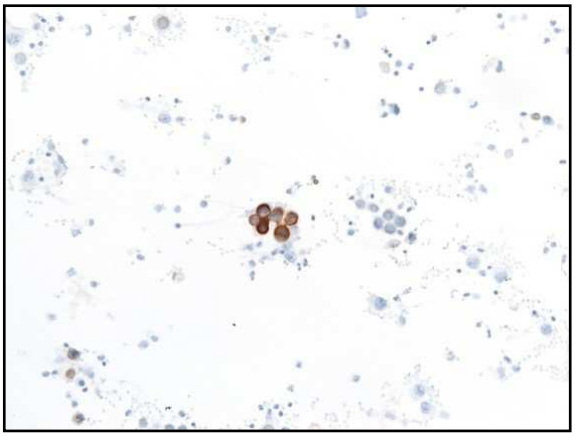


Fig 3: CK 5/6 on cytospin prepared from sample 112 T

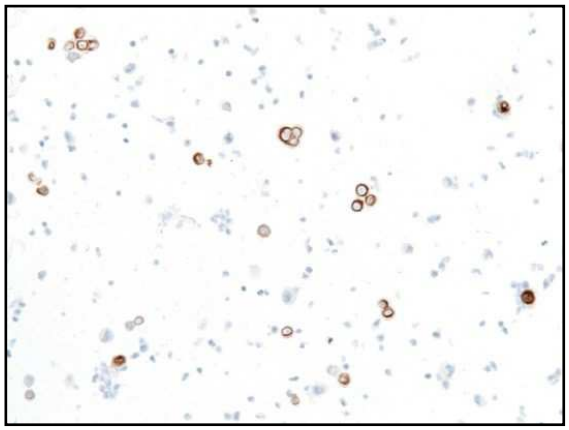


Fig 4: CK 5/6 on cell block prepared from sample 112 T

Assessment outcomes

Overview

The current level of requests for the two slide options is **CB** = 59 labs (70%), **CS** = 25 labs (30%). Although the actual submission rate was only 81 labs out of 84 registered participants, these percentages were maintained.

The total number of failed submissions was 18 (6%), with those assessed as borderline 38 (12%), and those passing the assessment was 260 slides (82%).

Breakdown of failed slides: **R**=8, **S**=3, (S100); **T**=5, **U**=2 (CK 5/6).

Cytokeratin 5/6 (T & U)

NEQAS slides (**T**)

Five slides failed on the NEQAS CK 5/6 samples; three of these were on cytospins, and two on cell blocks. Statistically, this equates to a failure rate of 8% for cytospins, and 5% for cell blocks.

All of the five slides (**T**) were failed due to poor demonstration; too weak, or absent (see fig 9) of epithelial cells in the samples. Three of the five slides were stained using the Dako CK 5/6, clone D5/16B4, which was the most popular antibody employed, used by 43 out of the 76 labs (57%) who returned their methodology. The dilutions ranged from 1:20 (CB), 1:50 (CS) to 1:100 (CS). Interestingly, the lab who stained at 1:100 on a CS stated that they had also performed a retrieval step, using ER1 for 10 mins. The other two markers were a Cell Marque 356 M-18 clone (CB with ER1), and a Novocastra CK5 antibody (CS no RT).

In-house slides (**U**)

The two in-house slides (**U**) that failed were on a Thin Prep sample, using the Dako CK 5/6 as above (no dilution given), which showed excessive background and non-specific staining; the second was on a FFPE section (no protocol entered), which had noticeable section lifting, weak demonstration, and pronounced background staining.

S100 (R & S)

Introduction

This run was essentially designed to investigate the participants' ability to stain melanoma cells with S100 in the cell block and cytospin, in order to establish some trends and recommendations, given that for the majority of labs S100 is not usually their marker of choice. In recent runs, only up to around 10% of participants returned a NEQAS slide stained using S100 as their preferred melanoma marker.

Overview

There were 80 of the 81 participants submitting stained slides for S100. A single, non-UK participant, did not return their cell block section stained with S100.

For the NEQAS S100 (**R**) samples (N =80):

- 8 slides failed –10%
- 15 slides were borderline –19%
- 57 slides were assessed as passed - 71%

The average score for S100 NEQAS (**R**) slides = 13.9, for the S100 in-house (**S**) = 15.2 (for all S100: **R** & **S** = 14.5).

Cell Blocks (CB) v Cytospins (CS)
A total of 8 S100 NEQAS (**R**) slides failed the assessment, six were on **CB** (10.7%) and two **CS** (8.3%).

The average scores for each of the two samples was:

- NEQAS CB S100 (**R**) = 13.4 Median = 13.5
- NEQAS CS S100 (**R**) = 15.1 Median = 16.0

Summary table for all S100 NEQAS slides (**R**)

Slides	NEQAS (R)		NEQAS (R)	
	Cell block	Cytospins	Cell block	Cytospins
Pass (N/%)	36	21	64.3%	87.5%
Borderline (N/%)	14	1	25.0%	4.2%
Fail (N/%)	6	2	10.7%	8.3%

NEQAS slides (**R**)

Best performing slides
Seven out of the 80 slides scored between 18 - 20 (9%); 4/24 were on cytospins (17%), and 3/56 were on cell blocks (5%).

Summary table—best performing slides

Sample	Primary	Dilution	Pretreatment	Automation	2nd Layer
Cytospin	Dako Poly	Not given	None	Ventana Benchmark ULTRA	Not Given
Cytospin	Dako Poly	1000	None	Ventana Benchmark GX	Ventana iView
Cytospin	Leica RTU	RTU	None	Leica Bond III	Leica Bond Refine
Cytospin	Dako Poly	200	ER1 5 mins	Leica Bond Max	Leica Bond Refine
Cell Block	Novocastra NCL	500	None	Leica Bond III	Leica Bond Refine
Cell Block	Dako RTU	RTU	PT Link 20 mins	Dako Autostainer Link 48	Dako EnVision FLEX+
Cell Block	Dako Poly	4000	Ventana CC1 mild	Ventana Benchmark ULTRA	Ventana UltraView Kit

Borderline NEQAS S100 slides (**R**)
When examining the assessors' comments, the main reasons for borderline scores was either weak staining of melanoma cells (8) or non-specific staining (6). Where there was weak demonstration of melanoma cells, half of these were *probably* due to lack of or insufficient pretreatment/retrieval methods. This though can only be taken at face value. As we reported in the write up in the Run 111 Journal, we are not always convinced that the details relating to antigen retrieval are accurate, given that it relies on labs selecting the correct option. See page 4: http://www.ukneqasiccish.org/wp/wp-content/uploads/2016/01/cyto_106_109.pdf

N.B. Only 75 (94%) laboratories entered their methodologies, out of the 80 that returned slides. It is worth stressing at this point, that some of the primary antibodies used to stain the slides, have only been employed by one or two participants, and therefore any statistical interpretations must be viewed in such light.

Failed NEQAS S100 slides (**R**)
All of the 8 slides that were assessed as inadequate for diagnostic purposes, and therefore scored ≤ 9/20 had either absence, very weak, or weak staining as the main comment. For two of the six slides stained on cell block sections, additional comments of lack of retrieval or insufficient pretreatment was also included. A third cell block slide had entered a retrieval method (CC1 mild), but had indicated that this was not used on the NEQAS slide. A single slide, cell block, had excessive counterstain, and another slide, cytospin, was marked down due to poor morphology. Only one of the cell block sections had non-specific staining at an intensity considered to have been detrimental to diagnosis.

Summary table - Markers used on failed slides (N = 8):

Antibody	Failed (N)	Total Users (N)	% Users (75)	Failure rate
Dako S100 polyclonal	3	44	59%	7%
Novocastra NCL-S100p	1	7	9%	14%
Leica RTU PA00900	1	3	4%	33%
S100 4C4.9 monoclonal*	1	3	4%	33%
Cell Marque S100 (16/f5) MCA	1	1	1%	100%
No data entered	1	1	1%	100%

*Supplier not given, widely available (see Figure 5 below).

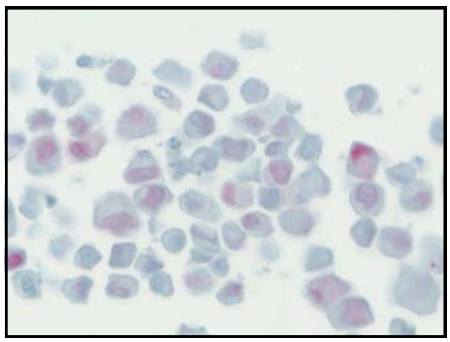


Fig 5: Failed cell block section. S100 4C4.9 clone, weak staining with some cells unstained.

As pointed out above, giving any statistical data from such a small sample set is inadvisable; e.g. 3 slides stained using the Dako polyclonal, which is used by 59% of participants constitutes a failure rate of only 7%, when compared to the Novocastra poly which is used by 7 labs, and therefore constitutes a failure rate of 14%. More interestingly is the combination of either CC1 mild and the Ventana Benchmark ULTRA (3 labs failed : all on CB), or the Leica Bond Max without retrieval (3 labs failed : 2 CS, 1 CB) out of the 7 labs who returned their protocols.

Previous use and results for S100
When viewing the markers used for Runs 110 and 111, 8 labs had chosen to use S100 as their preferred marker, either for one or both of these runs, even with a choice of HMB45/Melan-A/S100, although in practice only one lab had actually used S100 for both previous runs.

Three labs (2 CB, 1 CS) had moved from S100 to either Melan-A or HMB45, and four labs (2 CB, 2 CS) had changed from HMB45 or Melan-A to S100. The reasons for these changes are not registered, but may be due to requirements or availability of reagents linked to automation.
Interestingly, all those changing from either Melan-A or HMB45 to S100 scored *worse*, whereas 2/3 of those changing from S100 to Melan-A *improved* their scores.

**Summary table– Labs who changed between HMB45 or MelanA (Blue) and S100 (Tan) during runs 110—112

Run/Lab	Sample	Run 110	Score	Run 111	Score	Run 112	Score
1	CB	Melan A	19	Novocastra NCL	16	Leica RTU	16
2	CB	Dako poly	15	Melan A	12	Dako poly	12
3	CB	Melan A	19	Dako poly	8	Dako poly	16
4	CB	Ventana 4C4.9	8	Melan A	12	Ventana 4C4.9	10
5	CS	Dako poly	18	Dako poly	13	Dako poly	16
6	CS	Dako poly	15	Melan A	16	Dako poly	16
7	CS	HMB45	17	Dako poly	12	Dako poly	15
8	CS	Melan A	17	Leica RTU	5	Leica RTU	7

Conclusions

- Cytospins performed better than the cell blocks
- Although *failure* rates comparable CB = 10.7%: CS = 8.3%
- Cytospins better without pre-treatments
- Cell blocks need retrieval methods for best results
- Cell blocks were prone to weak and non-specific staining….
- ...leading to a 25% borderline rate for the cell block samples
- High % of these used a Ventana RTU on XT or ULTRA (67%)
- Only one marker was used by > 10% labs: Dako polyclonal...
- ...must be wary of giving meaningful comparison of markers
- Several markers used by only one or two participants
- Protocols must be used as a guide rather than absolute
- **Labs changing HMB45/Melan-A for S100 tend to do worse
- Melanoma markers average score: on NEQAS (**R**) samples:
  - Run 110 = 15.9 - HMB45/Melan-A/S100
  - Run 111 = 15.8 - HMB45/Melan-A/S100
  - Run 112 = 13.4 - S100 only
- **S100 performs less well than Melan-A or HMB45 on cytological melanoma samples**

Selected Images showing Optimal and Sub-optimal Immunostaining

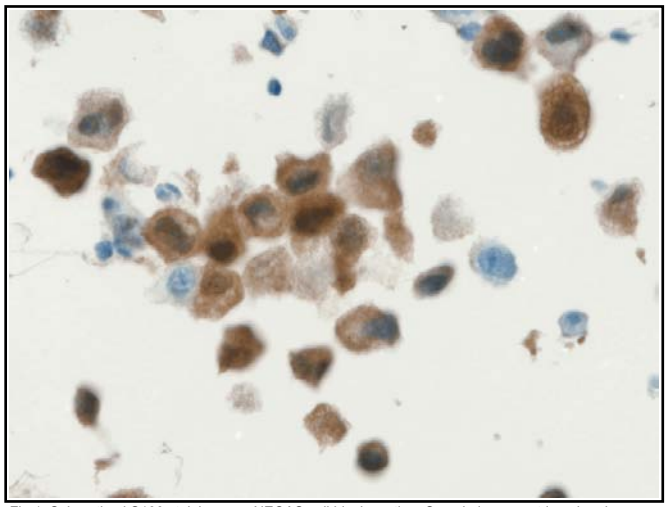


Fig 1. Sub-optimal S100 staining on a NEQAS cell block section. Sample is over retrieved and there is some evidence of non-specific staining. Slide assessed as a low pass. Dako polyclonal, 1:4000, 20 mins, with hot Dako PT Link 20 mins, on a Dako Autostainer Link 48, and the RTU Dako EnVision FLEX kit.

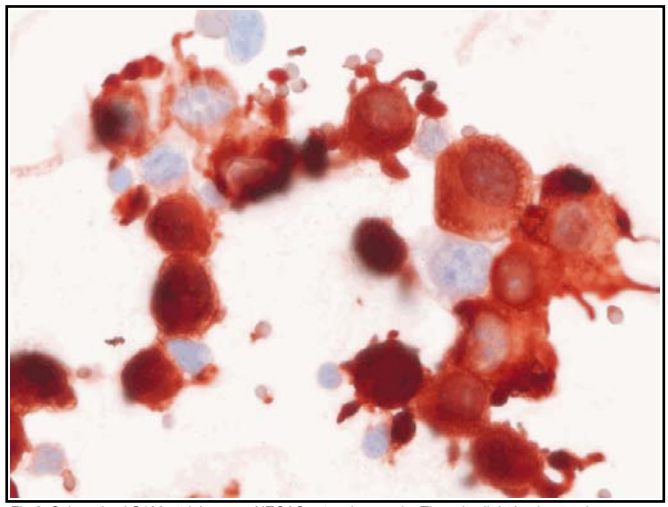


Fig 2. Sub-optimal S100 staining on a NEQAS cytospin sample. There is slight background staining, and leeching of the chromogen, plus a pale counterstain. Again assessed as adequate for diagnostic use. Dako poly, 1:500, 15 mins, no RT, on a Leica Bond Max, with a prediluted Polymer Refine kit.

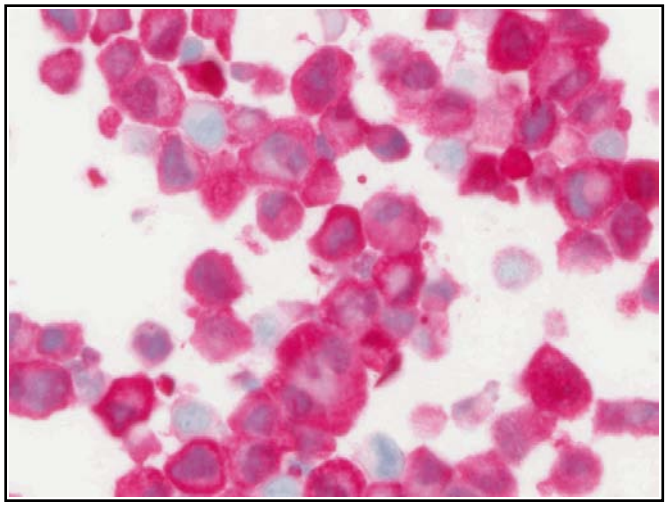


Fig 3. Excellent staining on a NEQAS CB, there is only very slight background staining, which is not always the case for APAAP methods. Dako polyclonal, 1:300, 32 mins, with Ventana CC1 standard, 32 min, on a Ventana Benchmark XT, using a RTU AP Red Detection Kit.

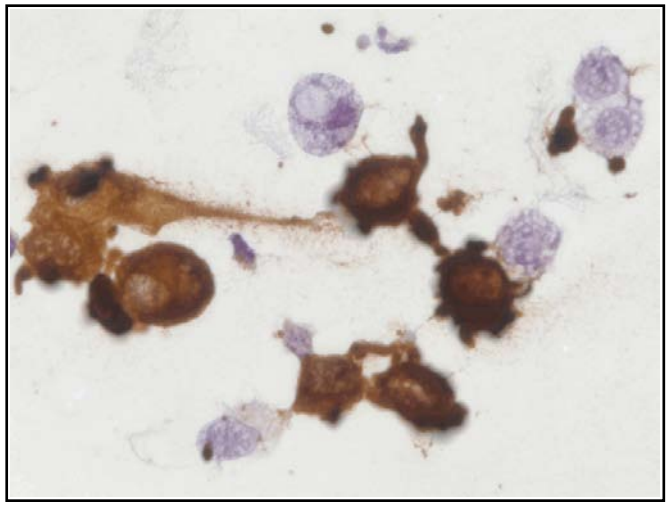


Fig 4. Sub-optimal S100 staining on a NEQAS cytospin sample. Staining is diffuse, and there is also morphological damage. Slide assessed as borderline. Novacastra polyclonal, 30 mins, no dilution given, no RT, Dako Autostainer Link 48, using a Dako Envision+ HRP rabbit kit, again no times given.

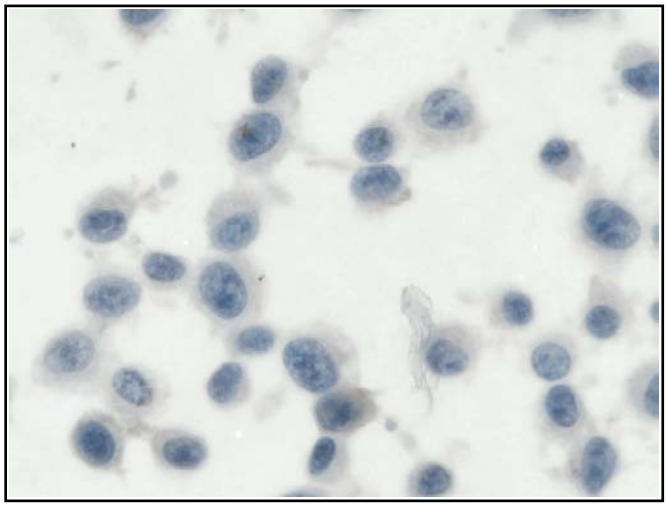


Fig 5. Poor S100 demonstration on a NEQAS cytospin. Staining is absent or insufficient for diagnostic use. This slide failed the assessment. Cell Marque polyclonal, 1:50, 15 mins, no RT, on a Leica Bond Max, using a RTU Leica Bond Polymer Refine kit, at 20°C.

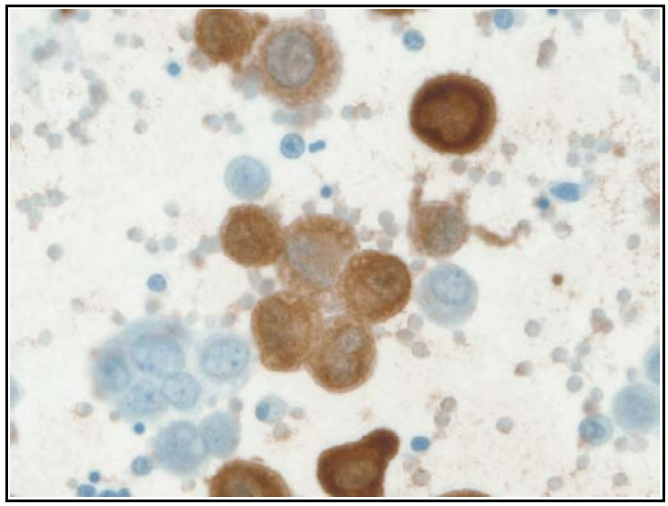


Fig 6. Excellent S100 staining from an in-house cytospin. Tumour cells are nicely demonstrated and all other cells are negative, or with only a hint of staining, but overall the slide is clean. Dako poly, dilution given as 1:30000, 16 mins, no RT, on a Ventana Benchmark GX.

Selected Images showing Optimal and Sub-optimal Immunostaining

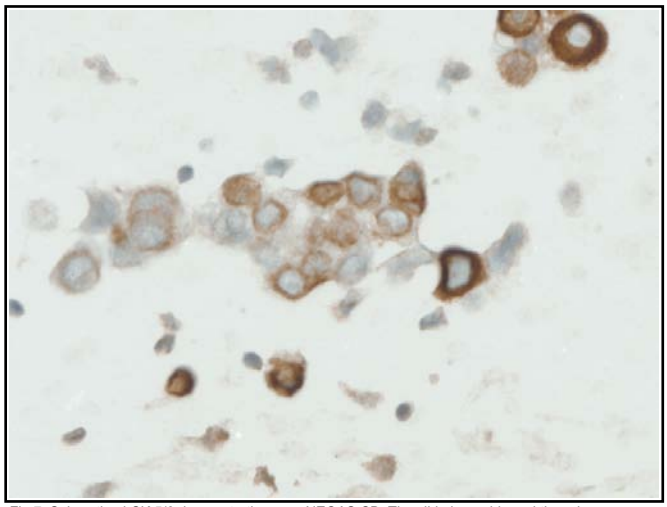


Fig 7. Sub-optimal CK 5/6 demonstration on a NEQAS CB. The slide is muddy and there is some background staining, but assessed as just adequate for diagnostic purposes. Prediluted Ventana CK 5&6, 16 mins, with the Ventana CC1 32mins, on a Benchmark ULTRA, and a RTU Ventana OptiView Kit.

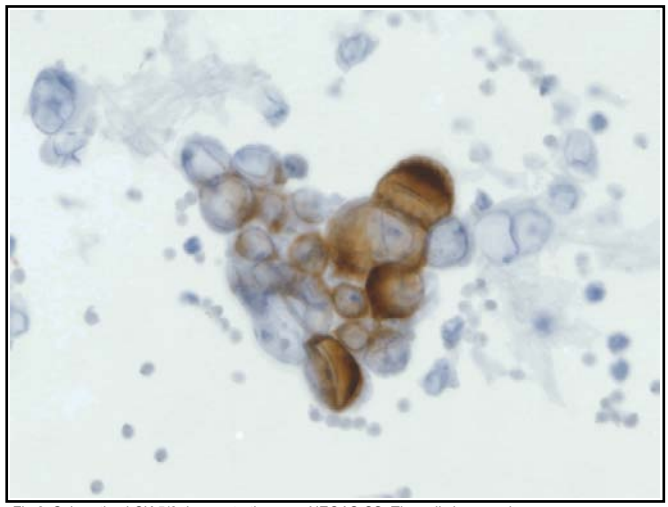


Fig 8. Sub-optimal CK 5/6 demonstration on a NEQAS CS. The cells have undergone morphological damage. Melanoma cells can be seen, but the slide was still assessed as borderline. Invtitrogen CK 5/6, 1:25, 20 mins, on a Ventana Benchmark XT, with a RTU Ventana UltraView Kit, no time given.

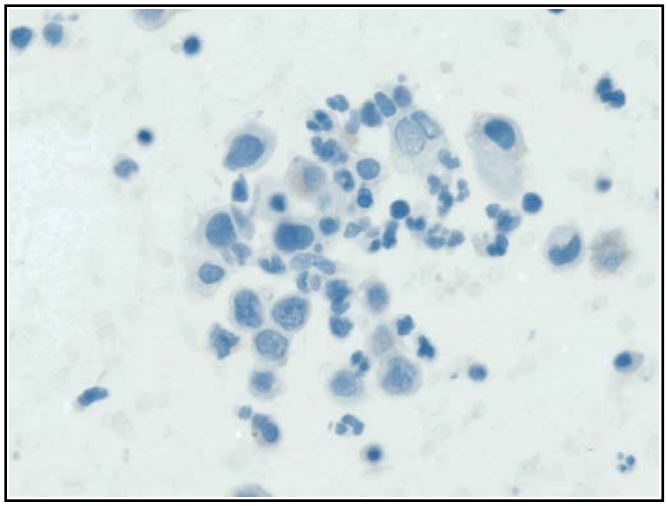


Fig 9. Poor CK 5/6 staining on a NEQAS CB. There is little or no staining, the slide failed, and not considered safe for diagnostic purposes. Cell Marque 356 M-18, diluted 1:2, 30 mins, with a Leica ER1 30 mins, on a Leica Bond Max, with a Leica Bond Polymer Refine for 15 mins.

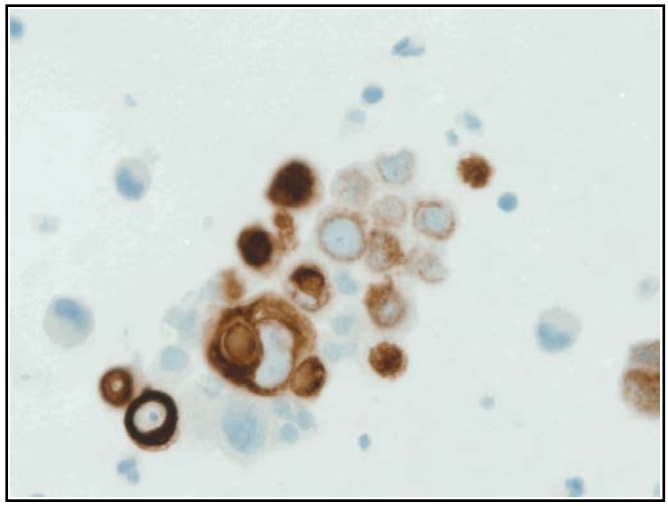


Fig 10. Excellent CK 5/6 staining on a NEQAS CB. There is good crisp staining of the carcinoma cells, the background is clean and the counterstain is of a nice intensity. Ventana CK 5/6, 8 mins, Ventana CC1 64 mins, Ventana Benchmark ULTRA, Ventana OptiView Kit.

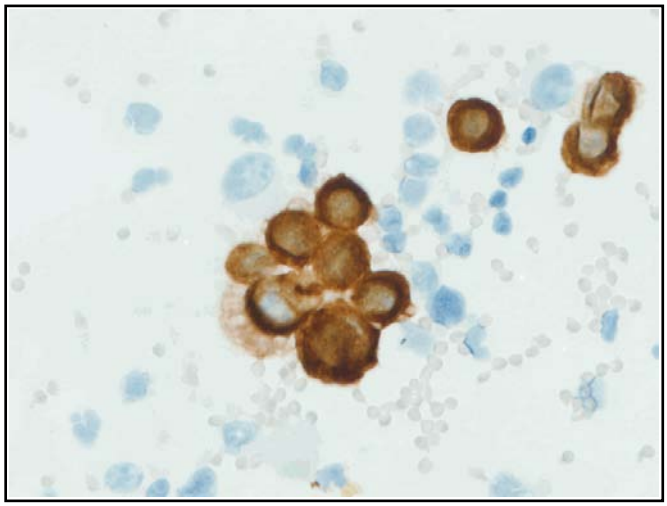


Fig 11. Excellent CK 5/6 staining on a NEQAS CS. Tumour cells are nice demonstrated and the rest of the cell population is clear. Beckton Dickson CAM 5.2, prediluted (to 1:500), no RT, on a Ventana Benchmark ULTRA, using a prediluted Ventana UltraView Kit for 8 mins.

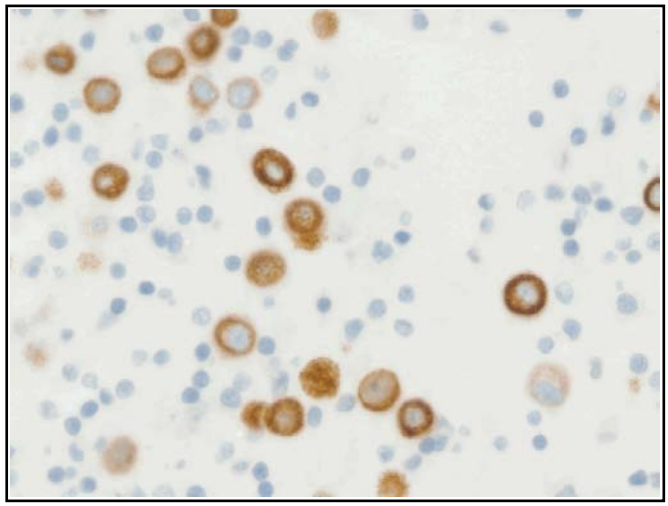
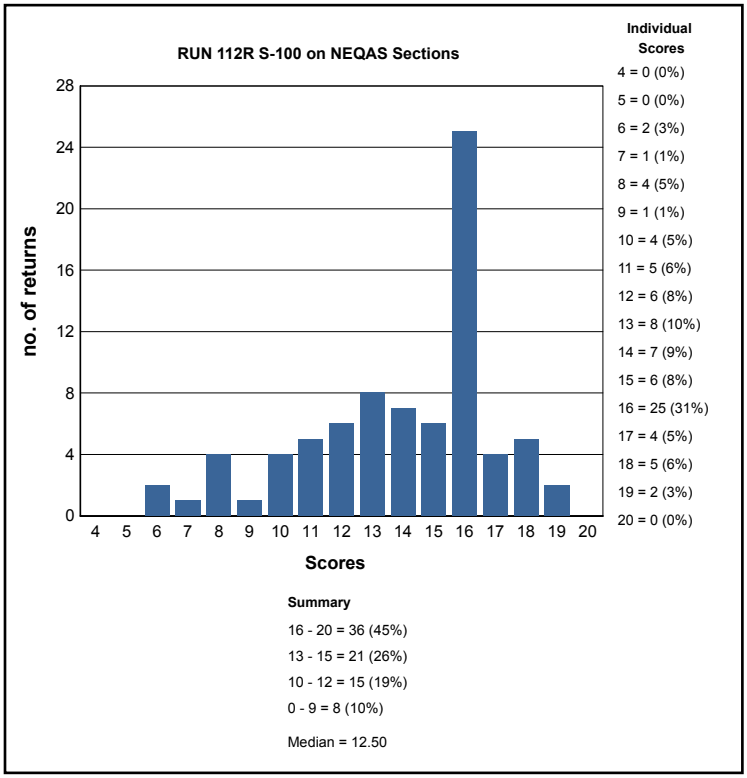
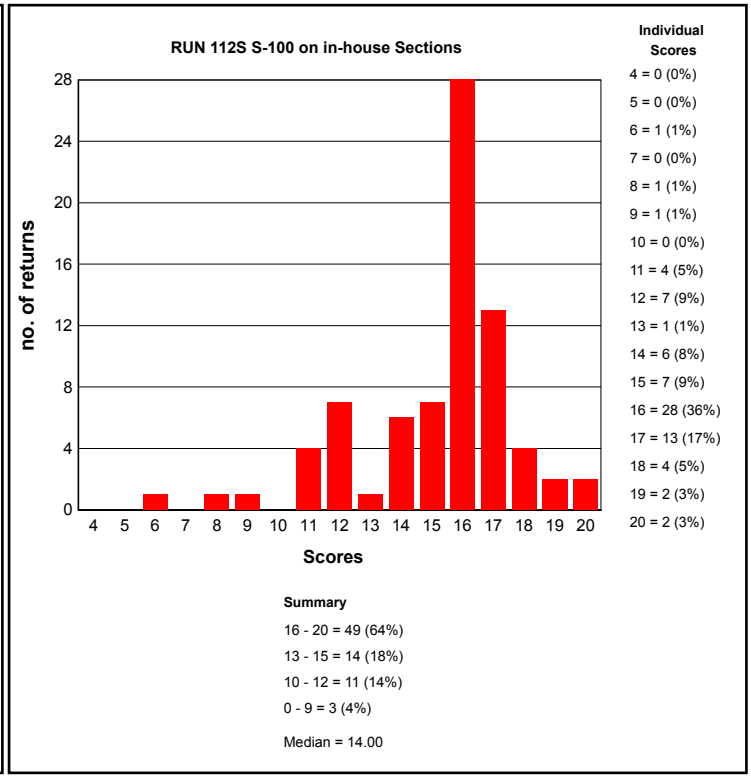
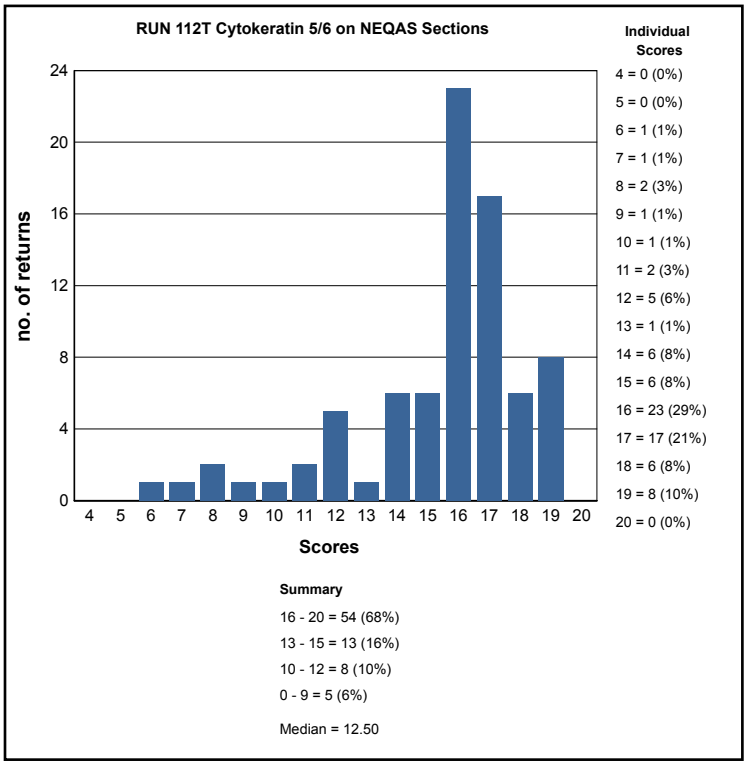


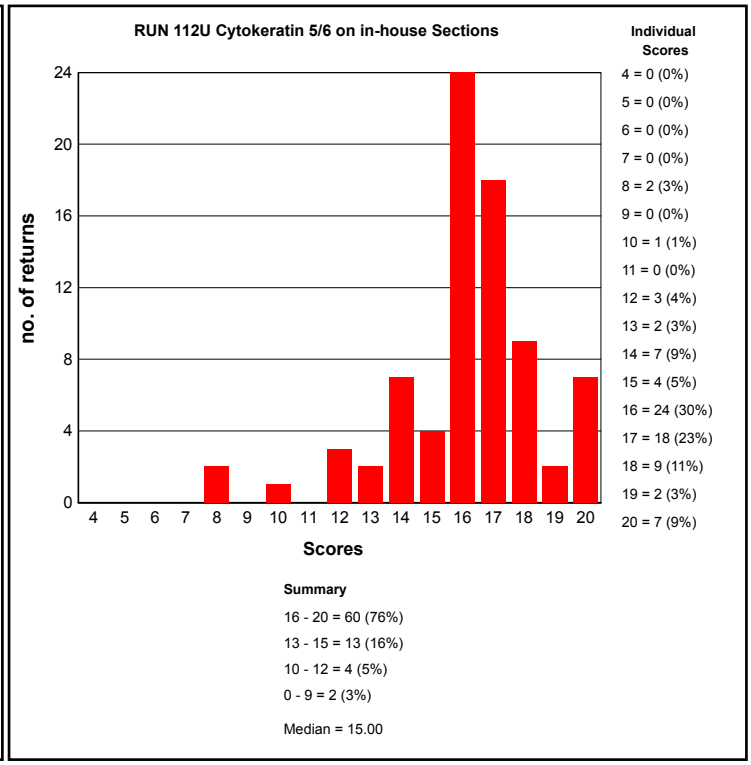
Fig 12. Excellent staining on an in-house CB from a pleural effusion. Staining is crisp with little or no staining of the cell block matrix. Dako M7237 CK 5/6, 1:50, 32 mins, no RT, Ventana Benchmark ULTRA, using a RTU OptiView DAB IHC Detection Kit, 8 mins.

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: 112

Primary Antibody : S-100

Antibody Details	N	%
Dako Omnis RTU (Poly)	1	100
Dako RTU IR504 (poly)	6	83
Dako Z0311 (S100 poly)	44	75
Leica RTU PA00900 (poly)	3	67
Novocastra NCL-S100p (S100 poly)	7	57
Other	7	71
Ventana 760 2523 (S100 poly)	1	100
Ventana 790 2914 (S100 poly)	6	33

Cytology Run: 112

Primary Antibody : Cytokeratin 5/6

Antibody Details	N	%
Chemicon MAB 1620 CK 5/6	1	100
Dako M7237 CK5/6 (clone D5/16B4)	43	84
Other	32	81

Cytology Run: 112

Primary Antibody : S-100

Antigen Retrieval	N	%
YES	29	36
NO	52	64
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	29	
Not Specified	0	

Cytology Run: 112

Primary Antibody : Cytokeratin 5/6

Antigen Retrieval	N	%
YES	37	46
NO	44	54
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	37	
Not Specified	0	

Cytology Run: 112

Heat Mediated Retrieval

Cytology Run: 112

Heat Mediated Retrieval

Cytology Run: 112

Enzyme Mediated Retrieval

Cytology Run: 112

Enzyme Mediated Retrieval

Cytology Run: 112

Detection	Cytokeratin 5/6		S-100	
	N	%	N	%
AS PER KIT	6	100	6	33
Dako EnVision FLEX+ (K8002/12)	10	90	9	100
Dako Envision+ HRP mouse K4004/5/6/7	1	100	0	0
Dako Envision+ HRP rabbit K4008/9/10/11	0	0	1	0
Leica Bond Polymer Refine (DS9800)	20	70	20	70
None	1	100	1	100
NOT APPLICABLE	1	0	0	0
Other	4	75	4	75
Ventana iView system (760-091)	3	67	3	100
Ventana OptiView Kit (760-700)	8	100	7	57
Ventana UltraView Kit (760-500)	17	88	18	61

Cytology Run: 112

Automation	Cytokeratin 5/6		S-100	
	N	%	N	%
Dako Autostainer Link 48	11	91	11	91
Dako Autostainer Plus Link	1	100	1	100
Dako Omnis	2	100	1	100
Leica Bond Max	9	67	10	50
Leica Bond-III	12	75	12	92
Ventana Benchmark GX	3	67	3	100
Ventana Benchmark ULTRA	22	95	23	57
Ventana Benchmark XT	15	73	14	64

Cytology Run: 112

Chromogen	Cytokeratin 5/6		S-100	
	N	%	N	%
AS PER KIT	10	100	10	70
DAKO DAB+	1	100	1	0
Dako EnVision Plus kits	0	0	1	100
Dako FLEX DAB	9	89	8	100
Leica Bond Polymer Refine kit (DS9800)	20	75	21	71
Other	5	60	4	75
Ventana DAB	8	100	10	70
Ventana Enhanced Alk. Phos. Red Detection Kit	0	0	1	0
Ventana iview	3	33	2	100
Ventana Ultraview DAB	20	85	17	59

BEST METHODS - Gold Standard Antibody

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

S-100 - Method 1

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

Primary Antibody:	Novocastra NCL-S100p (S100 poly) , 15 Mins, 25 ºC Dilution 1: 500
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Optimax Wash Buffer
HMAR:	
EAR:	VBS Bond Enzyme 1, 37 ºC. Digestion Time NEQAS: 10 Mins. In-House: 10 Mins
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, 25 ºC

S-100 - Method 2

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

Primary Antibody:	Dako Z0311 (S100 poly) , 32 Mins
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	
EAR:	Ventana Protease 1 (760-2018)
Chromogen:	Ventana Ultraview DAB
Detection:	

S-100 - Method 3

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

Primary Antibody:	Dako Z0311 (S100 poly) , 32 Mins, 42 ºC Dilution 1: 1000
Automation:	Ventana Benchmark GX
Method:	Ventana iView Kit
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	
EAR:	
Chromogen:	Ventana iview
Detection:	Ventana iView system (760-091)

S-100 - Method 4

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

Primary Antibody:	Dako Z0311 (S100 poly) , RT ºC Dilution 1: 1:200
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Other
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800) , RT ºC

BEST METHODS - Secondary Antibody

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

Cytokeratin 5/6 - Method 1

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

Primary Antibody:	Dako M7237 CK5/6 (clone D5/16B4) , 20 Mins, 20 ºC Dilution 1: 50
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: FLEX TRS HIGH PH
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 20 ºC., Time 1: 10 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 20 Mins, 20 ºC Prediluted

Cytokeratin 5/6 - Method 2

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

Primary Antibody:	Dako M7237 CK5/6 (clone D5/16B4) , 15 Mins, 25 ºC 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), Time 1: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, 25 ºC Prediluted

Cytokeratin 5/6 - Method 3

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

Primary Antibody:	Dako M7237 CK5/6 (clone D5/16B4) , 32 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

Cytokeratin 5/6 - Method 4

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

Primary Antibody:	Dako M7237 CK5/6 (clone D5/16B4) , 30 Mins, 21 ºC Dilution 1: 25
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: DAKO 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

Alimentary Tract Module: GIST Run 112

Suzanne Parry

	First Antibody	Second Antibody
Antigens Assessed:	CD117	DOG-1
Tissue Sections circulated:	Appendix, GIST & Desmoid Tumours	Appendix, GIST & Desmoid Tumours
Number of Registered Participants:	118	
Number of Participants this Run	112 (95%)	

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, & 6)**
- Good localisation of CD117 to mast cells in the appendix and desmoid sections (Fig 1 desmoid not shown)
- Good localisation of CD117 to interstitial cells of Cajal (Fig 6A)
- Good localisation of CD117 to cells of the GIST (Fig 2)
- No staining of the desmoid tumour

Features of Sub-optimal Immunostaining: **(See Figs 3, 4 & 5)**
- Weak and/or patchy staining of the tumour cells of the GIST (Fig 4)
- Little or no staining of the mast cells
- Excessive background or non specific staining (Figs 3 & 5)
- Staining of the desmoid tumour (Fig 5)

Second Antibody: DOG-1

"Discovered on GIST 1" (DOG-1) antibody was initially described in 2004[4] and has now started to be recognized as a more specific marker of GISTs than CD117 [4,5,6]. A recent study has shown DOG-1 sensitivity of 99% and found to be strong and easier to interpret than CD117[6]. The groups also showed that seven CD117 negative GIST cases were found to be DOG-1 positive, and six of the tumours had PDGFRA mutations. Furthermore, DOG-1 is not expressed in Kaposi sarcomas of the GI tract which have been shown to stain for CD117[7]. In more cases, combined CD117 and DOG-1 immunohistochemistry has been suggested as a sufficient panel to confirm diagnosis, with CD117 and DOG-1 negative cases tested further with a panel of antibodies, including SMA, Desmin, S100. Molecular analysis should be considered[6].

Features of Optimal Immunostaining (See Figs 7, 8 & 12)
- Good localisation of DOG-1 to cells of the GIST (Figs 7 & 8)
- Good localisation of DOG-1- to the interstitial cells of Cajal
- No staining of desmoid tumour

Features of Sub-optimal Immunostaining (See Figs 8 & 9)
- Weak and/or patchy staining of the tumour cells of the GIST (Figs 10 & 11B)
- Excessive background or non specific staining (Fig 9)
- Staining of the desmoid tumour
- Staining of the mast cells (Note: Mast cells are not expected to stain with DOG-1)

Tissue Distribution and Assessment Procedure

Composite slides comprising of a GIST, appendix and desmoid tumour were distributed to all participants for them to stain with the requested antibodies (CD117 and DOG-1) using their routine protocol. The circulated tissue was sequential sections from the same tissue block used for both the requested antibodies.
Assessment was carried out around a multi-header microscope with each slide being assessed by 4 independent assessors and a microscope lead. All 3 samples on the slide were viewed, and then a final overall score out of 5 was given by each individual assessor. These four scores were then combined to give an overall score out of 20.

Assessment Summary:

Results from the **CD117** assessment showed acceptable pass, borderline pass and fail rates of 83%, 13% and 4% respectively. These results were slightly higher than the previous assessment (Run 111), where 78% of participants achieved an acceptable pass. Similarly to previous assessment runs, weak demonstration of staining in the GIST tumour was the main reason for a failed result (scores of ≤9/20) or a borderline result (scores of 10-12/20). Background and non-specific staining, particularly in the desmoid, tumour were also reasons why labs were allocated lower marks. It became apparent during the assessment that an inappropriate antibody dilution factor or antigen retrieval protocol was the main cause of sub-optimal results. The Dako polyclonal CD117 antibody remains the most popular choice of antibody: In this assessment, 82% (N=92) of labs used the Dako polyclonal CD117 antibody, which showed an acceptable pass rate of 85%. The Ventana 9.7 clone was the next most popular antibody, used by 11 (10%) of labs. This antibody showed a pass rate of 64%.

The **DOG-1** antibody was chosen as the secondary antibody for the Run 112 assessment. Slightly fewer labs (N=101) participated in the DOG-1 assessment, however, the results for this antibody showed similar pass rates to that of the CD117: 80% achieved an acceptable pass, a further 16% of labs received a borderline mark, and 4 labs (4%) failed the assessment. Again, weak staining was the main reason for failed or borderline passes. The Leica K9 clone was the most popular antibody, used by 69% (N=70) labs, and this showed a pass rate of 84%. The Ventana SP31 clone was the next most popular DOG-1 antibody, used by 14 labs (14%), and showed an acceptable pass rate of 57%. For the in-house samples, most labs provided composite slides including either normal appendix and a GIST or a GIST with normal areas of epithelium. This was very encouraging to see labs using ideal control material. The standard of staining on the in-house tissue was similar to that seen on the UK Neqas distributed material.

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. Hasegawa T et al, Gastrointestinal stromal tumors: consistent CD117 immunostaining for diagnosis, and prognostic classification based on tumor size & MIB-1 grade. Hum Pathol. 2002 Jun;33(6):669-76.
5. Rudolph et al, Gastrointestinal mesenchymal tumors - immunophenotypic classification and survival analysis. 2002 Sep;441(3):238-48. Epub 2002 Jul 6.
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

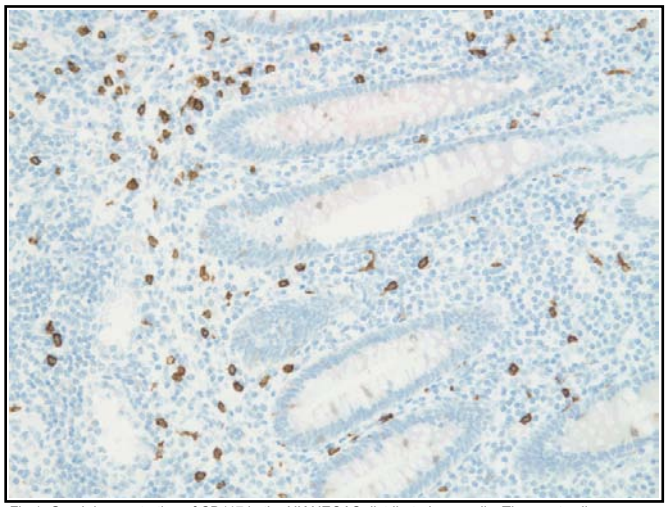


Fig 1. Good demonstration of CD117 in the UK NEQAS distributed appendix. The mast cells show distinct membranous staining, while the background remains clean. Stained with the Dako polyclonal antibody, 1:200, on the Dako Autostainer with antigen retrieval in the PT link for 20 mins, FLEX detection kit.

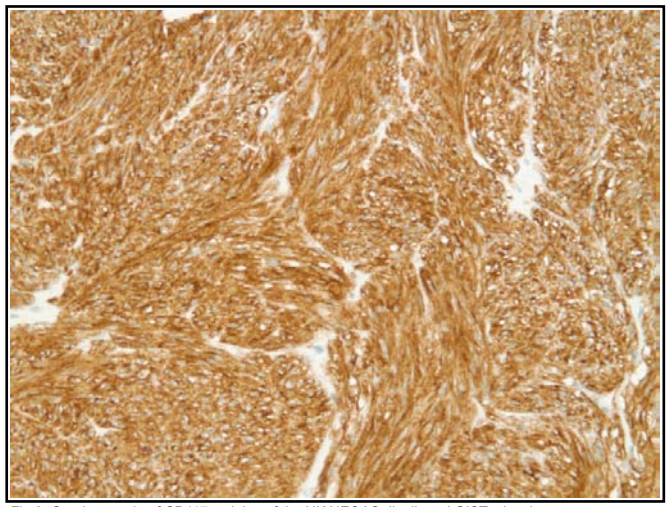


Fig 2. Good example of CD117 staining of the UK NEQAS distributed GIST, showing strong cytoplasmic and membranous staining in the tumour cells. Section stained with the Ventana pre-diluted 9.7 clone antibody on the Ventana ULTRA with CC1 pre-treatment for 64 minutes, Optiview detection kit.

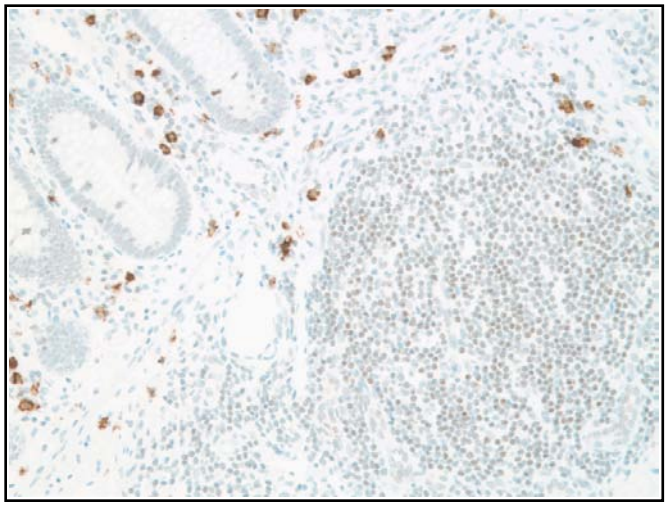


Fig 3. Sub-optimal demonstration of CD117 in the UK NEQAS distributed appendix. Although the mast cells are staining as expected, there is also non-specific staining of the lymphocytes. Section stained with the Ventana pre-diluted 9.7 clone antibody on the Ventana Benchmark XT with CC1 pre-treatment for 32 minutes and Optiview detection kit.

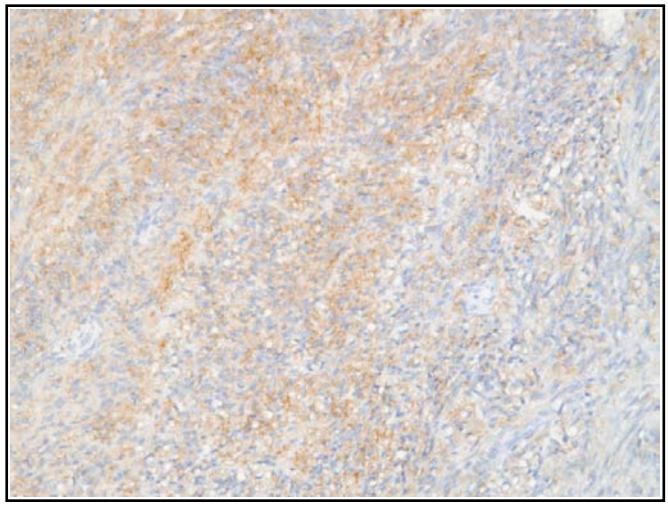


Fig 4. Poor CD117 staining in the UK NEQAS distributed GIST section (compare to Fig 2). The staining is very weak and patchy. The appendix section on the same slide was also negative in the mast cells which are expected to be positive. Stained with the Dako polyclonal antibody, 1:50, no pre-treatment on the Autostainer.

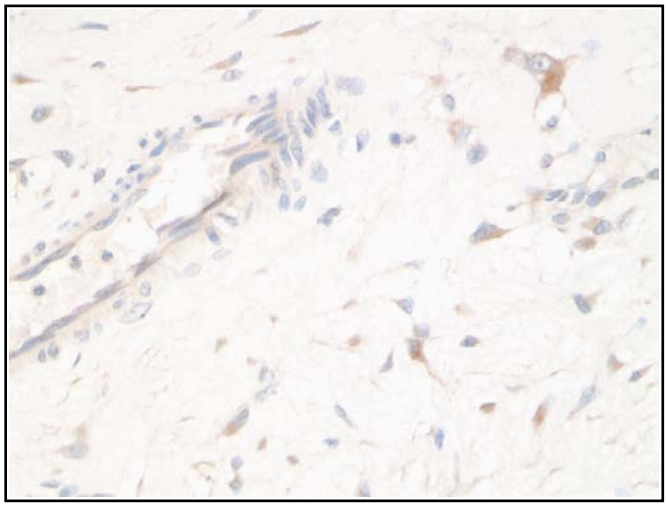


Fig 5. Sub-optimal CD117 staining in the UK NEQAS distributed desmoid tumour. The example shows excessive background and non-specific staining. The slide was stained using the Dako polyclonal antibody, on the Leica Bond III with ER2 antigen retrieval for 20 minutes.

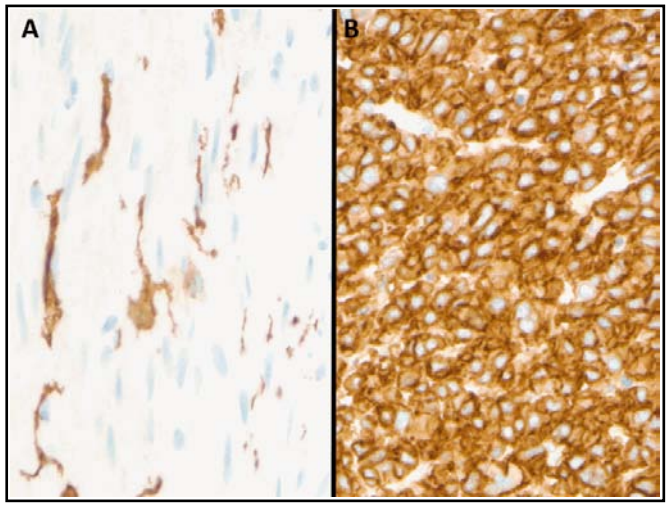


Fig 6. Good example of an in house control submitted for assessment of CD117: The section showed good strong staining of the Cells of Cajal in the muscle layer (A) as well as strong distinct staining in the GIST (B). Stained with the Dako polyclonal antibody, 1:300, on the Ventana ULTRA , CC1 standard pre-treatment.

Selected Images showing Optimal and Sub-optimal Immunostaining

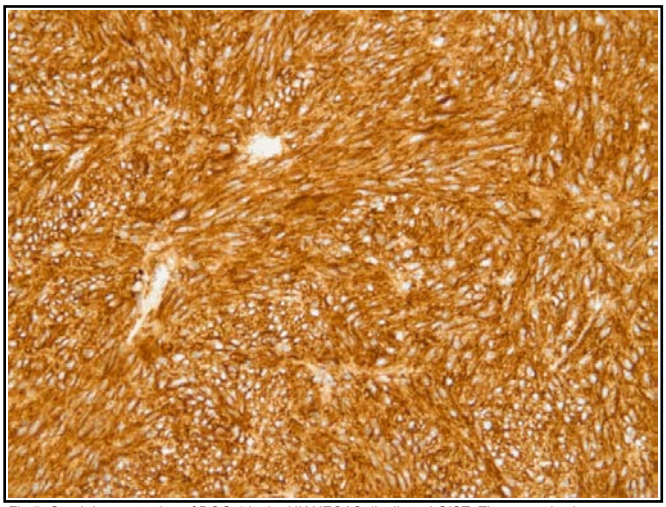


Fig 7. Good demonstration of DOG-1 in the UK NEQAS distributed GIST: The example shows strong crisp staining in the tumour cells. Section stained with the Leica K9 antibody, 1:20, on the Dako Autostainer with pre-treatment in the PT link, high pH buffer for 20 minutes.

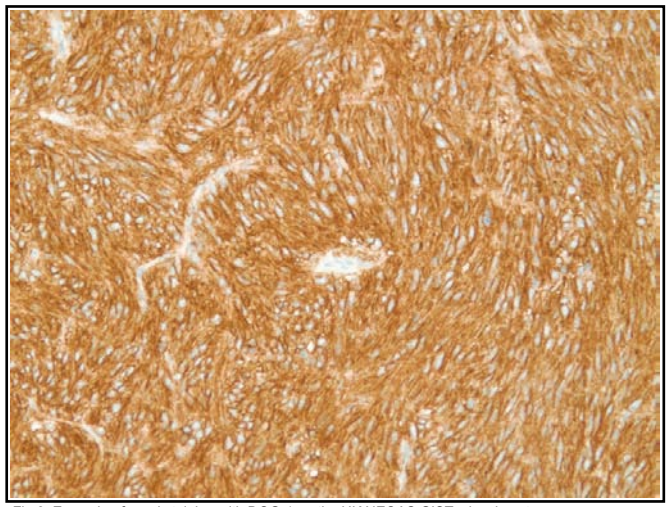


Fig 8. Example of good staining with DOG-1 on the UK NEQAS GIST, showing strong expression in the tumour cells. Stained with the Leica K9 antibody, 1:50, on the Ventana ULTRA with CC1 pre-treatment for 64 minutes and UltraView detection kit.

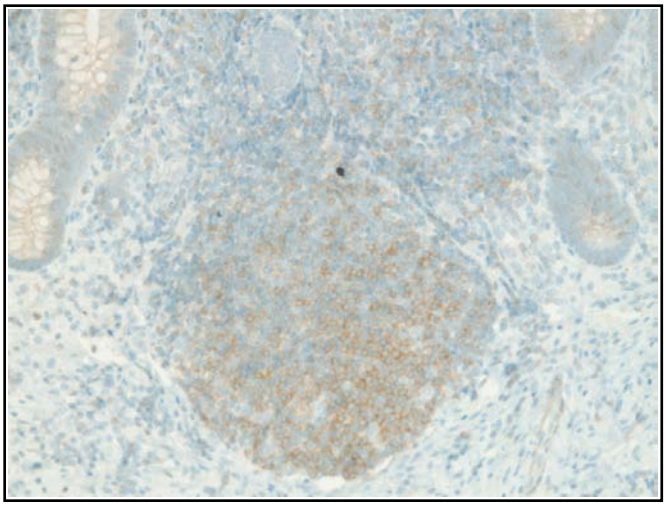


Fig 9. Sub-optimal demonstration of DOG-1 in the UK NEQAS distributed appendix. This tissue should be negative with DOG-1, but the example shows non-specific staining in the lymphocytes. Stained with the Ventana SP31 pre-diluted antibody on the Benchmark XT with CC1 retreival for 56 minutes.

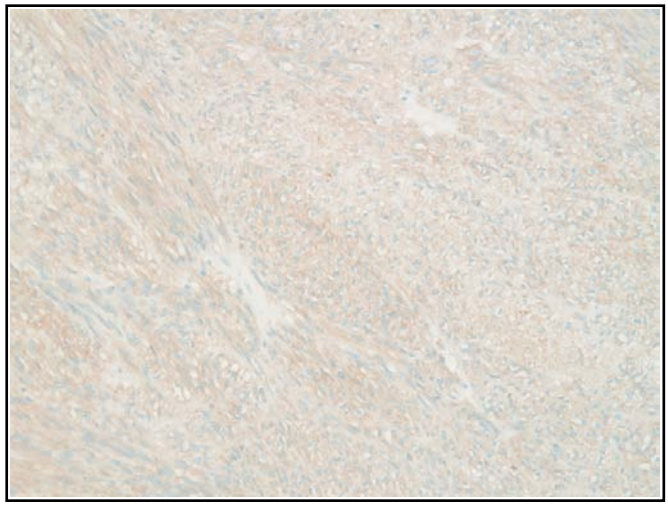


Fig 10. Poor demonstration of DOG-1 in the UK NEQAS GIST (compare to Figs 7&8). The staining is much weaker than expected, most likely due to insufficient antigen retrieval. Section stained with the Ventana SP31 pre-diluted antibody on the ULTRA and pre-treatment with CC1 for 32 minutes.

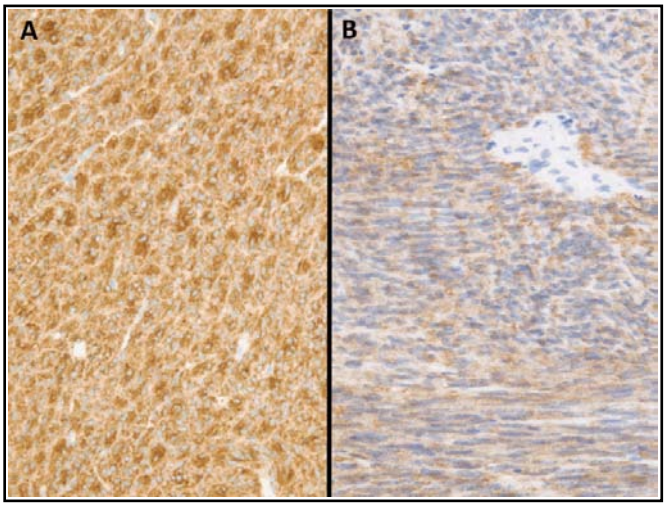


Fig 11. Two examples of sub-optimal staining in the UK NEQAS GIST. (A) shows heat artefact possibly caused by excessive antigen retrieval. In (B) the staining is weak and diffuse; stained with the Leica K9 antibody (no dilution) provided.

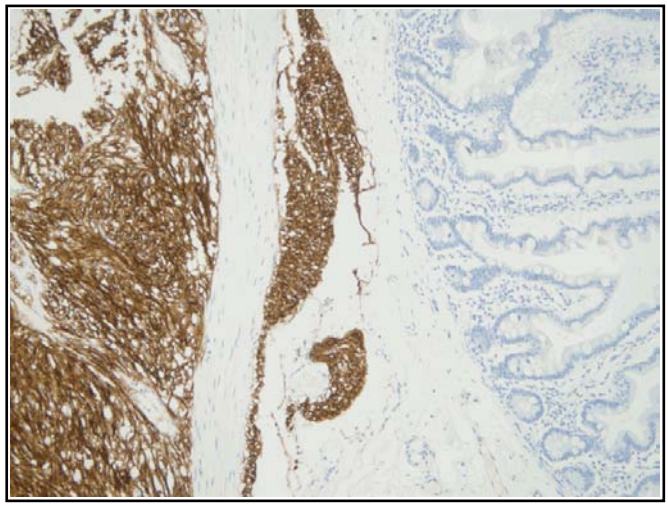
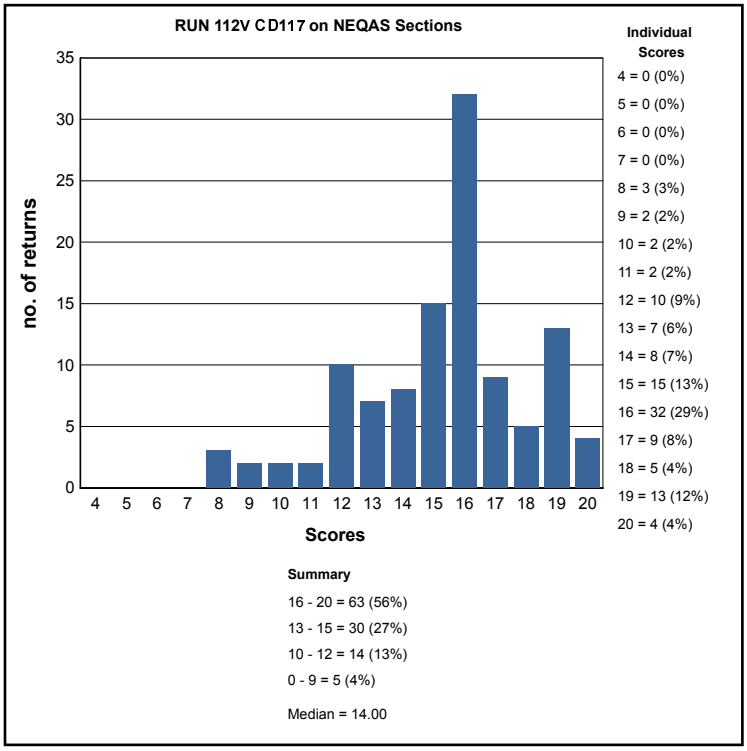
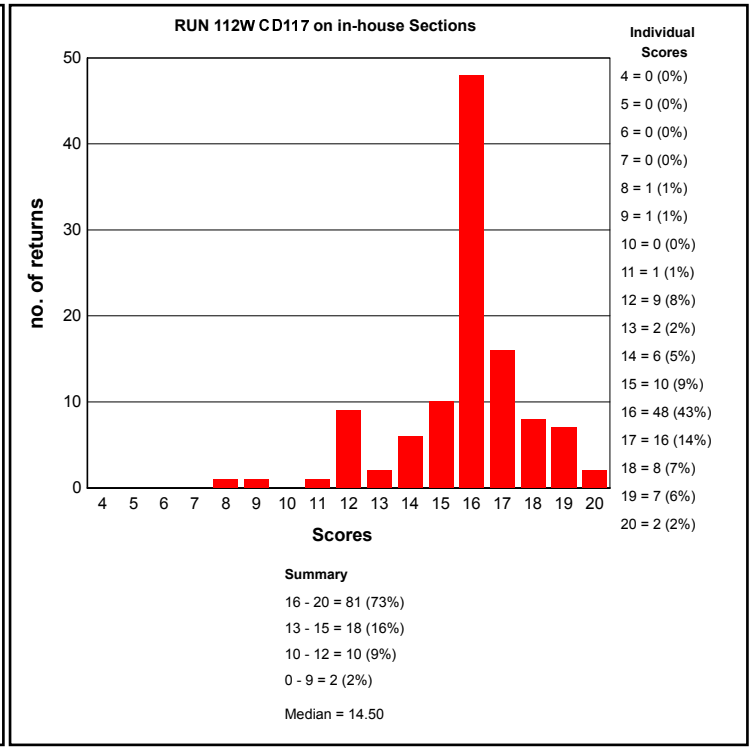
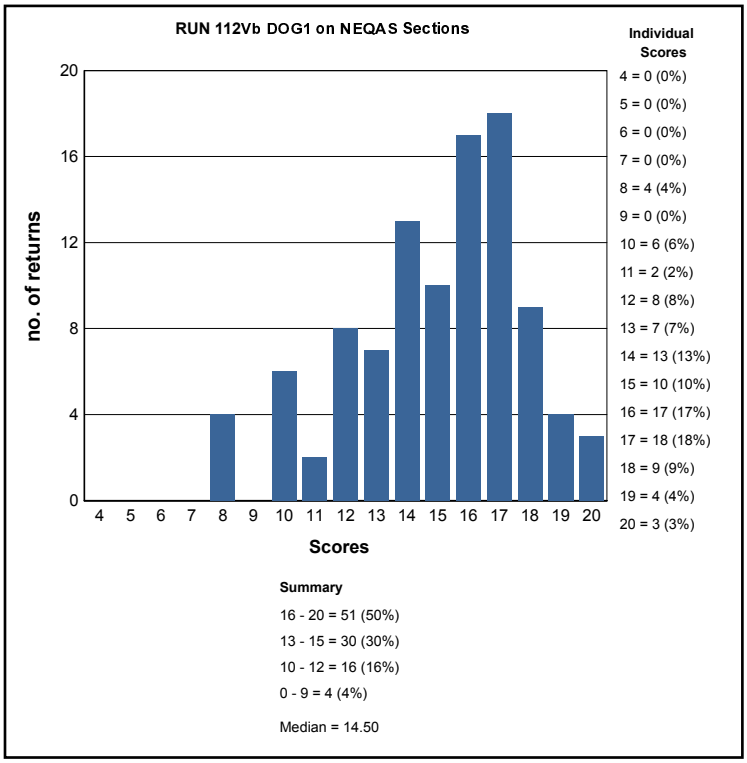


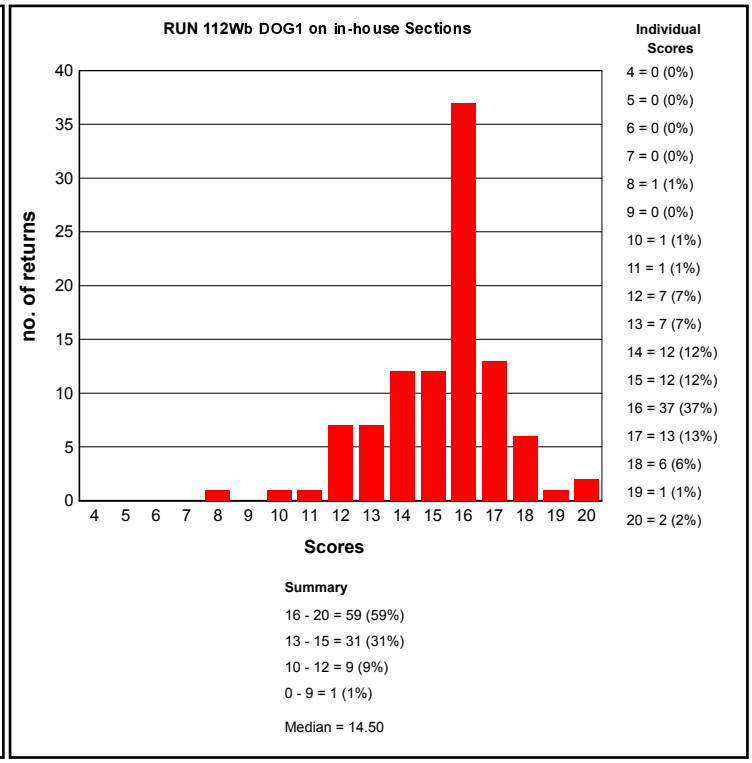
Fig 12. Good example of the in-house control stained with DOG-1. The section includes both normal epithelium and a strongly stained GIST. The background is also clean. Stained with the Leica K9 antibody, 1:50, on the BondMax and ER1 retrieval for 20 minutes.

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: 112

Primary Antibody : CD117

Antibody Details	N	%
Cell Marque 117R/S-xx (YR145)	4	100
Dako A4502 (rb poly)	92	85
Epitomics AC-0029 (EP10)	1	100
Leica/Novocastra NCL-CD117 (T595)	1	100
Other	2	100
Spring Bioscience M3264 (SP26)	1	0
Ventana 790-2951 (9.7)	11	64

Alimentary Tract Pathology Run: 112

Primary Antibody : DOG1

Antibody Details	N	%
Abcam TMEM16A (ab53212)	1	100
Biocare CM 385 (1.1)	1	0
Cell Marque 244R-14/15/16 (SP31)	2	100
Cell Marque 244R-17/18 (SP31)	3	67
Leica NCL-L-DOG-1 (K9)	52	85
Leica PA0219 (K9)	18	83
Menarini MP-385-CM01/1	1	100
Other	4	100
Spring Biosciences M3311 (SP31)	1	100
Thermo RM-9132-R7 (SP31)	1	100
Ventana (SP31) 760-4590	14	57

Alimentary Tract Pathology Run: 112

Heat Mediated Retrieval	CD117 N	CD117 %	DOG1 N	DOG1 %
Dako Omnis	0	0	1	100
Dako PTLink	13	100	12	83
Lab vision PT Module	1	100	1	100
Leica ER1 10 mins	1	100	0	0
Leica ER1 20 mins	5	80	7	86
Leica ER1 30 mins	3	33	0	0
Leica ER2 10 mins	1	0	0	0
Leica ER2 20 mins	18	100	21	81
Leica ER2 30 mins	9	100	6	100
None	4	50	1	100
Pressure Cooker	1	100	1	100
Steamer	0	0	1	0
Ventana CC1 16mins	0	0	1	100
Ventana CC1 24mins	0	0	2	100
Ventana CC1 32mins	7	86	6	67
Ventana CC1 36mins	7	29	4	50
Ventana CC1 40mins	2	50	0	0
Ventana CC1 48mins	3	67	1	100
Ventana CC1 52mins	1	100	1	0
Ventana CC1 56mins	4	100	3	100
Ventana CC1 64mins	9	89	11	73
Ventana CC1 88mins	1	100	1	100
Ventana CC1 8mins	0	0	1	100
Ventana CC1 mild	8	75	5	60
Ventana CC1 standard	14	86	11	82
Ventana CC2 32mins	0	0	1	100

Alimentary Tract Pathology Run: 112

Enzyme Mediated Retrieval	CD117 N	CD117 %	DOG1 N	DOG1 %
AS PER KIT	2	100	1	100
NOT APPLICABLE	70	80	60	77
Other	1	100	0	0

Alimentary Tract Pathology Run: 112	CD117		DOG1	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	7	86	8	88
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)	0	0	1	0
Dako EnVision FLEX+ (K8002/12)	7	100	7	86
Dako Envision+ HRP mouse K4004/5/6/7	0	0	1	0
Dako Envision+ HRP rabbit K4008/9/10/11	1	100	0	0
Leica Bond Intense R Detection (DS9263)	0	0	1	100
Leica Bond Polymer Refine (DS9800)	36	89	31	84
None	0	0	1	100
Other	4	75	3	100
Ventana iView system (760-091)	2	50	1	100
Ventana OptiView Kit (760-700)	21	81	15	87
Ventana UltraView Kit (760-500)	32	75	27	74

Alimentary Tract Pathology Run: 112	CD117		DOG1	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	1	100	1	100
Dako Autostainer Link 48	12	100	10	80
Dako Autostainer plus	1	100	1	100
Dako Autostainer Plus Link	2	50	2	100
Dako Omnis	0	0	1	100
LabVision Autostainer	0	0	1	0
Leica Bond Max	12	83	13	85
Leica Bond-III	26	92	22	86
Ventana Benchmark GX	1	100	1	100
Ventana Benchmark ULTRA	32	75	28	71
Ventana Benchmark XT	25	76	18	78

Alimentary Tract Pathology Run: 112	CD117		DOG1	
Chromogen	**N**	**%**	**N**	**%**
A. Menarini Liquid Stable DAB kit	1	100	0	0
AS PER KIT	17	94	14	93
BioGenex liquid DBA (HK-124-7K)	0	0	1	100
Dako EnVision Plus kits	2	100	2	100
Dako FLEX DAB	8	88	8	63
Leica Bond Polymer Refine kit (DS9800)	32	88	31	87
NOT APPLICABLE	1	100	1	0
Other	5	100	4	100
Ventana DAB	10	70	7	86
Ventana iview	2	50	1	100
Ventana Ultraview DAB	34	74	30	67

BEST METHODS - Gold Standard Antibody

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

CD117 - Method 1

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

Primary Antibody: Dako A4502 (rb poly)
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)

CD117 - Method 2

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

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

CD117 - Method 3

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

Primary Antibody:	Dako A4502 (rb poly) , 60 Mins, 37 ºC Dilution 1: 500
Automation:	Ventana Benchmark XT
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 88mins, Buffer: CC1
EAR:	
Chromogen:	AS PER KIT, 37 ºC., Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 37 ºC Prediluted

CD117 - Method 4

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

Primary Antibody:	Ventana 790-2951 (9.7) , 32 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	
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

DOG1 - Method 1

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

Primary Antibody:	Leica NCL-L-DOG-1 (K9) , 15 Mins, 37 ºC Dilution 1: 1:00
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800), RT ºC., Time 1: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, RT ºC Prediluted

DOG1 - Method 2

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

Primary Antibody:	Leica PA0219 (K9) Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 56mins
EAR:	
Chromogen:	Other
Detection:	Ventana OptiView Kit (760-700) Prediluted

DOG1 - Method 3

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

Primary Antibody:	Leica NCL-L-DOG-1 (K9) , 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

DOG1 - Method 4

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

Primary Antibody:	Leica NCL-L-DOG-1 (K9) , 20 Mins, 21 ºC Dilution 1: 20
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	AS PER KIT
HMAR:	Dako PTLink, Buffer: Dako High pH TRS
EAR:	
Chromogen:	AS PER KIT, 21 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	AS PER KIT , 20 Mins, 21 ºC Prediluted

Lynch Syndrome/HNPCC Run 112

Keith Miller and Suzanne Parry

	Gold Standard	Second Antibody
Antigens Assessed:	MLH1	PMS2
Tissue Sections circulated:	Normal Appendix & 2 Colonic Tumours	Normal Appendix & 2 Colonic Tumours
Number of Registered Participants:	85	
Number of Participants This Run:	79 (93%)	

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 immunopositivity 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 immunopositivity in tumour cell nuclei can be directly compared with the strong nuclear staining intensity in well fixed adjacent normal epithelium or lymphoid cells or stromal cells. Artefactually, distorted staining patterns due to poor fixation (affecting both stromal cells as well as tumour cells) in central parts of the tumour are irrelevant. It

should be borne in mind that geneticists may use the immunohistochemical expression pattern of MMR proteins to interpret mutations of uncertain significance, and that both false -negative and false-positive staining (or interpretation) may potentially cause misinterpretation of DNA sequence findings. This could result in a mutation being interpreted as pathogenic when it is, in fact, neutral in effect, and thus result in inappropriate predictive genetic testing being offered to the family. Hence, care is needed in both staining and interpretation.

Assessment Procedure:

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

Features of Optimal Immunostaining: (Figs 1, 2, 3, 4, 7, 8 &10)

Appendix: (Figs 1, 3, 7 & 8)

- 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 2B, 4B & 10B)

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

Tumour with loss of MMR protein: (Figs 2A, 4A & 10A)

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

Features of Sub-optimal Immunostaining: (Figs 5, 6, 9, 11 & 12)

Appendix: (Figs 5 & 9)

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

Tumour without loss of MMR protein: (Fig 6)

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

Tumour with loss of MMR protein: (Figs 11 & 12)

- 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 **MLH1** assessment were higher than the previous time this antibody was assessed, with 73% of labs achieving an acceptable pass, and a further 21% of participants receiving a borderline score (10-12/20). The lower scores were mostly caused by weak staining, however, 2 labs failed the assessment due to inappropriate false positive staining in the colonic tumour with loss of MLH1, which should have been negative for MLH1. Similarly to the recent previous assessments for MLH1 , the Ventana M1 clone was the most popular choice of antibody. This was used by 37% of participating labs in this assessment and showed an acceptable pass rate of 93%. The Novocastra/Leica and Dako ESO5 antibody clones were also popular, and these showed pass rates of 57% and 63% respectfully.

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

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4. Frayling IM, Happerfield L, Mattocks C, Oakhill K, Arends MJ. Application of Molecular Diagnostics to Hereditary Nonpolyposis Colorectal Cancer (chapter 32). In: "Molecular Diagnostics, For the Clinical Laboratorian" (2nd Edition). Eds: Coleman WB & Tsongalis GJ. Humana Press Inc., NJ. 2005. ISBN: 1-59259-928-1, ISBN13: 978-1-58829-356-5; ISBN10: 1-58829-356-4. pp 375 – 392.
5. Poulogiannis G, Frayling IM, Arends MJ. DNA Mismatch Repair Deficiency in Sporadic Colorectal Cancer and Lynch Syndrome. *Histopathology* 2010; 56: 167 - 179.a.
6. Vasen F, et al. Revised guidelines for the clinical management of Lynch syndrome (HNPCC): recommendations by a group of European experts. Gut 2013;62: 812-823.

Selected Images showing Optimal and Sub-optimal Immunostaining

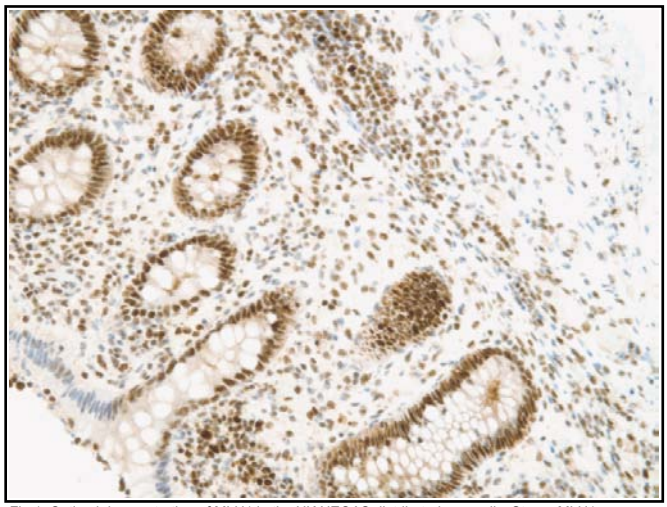


Fig 1. Optimal demonstration of MLH1 in the UK NEQAS distributed appendix. Strong MLH1 staining of the epithelial and stromal cells, with fading of staining intensity towards the luminal surface of the epithelial crypts. Stained with the Dako RTU ES05 antibody on the Autostainer with antigen retrieval in the PT link in high pH buffer.

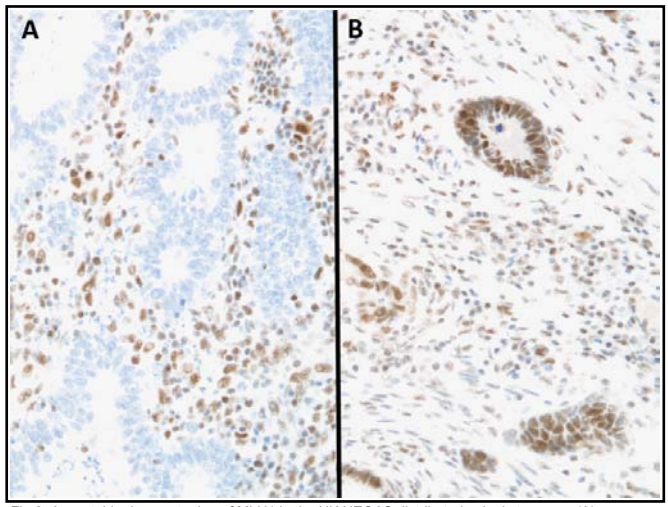


Fig 2. Acceptable demonstration of MLH1 in the UK NEQAS distributed colonic tumours. (A) with loss of MLH1 and (B) with normal expression of MLH1 show the expected staining pattern. Both sections also show staining in the stromal cells and lymphocytes, which act as an internal tissue control. (Same protocol as Fig 1).

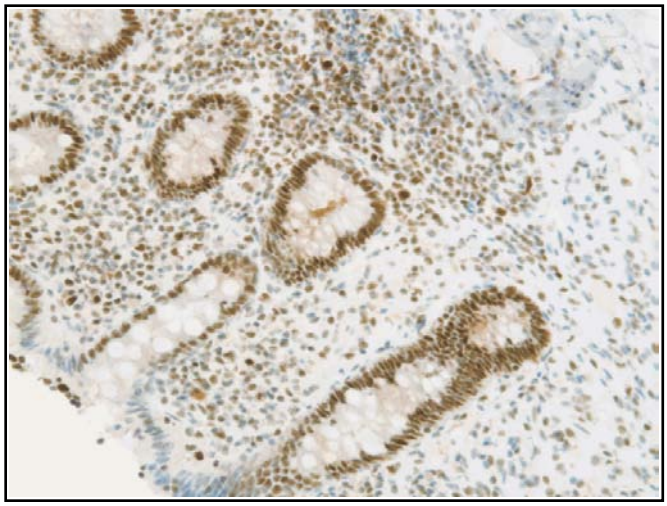


Fig 3. Good demonstration of MLH1 in the UK NEQAS distributed appendix. As with Fig 1 there is strong MLH1 staining of the epithelial stromal cells, with fading of staining intensity towards the luminal surface. Staining using the Novocastra ES05 antibody, 1:100, on the Dako Autostainer with pre-treatment in the PT link in high pH buffer for 20 minutes.

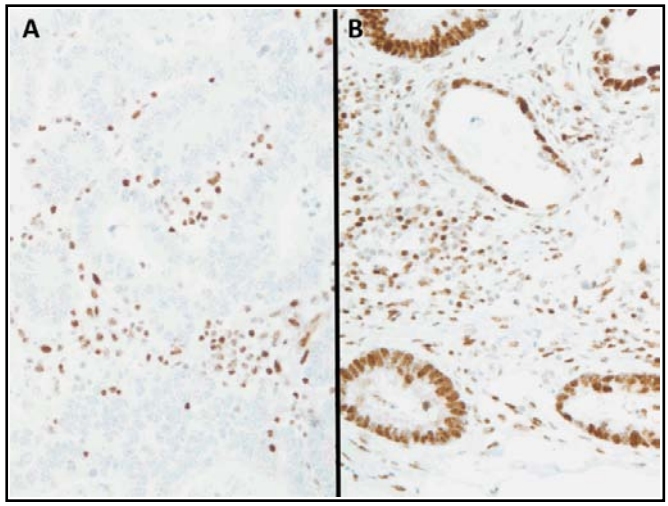


Fig 4. Good demonstration of MLH1 in the UK NEQAS distributed colonic tumours. As expected, tumour (A) is negative, while tumour (b) is positive for MLH1. Both sections show good strong staining of the intratumoural lymphocytes and stromal cells. Stained with the Ventana M1 antibody, CC1 antigen retrieval for 64 minutes and Optiview detection.

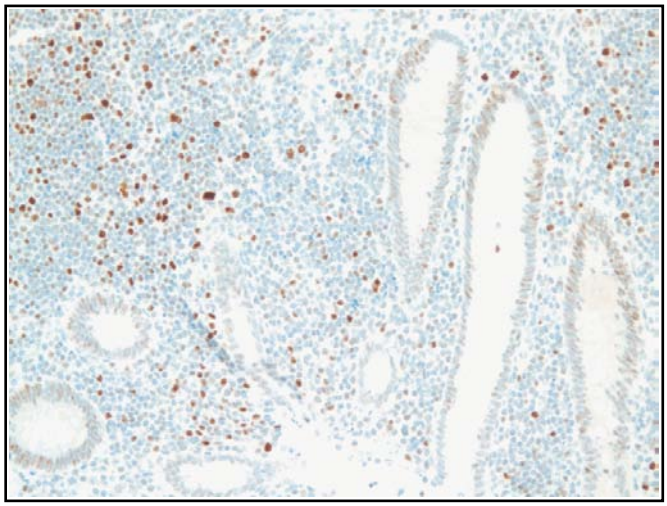


Fig 5. Weak demonstration of MLH1 in the UK NEQAS distributed appendix (compare to Figs 1&3). Although some lymphocytes are stained, the epithelial crypts are much weaker than expected. However, this was not a fail at assessment. Stained with the Dako ES05 antibody, 1:50, on the Ventana Benchmark XT with CC1 retrieval for 64 minutes.

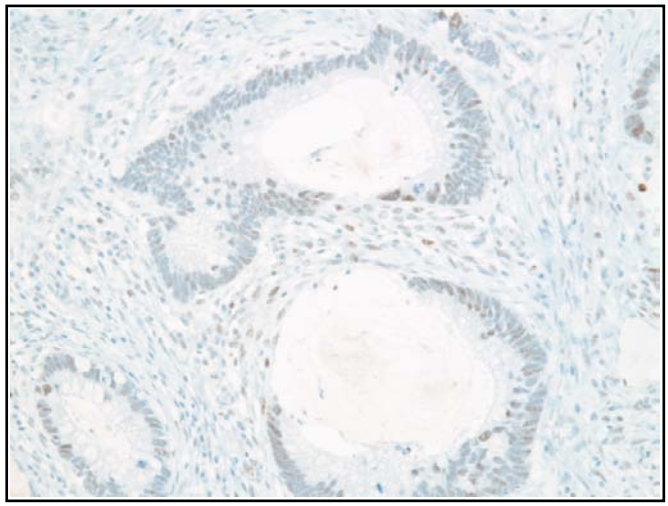


Fig 6. Poor demonstration of MLH1 on the UK NEQAS distributed colonic tumour with normal expression of MLH1. The tumour cells should be positive, however the staining in the example is very weak, with many tumour cells not staining at all. There is also very little staining of the stromal cells and lymphocytes. Stained with the Leica ES05 antibody, 1:100, on the Ventana

Selected Images showing Optimal and Sub-optimal Immunostaining

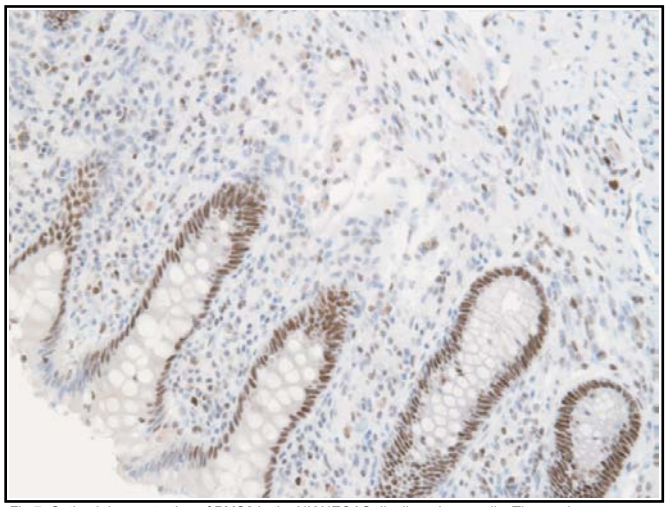


Fig 7. Optimal demonstration of PMS2 in the UK NEQAS distributed appendix. The section shows strong PMS2 staining of the epithelial and stromal cells, with fading of staining intensity towards the luminal surface of the epithelial crypts. Stained with the Dako EP51 antibody, 1:40, on the Leica bond III with ER2 antigen retrieval for 40 minutes.

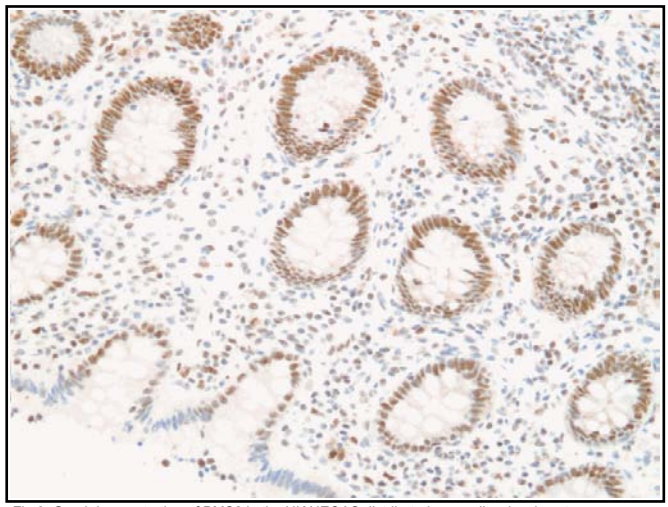


Fig 8. Good demonstration of PMS2 in the UK NEQAS distributed appendix, showing strong staining of the epithelial cells, which fades in intensity towards the luminal surface of the crypts. Stained with the Dako EP51 antibody, 1:80, on the Autostainer with pre-treatment in the PT link in high pH buffer for 20 minutes.

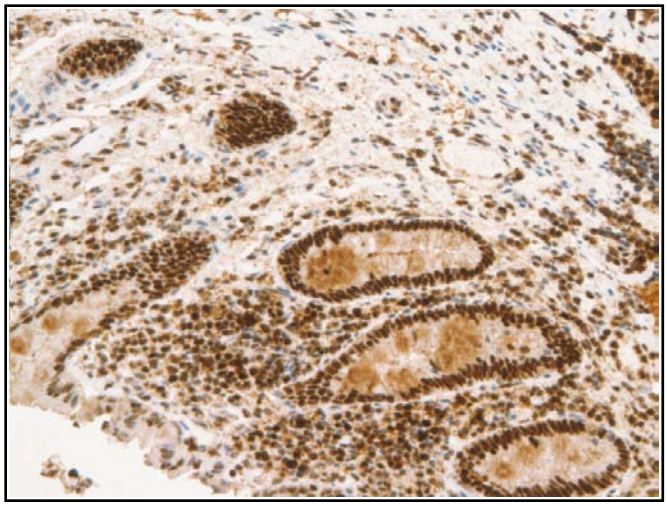


Fig 9. Unacceptable demonstration of PMS2 on the UK NEQAS distributed appendix. The image shows excessive staining and background staining. This is most likely to be caused by over antigen retrieval. Stained using the Ventana EPR3947 pre-diluted antibody on the Benchmark GX with CC1 antigen retrieval for 88 minutes.

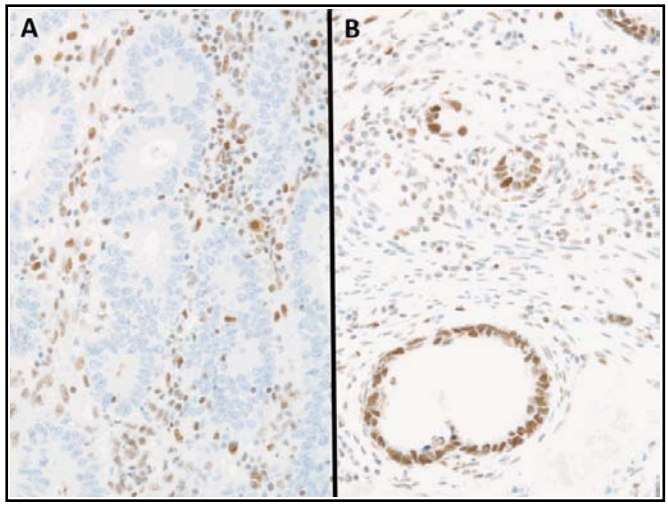


Fig 10. Optimal demonstration of PMS2 on the UK NEQAS distributed PMS2 colonic tumours: Both the negative tumour (A) and the positive tumour (B) show the expected level of staining. The intratumoural lymphocytes and stromal cells are staining as expected. (Same protocol as Fig 8).

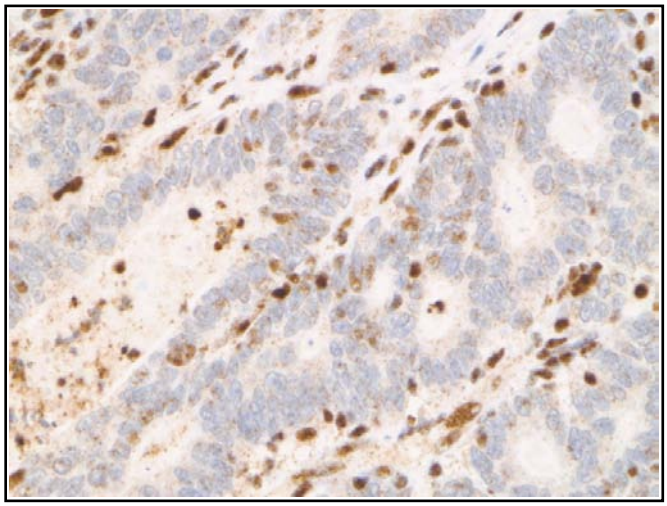


Fig 11. Unacceptable demonstration of PMS2 in the UK NEQAS distributed tumour with loss of PMS2. The section shows non-specific staining of some tumour cells which should be negative. This was most likely caused by excessive antigen retrieval. (Same protocol as Fig 9).

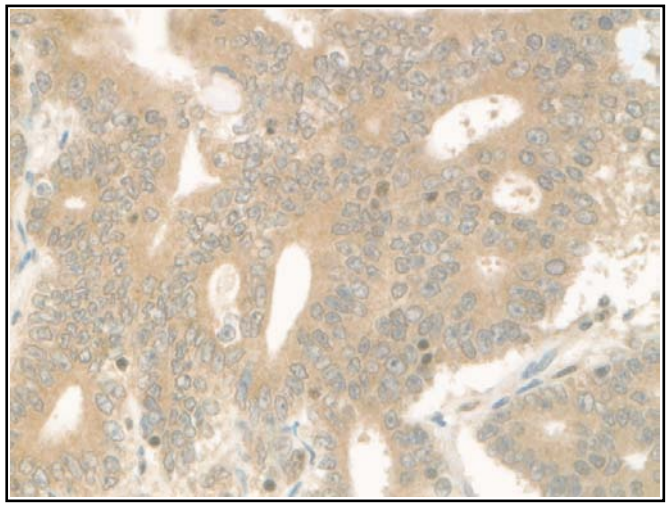
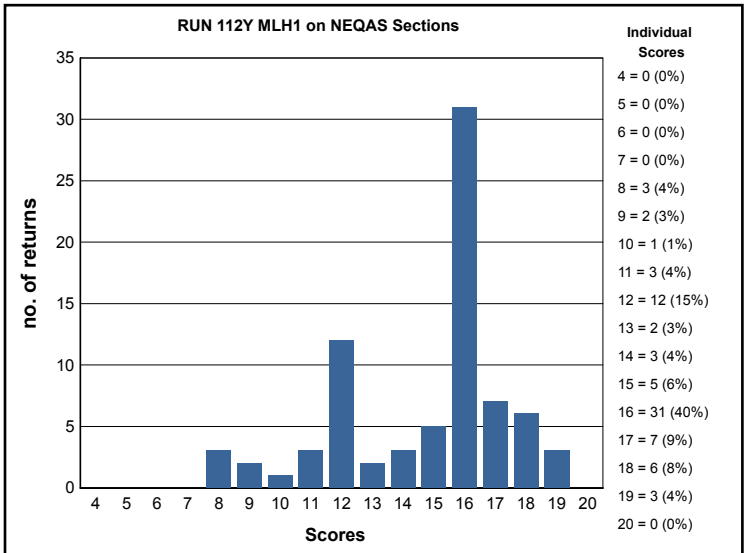
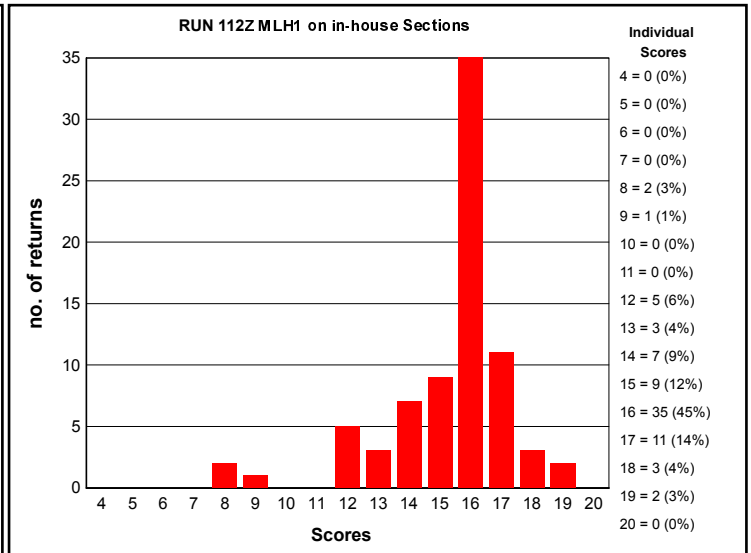
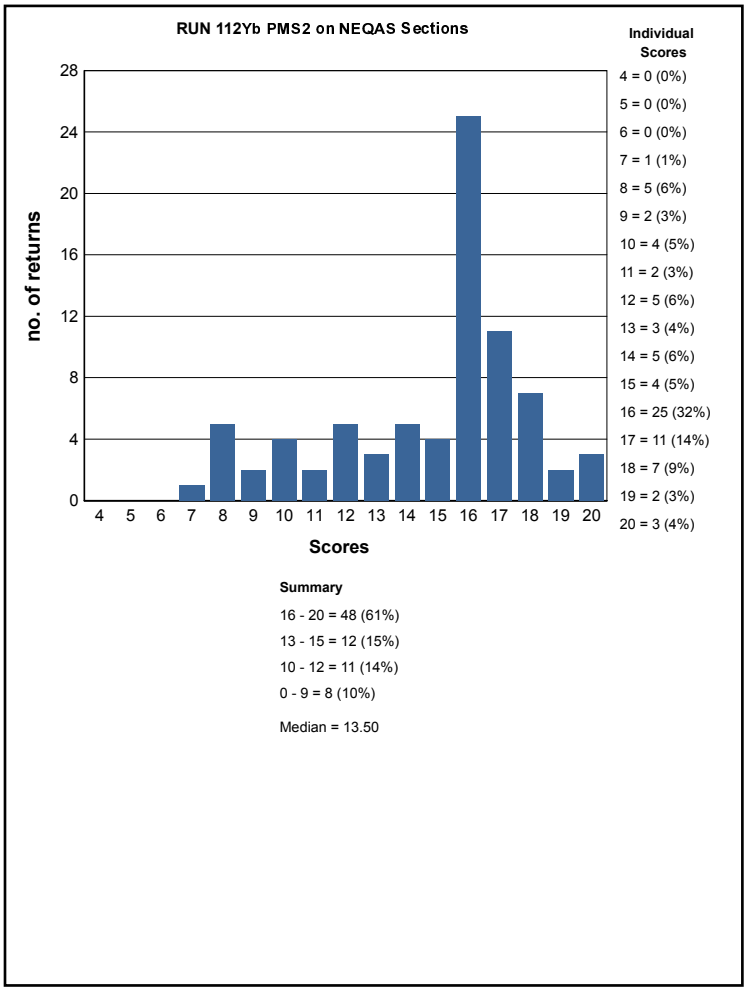


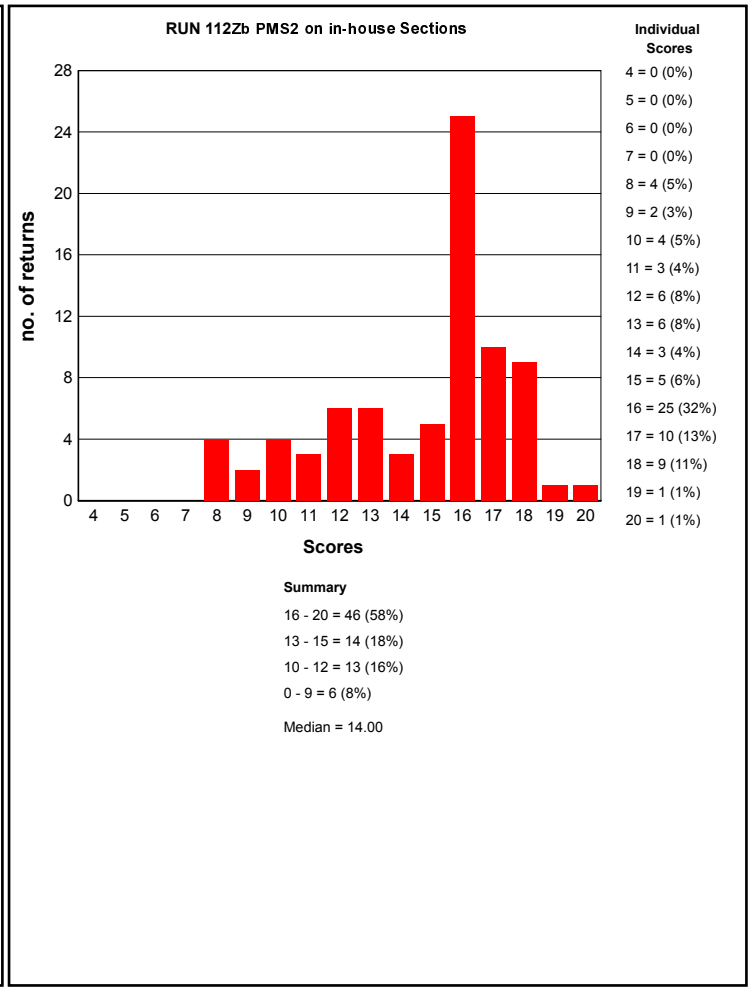
Fig 12. Sub-optimal staining in the UK NEQAS distributed tumour with loss of PMS2: Although the tumour is negative as expected, the section shows excessive cytoplasmic staining. Again, excessive antigen retrieval is the most likely cause of the inappropriate staining. Stained with Pharmingen 51-1327 GR antibody, 1:100, on the Leica BondMax, ER2 for 40 minutes.

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: 112

Primary Antibody : MLH1

Antibody Details	N	%
BD Pharmingen (G168-15)	8	38
BD Pharmingen (G168-728)	1	100
Biocare medical CM/PM 220 (G168-15)	1	100
Dako Flex RTU IR079/IS079 (ES05)	11	64
Dako M3640 (ES05)	8	63
Leica Bond RTU PA0610 (ES05)	2	50
Novocastra NCL-L-MLH1 (ES05)	16	63
Other	2	100
Ventana 790-4535 (M1)	29	93

Primary Antibody : PMS2

Antibody Details	N	%
BD Bio/Pharmingen 556415 (A16-4)	15	80
Cell Marque 288M -16 (MRQ28)	2	50
Cell Marque 288R -17/18 (EPR3947)	2	100
Dako M3647 (EP51)	15	93
Dako RTU FLEX IR087 (EP51)	10	70
Leica/Novoca NCL-L-PMS2 (MOR4G)	2	0
Other	1	100
Ventana 760-4531 (EPR3947)	32	72

HNPCC Run: 112

Heat Mediated Retrieval	MLH1 N	MLH1 %	PMS2 N	PMS2 %
Biocare Decloaking Chamber	1	100	1	100
Dako PTLink	9	89	8	88
Lab vision PT Module	1	100	1	100
Leica ER1 20 mins	3	0	0	0
Leica ER1 30 mins	1	0	0	0
Leica ER2 20 mins	6	50	9	78
Leica ER2 30 mins	8	63	7	57
Leica ER2 40 mins	8	88	10	90
None	0	0	1	100
Other	1	100	0	0
Steamer	0	0	1	100
Ventana CC1 32mins	5	80	0	0
Ventana CC1 36mins	1	100	0	0
Ventana CC1 40mins	4	75	1	100
Ventana CC1 48mins	3	67	4	50
Ventana CC1 56mins	2	100	1	100
Ventana CC1 64mins	15	73	11	91
Ventana CC1 72mins	0	0	2	100
Ventana CC1 80mins	1	100	2	50
Ventana CC1 88mins	1	0	2	50
Ventana CC1 92mins	2	100	9	78
Ventana CC1 extended	0	0	1	100
Ventana CC1 standard	7	71	4	25
Ventana CC2 64mins	0	0	1	0
Ventana CC2 80mins	0	0	1	100
Ventana CC2 92mins	0	0	1	100
Ventana CC2 extended	0	0	1	0

HNPCC Run: 112

Enzyme Mediated Retrieval	MLH1 N	MLH1 %	PMS2 N	PMS2 %
AS PER KIT	2	50	1	100
NOT APPLICABLE	37	68	37	84

HNPCC Run: 112	MLH1		PMS2	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	3	100	3	100
Biocare polymer (M4U534)	1	100	1	100
Dako EnVision FLEX (K8000/10)	0	0	1	100
Dako EnVision FLEX+ (K8002/12)	7	86	4	100
Dako Envision+ HRP mouse K4004/5/6/7	1	100	1	100
Leica Bond Polymer Define (DS9713)	0	0	1	0
Leica Bond Polymer Refine (DS9800)	25	60	24	83
None	0	0	2	50
Other	2	50	2	50
Ventana OptiView (760-700) + Amp. (7/860-099)	2	100	3	100
Ventana OptiView Kit (760-700)	29	72	29	79
Ventana UltraView Kit (760-500)	8	75	6	0

HNPCC Run: 112	MLH1		PMS2	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	0	0	1	100
Dako Autostainer Link 48	7	86	6	83
Dako Autostainer plus	2	100	2	100
Dako Autostainer Plus Link	1	100	2	100
LabVision Autostainer	0	0	1	100
Leica Bond Max	11	36	9	67
Leica Bond-III	15	80	17	82
Menarini - Intellipath FLX	1	100	1	100
None (Manual)	1	0	0	0
Ventana Benchmark GX	1	100	1	0
Ventana Benchmark ULTRA	25	76	27	74
Ventana Benchmark XT	14	71	12	67

HNPCC Run: 112	MLH1		PMS2	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	15	73	16	81
Dako EnVision Plus kits	2	100	2	50
Dako FLEX DAB	5	80	6	100
Leica Bond Polymer Refine kit (DS9800)	25	64	24	83
Other	6	67	8	75
Ventana DAB	14	79	14	86
Ventana Ultraview DAB	12	75	9	22

BEST METHODS - Gold Standard Antibody

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

MLH1 - Method 1

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

Primary Antibody: Novocastra NCL-L-MLH1 (ES05) , 15 Mins Dilution 1: 1/40
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 40 mins
EAR:
Chromogen: Leica Bond Polymer Refine kit (DS9800), Time 1: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins

MLH1 - Method 2

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

Primary Antibody: Novocastra NCL-L-MLH1 (ES05) , 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

MLH1 - Method 3

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

Primary Antibody:	Dako Flex RTU IR079/IS079 (ES05) , 30 Mins, 20 ºC Prediluted
Automation:	Dako Autostainer Plus Link
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: high pH target retrieval solution
EAR:	
Chromogen:	Dako FLEX DAB, 20 ºC., Time 1: 10 Mins, Time 2: 10 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 30 Mins, 20 ºC Prediluted

MLH1 - Method 4

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

Primary Antibody:	Ventana 790-4535 (M1) , 16 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 56mins, Buffer: CC1
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

BEST METHODS - Secondary Antibody

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

PMS2 - Method 1

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

Primary Antibody:	Dako M3647 (EP51) , 15 Mins Dilution 1: 1/40
Automation:	Leica Bond-III
Method:	AS PER KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 40 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

PMS2 - Method 2

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

Primary Antibody:	BD Bio/Pharmingen 556415 (A16-4)
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)

PMS2 - Method 3

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

Primary Antibody:	Dako M3647 (EP51) , 21 ºC Dilution 1: 80
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	AS PER KIT
HMAR:	Dako PTLink, Buffer: Dako High pH TRS
EAR:	
Chromogen:	AS PER KIT, 21 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	AS PER KIT , 21 ºC Prediluted

PMS2 - Method 4

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

Primary Antibody:	Dako RTU FLEX IR087 (EP51) , 12 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins, Buffer: CC1
EAR:	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

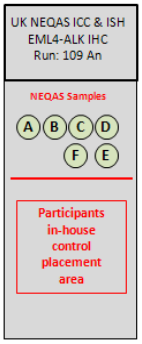
ALK NSCLC IHC Module Run 112

Merdol Ibrahim, Suzanne Parry, Dawn Wilkinson, Neil Bilbe, David Allen, Steven Forrest, Perry Maxwell, Tony O'Grady, Jane Starczynski, Phillipe Taniere, John Gosney, Keith Kerr, Erik Thunnissen & Keith Miller

Sample and Slide Distribution

Antibody assessed	ALK
Samples Circulated	Composite slide (see table below)
Number Participants	47

Distributed slide layout and Table 1 below illustrate the positioning of the distributed samples along with their pre-



Sample code	Sample	FISH status (Vysis)	IHC status (Roche D5F3)
A	NSCLC adenocarcinoma	+ve (inversion + deletion)	+ve
B	NSCLC adenocarcinoma	-ve	-ve
C	Appendix	-ve	+ve for ganglion cells & axons
D	Cell line:	-ve	-ve
E	Cell line:	+ve (inversion)	Approx. 50% +ve & 50% -ve
F	Cell line:	+ve (inversion)	Approx. 50% +ve & 50% -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

Update to the Assessment Criteria

After the initial pre-pilot assessment (Run 108), the assessment procedure has been further updated to include:

Same slide NEQAS and In-house controls

The UK NEQAS ICC & ISH distributed samples were placed and orientated as shown in in the 'Distributed slide layout and Table1. The microscope slide also had an area for participants to cut and place their appropriate in-house control material alongside the NEQAS sample. This set-up made sure that both the NEQAS and in-house controls had the same methodology applied to all samples. The request to include in-house samples also provided information on the selection and type of ALK in-house control that participants are using (see results below).

Interpretation criteria incorporating staining intensity

During the pre-pilot assessment the scoring criteria for ALK IHC employed a simple '+ve'/'-ve' (positive/negative) interpretation for each of the NEQAS distributed samples. This has now been updated for the IHC positive samples to also include feedback on the intensity of the observed staining including 3+ (high), 2+ (medium) and 1+ (low). Assessors were therefore asked to score each of the ALK IHC positive samples as: +ve (3+) OR +ve (2+) OR +ve (1+). This allowed more informative feedback on the intensity of staining on the ALK positive samples but also reflected the different scoring system employed by participants themselves (see Table 2)

Assessment process

- An assessment panel/team consisted of 4 assessors and a microscope driver (lead).
- Each of the two assessment teams scored all the samples independently, providing interpretation of the samples along with comments highlighting if there were interpretation and technical issues.
- Each of the 4 assessors within each team scored the slides with an overall possible score out of 5, with marks summed together to give a final score out of a maximum of 20:

Table 2: Assessment interpretation

- During this assessments all participants slides were scored twice to make sure the panel were consistent in their scoring.

Results

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

Features of Acceptable Staining
- ALK IHC positive tumour sample (sample A) (Fig. 1) should demonstrate granular cytoplasmic staining in the majority of the tumour nuclei.
- ALK IHC negative tumour sample (Fig 2; sample B) and negative cell lines (Fig. 4; sample D) should show no ALK IHC staining
- Appendix (sample C) (Fig 3) should show staining in ganglion cells and axons
- ALK IHC positive cell line samples (sample E & F; Figs. 5 & 6) should demonstrate granular cytoplasmic staining in approximately 50% of the sample preparation.

Features of Unacceptable Staining
- Non-specific / Excessive non-specific staining (Fig 7 & 8)
- False negative or absence of ALK staining where tumour cells should be staining positive
- Absence of staining in appendix

Pass Rates & Methodologies

In this assessment, on the NEQAS distributed samples (See 'Pass Rate Graphs') there was an overall pass rate of 87% (n=41) with 9% (n=4) achieving borderline acceptability and 4% (n=2) who's samples were found to be unacceptable.

The reasons for the borderline and unacceptable results were mainly due to non-specific staining (See Figure 7 & 8) as well as staining being weak in some of the samples possibly due to the detection system employed (see Table 2).

Once again there were 3 main antibody clones used (D5F3, ALK1 and 5A4) used either as part of an assay (Roche diagnostics) or lab devised methods:

- The Roche D5F3 (Tables 1 & 2) was the main antibody used accounting for 72% (n=34) of users, with 87% demonstrating either an acceptable or excellent staining quality on the NEQAS distributed samples. The majority of participants used the recommended procedure for the Roche D5F3 assay but it was also noted that two participants appeared to use the assay on Dako platforms. Whether this was an error on the part of the participant incorrectly entering data is not known. It was also noted that there were differences in the entered detection system with some participants appearing not to use the 'OptiView amplification' step but again this may be due to erroneous data entry. The few of the participants who had unacceptable scores was due to non-specific punctate staining (Fig 7 & 8) which gave a false-positive interpretation.
- The Leica Biosystems 5A4 clone (Tables 1 & 2) was used by 3 participants (down from 5 in the previous assessment) in a concentrate. The concentrate 5A4 clone was used on both the Dako and the Ventana automated platforms with their respective detection systems. Overall there was a 100% pass rate using this antibody clone.
- The cell signalling D5F3 clone (Table 1 and 2) was used by 2 participants and had a 100% pass rate
- The Dako ALK1 (Table 1 & 2) (not recommended by Dako for NSCLC) was used by two participants with both achieving a borderline acceptable mark, due to very weak staining.

Scoring Systems Employed by Participants

Of the 39 participants who submitted scoring methodology data, 89% used a simple +ve/-ve scoring methods with 11% using an intensity based scoring method (3+,2+,1+ and Neg.).

In-house Controls

The scoring of in-house controls is not solely based on the quality of ALK staining but the choice and suitability of the controls in gauging the sensitivity and specificity of ALK in the lung setting. Assessors also look at the quality and preservation of the submitted samples.

Forty six out of forty seven (98%) participants submitted in-house controls alongside the NEQAS distributed sample (see 'Pass Rate Graphs'), and of these participants 36% (n=16) were assed as having submitted an acceptable in-house control, 54% (n=25) were scored as borderline acceptable and 11% (n=5) as having submitted unacceptable in-house controls.

As indicated in the table below participants not submitting an appropriate control or solely a single section are scored a maximum 'borderline' score (10-12/20).

Tissue types submitted

Table 4 shows the tissue types submitted.
- 21% of participants did not submit an in-house control and were marked down for their non-submission.
- 31% submitted only a single in-house control, which was deemed to be unacceptable so a maximum borderline score (12/20) was given. 53% of these participants submitted a single NSCLC ALK positive sample, with 20% submitting a single Appendix section,
- 21% submitted at least 2 controls with 56% including both an ALK positive and negative control
- 26% submitted at least three or more in-house controls with a variety of tissue types

Lymphoma control

Lymphoma as a control in the lung setting for ALK IHC is not recommended and gives a false security of the sensitivity of the test as previously described (See journal run 111).

Appendix control

Appendix appears to be useful to gauge the sensitivity of ALK IHC, but is not recommended as a 'single tissue control' and where used should be used alongside a multi-tissue control (see below)

Submission on in-house controls for assessments

- Participants in-house control tissue should consist of composite control as outlined above
- Participants submitting solely a single section e.g. a single positive/negative lung control, appendix, lymphoma etc will be scored a maximum 'borderline' score (10-12/20).

Recommended ALK IHC controls

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
c. Appendix may also be used alongside the lung tumour controls (see Fig 6): Gauges both sensitivity and specificity

- Commercially available control material (e.g. cell lines, xenografts etc) 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.

Acknowledgements

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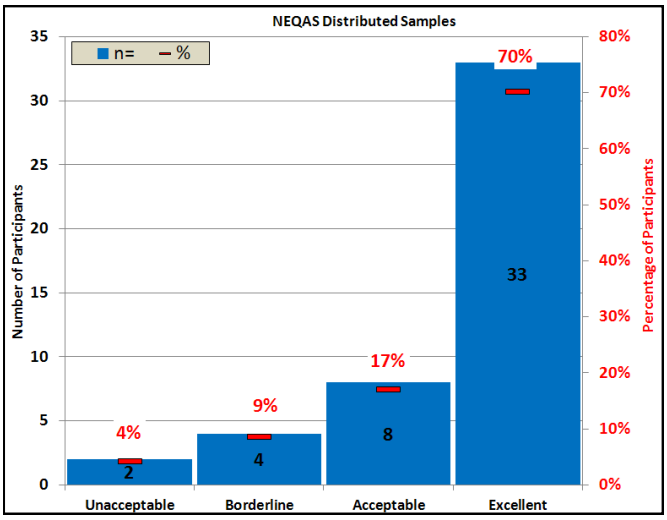
Pass Rates Graphs: Below graphs show (A) NEQAS and (B) Participant in-house pass rates

A. NEQAS Sample Pass Rates



B. IN-HOUSE Sample Pass Rates

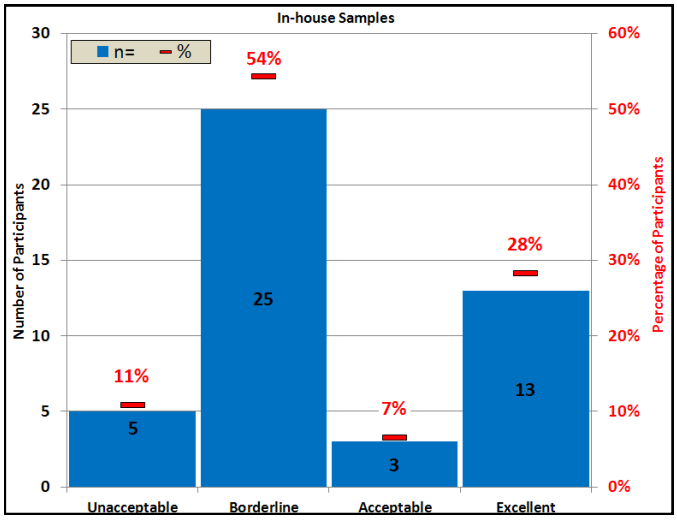


Table 1: Antibodies Submitted & Pass Rates (n=43)

Antibody	n=	% of total methods submitted	Excellent	Acceptable	Borderline	Unacceptable
Cell Signalling Tech. D5F3	2	5%	1 (50%)	1 (50%)	-	-
Dako IR/IS 641 (ALK1)	1	3%	-	-	1 (100%)	-
Dako M7195 (ALK1)	1	3%	-	-	1 (100%)	-
Novocastra NCL-ALK (5A4)	3	15%	2 (67%)	1 (33%)	-	-
Thermo/Neomarkers (5A4)	2	3%	2 (100%)	-	-	-
Ventana/Roche (D5F3)	34	72%	26 (76%)	4 (12%)	2 (6%)	2 (6%)

Table 2: Pass rates with Associated Antibody, Automated Platform and Detection Systems

Primary antibody	Automation Instrument	Detection kit	Excellent	Acceptable	Borderline	Unacceptable
	LabVision Autostainer	Dako Envision HRP/DAB (K5007)	-	1 (100%)	-	-
Cell Signalling Tech. D5F3	Ventana Benchmark XT	Ventana OptiView Kit (760-700)	1 (100%)		-	-
Dako IR/IS 641 (ALK1)	Leica Bond-III	Leica Bond Polymer Refine (DS9800)	-		1 (100%)	-
Dako M7195 (ALK1)	Dako Autostainer Link 48	DAKO Envision FLEX+ mouse Linker	-		1 (100%)	-
	Dako Autostainer Link 48	Dako EnVision FLEX+ (K8002/12)	2 (100%)	-		-
Novocastra NCL-ALK (5A4)	Ventana Benchmark ULTRA	Ventana OptiView (760-700) + Amp. (7/860-099)	-	1 (100%)		-
	Leica Bond Max	Leica Bond Polymer Refine (DS9800)	1 (100%)	-	-	-
Thermo/Neomarkers (5A4)	Ventana Benchmark XT	Ventana OptiView Kit (760-700)	1 (100%)	-	-	-
	Dako Autostainer	Dako EnVision FLEX+ (K8002/12)	-	1 (100%)	-	-
	Dako Autostainer plus	DAKO Envision FLEX+ mouse Linker	-	-	-	1 (100%)
		Ventana OptiView (760-700) + Amp. (7/860-099)	2 (100%)	-	-	-
	Ventana Benchmark ULTRA	Ventana OptiView Kit (760-700)	4 (80%)	-	-	1 (20%)
		Ventana OptiView (760-700) + Amp. (7/860-099)	6 (75%)	2 (25%)	-	-
Ventana/Roche (D5F3)	Ventana Benchmark XT	Ventana OptiView Kit (760-700)	14 (82%)	1 (6%)	2 (12%)	-

Table 3: Scoring systems used for respective antibody clones

		Scoring Methods Used	
Antibody (clone)	**n =**	**+ve / -ve**	**3+,2+,1+, neg.**
Ventana/Roche (D5F3)	27	94%	6%
Novocastra NCL-ALK (5A4)	6	100%	-
Cell Signalling Tech. D5F3	2	-	100%
Dako IR/IS 641 (ALK1)	1	0%	100%
Dako M7195 (ALK1)	1	100%	-
Thermo/Neomarkers (5A4)	1	100%	-
	Overall	**89%**	**11%**

Table 4: Controls and tissue types submitted

no. of in-house controls submitted per participant	no. of participants	%	Tissue types submitted
0	9	21%	Na
1	13	31%	53%: NSCLC (+ve) 20%: Appendix (+ve) 13%: Other (+ve)
2	9	21%	56%: NSCLC (+ve) + NSCLC (-ve) 11%: Appendix (+ve) + Other (+ve) 11%: NSCLC cell line (+ve) + NSCLC cell line (-ve) 11%: NSCLC (+ve) + Lymphoma (+ve) 11%: NSCLC (+ve) + Other (-ve)
3	6	14%	33%: NSCLC (+ve) + NSCLC (-ve) + Appendix (+ve) 17%: NSCLC (+ve) + Lymphoma (-ve) + Other (-ve) 17%: 2 x ALCL (+ve) + Appendix (+ve) 17%: NSCLC (+ve) + NSCLC (-ve) + SCLC (-ve) 17%: NSCLC (+ve) + Lymphoma (+ve) + Other (-ve)
>3	5	12%	Nd

Selected Images showing Optimal and sub-optimal Immunostaining

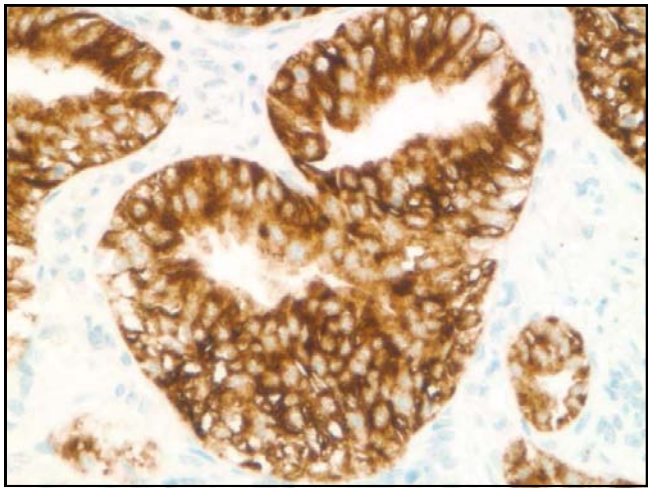


Fig 1. Good demonstration of ALK IHC in the UK NEQAS distributed positive tumour (sample A). The section shows strong membranous and cytoplasmic staining of the neoplastic cells. Stained using the Ventana D5F3 antibody on the Benchmark XT, CC1 for 92 minutes and Optiview detection kit.

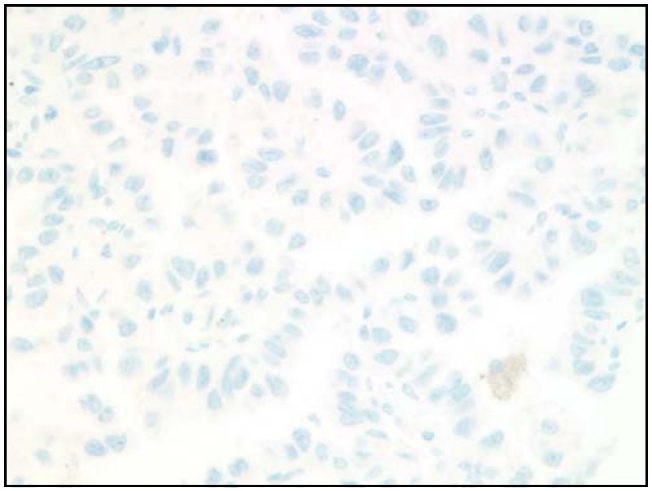


Fig 2. Acceptable result in the UK NEQAS distributed tumour (sample B) stained with ALK IHC. As expected, the sample is negative for ALK (Same protocol as Fig 1).

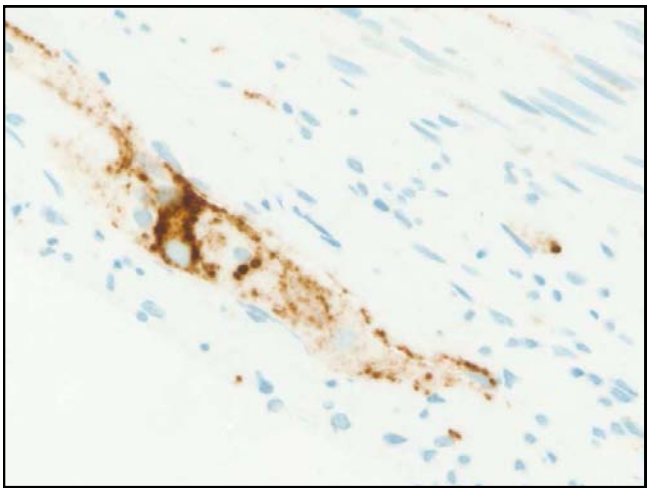


Fig 3. Good demonstration of ALK in the UK NEQAS distributed appendix (sample C). This tissue acts as an additional control to help gauge the sensitivity of the assay, and shows strong positive staining of the ganglion cells and axons. (Same protocol as Figs 1&2).

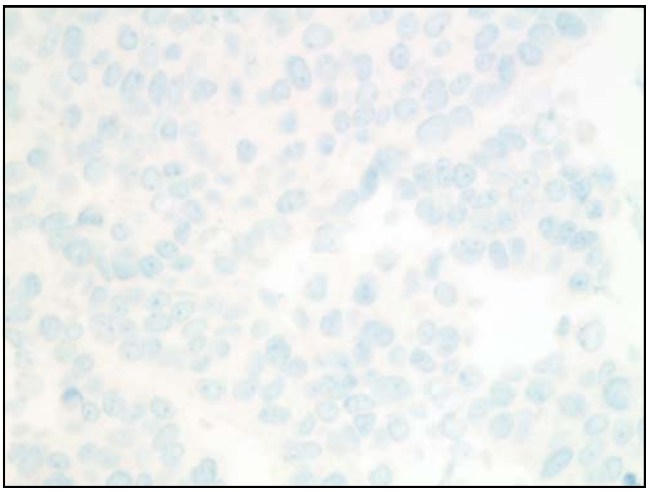


Fig 4. Acceptable demonstration of ALK in the UK NEQAS distributed negative cell line (sample D). As expected the tumour cells are negative and the section shows no background staining. (Same protocol as Figs 1-3).

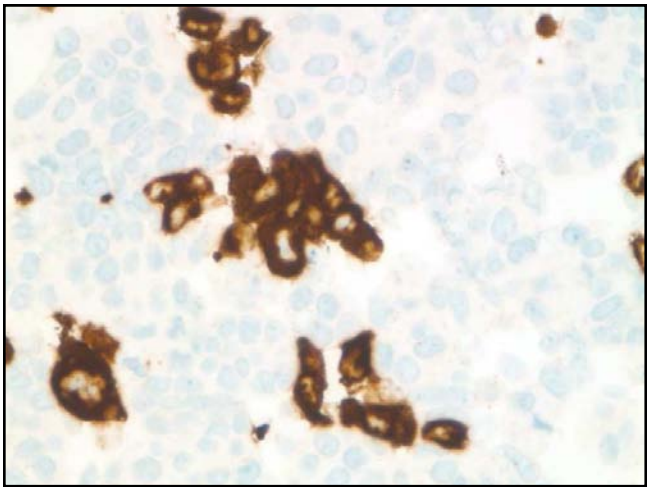


Fig 5. Optimal demonstration of ALK IHC in the UK NEQAS distributed cell line (sample E). This cell line consists of a mixture of positive and negative tumour cells. The example shows good strong membranous staining of the positive tumour cells, while the negative tumour cells remain clean. (Same method as Figs 1-4).

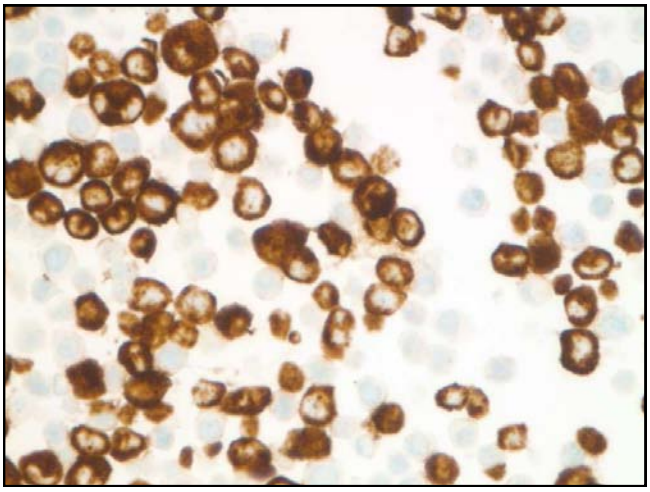


Fig 6. Optimal demonstration of ALK IHC on the UK NEQAS positive cell line (sample F). The example shows strong membranous staining of tumour cells. (Same protocol as Figs 1-5).

Selected Images showing Optimal and sub-optimal Immunostaining

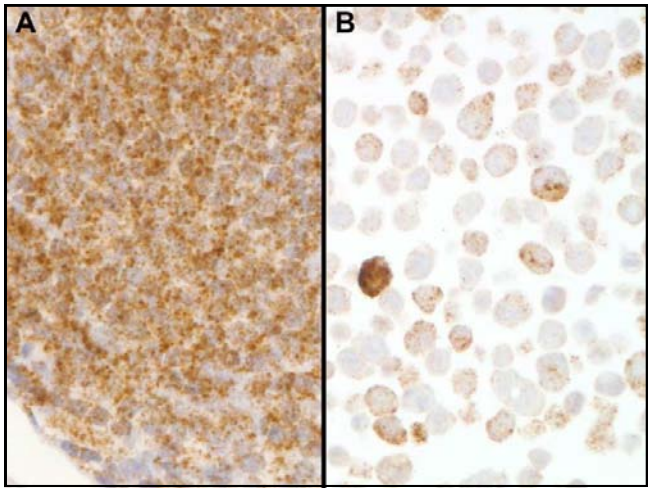


Fig 7. Sub-optimal staining with ALK on the UK NEQAS distributed samples C (appendix) and D (negative cell line). Both sections show excessive non-specific deposits. As with previous observations at UK NEQAS IHC, the excessive tyramide staining has often been associated with excessive baking of slides, in particular, extra heating on the hotplate.

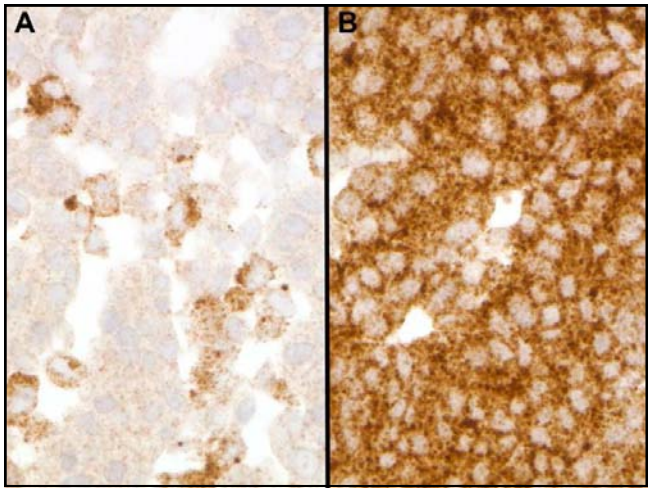


Fig 8. Poor demonstration of ALK on the UK NEQAS distributed samples E and F. Although the expression levels of both are as expected, the examples show excessive non-specific deposits. Stained using the Ventana D5F3 assay with OptiView detaection on the Benchmark XT.

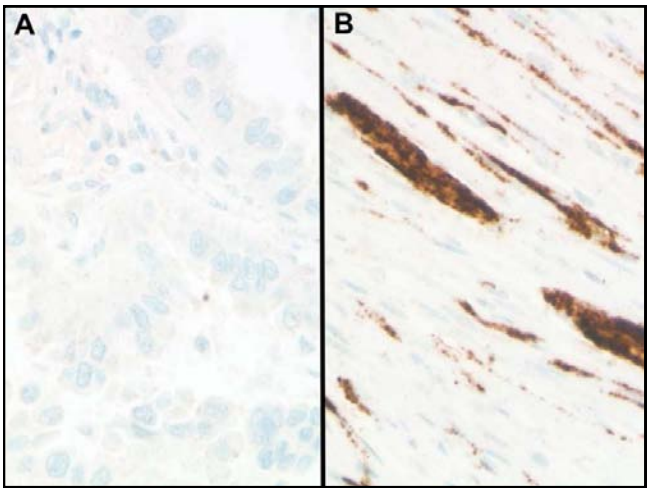


Fig 9. Two examples of in-house tissue submitted for ALK IHC assessment:. Both (A) negative tumour and (B) normal appendix, show the expected level of expression. The appendix was included in the multi-tissue block, as this is a useful control to help gauge the assay sensitivity. However, UK NEQAS does not recommend using appendix alone as an in-house control.

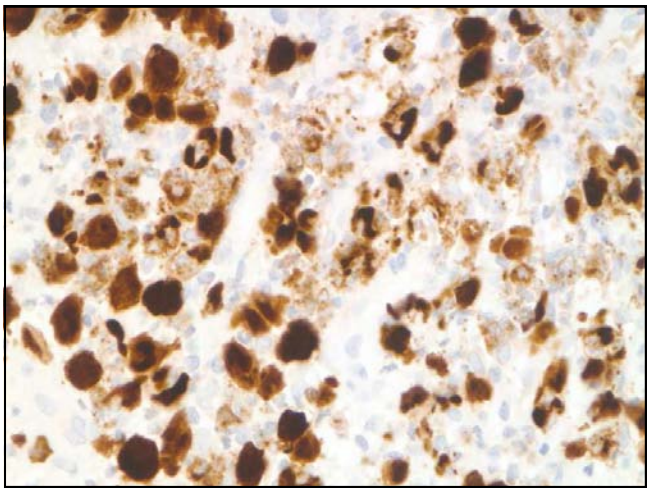


Fig 10. Good example of an in-house ALK positive NSCLC. The section shows strong staining of tumour cells with a clean background. Stained using the Novocastra 5A4 clone on the Ventana Benchmark ULTRA with OptiView detection.

HER2 ISH Module: Interpretive Run 41

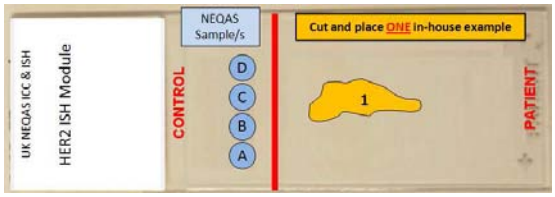
Merdol Ibrahim and Suzanne Parry

Gene Assessed:	HER2
Sections Circulated:	Composite slide consisting of 4 breast carcinoma sections (see table below)
Number of Registered Participants:	175
Number of Participants This Run	147 (84%)

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

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

Tissue Section Positioning: NEQAS samples were positioned on the 'control' section of the slide. Participant requested to place a single 'in -house' example placed as shown below.



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.

Assessment Procedure

The assessment of slides 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 (Table 1: **Statistical Approach to the Scoring System**).

Assessment Results

A full breakdown of pass rates is shown ion Table 2. Similar to the previous assessments, all participants taking part in the module are using a dual probe (ratio) method. 56% of labs are used FISH, with 44% using a CISH method (Table 2), which was similar to the previous Run (40). The Pathvysion Vysis kit still remains the most popular FISH method, and this was used by 34% Labs (Table 2). The Ventana DDISH was the most favoured chromogenic ISH technique, which was used by 33% of Labs (accounting for 77% of all CISH methods).

Overall the acceptable pass rate, irrespective of method, was 67% (≥30/36). A further 23% received a borderline pass (24-29/36), and 5 labs (6%) failed the assessment (scores of <24/36). The acceptable pass rate for the FISH users was 78% compared to 53% for those labs using the chromogenic method.

When comparing the two most popular methods (PathVysion Vysis vs Ventana DDISH) (Table 2), it was once again obvious that there was a larger percentage of unacceptable results with the DDISH method (4% vs 22%). The reasons for such a discrepancy were obvious when the samples were assessed in the technical module (See 'ISH technical' write-up).

HER2 ISH Method and Probe Enumeration

The table on the next page (table 3) shows the various ISH methods employed along with mean HER2, Cen17 counts and ratio calculations. See 'ISH technical' write-up for further information

Frequency Histograms

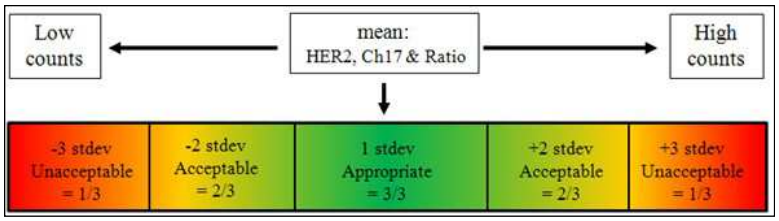
Frequency histograms of the assessment results are also provided showing the distribution of HER2 copy and Cen17 counts and ratio calculations. Participants are encouraged to use the provided frequency histograms to gauge their performance and variability in counts for each of the distributed samples.

References

1. Rakha EA, Pinder SE, Bartlett JM, Ibrahim M et al. et al., (2014) Updated UK Recommendations for HER2 assessment in breast cancer. J Clin Pathol. 68 (2):93-99.
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.

Table 1: Statistical Approach to the Interpretive ISH Scoring System

1. All participant data is initially evaluated to exclude '**outliers**'. An outlier is defined as those scores that are 1.5 times the interquartile range (IQR) outside of the lower and upper quartiles.
2. A mean score is then generated for the submitted HER2 copy, chromosome 17 and ratios.
3. Counts for HER2 copy, Cen17 and ratio are scored individually such that results within +/- 1 standard deviation (stdev) of the mean are score 3/3, +/- 2 stdev = 2/3 and ≥ +/- 3 stdev = 1/3. A non submission is scored 0/3.



4. For each of the samples there is therefore a possible score out of 9 (those using a ratio method) and 3 (those using a single copy method). As NEQAS ICC & ISH distributes 4 samples there is a possible score out of 36 (those using a ratio method) and 12 (those using a single copy method). (score = 3).

Dual Probe: Ratio Scoring Method

- For each samples (A to D) marks are awarded for HER2 copy, Cen17& Ratio by comparing participants score to standard deviation (stdev) from the mean score, such that: (a) 3/3 = within 1 stdev from the mean, (b) 2/3 = within 2 stdev from the mean (c) 1/3 = > 3 stdev from the mean. A possible score 9/9 marks is attainable for each sample.
- Individual sample scores are summed to give a possible score out of 36.
- Score interpretation: 36/36 = Excellent ; 30-35/36 = Acceptable ; 24-29/36 = Borderline ; <24/36 = Unacceptable

Single Probe: HER2 Copy Scoring Method

- For each sample (A to D) marks are awarded for HER2 copy counts by comparing your counts with the standard deviation (stdev) from the mean score, such that: (a) 3/3 = within 1 stdev from the mean, (b) 2/3 = within 2 stdev from the mean (c) 1/3 = > 3 stdev from the mean. A possible score 3/3 marks is attainable for each sample
- Individual sample scores are summed to give a possible score out of 12.
- Score interpretation: 12/12 = Excellent ; 10-11/12 = Acceptable ; 8-9/12 = Borderline ; <8/12 = Unacceptable

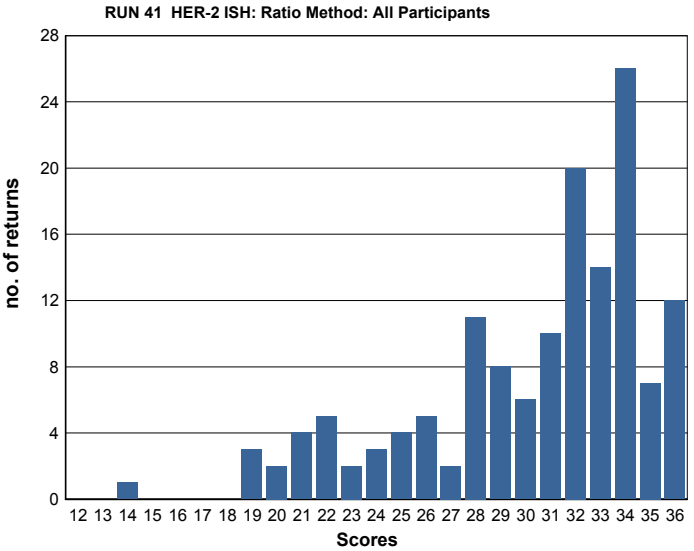
Table 2: Breakdown of Pass Rates

Method	no. of labs	% of users	Pass	Borderline	Unacceptable
Ratio: Dako DuoCISH	1	1%	100%	-	-
Ratio: Dako IQFISH pharmDX	9	6%	67%	33%	-
Ratio: Dako Pharm Dx	3	2%	67%	33%	-
Ratio: Kreatech Probes	4	3%	100%	-	-
Ratio: Leica HER2 FISH TA9217	7	5%	86%	14%	-
Ratio: Other - CISH	1	1%	-	100%	-
Ratio: Other - FISH	4	3%	50%	50%	-
Ratio: Pathvysion Vysis Kit	50	34%	80%	16%	4%
Ratio: Ventana BDISH 800-098/505	7	5%	57%	43%	-
Ratio: Ventana DDISH (780/800-4422)	49	33%	53%	24%	22%
Ratio: Ventana Inform Silver ISH	4	3%	50%	50%	-
Ratio: Zytovision ZytoDot 2C	2	1%	50%	50%	-
Ratio: Zytovision ZytoLight	6	4%	83%	-	17%

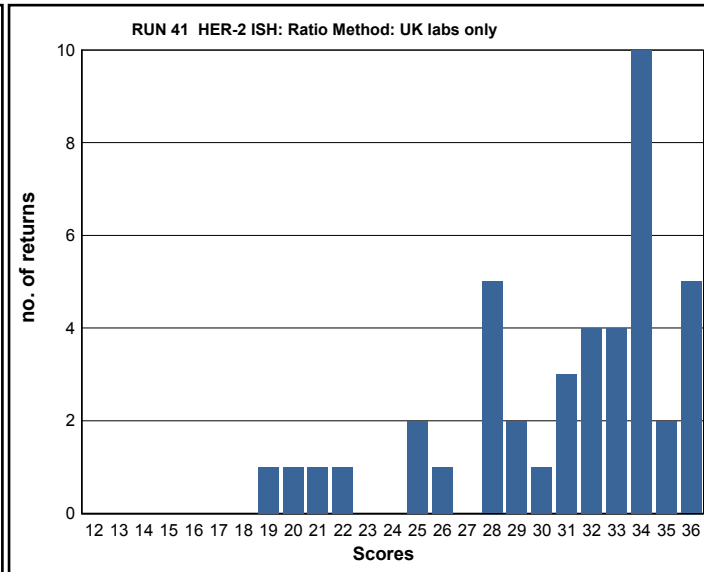
Table 3: Methods and mean counts/ratios (n/a= not applicable):

		Sample A: mean (stdev)			Sample B: mean (stdev)			Sample C: mean (stdev)			Sample D: mean (stdev)		
METHOD	n=	**Copy No**	**cen17**	**Ratio**	**Copy No**	**cen17**	**Ratio**	**Copy No**	**cen17**	**Ratio**	**Copy No**	**cen17**	**Ratio**
Ratio: Pathvysion Vysis Kit	50	3.66 (0.57)	2.71 (0.43)	1.37 (0.21)	3.72 (0.88)	2.83 (0.6)	1.31 (0.27)	1.99 (0.56)	2.08 (0.55)	0.94 (0.24)	2.79 (0.55)	2.13 (0.4)	1.31 (0.24)
Ratio: Ventana DDISH (780/800-4422)	47	3.66 (0.85)	2.27 (0.7)	1.72 (0.58)	3.60 (1.01)	2.63 (0.79)	1.42 (0.44)	2.27 (0.75)	1.76 (0.55)	1.28 (0.34)	3.12 (0.6)	2.00 (0.42)	1.63 (0.42)
Ratio: Dako IQFISH pharmDX	9	3.83 (0.68)	2.82 (0.32)	1.37 (0.19)	3.83 (0.6)	3.01 (0.3)	1.27 (0.12)	2.05 (0.38)	2.18 (0.36)	0.95 (0.13)	2.92 (0.44)	2.04 (0.35)	1.45 (0.16)
Ratio: Leica HER2 FISH TA9217	7	3.78 (0.39)	2.78 (0.33)	1.38 (0.2)	3.96 (0.22)	2.85 (0.54)	1.45 (0.37)	1.87 (0.12)	2.03 (0.28)	0.94 (0.12)	2.83 (0.35)	2.53 (0.59)	1.36 (0.24)
Ratio: Ventana BDISH 800-098/505	7	3.37 (0.65)	2.3 (0.34)	1.5 (0.34)	3.98 (0.99)	2.8 (0.65)	1.47 (0.46)	2.19 (1.05)	1.76 (0.31)	1.33 (0.37)	2.76 (0.38)	2.07 (0.42)	1.38 (0.22)
Ratio: Zytovision ZytoLight	6	3.75 (0.4)	2.55 (0.61)	1.55 (0.4)	3.63 (0.51)	2.76 (0.64)	1.35 (0.19)	2.22 (0.28)	2.15 (0.5)	1.07 (0.23)	2.71 (0.31)	2.00 (0.39)	1.38 (0.14)
Ratio: Kreatech Probes	4	3.57 (0.39)	2.4 (0.43)	1.5 (0.16)	3.47 (0.37)	2.62 (0.49)	1.35 (0.25)	1.91 (0.16)	2.1 (0.04)	0.91 (0.08)	2.58 (0.24)	1.88 (0.14)	1.39 (0.08)
Ratio: Other - FISH	4	3.7 (0.81)	2.38 (0.58)	1.62 (0.22)	4.21 (0.57)	2.93 (0.4)	1.44 (0.08)	2.09 (0.12)	1.94 (0.21)	1.07 (0.07)	3.14 (0.85)	1.78 (0.29)	1.81 (0.67)
Ratio: Ventana Inform Silver ISH	4	4.95 (0.9)	2.72 (0.65)	1.88 (0.43)	4.01 (0.71)	3.3 (0.52)	1.18 (0.24)	2.22 (0.17)	1.83 (0.28)	1.22 (0.17)	3.16 (0.34)	2.21 (0.27)	1.45 (0.25)
Ratio: Dako Pharm Dx	3	4.01 (0.3)	2.68 (0.19)	1.51 (0.21)	3.78 (0.1)	2.65 (0.39)	1.45 (0.2)	1.9 (0.06)	2.31 (0.3)	0.83 (0.09)	2.83 (0.02)	2.00 (0.32)	1.44 (0.24)
Ratio: Zytovision ZytoDot 2C	2	4.02 (1.95)	2.09 (0.01)	1.92 (0.92)	3.15 (0.57)	2.1 (0.14)	1.49 (0.17)	2.14 (0.65)	1.46 (0.06)	1.46 (0.39)	2.85 (0.07)	1.86 (0.13)	1.54 (0.07)
Ratio: Dako DuoCISH	1	3.35 (n/a)	2.17 (n/a)	1.54 (n/a)	3.51 (n/a)	2.77 (n/a)	1.26 (n/a)	1.98 (n/a)	2.34 (n/a)	0.84 (n/a)	2.98 (n/a)	2.25 (n/a)	1.32 (n/a)
Ratio: Other - CISH	1	3.8 (n/a)	1.6 (n/a)	2.38 (n/a)	3.35 (n/a)	2.05 (n/a)	1.63 (n/a)	1.7 (n/a)	1.35 (n/a)	1.26 (n/a)	3.1 (n/a)	1.9 (n/a)	1.63 (n/a)

GRAPHICAL REPRESENTATION OF PASS RATES (ratio methods only)



Excellent: 36-36/36 = 12 (8%)
Acceptable: 30-35/36 = 83 (57%)
Borderline: 24-29/36 = 33 (23%)
Unacceptable: <24/36 = 17 (12%)



Excellent: 36-36/36 = 5 (12%)
Acceptable: 30-35/36 = 24 (56%)
Borderline: 24-29/36 = 10 (23%)
Unacceptable: <24/36 = 4 (9%)

METHODS USED and PASS RATES

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

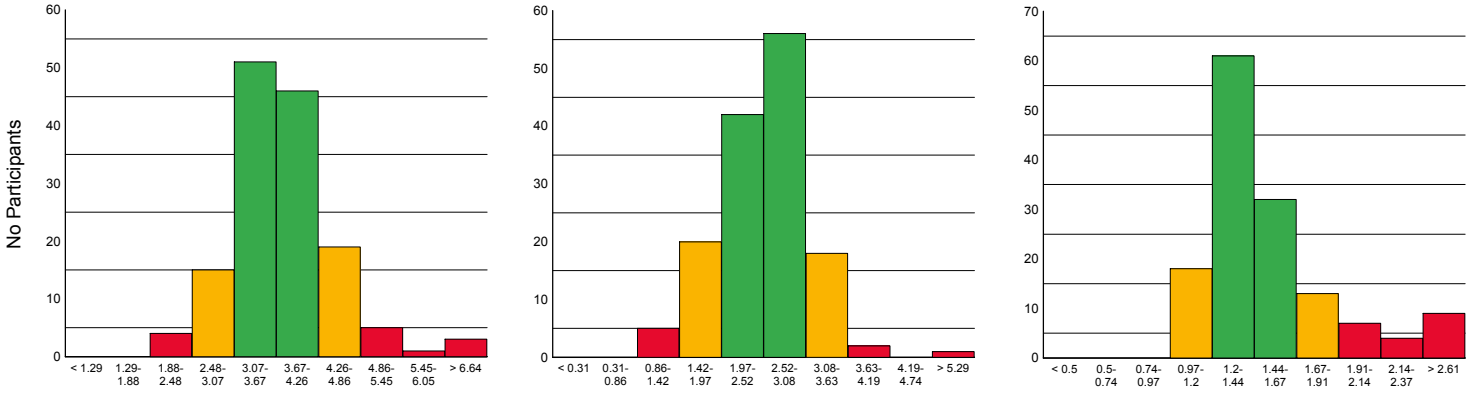
Ratio Method	N	% Pass (score ≥ 30/36)
Ratio: Dako DuoCISH	1	100%
Ratio: Dako IQFISH pharmDX	9	67%
Ratio: Dako Pharm Dx	3	67%
Ratio: Kreatech Probes	4	100%
Ratio: Leica HER2 FISH TA9217	7	86%
Ratio: Other - CISH	1	0%
Ratio: Other - FISH	4	50%
Ratio: Pathvysion Vysis Kit	50	78%
Ratio: Ventana BDISH 800-098/505	7	57%
Ratio: Ventana DDISH (780/800-4422)	47	51%
Ratio: Ventana Inform Silver ISH	4	50%
Ratio: Zytovision ZytoDot 2C	2	0%
Ratio: Zytovision ZytoLight	6	83%

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

FREQUENCY HISTOGRAMS

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

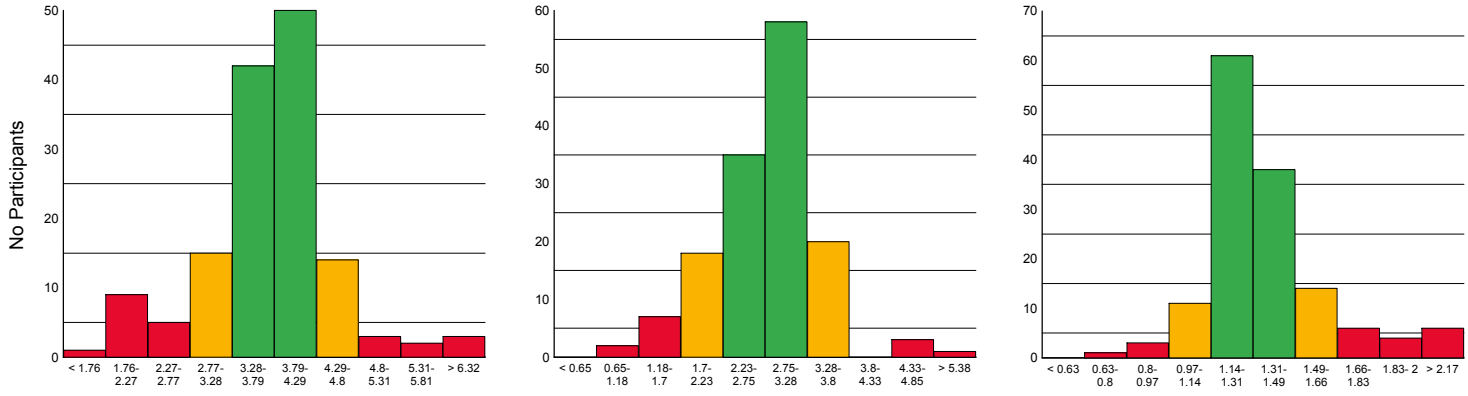
Green = mean +/- 1 Stdev Amber = mean +/- 2 Stdev Red = mean ≥ +/- 3 stdev



Sample A: Av. HER2 copy

Sample A: Av. Chr17

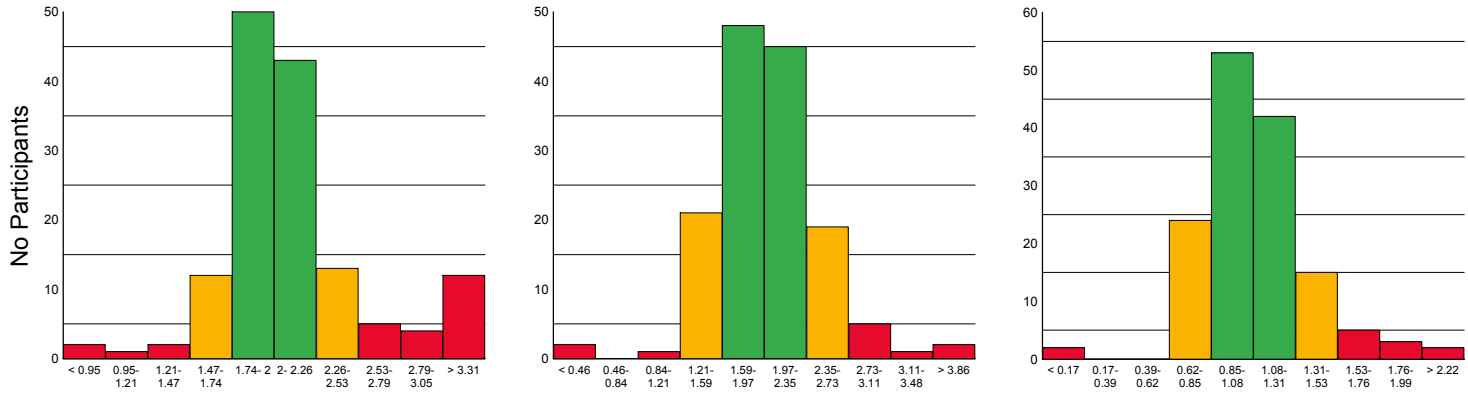
Sample A: Ratio



Sample B: Av. HER2 copy

Sample B: Av. Chr17

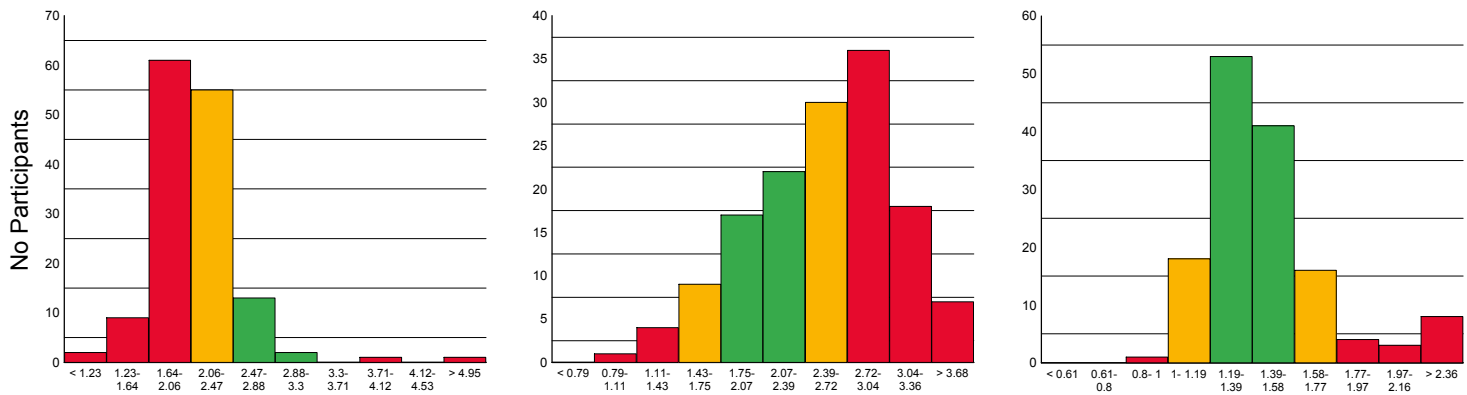
Sample B: Ratio



Sample C: Av. HER2 copy

Sample C: Av. Chr17

Sample C: Ratio



Sample D: Av. HER2 copy

Sample D: Av. Chr17

Sample D: Ratio

HER2 ISH Module: Technical (Pilot) Run 41

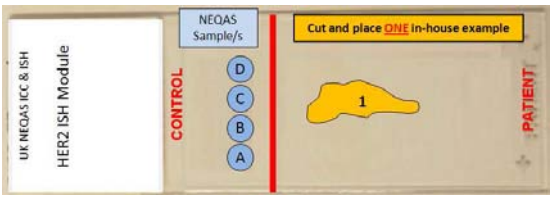
Merdol Ibrahim and Suzanne Parry

Gene Assessed:	HER2
Sections circulated:	Composite slide consisting of 4 breast carcinoma sections (see table below)
Number of Registered Participants:	175
Number of Participants Taking Part this Run	142 (80%) (73 Fluorescent and 69 Chromogenic)

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: NEQAS samples were positioned on the 'control' section of the slide. Participant requested to place a single 'in -house' example placed as shown 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



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

A single In-house 'example' is requested for this module, which participants are required to place alongside the UK NEQAS distributed slides (See above: 'Tissue Section Positioning').

Results Summary

CISH Results

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

The overall results from the CISH technical assessment saw similar pass rates to the previous assessment run (40): 48% of labs received an acceptable pass, and a further 28%. 17 labs (25%) failed the assessment. 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. Another reason for failure was due to excessive signals obscuring the nuclei and signals outside of the nuclei, which made the sections uninterpretable. 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 and the slide was deemed still readable, the section 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 (84%) 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. The data from the 1 lab shown in the report graphs show 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 because the signals were weak in some of the cores. This again is another CISH methodology recognised by the assessment team, as the HER2 signals are green and the Cen17 signals are red.

Important: Laboratories experiencing problems with their ISH methods 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 higher rates than the previous assessment run (40): 71% of labs received an acceptable pass, and a further 8% achieved a borderline, The current run showed a fail rate of 21%. The main reason for unacceptable results was due to weak or no signals. We

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 **Individual comments are also provided on participant reports**
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 **Individual comments are also provided on participant reports**
Score = 0	Slide Not submitted for assessment

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 60% of labs for this assessment. Labs using this method showed an acceptable pass rate of 52%, which was much lower than the previous assessment. A further 14% of labs using the Vysis kit received a borderline, and 34% of Vysis users failed the assessment. The Dako IQFISH and the Leica FISH kits were the next popular choices of kits, with 14% of FISH submissions stained with the Dako IQFISH and 10% with the Leica kit. Both of these methods showed a very good acceptable pass rates of 100% A variety of other kits were used, including the Dako Pharm Dx, Kreatech Probes and the Zytovision ZytoLight, all of which performed well, although the numbers of users of these kits is 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

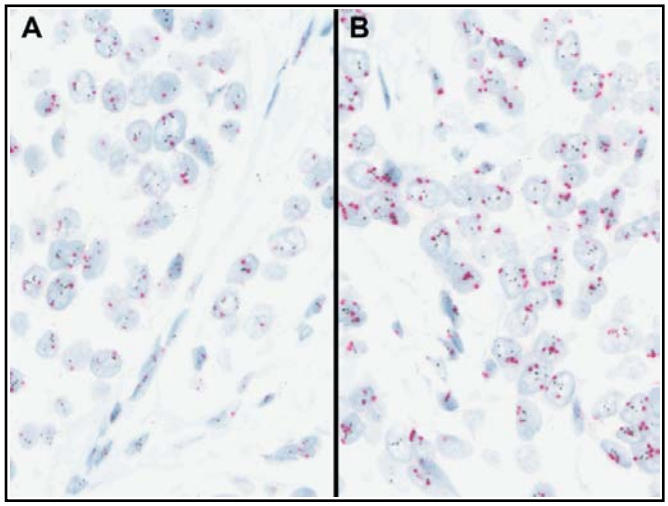


Fig 1. Acceptable Ventana DDISH in the UK NEQAS distributed non-amplified samples 'A' and 'B' showing distinct HER2 signals (black) and Chr17 signals (red).

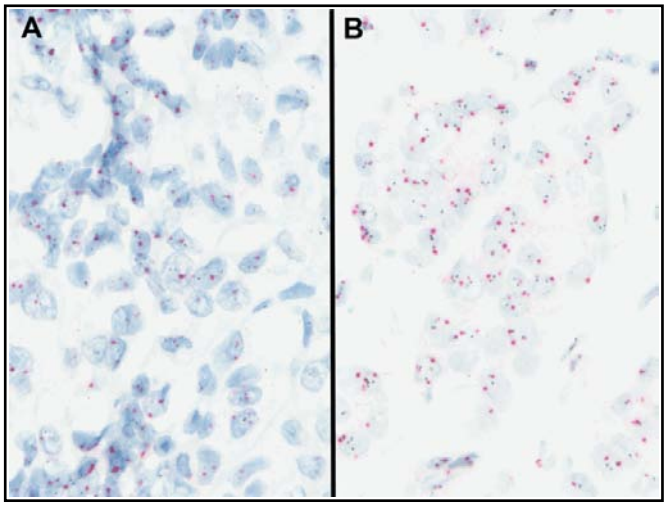


Fig 2. Acceptable Ventana DDISH in the UK NEQAS distributed non-amplified samples 'C' and 'D'. The sections show strong HER2 signals (black) and Chr17 signals (red) and the expected copy numbers per cell.

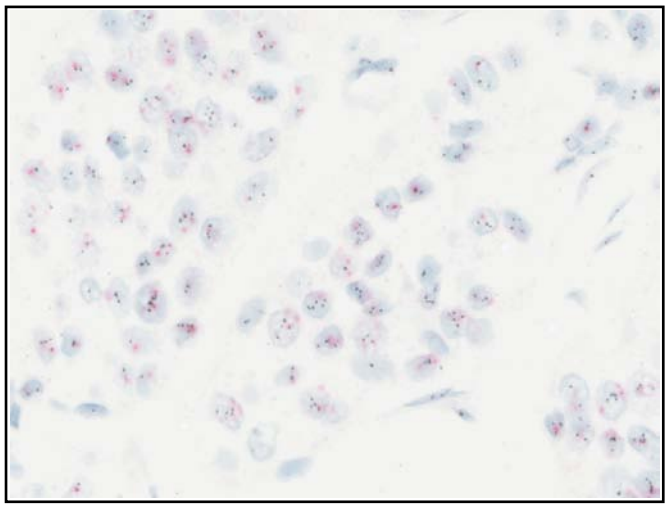


Fig 3. Borderline pass demonstration of Ventana DDISH in the UK NEQAS non-amplified sample 'A'. Although the signals are visible, the Chr17 signals are leaching asking it slightly more difficult to read.

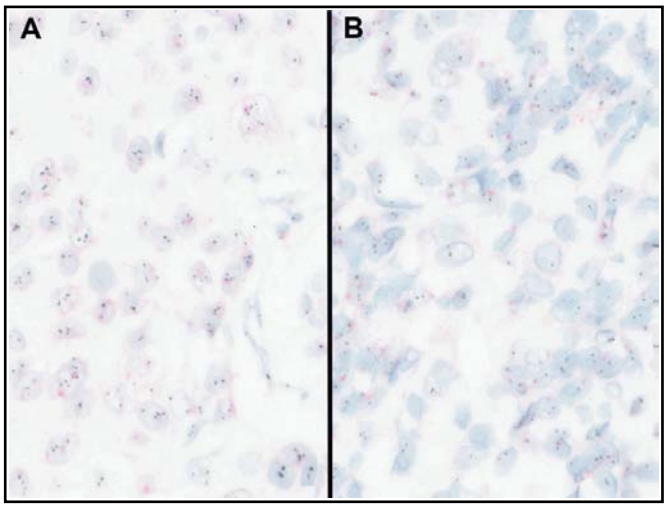


Fig 4. Two examples showing unacceptable Ventana DDISH staining in the UK NEQAS samples: Both the non-amplified samples 'A' and 'B' show weak or no Chr17.

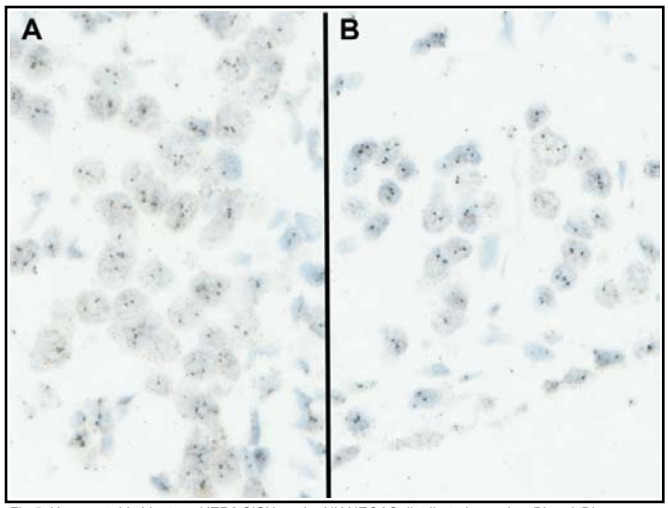


Fig 5. Unacceptable Ventana HER2 SISH on the UK NEQAS distributed samples 'B' and 'D'. Both sections have excessive silver deposit.

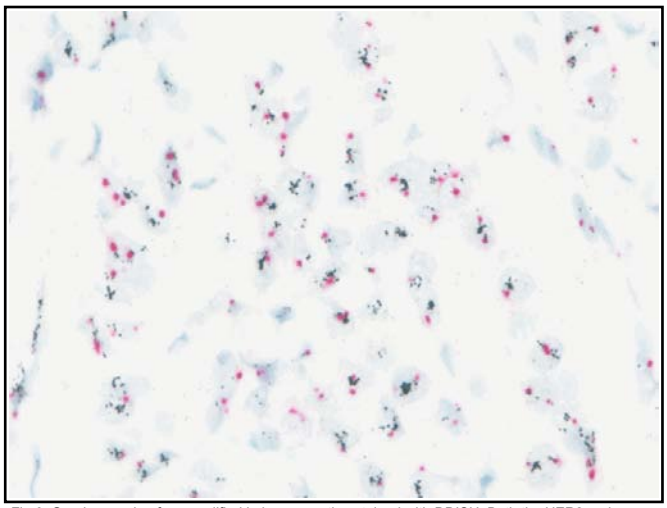
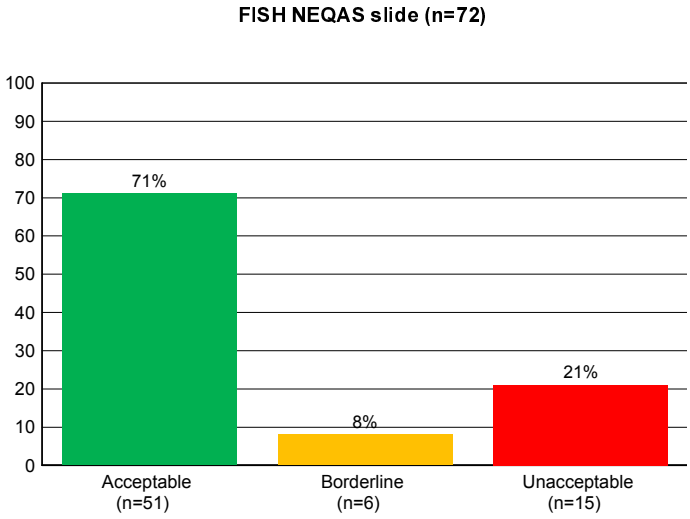
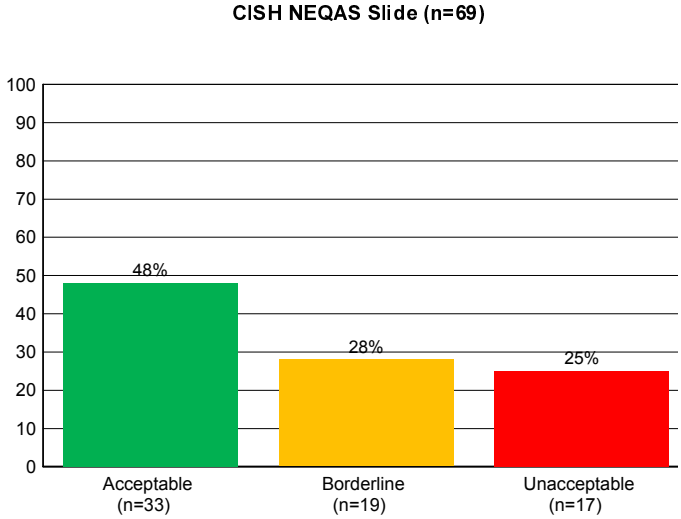


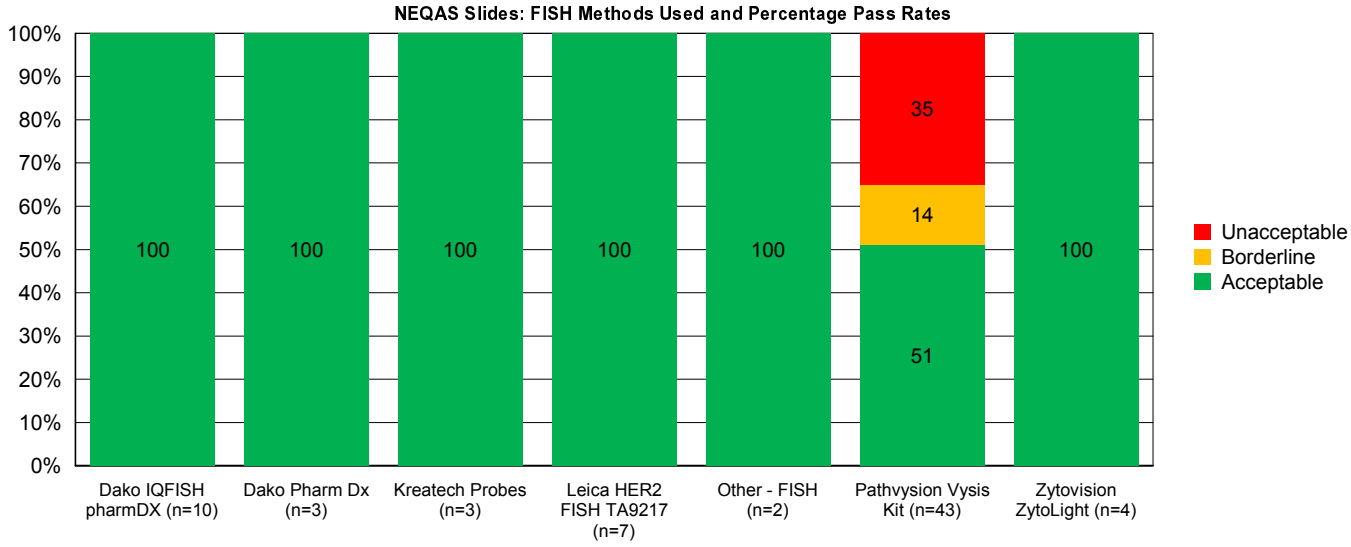
Fig 6. Good example of an amplified in house section stained with DDISH. Both the HER2 and Chr17 signals are strong and distinct.

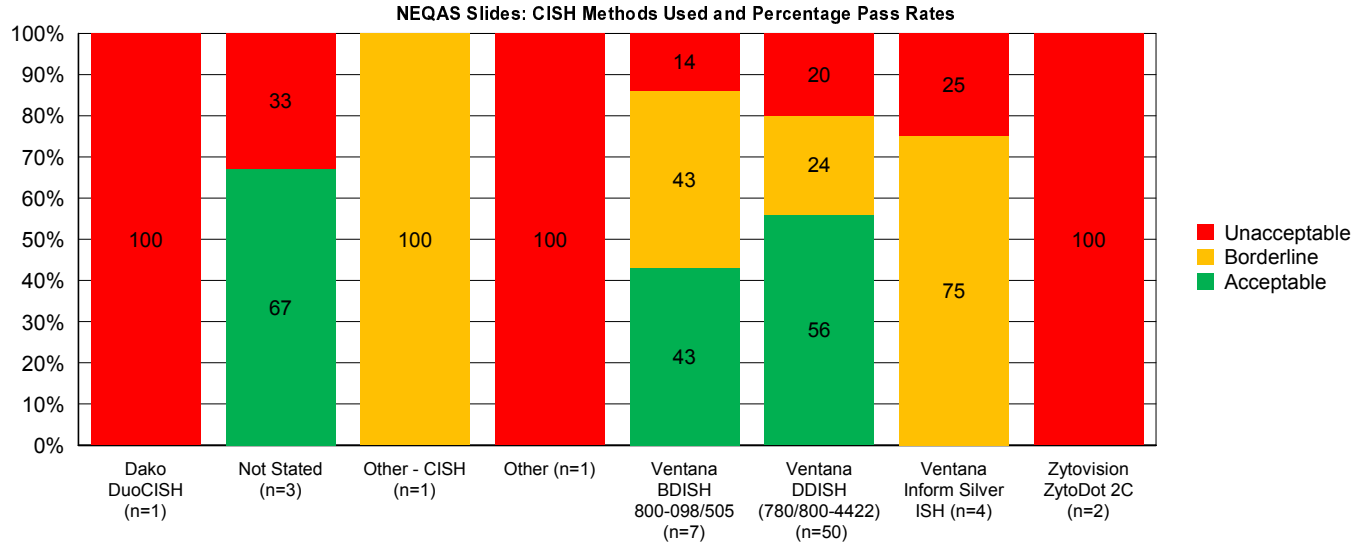
Technical ISH: Pass Rates and Methods

Overall Pass Rates









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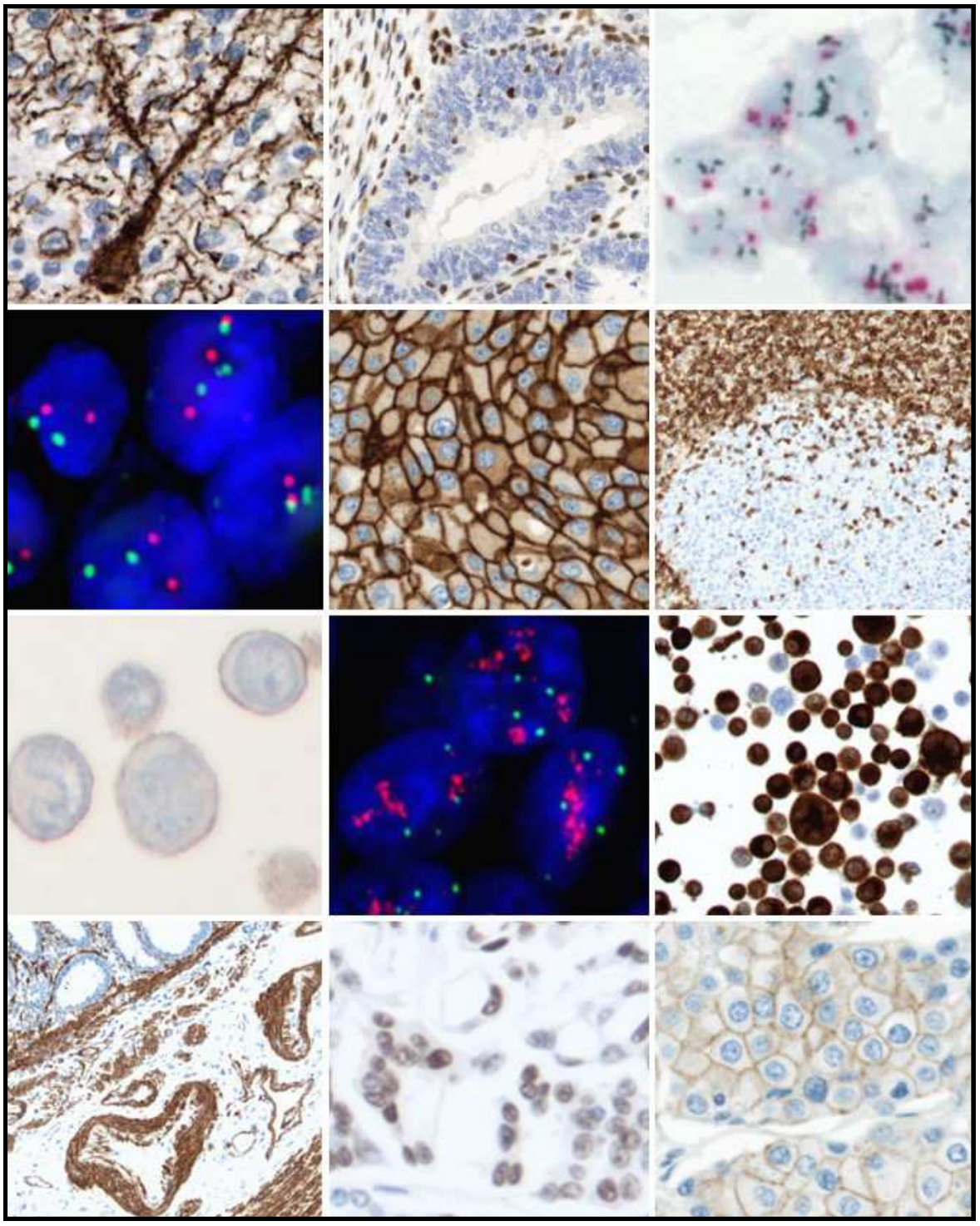

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