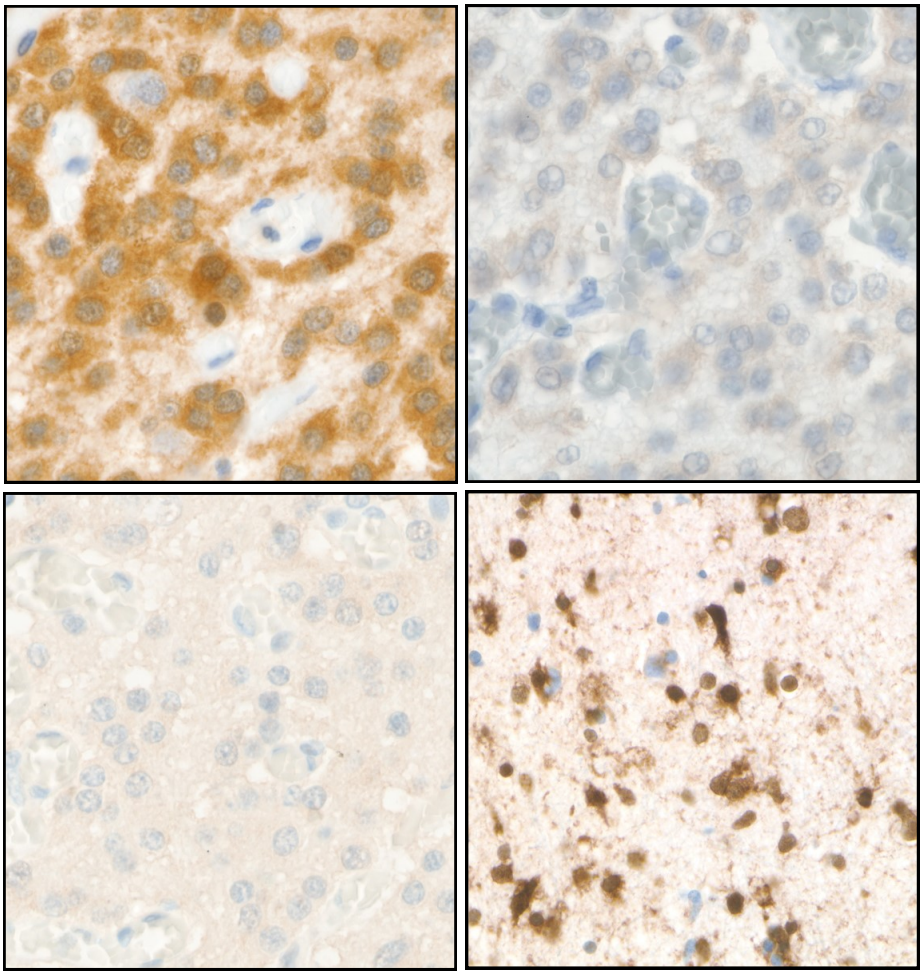
UK NEQAS
International Quality Expertise

Immunocytochemistry

Improving Immunocytochemistry for Over 25 Years

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

Assessment Dates: 29th March — 15th April 2016



Cover Photo: Taken from the Neuropathology Module 2nd Antigen:

Top Left: Optimal IDH-1 staining on the NEQAS tumour sample (J)
Top Right: Suboptimal staining of oligodendroglioma on the NEQAS slide (J)
Bottom Left: Poor demonstration of IDH-1 in the tumour in the NEQAS section (J)
Bottom Right: Excellent IDH-1 staining of a glioma from an in-house control sample (K)

Participants Meeting 2016 -September 9th
Further details: www.ukneqasiccish.org/participant-meeting-2016

Articles / Reports

Immunocytochemistry Modules

In situ Hybridisation Modules

General Information

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Data shown in this article is collated from UK NEQAS ICC & ISH assessments and is presented and described 'as collected', and does not either 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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Poundbury Cancer Institute
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ASSESSORS

UK NEQAS ICC has over 100 Assessors (scientists and pathologists) from the UK, Australia, Canada, Denmark, Germany, Hungary, Ireland, Portugal, Slovenia, South Africa, Sweden and Switzerland.
The list below shows assessors who took part in the current assessment.

United Kingdom
Mr D Allen, London
Prof M Arends, Edinburgh
Mr N Bilbe, London
Mr D Blythe, Leeds
Ms A Clayton, Preston
Dr C Cardozo, Preston
Mr A Dodson, London
Mr I Downie, Glasgow
Mr D Fish, Warwick
Mrs S Forrest, Liverpool
Mr S Forrest, Liverpool
Dr I Frayling, Cardiff
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Ms S Moliveira, Lisbon

Slovenia
Dr D Vidovic, Maribor

Switzerland
Dr P-A Diener, St. Gallen
Dr L Tornillo, Basel

Journal layout and design prepared by UK NEQAS ICC & ISH

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**

Scheme News and Updates

UK NEQAS
Immunocytochemistry & In-Situ Hybridisation

① We are now ISO 17043 Accredited

Following a visit by UKAS in February 2016, UK NEQAS ICC & ISH is now fully accredited to ISO 17043.

The list of accredited modules can be found on the UKAS Accredited PT Providers link: Click on the image on the left or the following link:
www.ukas.com/list-all-organisations/?org_type=13&org_cat&cpage=3

② ALK NSCLC IHC Module is now Live

The ALK for NSCLC module has been given approval by the Techniques in Cellular Pathology Steering Committee to be removed from Pilot to a Live module as from April 2016, Run 114.

All UK Clinical laboratories who subscribe to this module will now be Poor Performed in line with the other Biomarkers. Due to the direct impact that the results of assays for biomarkers have on patient management, more stringent performance monitoring mechanisms are employed: See section 2.8 of the Participants Manual (**www.ukneqasiccish.org/participant-manuals**).

This module will be formally requested to be included in our ISO 17043 repertoire.

③ Breast HER2 ISH Technical Module is now Live

Laboratories who take part in the Breast Her2 ISH module (Interpretive) must also submit their slide for the ISH Technical module. The two modules will now be combined and also be subject to Poor Performance monitoring. The scheme will contact participants with poor performance details.

④ User Survey 2016

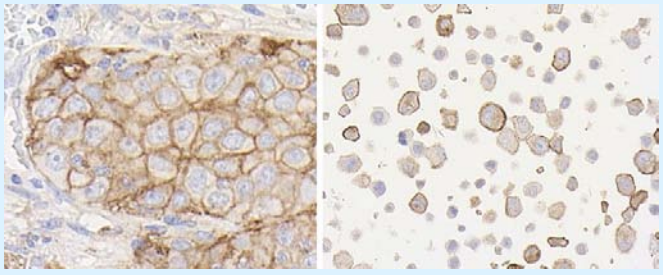
The return period for the latest User Survey has now closed. We will publish the write up in the next Journal - Run 114.

⑤ PD-L1 NSCLC IHC Module

A PD-L1 NSCLC IHC module is now being prepared. We have been busy testing samples in preparation.

We hope to have the pre-pilot assessment running by the third quarter in 2016. All participants will be invited to take part in due course and we hope to make this module freely available in the first instance.

A separate survey will be distributed to all participants who would like to take part.



⑥ Participant Meeting 2016

We can confirm that the next Participant Meeting will be held on **Friday, 9th September** at the Grange Hotel, Holborn, London. Further details can be found on the UK NEQAS ICC & ISH website: **www.ukneqasiccish.org/participant-meeting-2016**

Registrations will be open until late August. We will be charging a small fee this year of £50. Depending on the level of sponsorship we may reduce or revise this. A draft agenda has been drawn up and will appear on the website soon.

⑦ Looking for an IHC Best Method?

Don't forget we have a whole database of methods to help you get started: **www.ukneqasiccish.org/best-methods**

We will be adding more data in the next few weeks.

⑧ Scheme Documentation

Due to various changes, such as those detailed above, we will be periodically updating the scheme's documentation on the website; e.g. Participant Manual and Poor Performance Criteria, please check that you have the latest versions where applicable.

Change of Address:

Please remember that the scheme has moved. Our new address is: **Room 127, Finsbury Business Centre**
40 Bowling Green Lane, London EC1R 0NE. UK

Tel: +44 (0) 207 415 7065
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For further details visit: www.ukneqasiccish.org

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Julie Williams and Suzanne Parry

	Gold Standard	Second Antibody
Antigens Assessed:	SMA	CK 5/6
Tissue Sections circulated:	Normal Appendix & Leiomyosarcoma	Lung: Squamous cell carcinoma & Adenocarcinoma
Number of Registered Participants:	330	
Number of Participants this Run	311 (94%)	

Introduction

Gold Standard: SMA

The alpha-isoform of smooth muscle actin (αSMA) belongs to a group of cytoplasmic actins, of which there are six major different isoforms (Roholl et al.). It is useful for the demonstration of myogenic differentiation. Antibodies to SMA label smooth muscle cells, which are found in vascular walls, intestinal muscularis, and muscularis propria and in the stroma of various other tissues (Skalli et al., Mason & Gatter, Rizeq et al.). It also reacts with myoepithelial cells in tissues such as breast and salivary glands. The main diagnostic use of SMA is in a panel of antibodies to demonstrate leiomyomas and leiomyosarcomas (Oda et al.). It is also used to identify the loss of myoepithelial cells around the ducts in invasive breast carcinomas (Lazard et al.).

Features of Optimal Immunostaining:
Appendix: (Figs1 & 5)
- Strong staining of the smooth muscle layers of the muscularis propria and around the vessels in the submucosa
- Minimal background staining

Leiomyosarcoma: (Fig 2)
- Strong cytoplasmic staining of the tumour cells
- Minimal background staining

Features of Suboptimal Immunostaining: (Figs 3, 4 & 6)
- 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. Roholl PJM, Elbers HRJ, Prinsen I, Claessens JAJ, van Unnik JAM. Distribution of actin isoforms in sarcomas: an immunohistochemical study. Hum Pathol 1990;21:1269-74.
2. Oda Y, Miyajima K, Kawaguchi K, Tamiya S, Oshiro Y, Hachitanda Y, Oya M, Iwamoto Y, Tsuneyoshi M. Pleomorphic leiomyosarcoma: clinicopathologic and immunohistochemical study with special emphasis on its distinction from ordinary leiomyosarcoma and malignant fibrous histiocytoma. Am J Surg Pathol. 2001 Aug;25(8):1030-8.

Second Antigen: CK 5/6

Cytokeratin 5/6 (CK 5/6) is a very useful antibody in diagnostic pathology. It is particularly valuable in the differential diagnosis of carcinomas and the distinction between low differentiated squamous cell carcinoma and adenocarcinoma:
Characteristically squamous carcinomas stain strongly and diffusely, but generally adenocarcinomas stain focally, weakly, or not at all. CK5 expression in the mesothelium also makes this antibody a helpful marker in the differentiation between epithelioid mesothelioma and lung carcinoma when used with other antibodies against mesothelioma markers (Clover et al.) CK5/6 is also useful in the breast pathology setting and in the interpretation of problematic breast biopsies to help distinguish between DCIS or atypical hyperplasia and florid hyperplasia (Rabban et al., Otterbach et al.).

Features of Optimal Immunostaining:
Lung Squamous Cell Carcinoma: (Figs 7 & 9)
- Strong cytoplasmic staining of the lung squamous cell carcinoma.
- No background staining

Lung Adenocarcinoma:
- No staining
- No background staining

Suboptimal Immunostaining: (Figs 8 & 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 lung adenocarcinoma

References:
1. J Clover, et al. Anti-cytokeratin 5/6: a positive marker for epithelioid mesothelioma. Histopathol 31: 140-3, 1997
2. J Rabban, et al. Solid papillary ductal carcinoma in situ versus usual ductal hyperplasia in the breast: a potentioally difficult distinction resolved by cytokeratin 5/6.
3. F Otterbach et al. Cytokeratin 5/6 immunohistochemistry assists the differential diagnosis of atypical proliferations of the breast. Histopathol 37: 232-240, 2000

Assessment Summary:

SMA

311 laboratories submitted their slides for the SMA assessment. All, apart from 2 labs also submitted their in-house control sections. The results were similar to the last time SMA was assessed (Run 111) as shown in the table below:

SMA Pass Rates:		
	Run 111	**Run 113**
Acceptable	83% (N=267)	84% (N=261)
Borderline	13% (N=41)	14% (N=42)
Unacceptable	3% (N=10)	3% (N=8)

The reason for failure was either due to very weak staining or excessive background staining. Both of these issues were mostly due to inappropriate antibody dilution and pre-treatment. As an example, one of the labs showing weak staining is using a dilution of 1:5000 with the Dako 1A4 clone and no antigen retrieval.
It is likely that the combination of a high dilution of antibody with insufficient pre-treatment was the reason for the weak staining seen. Other slides showing excessive background were mostly due to excessive antigen retrieval or a lengthy incubation time with the antibody. Similarly to the previous runs, the Dako 1A4 clone remains the most popular antibody, used by 187 participants and showed an acceptable pass rate of 79% in the current assessment. There was an increase in the users of the Ventana 1A4 clone, with now 39 labs using this clone compared to 33 participants in the previous SMA assessment. This antibody showed an acceptable pass rate of 82%. The Leica/Novocastra asm-1 antibody was also popular, used by 25 laboratories in the current assessment, and showed an acceptable pass rate of 84%. The in-house material showed very similar pass rates to the UK NEQAS distributed sections.

Julie Williams and Suzanne Parry

Only 4 labs failed on their own in house control; 3 of these labs also failed on the Neqas material for the same reasons as their in-house tissue. Most labs are using appendix as their in-house control material, which is ideal to demonstrate SMA staining. Other labs used tonsil, colon, breast or multi-tissue controls containing elements positive for SMA.

CK5/6

304 laboratories submitted slides for the CK5/6 assessment, and of these, all apart from 3 labs also submitted their in-house material. Similarly to the SMA assessment, the pass rates were very good. These are summarised in the table below:

CK 5/6 Pass Rates Run 113:	
Acceptable	95% (N=290)
Borderline	3% (N=8)
Unacceptable	2% (N=6)

It was noted that several labs submitted a CK5 antibody, (CK5 alone does not cross-react with CK6 and therefore showed a slightly lower expression than expected) instead of a CK5/6 antibody. Some labs also submitted other CK cocktails, including CK5/6/8/18 and CK5/6/7.
All the slides that failed the assessment showed very weak or little demonstration of CK5/6 and were therefore considered unacceptable.

The in-house results showed similar pass rates to the Neqas distributed slides, with 96% receiving an acceptable pass, a further 4% achieved a borderline pass, and only 2 labs (1%) failed the assessment. Again, the reason for failure on the in-house tissue was due to very weak staining. Dako's D5/16 B4 clone was the most popular choice of antibody clone

This was used by 167 labs and showed a pass rate of 95%. The Ventana D5/16B4 antibody was also a popular choice, used by 47 laboratories, and showed an acceptable pass rate of 100%. The Leica/Novocastra CK5 only XM-26 clone was used by 44 participants, and this showed an acceptable pass rate of 100%. A range of tissues were used as the participant's in-house tissues, including tonsil, skin, prostate and mesothelioma, and many labs using multi-tissue controls.

Selected Images showing Optimal and Sub-optimal Immunostaining

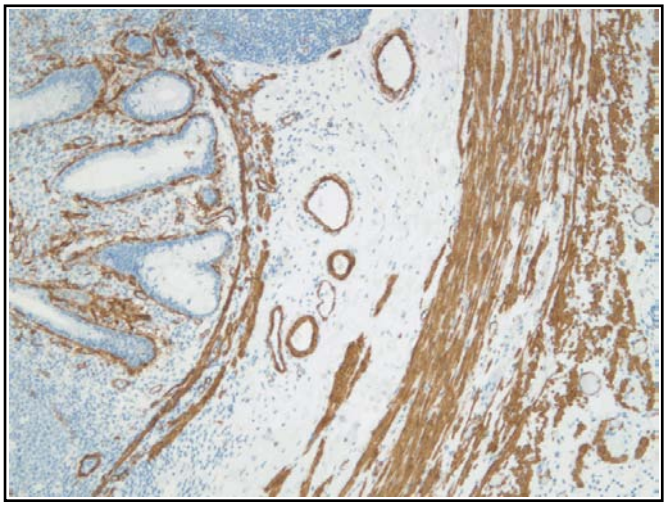


Fig 1. Optimal demonstration of SMA in the UK NEQAS appendix section, showing strong staining of the smooth muscle layers in the muscularis propria and muscualris mucosae. Stained with the Dako 1A4 clone, 1:200, on the Dako Omnis platform with 30 minutes antigen retrieval in high pH buffer, FLEX detection kit.

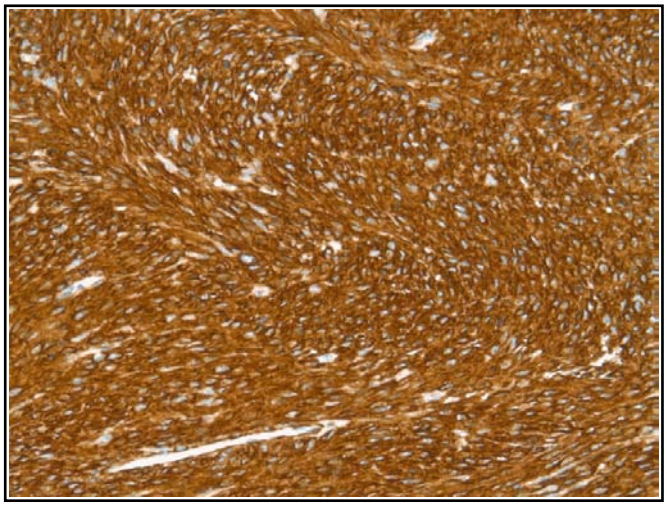


Fig 2. Good demonstration of SMA on the UK NEQAS distributed leiomyosarcoma section: The staining is strong and crisp with a nice clean background (same protocol as Fig 1).

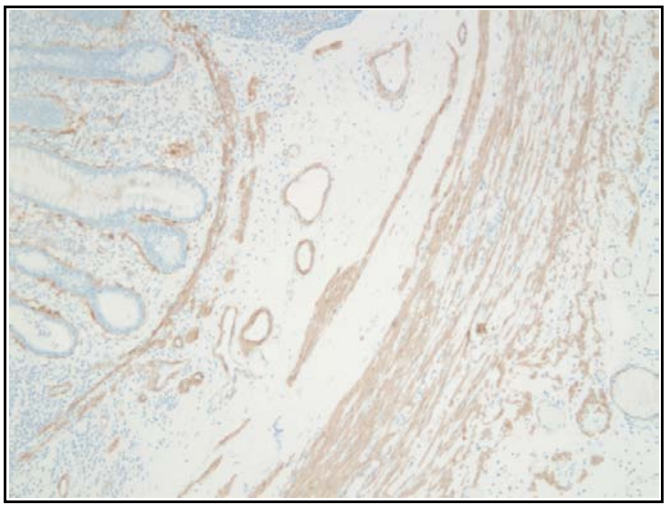


Fig 3. Borderline acceptable SMA on the UK NEQAS appendix section (compare to Fig 1). Although the smooth muscle layers in the muscularis propria and muscualris mucosae are demonstrated, the staining is weak and some of the finer fibres and myofibroblasts are not staining. Stained with the Dako 1A4 clone, 1:400 on the Ventana ULTRA and no pre-treatment.

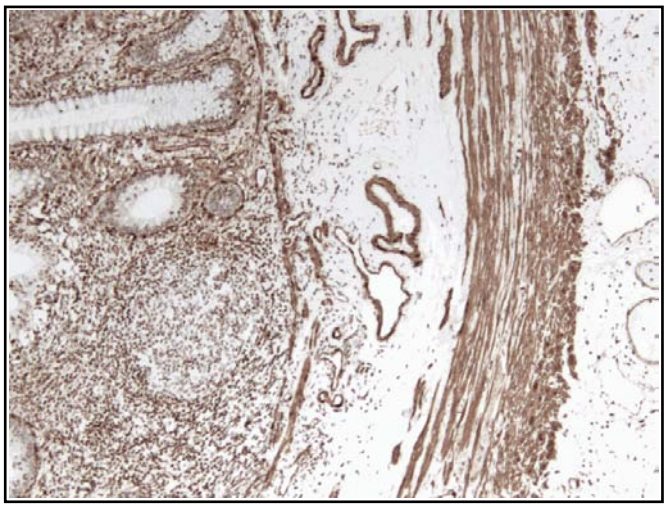


Fig 4. Unacceptable demonstration of SMA on the UK NEQAS appendix section. There is inappropriate staining of many cells not expected to stain. Stained using the Dako 1A4 clone, 1:5000 on the Ventana ULTRA with CC1 retrieval for 32 minutes and OptiView detection.

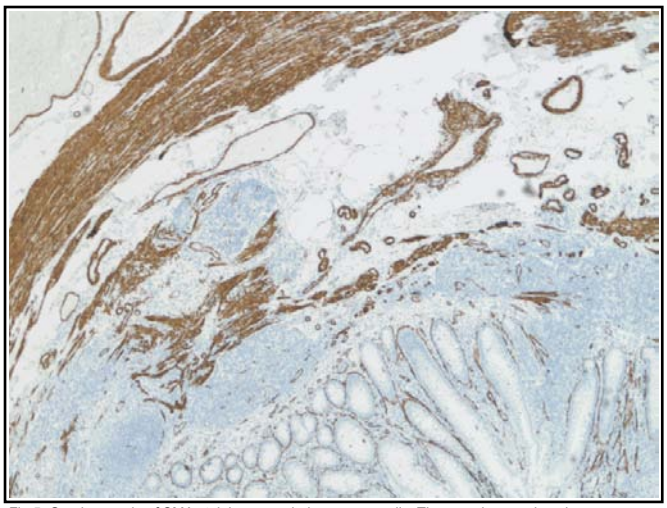


Fig 5. Good example of SMA staining on an in-house appendix. The sample was placed as requested alongside the NEQAS distributed sample on the same slide. Same protocol as in Fig 1.

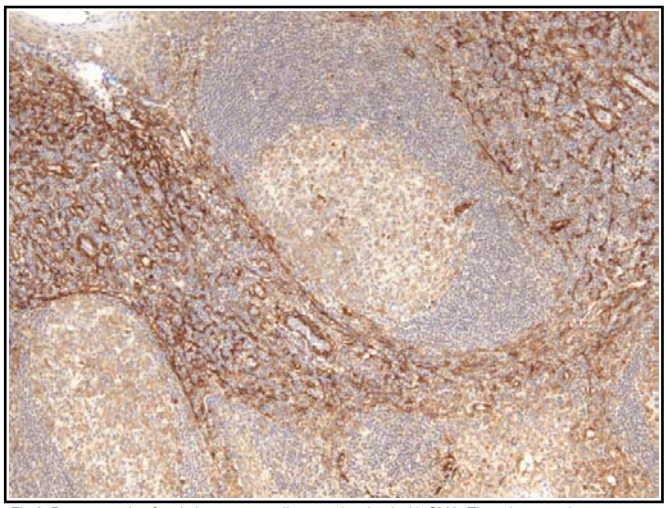


Fig 6. Poor example of an in house appendix control stained with SMA. There is excessive background and non-specific staining. Stained using the Leica/Novocastra pre-diluted antibody on the BondMax with retrieval in ER2 for 10 minutes.

Selected Images showing Optimal and Sub-optimal Immunostaining

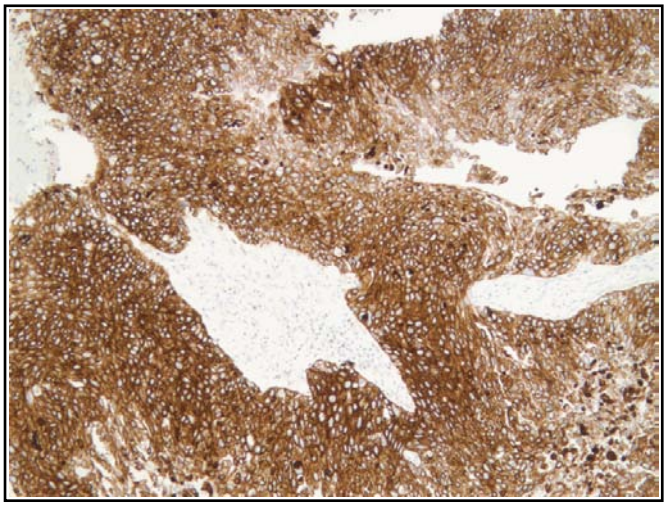


Fig 7. Optimal demonstration of CK5/6 in the UK NEQAS distributed lung tumour. Example demonstrates moderate to strong cytoplasmic staining of the neoplastic cells, while the background remains clean. Stained using the Dako D5/16 B4 clone, 1:50, on the Ventana Benchmark XT with CC1 standard antigen retrieval.

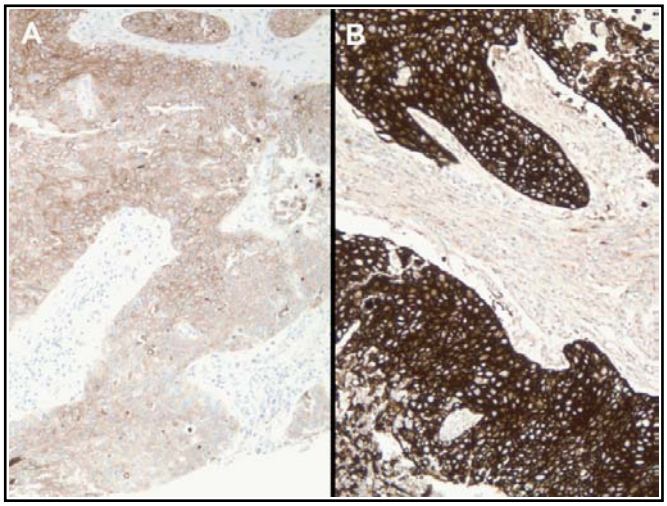


Fig 8. Two examples of sub-optimal CK5/6 staining on the UK NEQAS distributed lung tumour sample. Although both examples show staining of the neoplastic cells (A) the staining is weak, while (B) the staining is too strong and there is some background staining, most likely due to excessive pre-treatment (ER2 for 30 minutes on the Bond III).

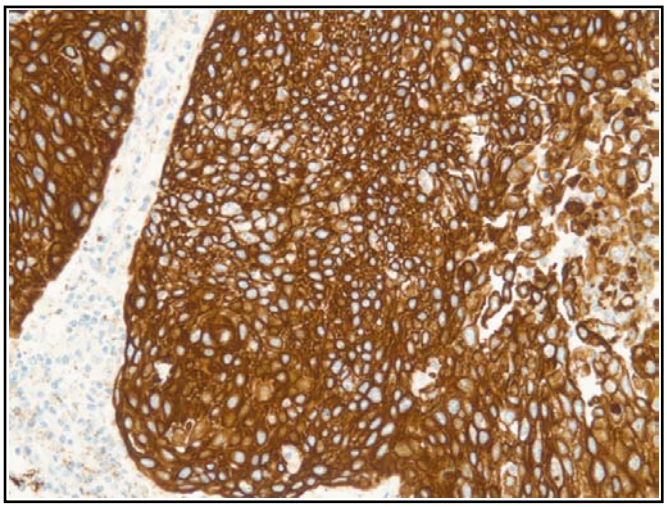


Fig 9. Good example of CK5/6 staining on the UK NEQAS distributed lung tumour, showing strong cytoplasmic staining of the neoplastic cells. Section stained with the Dako Omnis RTU D5/16 B4 antibody on the Dako Omnis with Flex detection.

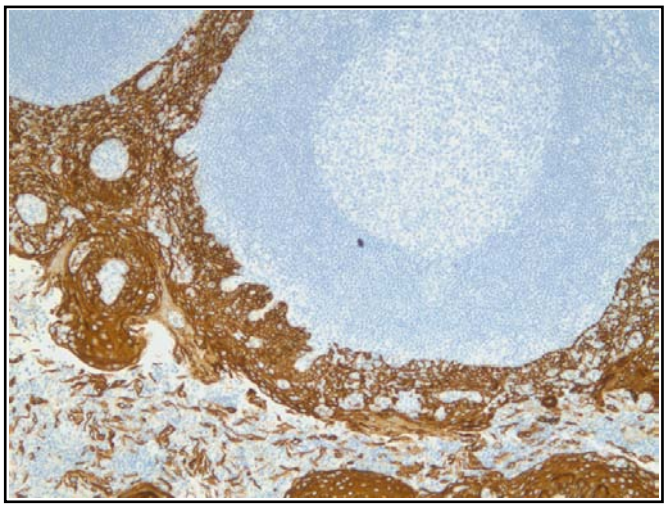


Fig 10. Good demonstration of CK5/6 on the UK NEQAS tonsil, showing strong cytoplasmic staining (same protocol as Fig 9).

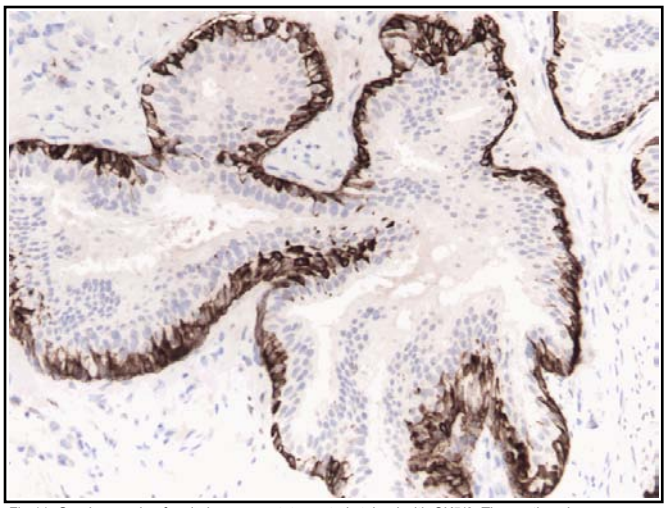


Fig 11. Good example of an in-house prostate control stained with CK5/6. The section shows strong cytoplasmic staining of virtually all the basal cells. Stained using the Dako D5/16 B4 clone on the Leica BondMax with ER2 antigen retrieval.

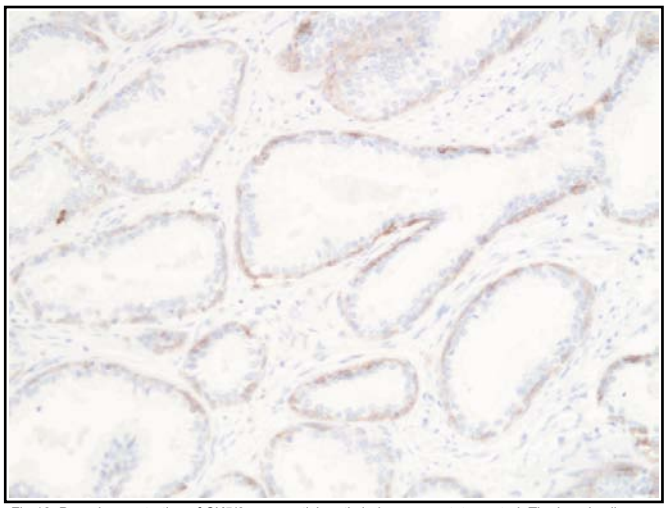
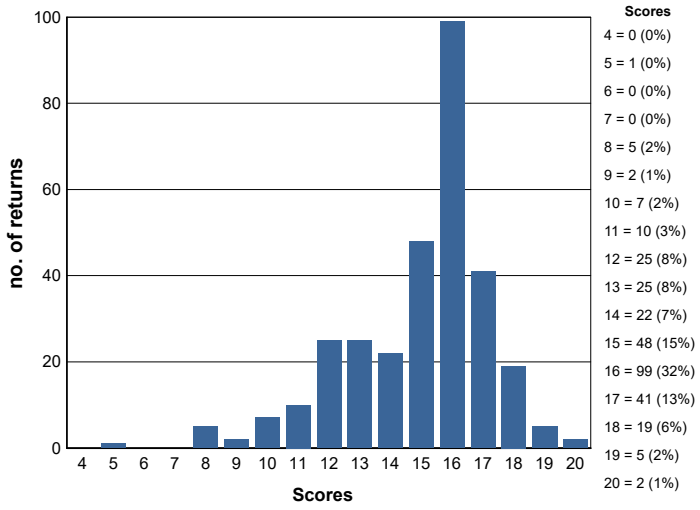


Fig 12. Poor demonstration of CK5/6 on a participant's in-house prostate control: The basal cell staining is very weak. Stained using the Dako D5/16 B4 clone on the Ventana XT with CC1 standard antigen retrieval.

GRAPHICAL REPRESENTATION OF PASS RATES

RUN 113A Smooth Muscle Actin (SMA) on NEQAS Sections

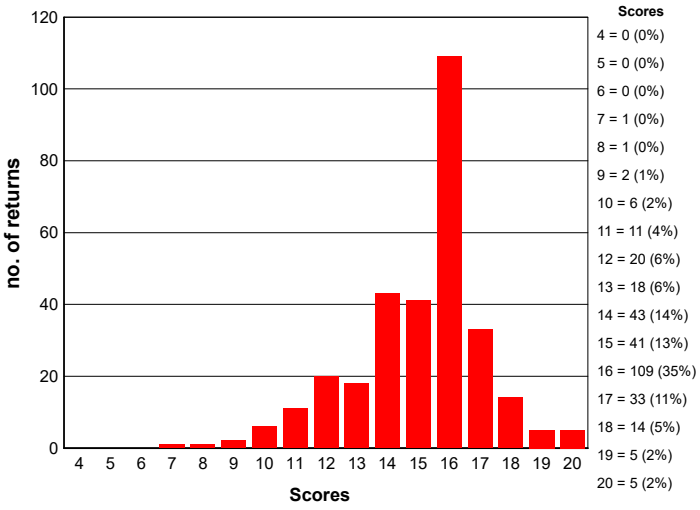


Individual Scores
4 = 0 (0%)
5 = 1 (0%)
6 = 0 (0%)
7 = 0 (0%)
8 = 5 (2%)
9 = 2 (1%)
10 = 7 (2%)
11 = 10 (3%)
12 = 25 (8%)
13 = 25 (8%)
14 = 22 (7%)
15 = 48 (15%)
16 = 99 (32%)
17 = 41 (13%)
18 = 19 (6%)
19 = 5 (2%)
20 = 2 (1%)

Summary
16 - 20 = 166 (53%)
13 - 15 = 95 (31%)
10 - 12 = 42 (14%)
0 - 9 = 8 (3%)

Median = 13.50

RUN 113B Smooth Muscle Actin (SMA) on in-house Sections

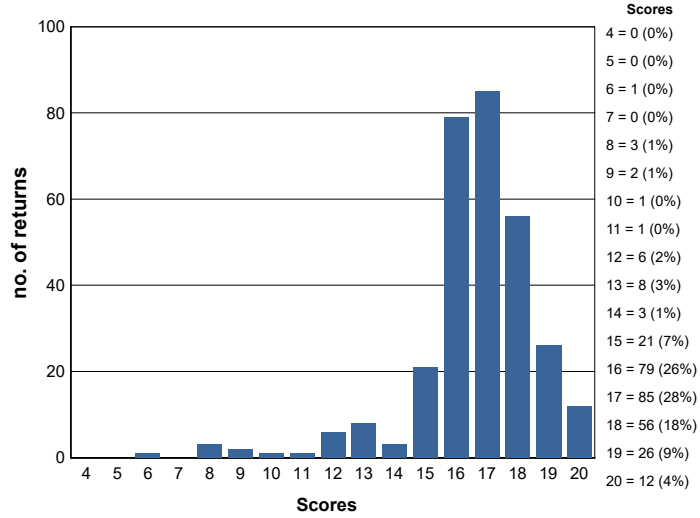


Individual Scores
4 = 0 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 1 (0%)
8 = 1 (0%)
9 = 2 (1%)
10 = 6 (2%)
11 = 11 (4%)
12 = 20 (6%)
13 = 18 (6%)
14 = 43 (14%)
15 = 41 (13%)
16 = 109 (35%)
17 = 33 (11%)
18 = 14 (5%)
19 = 5 (2%)
20 = 5 (2%)

Summary
16 - 20 = 166 (54%)
13 - 15 = 102 (33%)
10 - 12 = 37 (12%)
0 - 9 = 4 (1%)

Median = 13.50

RUN 113C CK 5/6 on NEQAS Sections

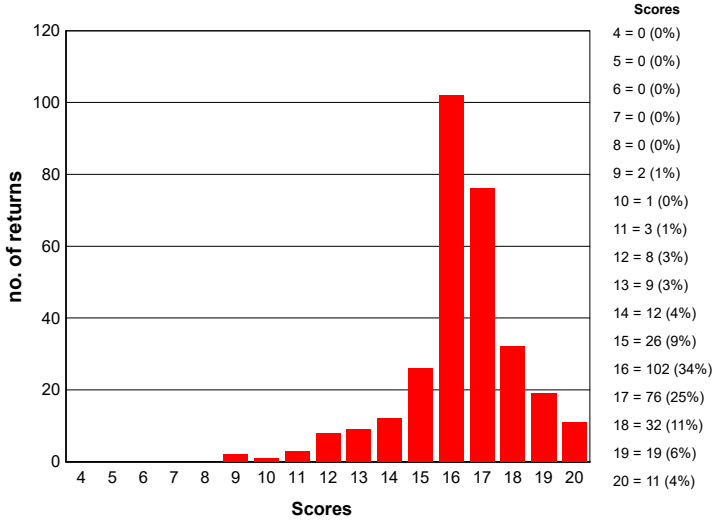


Individual Scores
4 = 0 (0%)
5 = 0 (0%)
6 = 1 (0%)
7 = 0 (0%)
8 = 3 (1%)
9 = 2 (1%)
10 = 1 (0%)
11 = 1 (0%)
12 = 6 (2%)
13 = 8 (3%)
14 = 3 (1%)
15 = 21 (7%)
16 = 79 (26%)
17 = 85 (28%)
18 = 56 (18%)
19 = 26 (9%)
20 = 12 (4%)

Summary
16 - 20 = 258 (85%)
13 - 15 = 32 (11%)
10 - 12 = 8 (3%)
0 - 9 = 6 (2%)

Median = 13.50

RUN 113D CK 5/6 on in-house Sections



Individual Scores
4 = 0 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 0 (0%)
8 = 0 (0%)
9 = 2 (1%)
10 = 1 (0%)
11 = 3 (1%)
12 = 8 (3%)
13 = 9 (3%)
14 = 12 (4%)
15 = 26 (9%)
16 = 102 (34%)
17 = 76 (25%)
18 = 32 (11%)
19 = 19 (6%)
20 = 11 (4%)

Summary
16 - 20 = 240 (80%)
13 - 15 = 47 (16%)
10 - 12 = 12 (4%)
0 - 9 = 2 (1%)

Median = 14.50

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

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

General Pathology Run: 113

Primary Antibody : Smooth Muscle Actin (SMA)

Antibody Details	N	%
Dako M0851 SMA (Clone 1A4)	172	85
Thermo Scientific/Neomarkers MS 113-P (1A4)	2	50
Novocastra NCL-SMA (asm-1)	17	76
Ventana 760 2833 (1A4)	39	82
Ventana (HUC1-1) 760-2502	2	100
Novocastra RTU-SMA (asm-1)	3	67
Leica/Novocastra PA0943 (asm-1)	22	100
Ventana 760-2601 (HHF35)	4	100
A. Menarini MP-001C/Px (1A4)	1	100
Dako M0635 SMA (Clone HHF35)	3	33
Dako IR611 RTU Flex Link (1A4)	15	73
Cell Marque 201M (Clone HHF35)	1	0
Cell Marque 202M (Clone 1A4)	9	78
Invitrogen 18-0106 (1A4)	3	100
Sigma (1A4) A2547	10	100
Other	3	100

General Pathology Run: 113

Primary Antibody : CK 5/6

Antibody Details	N	%
Biogenex (EPR1600Y & EPR1602Y)	3	100
Cell Marque CMA775/6/7 (D516B4)	3	100
Cell Marque CMC775/6/7 (D516B4)	5	80
Chemicon MAB1620 (D5/16B4)	1	100
Dako RTU Link IR780 CK5/6 (D5/16B4)	22	91
Dako IS780 RTU Plus CK 5/6 (D5/16B4)	14	100
Dako M7237 (D5/16 B4)	131	93
Invitrogen/Zymed 08-0267 (D5/16B4)	1	100
Invitrogen/Zymed 18-0267 (D5/16B4)	4	100
Leica NCL-CK5 (XM-26)	38	100
Leica PA0568 RTU (XM-26)	6	100
Other	8	75
Ventana 760-4253 (D5/16B4)	15	100
Ventana 790-4554 (D5/16B4)	32	100
Dako Omnis GA780 RTU CK5/6 (D5/16B4)	4	100

General Pathology Run: 113

Heat Mediated Retrieval	CK 5/6 N	CK 5/6 %	Smooth Muscle Actin (SMA) N	Smooth Muscle Actin (SMA) %
_Leica BondMax ER1	2	100	0	0
_Leica BondMax ER2	2	100	0	0
_Ventana Benk XT CC1 (8mins)	0	0	1	100
_Ventana Benk XT CC1 (Mild)	1	100	0	0
_Ventana Benk XT CC1 (Standard)	1	100	0	0
Biocare Decloaking Chamber	1	100	1	100
Dako Omnis	9	100	8	100
Dako Pascal	1	100	1	100
Dako PTLink	36	97	38	76
Lab vision PT Module	4	100	2	50
Leica ER1 10 mins	0	0	9	67
Leica ER1 20 mins	4	75	4	75
Leica ER1 30 mins	9	89	4	100
Leica ER2 10 mins	0	0	6	83
Leica ER2 20 mins	48	92	18	94
Leica ER2 30 mins	15	87	3	100
Leica ER2 40 mins	3	67	0	0
Microwave	3	100	4	50
None	5	100	110	89
Other	3	100	2	50
Pressure Cooker	4	100	3	100
Pressure Cooker in Microwave Oven	1	100	1	100
Steamer	2	50	2	100
Ventana CC1 16mins	0	0	8	88
Ventana CC1 20mins	0	0	1	100
Ventana CC1 24mins	5	100	3	67
Ventana CC1 32mins	13	100	5	60
Ventana CC1 36mins	3	67	2	50
Ventana CC1 40mins	4	100	1	100
Ventana CC1 48mins	5	100	1	0
Ventana CC1 52mins	1	100	0	0
Ventana CC1 56mins	7	100	1	0
Ventana CC1 64mins	40	100	9	78
Ventana CC1 8mins	1	100	23	91
Ventana CC1 92mins	0	0	1	100
Ventana CC1 extended	2	100	1	100
Ventana CC1 mild	4	100	12	67
Ventana CC1 standard	33	100	10	80
Ventana CC2 64mins	1	100	0	0
Ventana CC2 standard	1	100	0	0
Water bath 68 OC	1	100	1	100
Water bath 95-98 OC	0	0	1	0

General Pathology Run: 113

Enzyme Mediated Retrieval	CK 5/6 N	CK 5/6 %	Smooth Muscle Actin (SMA) N	Smooth Muscle Actin (SMA) %
AS PER KIT	3	100	5	80
NOT APPLICABLE	119	96	174	81
Other	2	100	1	100
VBS Bond Enzyme 1	2	100	2	50
Ventana Protease	2	100	2	100
Ventana Protease 1 (760-2018)	4	100	0	0

General Pathology Run: 113	CK 5/6		Smooth Muscle Actin (SMA)	
Detection	**N**	**%**	**N**	**%**
A Menerini Polymer (MP-XCP)	0	0	1	100
AS PER KIT	23	91	22	86
BioGenex SS Polymer (QD 420-YIKE)	1	100	1	100
Dako EnVision FLEX (K8000/10)	10	90	13	69
Dako EnVision FLEX+ (K8002/12)	25	100	24	83
Dako Envision HRP/DAB (K5007)	5	100	7	100
Dako Envision+ HRP mouse K4004/5/6/7	1	100	2	0
Dako Envision+ HRP rabbit K4008/9/10/11	1	100	1	100
Dako rb-a-mo Ig (E0354)	1	100	0	0
ID Labs Universal mo kit HRP (IDSTM001)	0	0	1	0
LabVision UltraVision LP HRP (TL 125 HLJ)	0	0	1	0
LabVision UltraVision ONE Polymer (TL-012/5-HDJ/T	1	100	0	0
Leica Bond Polymer Define (DS9713)	0	0	1	100
Leica Bond Polymer Refine (DS9800)	78	87	91	92
MenaPath X-Cell Plus (MP-XCP)	1	100	2	50
None	1	100	3	67
NOT APPLICABLE	1	100	1	100
Novocastra Novolink PDS (RE7-140/150/280/290-K)	1	100	1	100
Other	12	92	8	50
Ventana iView system (760-091)	7	100	5	80
Ventana OptiView (760-700) + Amp. (7/860-099)	1	100	4	100
Ventana OptiView Kit (760-700)	42	100	40	83
Ventana UltraView Kit (760-500)	75	99	75	83

General Pathology Run: 113	CK 5/6		Smooth Muscle Actin (SMA)	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	1	100	1	100
Dako Autostainer	2	100	2	100
Dako Autostainer Link 48	29	100	37	81
Dako Autostainer plus	6	67	2	50
Dako Autostainer Plus Link	3	100	3	33
Dako Omnis	9	100	8	100
LabVision Autostainer	3	100	6	50
Leica Bond Max	48	88	43	95
Leica Bond X	1	100	0	0
Leica Bond-III	47	87	55	89
Menarini - Intellipath FLX	2	100	3	67
None (Manual)	8	75	9	78
Other	1	100	0	0
Shandon Sequenza	2	100	3	67
Ventana Benchmark GX	3	100	3	100
Ventana Benchmark ULTRA	76	99	81	83
Ventana Benchmark XT	55	100	53	79

General Pathology Run: 113	CK 5/6		Smooth Muscle Actin (SMA)	
Chromogen	**N**	**%**	**N**	**%**
A. Menarini Liquid Stable DAB kit	0	0	1	100
AS PER KIT	38	95	31	87
BioGenex Liquid DAB (HK153-5K)	1	100	1	100
Dako DAB K3468	1	100	0	0
DAKO DAB+	1	100	1	0
Dako DAB+ Liquid (K3468)	3	100	4	75
Dako EnVision Plus kits	1	100	3	67
Dako FLEX DAB	35	97	40	80
Dako REAL EnVision K5007 DAB	5	80	7	100
Dako REAL K5001 DAB	1	100	0	0
Leica Bond Polymer Refine kit (DS9800)	78	87	89	91
menapath xcell kit DAB (MP-860)	2	100	2	50
Other	19	100	19	68
Sigma DAB (D5637)	1	0	1	100
Ventana DAB	21	100	20	80
Ventana Enhanced Alk. Phos. Red Detection Kit	0	0	1	100
Ventana iview	5	100	4	100
Ventana Ultraview DAB	85	99	84	81
Vision BioSystems Bond X DAB	1	0	0	0

BEST METHODS - Gold Standard Antibody

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

Smooth Muscle Actin (SMA) - Method 1

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

Primary Antibody: Dako M0851 SMA (Clone 1A4) , 15 Mins, RT ºC Dilution 1: 600

Automation: Leica Bond-III

Method: Leica BondMAx Refine KIT

Main Buffer: Bond Wash Buffer (AR9590)

HMAR: Leica ER1 10 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

Smooth Muscle Actin (SMA) - Method 2

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

Primary Antibody:	Dako M0851 SMA (Clone 1A4) , 20 Mins, 32 ºC Dilution 1: 200
Automation:	Dako Omnis
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis, PH: 9
EAR:	
Chromogen:	Dako FLEX DAB, 32 ºC., Time 1: 5 Mins
Detection:	Dako EnVision FLEX (K8000/10) , 30 Mins, 32 ºC

Smooth Muscle Actin (SMA) - Method 3

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

Primary Antibody:	Dako M0851 SMA (Clone 1A4) , 15 Mins, 37 ºC Prediluted
Automation:	Dako Omnis
Method:	Dako FLEX+ kit
Main Buffer:	Dako Wash Buffer (S3006)
HMAR:	Dako Omnis
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB
Detection:	Dako EnVision FLEX+ (K8002/12) Prediluted

Smooth Muscle Actin (SMA) - Method 4

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

Primary Antibody:	Ventana 760 2833 (1A4) , 24 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark GX
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 8mins
EAR:	
Chromogen:	AS PER KIT, 37 ºC., Time 1: 8 Mins, Time 2: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 37 ºC Prediluted

BEST METHODS - Secondary Antibody

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

CK 5/6 - Method 1

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

Primary Antibody:	Dako Omnis GA780 RTU CK5/6 (D5/16B4) , 12.5 Mins, 32 ºC Prediluted
Automation:	Dako Omnis
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis, Buffer: Target Retrieval Solution High
EAR:	
Chromogen:	Dako FLEX DAB, Time 1: 5 Mins
Detection:	Dako EnVision FLEX (K8000/10) , 20 Mins, 32 ºC Prediluted

CK 5/6 - Method 2

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

Primary Antibody:	Dako M7237 (D5/16 B4) , 32 Mins, 36 ºC Dilution 1: 80
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700)

CK 5/6 - Method 3

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

Primary Antibody:	Leica NCL-CK5 (XM-26) , 19 Mins Dilution 1: 100
Automation:	Ventana Benchmark XT
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 32mins
EAR:	
Chromogen:	Ventana DAB
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099)

CK 5/6 - Method 4

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

Primary Antibody:	Cell Marque CMC775/6/7 (D516B4) , 50 Mins, 22 ºC Dilution 1: 60
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX kit
Main Buffer:	AS PER KIT, PH: 7.6
HMAR:	Dako PTLink, Buffer: as per kit, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT, 22 ºC., Time 1: 10 Mins
Detection:	Dako EnVision FLEX (K8000/10) , 20 Mins, 22 ºC Prediluted

The Breast Hormonal Receptor Module Run 113

Merdol Ibrahim and Suzanne Parry

Antigen Assessed:	Oestrogen Receptor (ER)
Tissue Sections circulated:	Composite slide consisting of 3 breast carcinomas with different levels of receptor expression and normal tonsil.
Number of Registered Participants:	289
Number of Participants This Run	271 (94%)

Circulated Tissue
The table below shows the staining characteristics of the tissue sections circulated during Run 113. This composed of three infiltrating ductal carcinomas (IDCs) with differing levels of receptor expression along with a section of tonsil. The staining of the breast tumours were characterised using the Leica 6F11, Ventana SP1 and Dako EP1 clones.

Sections	% Positivity	Expected Staining Intensity	Allred / Quick Score
A. IDC	>95%	High	8
B. IDC	50-70%	Mid	6 (the expression level varied depending on the serial section received)
C. IDC	0%	Negative	0
E. Tonsil	≤ 3%	Weak to Medium	0 (Negative)

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

General Guideline Used in The Assessment of slides:

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

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

In-House Tissue Recommendations:

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

The required composite control should consist of the following samples:

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

Participants NOT using a composite control are scored a 'borderline' pass (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 three invasive breast tumours of varying ER expression level (samples A-C). A tonsil (sample D) was also included and used as a further negative control to help gauge the specificity of the test.

NEQAS tissue samples were tested by staining every 50th section from the relevant tissue blocks using the Leica 6F11 clone. This made sure that any heterogeneity in the tissue samples did not result in a participant being penalised. Furthermore, samples were also tested by the relevant commercial companies to further verify the expected level of staining and included Leica (clone 6F11), Dako (1D5/ER-2-123 clones), Dako (EP1 clone) and Ventana (SP1 clone).

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

Features of Optimal Immunostaining (Figs 1-3)

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

Features of Suboptimal Immunostaining (Figs 4-7)

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

NEQAS Section Assessment Results

271 laboratories submitted their slides for the ER assessment. The results are summarised in the table below:

ER Pass Rates Run 113:	
Acceptable	88% (N=239)
Borderline	7% (N=18)
Unacceptable	5% (N=14)

The results were an improvement on the previous assessment of ER (Run 111), and were more in keeping with the ER assessment results seen at Run 110 (as shown in the following table):

	ER Pass Rates:		
	Run 110	**Run 111**	**Run 113**
Acceptable	85% (N=193)	74% (N=204)	88% (N=239)
Borderline	9% (N=25)	15% (N=40)	7% (N=18)
Unacceptable	6% (N=17)	11% (N=30)	5% (N=14)

The borderline and failed marks for this assessment Run 113 were mostly due to weak staining, particularly in the mid-expressing tumour. A few labs failed due to inappropriate staining and excessive background, and one particular lab showed unexpected membrane staining rather than the expected nuclear staining (shown in Fig 6A).

The Ventana SP1 clone was the most popular antibody, used by 75 (28%) of participating labs. This clone showed an acceptable pass rate of 97%. The Leica 6F11 and Dako EP1 were also popular clones, both were used by 55 (20%) of participating labs. They showed acceptable pass rates of 81% and 93% respectively.

In-House Tissue Assessment Results

99% of participants also submitted their in-house controls for assessment. Overall these showed a lower acceptable pass rate, with more participants receiving a borderline pass. The results are summarised in the table below:

In-House Pass Rates Run 113:	
Acceptable	77% (N=208)
Borderline	22% (N=58)
Unacceptable	1% (N=3)

Many of the borderline passes were due to the lab not providing a composite control consisting of a high, a mid and a negative-expressing tumour, which is a requirement by UK NEQAS IHC. Other labs lost marks due to poor tissue quality, inappropriate non-specific staining or excessive background.

A few labs contacted UK NEQAS ICC to highlight that they are still having issues with their in-house control section lifting during staining. This problem first arose when the scheme changed slides and it now requires that labs 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 and at the same time as the NEQAS sections. Although a few labs are still having issues with adhesion, most labs have adjusted to the slides with no problems. Participants have been advised with the draining and baking of the sections once they have cut their own in-house tissue on the Neqas slide. From internal testing of the slides, it was noted that the draining of the sections thoroughly before baking is very important to ensure that the sections will not lift during staining. Any participant who did have issues with adhesion 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.

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

Another factor to take into consideration is that the sensitivity of a participant's assay may be acceptable on their own tissue section, which would have been validated prior to routine testing. However, the sensitivity of the assay still may not be optimal. A protocol should be in place that will stain effectively on both the NEQAS samples and the labs own samples.

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

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

Comparing NEQAS Sample Scores and In-house Control Scores.

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

- The **UK NEQAS** distributed samples are scored against the expected levels of staining as defined by; 1) initial patient diagnosis for ER; 2) re-tested and validated staining of every 50th section from the relevant tissue blocks using the Leica 6F11 clone; 3) re-tested and validated samples by the relevant commercial companies to further verify the expected level of staining. Such companies include the Novocastra/Leica (clone 6F11), Dako (clone EP1) and the Ventana (clone SP1) 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- (Allred 3-6) and a negative (Allred 0) ER-expressing tumour, participants are given a maximum score of 12/20. The presence of normal staining glands is also encouraged.

It is not possible to know the expected staining levels of the participants' in-house slides and whether there are cases showing false-positive/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.

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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

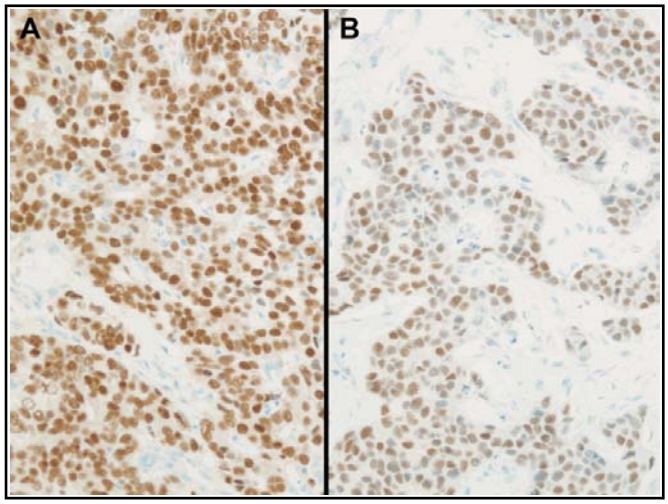


Fig 1. Optimally stained UK NEQAS distributed samples. (A) High ER expressing tumour shows intense staining in over 95% of neoplastic cells. (B) Mid-expressing tumour shows varying intensity of ER positivity in over 70% of neoplastic cells. Stained with the Ventana SP1 pre-diluted antibody on the ULTRA platform, CC1 retrieval for 52 mins with UltraView detection.

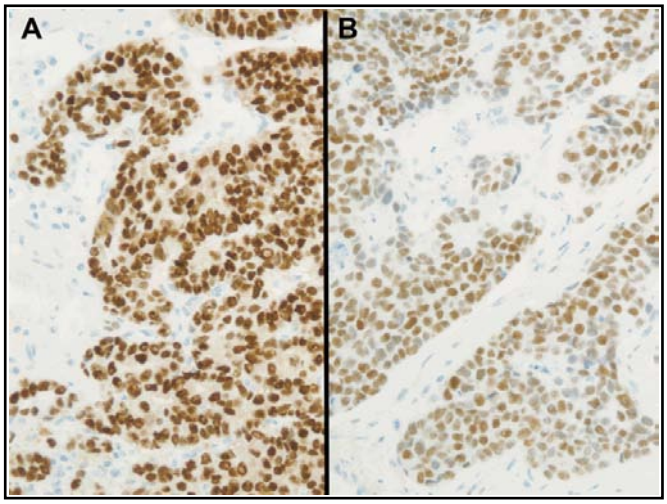


Fig 2. Optimal demonstration of ER in the UK NEQAS (A) high-an (B) mid- (B) ER expressing tumours. Both sections show the expected level of staining. Stained using the Dako RTU EP1 antibody on the Dako Autostainer, in a PT link with high pH retrieval.

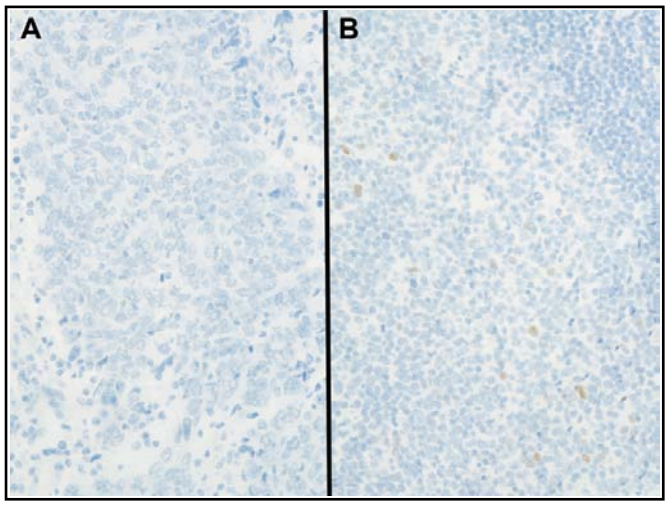


Fig 3. Optimal demonstration of ER in the UK NEQAS distributed samples. (A) ER negative tumour remains unstained, and as expected. (B) A small percentage of lymphocytes stained positive in the tonsil section. Same protocol as Fig 2.

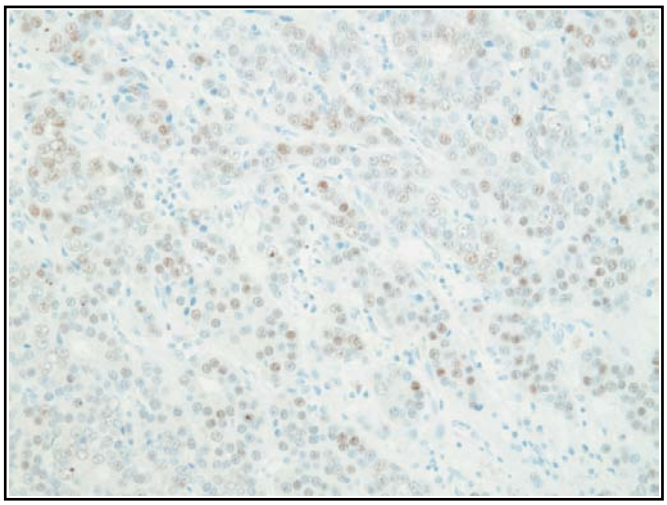


Fig 4. Unacceptable demonstration of ER in the UK NEQAS high-expressing tumour (compare to Figs 1A & 2A).The staining intensity is weak with less fewer cells staining positive than expected. Stained with the Leica/Novocastra 6F11 antibody, 1:40, and 16 minutes CC1 antigen retrieval on the Ventana Benchamrk XT.

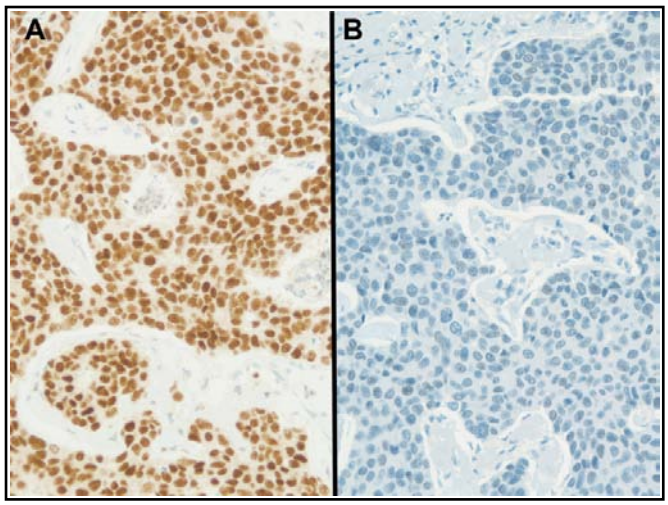


Fig 5. Two examples of unacceptable staining of the UK NEQAS mid-expressing tumour (compare to Figs 1B & 2B). (A) Staining is much higher than expected. B) Staining is very weak and slightly masked by the heavy counterstain. (A) Stained on the Ventana Benchmark ULTRA, SP1 pre-diluted antibody. (B) Stained with Leica 6F11 on Ventana Benchmark XT.

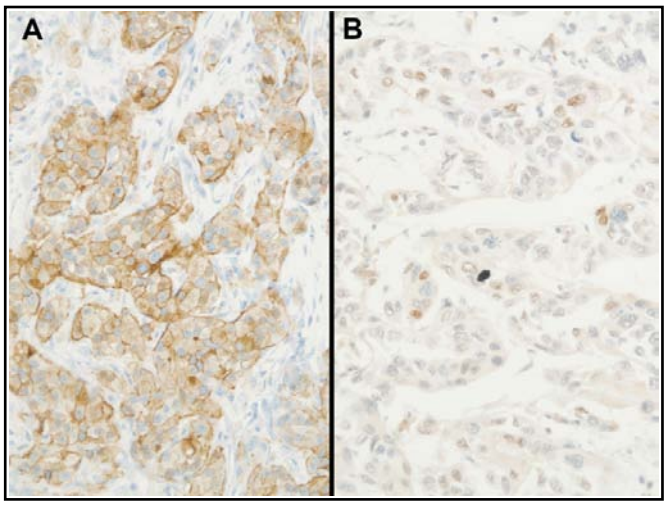


Fig 6. Poor staining of the UK NEQAS mid-expressing tumour. (A) Unexpected non-specific membranous staining, rather than the expected nuclear staining pattern. Stained using the Dako PharmDX Link anitbody and kit (1D5 + ER-2-123) on the Autostainer. (B) Weaker staining than expected, with non-specific cytoplasmic background staining.

Selected Images showing Optimal and Sub-optimal Immunostaining

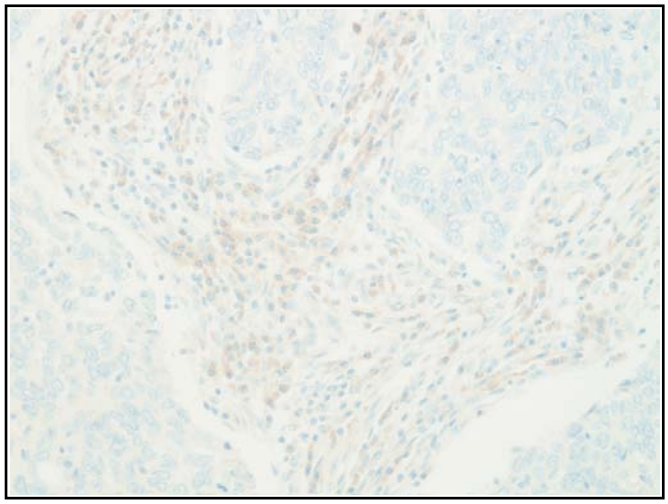


Fig 7. Inappropriate staining of plasma cells on the UK NEQAS negative expressing tumour. This was a feature commonly seen with sections stained using the Ventana detection kits. Assessors made an observational feedback comment, but labs were not deducted marks for this.

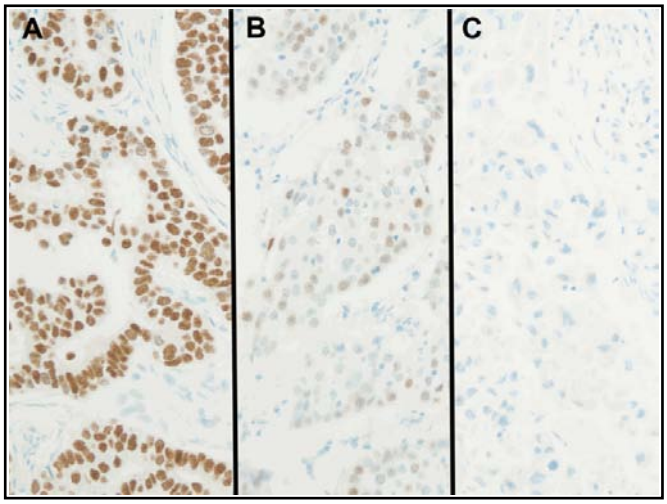
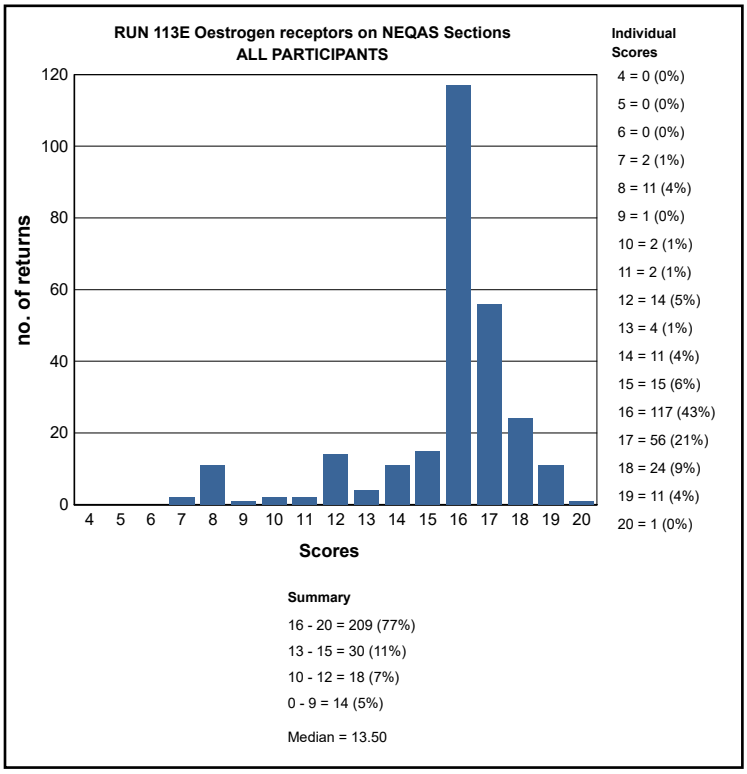
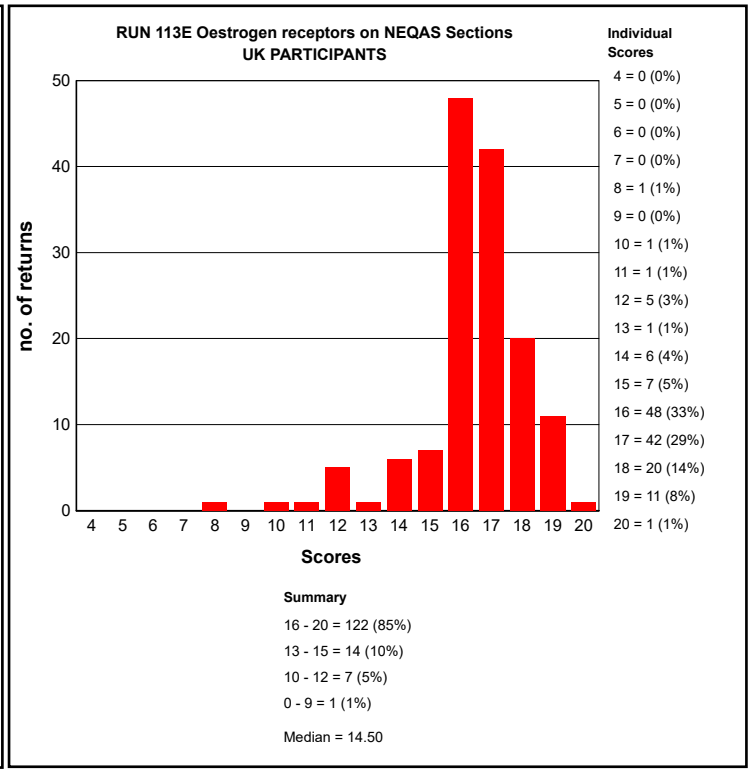
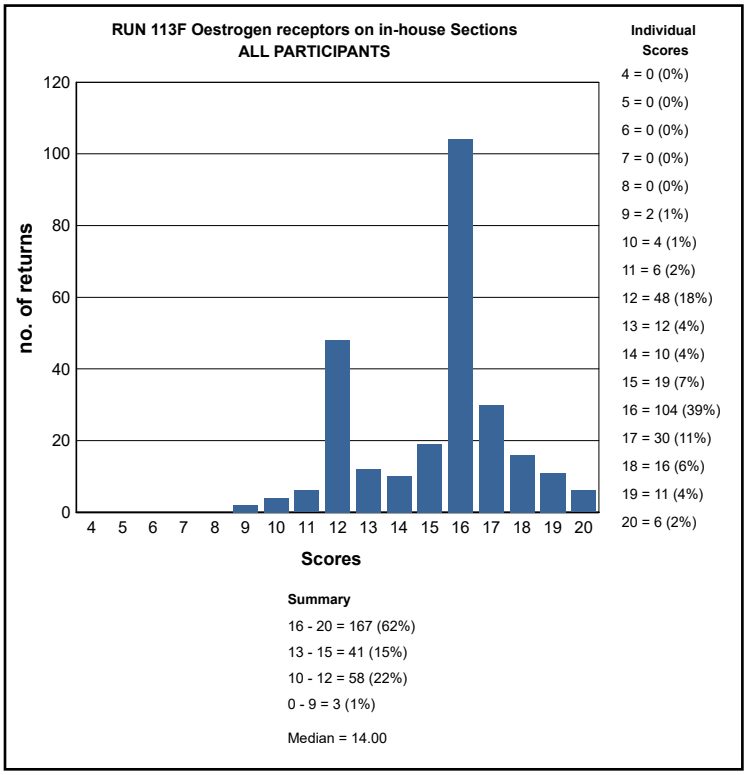


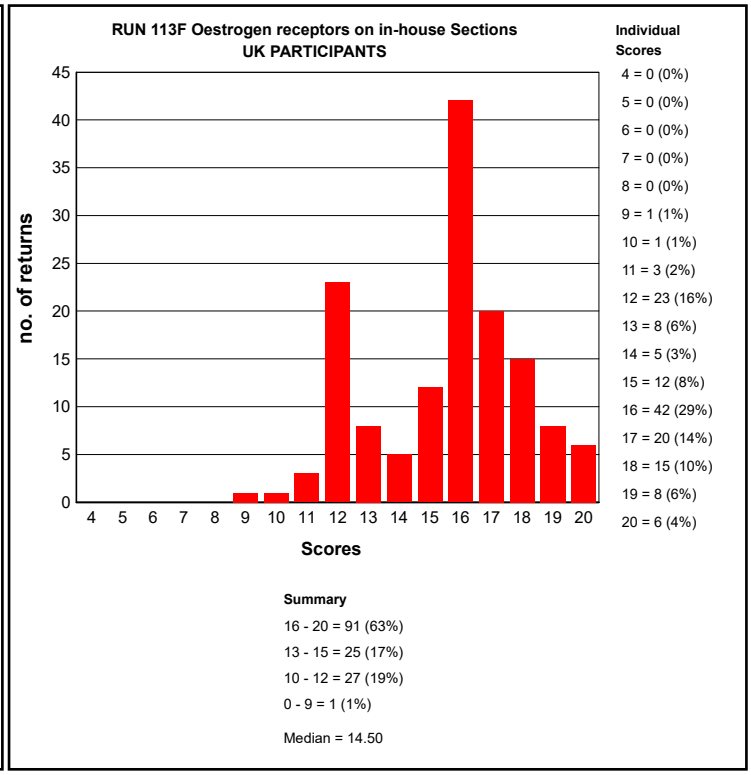
Fig 8. Excellent example and staining of an in-house ER control. The multi-block section contains high-, mid-, and negative-ER expressing tumours (A-C respectively). A control of known differing expression levels is important to gauge the sensitivity of the assay (same protocol as Fig 1)

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: 113

Primary Antibody : Oestrogen receptors

Antibody Details	N	%
Cell Marque 249-R (SP1)	2	100
Dako (EP1) M3643	29	83
Dako (EP1) RTU Auto Plus IS084	1	100
Dako (EP1) RTU FLEX IR084	25	96
Dako FLEX (1D5) IR/IS657	2	0
Dako IR151 Autostainer Link (SP1)	1	100
Dako M3634 (SP1)	3	100
Dako M7047 ER (1D5)	5	80
Dako SK310 pharmDX kit (Link) (1D5 + ER-2-123)	1	0
Leica Bond PA0151 (6F11)	5	60
Leica/Novocastra NCL-ER-6F11 (6F11)	10	80
Leica/Novocastra NCL-ER-6F11/2	13	92
Leica/Novocastra NCL-L-ER- 6F11	25	76
Leica/Novocastra RTU-ER-6F11	2	100
Other	3	33
Thermo Fisher/ Neomarkers RM 9101-S (SP1)	12	92
Vector VP-E613/4 (6F11)	4	100
Ventana 760-2132 (6F11)	1	100
Ventana 790-4324 (SP1)	75	97
Ventana 790-4325 (SP1)	36	97

Breast Steroid Hormone Receptor Run: 113

Automation	Oestrogen receptors N	%
Dako Autostainer Link 48	28	86
Dako Autostainer Plus Link	3	67
Dako Omnis	5	100
LabVision Autostainer	3	100
Leica Bond Max	31	77
Leica Bond-III	41	85
Menarini - Intellipath FLX	1	100
None (Manual)	5	40
Shandon Sequenza	2	100
Ventana Benchmark GX	7	86
Ventana Benchmark ULTRA	84	98
Ventana Benchmark XT	56	88

Breast Steroid Hormone Receptor Run: 113

Heat Mediated Retrieval	Oestrogen receptors N	%
Biocare Decloaking Chamber	1	100
Dako Omnis	5	100
Dako PTLink	30	87
Lab vision PT Module	3	100
Leica ER1 20 mins	11	55
Leica ER1 30 mins	15	80
Leica ER1 40 mins	5	100
Leica ER2 10 mins	3	100
Leica ER2 20 mins	29	93
Leica ER2 30 mins	6	67
Leica ER2 40 mins	2	50
Microwave	1	0
Other	1	100
Pressure Cooker	5	60
Ventana CC1 16mins	4	75
Ventana CC1 24mins	3	67
Ventana CC1 32mins	8	75
Ventana CC1 36mins	20	95
Ventana CC1 40mins	2	100
Ventana CC1 48mins	2	100
Ventana CC1 52mins	8	88
Ventana CC1 56mins	1	100
Ventana CC1 64mins	37	97
Ventana CC1 76mins	1	100
Ventana CC1 88mins	1	100
Ventana CC1 8mins	2	100
Ventana CC1 extended	5	100
Ventana CC1 mild	16	94
Ventana CC1 standard	33	94
Ventana CC2 64mins	1	100
Ventana CC2 mild	1	100
Water bath 95-98 OC	3	67

Breast Steroid Hormone Receptor Run: 113

Enzyme Mediated Retrieval	Oestrogen receptors N	%
AS PER KIT	7	100
NOT APPLICABLE	162	91
Ventana Protease 1 (760-2018)	1	100

Breast Steroid Hormone Receptor Run: 113

Detection	Oestrogen receptors N	%
AS PER KIT	12	92
Biocare polymer (M4U534)	1	100
Dako EnVision FLEX (K8000/10)	9	89
Dako EnVision FLEX+ (K8002/12)	19	84
Dako Envision HRP/DAB (K5007)	2	50
Dako Envision+ HRP mouse K4004/5/6/7	1	100
Dako rb-a-mo Ig (E0354)	1	100
Dako REAL HRP/DAB (K5001)	1	0
LabVision UltraVision LP HRP (TL 125 HLJ)	1	100
Leica Bond Polymer Refine (DS9800)	67	84
MenaPath X-Cell Plus (MP-XCP)	1	100
NOT APPLICABLE	2	50
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)	15	80
Ventana UltraView Kit (760-500)	123	94

Breast Steroid Hormone Receptor Run: 113

Chromogen	Oestrogen receptors N	%
AS PER KIT	20	90
Dako DAB K3468	1	100
DAKO DAB+	1	0
Dako EnVision Plus kits	2	50
Dako FLEX DAB	28	89
Dako REAL EnVision K5007 DAB	3	67
Dako REAL K5001 DAB	2	50
Leica Bond Polymer Refine kit (DS9800)	65	83
menapath xcell kit DAB (MP-860)	1	100
Other	8	75
Ventana DAB	7	86
Ventana Enhanced Alk. Phos. Red Detection Kit	1	100
Ventana iview	5	100
Ventana Ultraview DAB	123	93

BEST METHODS

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

Oestrogen receptors - Method 1

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

Primary Antibody: Dako (EP1) M3643 , 60 Mins, amb ºC Dilution 1: 120
Automation: Leica Bond Max
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER1 30 mins
EAR:
Chromogen: Leica Bond Polymer Refine kit (DS9800), amb ºC., Time 2: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 15 Mins, amb ºC Prediluted

Oestrogen receptors - Method 2

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

Primary Antibody: Leica/Novocastra NCL-ER-6F11 (6F11) Dilution 1: 1:50
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER1 20 mins
EAR:
Chromogen: Leica Bond Polymer Refine kit (DS9800)
Detection: Leica Bond Polymer Refine (DS9800)

Oestrogen receptors - Method 3

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

Primary Antibody: Ventana 790-4325 (SP1) , 8 Mins, 36 ºC Prediluted
Automation: Ventana Benchmark ULTRA
Method: Ventana UltraView DAB
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 36mins
EAR: NOT APPLICABLE
Chromogen: Ventana Ultraview DAB
Detection: Ventana UltraView Kit (760-500)

Oestrogen receptors - Method 4

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

Primary Antibody:	Dako (EP1) RTU FLEX IR084 , 30 Mins, 21 ºC Dilution 1: RTU
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	AS PER KIT
HMAR:	Dako PTLink, Buffer: trs high pH
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT, Time 1: 21 Mins, Time 2: 5 Mins
Detection:	AS PER KIT , 20 Mins, 21 ºC Prediluted

The Breast HER2 ICC Module Run 113

Keith Miller and Suzanne Parry

Antigen Assessed:	HER2
Sections Circulated:	Composite slide consisting of 4 breast carcinoma cell lines (see table below)
Number of Registered Participants:	348
Number of Participants this Run	292 (84%)

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.

With so many more cases now being tested worldwide, it is imperative that correct protocols and methodologies are followed and adhered to. The UK NEQAS HER2 IHC module assesses the technical quality of staining achieved by laboratories and provides feedback to laboratories to help improve the analytical phase of patient diagnosis.

Recommended HER2 testing guidelines are: The ASCO/CAP guidelines by Wolff et al.(2007), the UK guidelines by Walker et al., (2008), the ASCO/CAP guidelines by Wolff et al., 2013. , and the updated UK guidelines by Rakha et al., (2015). These publications provide invaluable guidelines covering interpretation, tissue fixation and antibody validation. The articles by Walker et. al. and Rhaka et al. also provide guidelines on the minimum number of tests per year that labs should be carrying out in order to provide a robust HER2 IHC service. The recommendations are 250 tests for HER2 IHC and 100 tests for HER2 ISH.

Participants who continue to have difficulty in producing acceptable staining results should be aware that the members of staff at UK NEQAS ICC are always willing to assist any laboratory struggling to meet the required standard of HER2 immunostaining, and would be happy to receive requests for assistance via e-mail in the first instance (see front of journal or the UK NEQAS ICC & ISH website for contact details).

In-House Control Tissue Recommendations

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

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

Assessment Summary:

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

Pass Rates Run 113:		
	Neqas Section	**In-House**
Acceptable	71% (N=207)	61% (N=174)
Borderline	11% (N=33)	20% (N=58)
Unacceptable	18% (N=52)	18% (N=52)

As with previous runs the most popular antibody was the Ventana 4B5, used by 61% of participants and showed an overall acceptable pass rate of 87%. 23 labs are using the Dako HercepTest (51% acceptable pass rate), and 19 labs are using the Leica Oracle kit 63% acceptable pass rate). 24% of participants used lab-devised methods, using a variety of antibodies, pre-treatment methods and platforms. These labs showed an acceptable pass rate of 14%. All data is based on the methodology information submitted by participants, but unfortunately not all methodology was provided by all labs.

References

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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.
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6. Wolff AC, et al. Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/ College of American Pathologists Clinical Practice Guideline Update. J Clin Pathol. 2013; 31 (31):3998-4013.
7. Rakha EA, et al. Updated UK rRecommendations for HER2 assessment in breast cancer. J Clin Pathol. 2015;68:93-99.

Acknowledgments

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

Selected Images showing Optimal and Sub-optimal Immunostaining

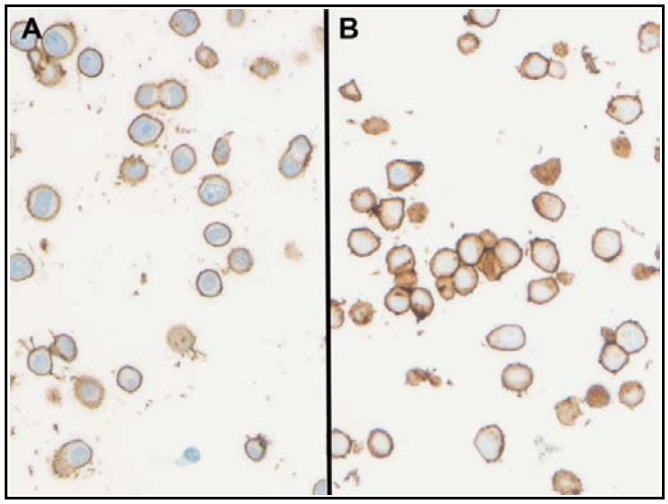


Fig 1. Appropriate staining of the UK NEQAS SK-BR3 (3+) cell line. (A & B) Strong and complete circumferential membrane. Stained using (A) the Leica Oracle kit, on a Bond III as per recommendations and (B) Ventana Pathway 4B5, CC1 retrieval on the Benchmark ULTRA with UltraView detection.

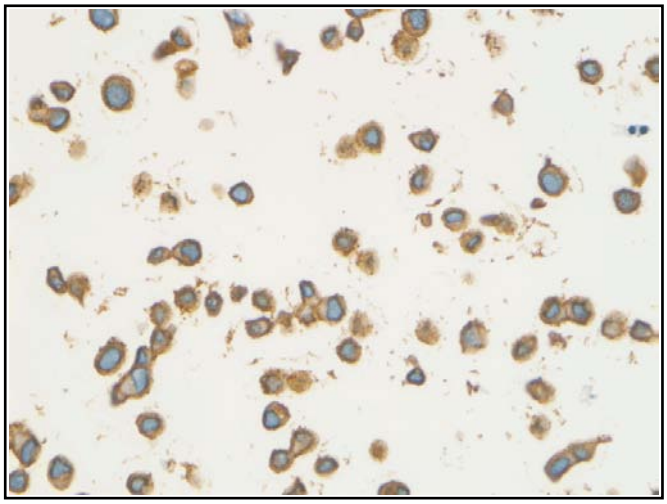


Fig 2. Unacceptable staining of the UK NEQAS 3+ cell line. The section shows excessive cytoplasmic staining, making it difficult to read the membranes. The staining was carried out using a lab-devised method with the Dako polyclonal antibody and antigen retrieval in the pressure cooker.

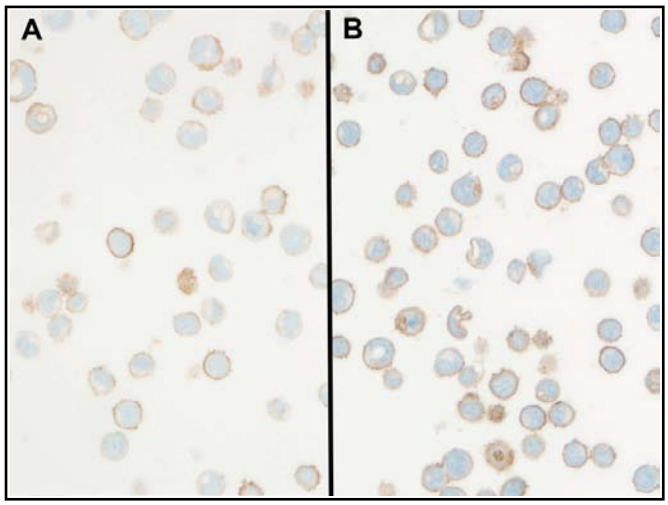


Fig 3. Two examples showing the expected level of staining of the UK NEQAS MDA-MB-453 (2+) cell line. (A & B) Majority of cells show weak to moderate complete membrane staining. Stained using (A) the Dako HercepTest on the Dako Autostainer with pre-treatment in the PT link. (B) Stained with the Ventana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

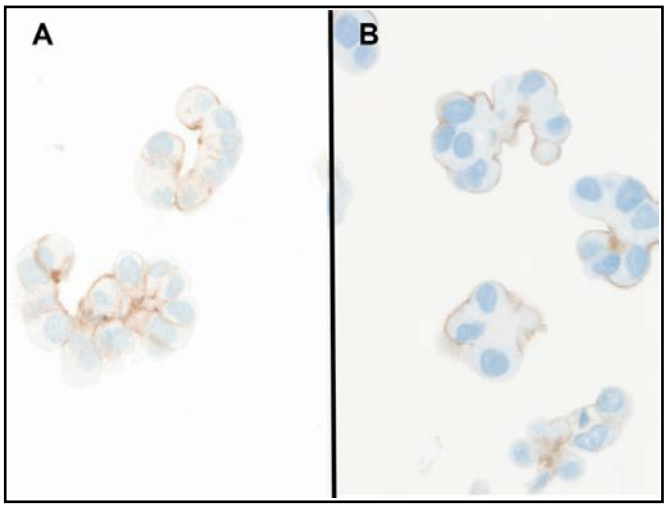


Fig 4. Unacceptable staining of the UK NEQAS 2+ distributed cell lines. (A) Excessive membrane staining, which is more representative of 3+ staining. (B) Morphology damage and excessive counterstain.

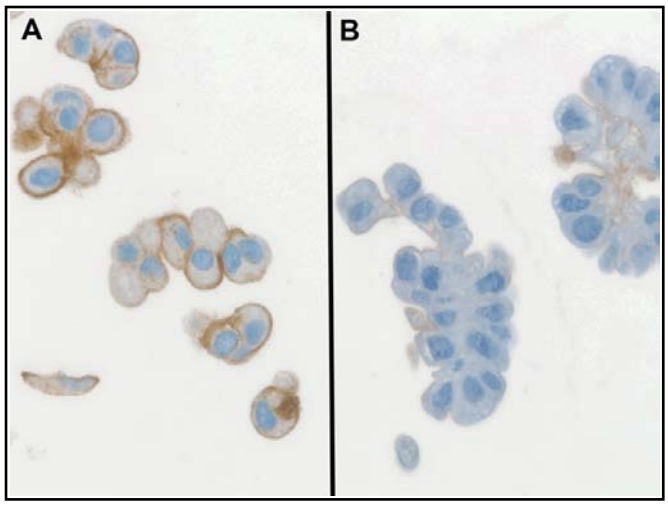


Fig 5. Staining of the UK NEQAS MDA-MB-175 (1+) cell line. (A)Expected level of staining, which is partial membranous. (B)Staining is too strong and more representative of 2+ staining. (C)Any possible staining is masked by the grey counterstain. This section also had water droplets covering most of the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

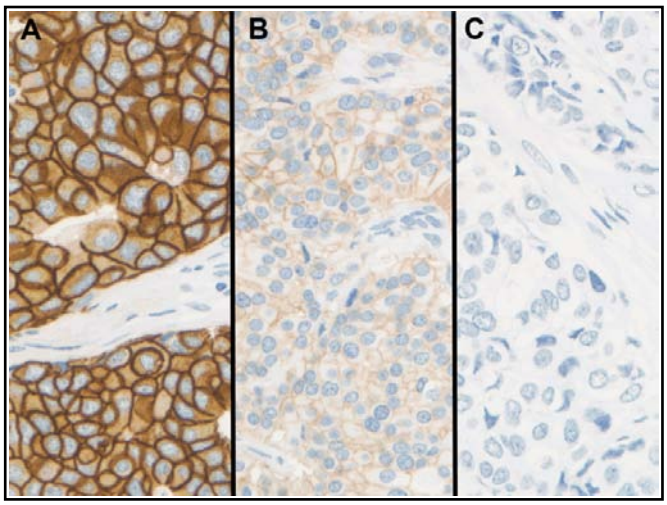
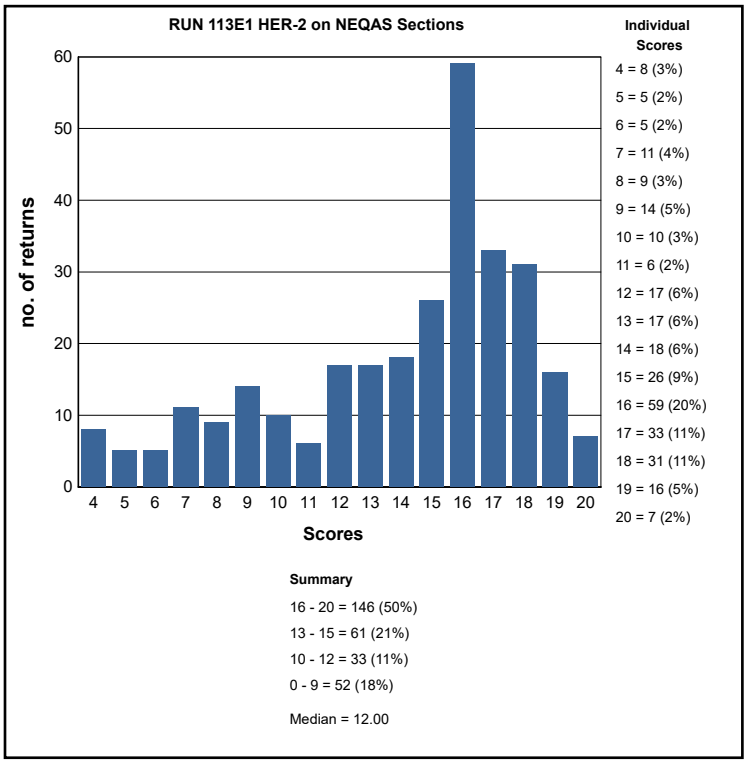
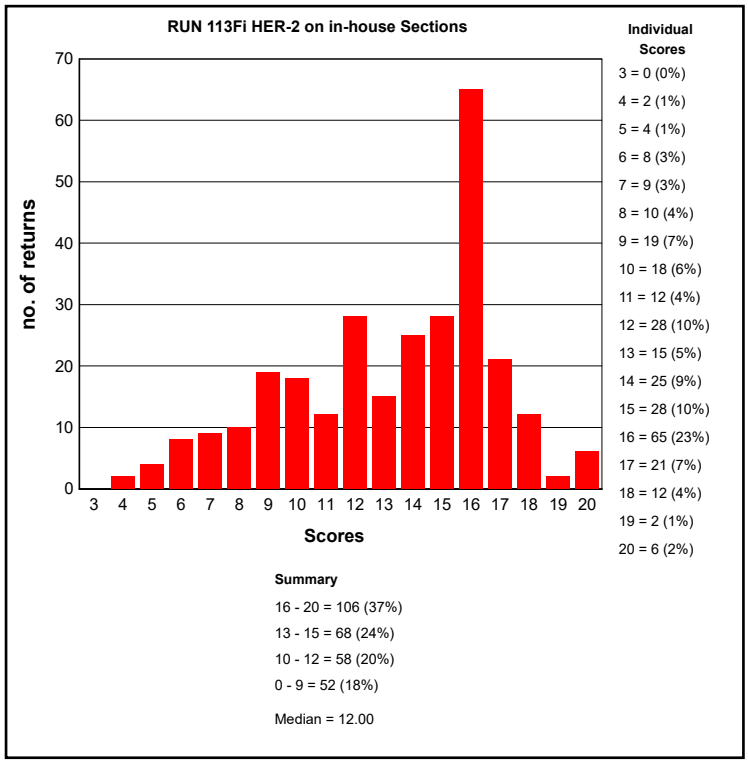


Fig 6. Good in-house breast controls optimally stained with HER2. (A) 3+ (B) 2+ (C) 1+ and (D) negative. Section stained with the Ventana 4B5 on the Benchmark ULTRA with CC1 retrieval for 36 mins and UltraView detection.

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: 113

Primary Antibody	N	%
Biocare CME 342 A,B (EP1045Y)	6	17
BioGenex (EP1045Y) rb mono	3	0
Biogenex AM134-5M (CB11)	3	33
Cell Marque 237R (SP3)	4	0
Cell Marque CMA 601 (CB11)	1	0
Dako A0485 C-erB-2 (poly)	22	45
Dako HercepTest K5204 (poly)	4	75
Dako HercepTest K5205 (poly)	1	0
Dako HercepTest K5207 (poly)	4	50
Dako Link HercepTest SK001 (poly)	9	78
Labvision / Neomarkers RM-9103 (SP3)	7	29
Leica Oracle HER2 Bond IHC (CB11)	19	63
Novocastra NCL-L-CB11 (CB11)	7	0
Novocastra RTU-CB11 (CB11)	1	0
Other	15	33
Ventana Confirm 790-4493 (4B5)	42	83
Ventana Pathway 790-100 (4B5)	13	85
Ventana Pathway 790-2991 (4B5)	123	93

Breast HER2 ICC Run: 113

Automation	N	%
BioGenex GenoMX 6000i	4	50
Dako Autostainer	3	33
Dako Autostainer Link 48	18	67
Dako Autostainer plus	4	25
Dako Autostainer Plus Link	3	67
Dako Omnis	2	100
LabVision Autostainer	2	0
Leica Bond Max	19	37
Leica Bond-III	19	63
Menarini - Intellipath FLX	2	0
None (Manual)	22	18
Other	5	20
Shandon Sequenza	1	0
Ventana Benchmark GX	10	70
Ventana Benchmark ULTRA	88	93
Ventana Benchmark XT	81	86

Breast HER2 ICC Run: 113

Heat Mediated Retrieval	N	%
Biocare Decloaking Chamber	12	8
Dako Omnis	2	100
Dako Pascal	1	0
Dako PTLink	23	61
Lab vision PT Module	1	0
Leica ER1 10 mins	4	50
Leica ER1 20 mins	7	14
Leica ER1 25 mins	17	71
Leica ER1 30 mins	1	0
Leica ER1 40 mins	1	100
Leica ER2 10 mins	1	0
Leica ER2 20 mins	2	50
Leica ER2 30 mins	2	50
Microwave	8	13
None	5	60
Other	3	67
Pressure Cooker	6	33
Steamer	1	0
Ventana CC1 16mins	3	100
Ventana CC1 20mins	3	67
Ventana CC1 24mins	2	100
Ventana CC1 32mins	13	92
Ventana CC1 36mins	37	100
Ventana CC1 40mins	2	100
Ventana CC1 48mins	1	0
Ventana CC1 52mins	6	83
Ventana CC1 56mins	8	88
Ventana CC1 64mins	6	83
Ventana CC1 8mins	5	40
Ventana CC1 mild	73	85
Ventana CC1 standard	12	100
Ventana CC2 36mins	2	100
Water bath 95-98 OC	11	55

Breast HER2 ICC Run: 113

Detection	N	%
AS PER KIT	24	63
Biocare polymer (M4U534)	1	100
Biocare SLAB (STU HRP 700H,L10)	3	0
BioGenex HRP (HK 519-06K)	2	50
BioGenex SS Polymer (QD 420-YIKE)	1	0
BioGenex SS Polymer (QD 430-XAKE)	1	0
Dako HerCep Test (K5204)	2	50
Dako EnVision FLEX (K8000/10)	10	50
Dako EnVision FLEX+ (K8002/12)	9	56
Dako Envision HRP/DAB (K5007)	6	17
Dako Envision+ HRP rabbit K4008/9/10/11	1	0
Dako HerCep Test Autor (K5207)	2	50
Dako HerCep Test Autor (SK001)	6	83
LabVision UltraVision ONE Polymer (TL-012/5-HDJ/T	1	0
Leica Bond Polymer Define (DS9713)	1	100
Leica Bond Polymer Refine (DS9800)	22	45
None	2	50
Other	11	36
Ventana iView system (760-091)	6	83
Ventana OptiView Kit (760-700)	11	64
Ventana UltraView Kit (760-500)	150	90

Breast HER2 ICC Run: 113

Enzyme Retrieval	N	%
AS PER KIT	13	77
NOT APPLICABLE	148	74
Other	3	33
Ventana Protease	1	100
Ventana Protease 1 (760-2018)	1	0

Breast HER2 ICC Run: 113

Chromogen	N	%
A. Menarini Liquid Stable DAB kit	1	0
AS PER KIT	46	70
BioGenex DAB (QD430)	1	100
BioGenex Liquid DAB (HK153-5K)	2	0
BioGenex liquid DBA (HK-124-7K)	1	0
Dako DAB+ Liquid (K3468)	2	50
Dako DAB+ REAL Detection (K5001)	3	0
Dako EnVision Plus kits	1	0
Dako FLEX DAB	20	55
Dako REAL EnVision K5007 DAB	5	20
LabVision DAB	1	0
Leica Bond Polymer Refine kit (DS9800)	25	44
Other	21	48
Ventana DAB	7	100
Ventana iview	4	75
Ventana Ultraview DAB	144	88

BEST METHODS

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

HER-2 - Method 1

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

Primary Antibody:	Ventana Pathway 790-2991 (4B5) , 16 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 mild
EAR:	
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500) Prediluted

HER-2 - Method 2

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

Primary Antibody:	Dako Link HercepTest SK001 (poly) , 30 Mins, 23 ºC Prediluted
Automation:	Dako Autostainer Link 48
Method:	Envision
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: AS PER KIT
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 23 ºC., Time 1: 10 Mins
Detection:	Dako HerCep Test Autor (SK001) , 30 Mins, 23 ºC Prediluted

HER-2 - Method 3

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

Primary Antibody:	Ventana Pathway 790-2991 (4B5)
Automation:	Ventana Benchmark XT
Method:	Ventana iView Kit
Main Buffer:	AS PER KIT
HMAR:	Ventana CC1 mild
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	Ventana iView system (760-091)

HER-2 - Method 4

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

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

The Gastric HER2 ICC Module Run 113

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

Assessment Procedure

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

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

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

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

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

Introduction

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 IHC2+/FISH+ expression. The initial development of the HER2 scoring criteria was developed as a precursor to the ToGA trial (Hoffman et al., 2008) with the study group modifying the breast HER2 IHC scoring algorithm to compensate for the incomplete membrane staining and greater tumour heterogeneity seen in gastric cancers. A different scoring system was also established for resection and core biopsies as illustrated in table 1. A more recent article by Rüschoff et al., (2010) has validated the scoring procedure further with a detailed approach to 'stepwise' HER2 IHC scoring in gastric cancers. The article also compared the Ventana 4B5 assay with the HercepTest and indicated "a tendency towards higher sensitivity for 4B5 detecting positive HER2 amplification" but "there was no difference between both test platforms with respect to the ISH-negative cases, with the one equivocal case testing negative at some sites and

as positive at others". Rüschoff and colleagues also used both fluorescent (Dako PharmDX or PathVysion Vysis) and chromogen (Ventana DDISH) to confirm their IHC findings.

Assessment Results

Features Of Acceptable Staining: (Figs 1, 2A, 3A & 5A)

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

Features Of Suboptimal or Unacceptable Staining: (Figs 2B, 3B, 4 & 5B)

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

Additional Comment:

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

Pass Rates

The pass rates for the UK NEQAS distributed samples were reasonably good and showed an improvement on the previous assessment Run 112 (summarised in the table below):

Gastric HER2 Pass Rates:				
	Run 110	Run 111	Run 112	Run 13
Acceptable	87% (N=44)	82% (N=53)	65% (N=42)	73% (N=48)
Borderline	10% (N=4)	8% (N=5)	20% (N=13)	17% (N=11)
Unacceptable	4% (N=2)	11% (N=7)	15% (N=10)	9% (N=6)

However, 6 labs (9%) still failed the assessment, and this was due to either weak membrane staining or over-staining. In the clinical setting false negative results will lead to suitable patients not being put forward for Herceptin therapy, and equally concerning is that many patients may be over-treated: For instance, a HER2 IHC result of 3+ will automatically mean that the patient is put forward for Herceptin therapy. Also, as IHC 2+ cases are also reflex tested, incorrect over-staining could mean that more samples than necessary are being put forward for in-situ hybridisation reflux testing.

Most labs are using the recommended standardised protocols for their particular automated systems, such as CC1 Mild antigen retrieval on the Ventana XT with the 4B5 pre-diluted antibody clone. However, a few labs are not using the recommended protocols, and this may have been the cause of either weaker or stronger membrane staining than expected. All participants apart from 2 labs also submitted their in-house controls.

The in-house results are summarised in the following table:

In-House Pass Rates Run 113:	
Acceptable	67% (N=42)
Borderline	30% (N=19)
Unacceptable	3% (N=2)

The overall pass rates on the in-house controls was slightly higher than on the Neqas samples. However, the acceptable pass level (scores of ≥13-20/20) was slightly lower than on the Neqas material, and therefore more labs received a borderline pass on the in-house samples. Several of these borderline passes were given because the labs did not submit ideal requested composite control material, consisting of a 3+, 2+ and 1+/0 expressing gastric/breast control tumour sample. These labs were therefore given a maximum score of 12/20. Other labs were marked down for poor tissue quality or fixation. 1 of the sections that failed the assessment showed excessive cytoplasmic staining and the other failed slide had severe folding, and therefore both of theses slides were considered uninterpretable.

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 88% of participants and showed an acceptable pass rate of 78% on the UK NEQAS distributed section. Only 1 of the 5 labs that are using the standardised Dako HercepTest received an acceptable pass. The Leica Oracle kit was used by 1 lab and unfortunately this slide failed the assessment due to very weak HER2 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 also acceptable for labs to submit a heterogeneous in-house control with areas of both 3+ and 2+ membrane expression for example, as long as the participant clearly indicates the areas where the assessment should be carried out.

References:

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

Acknowledgments

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

Selected Images showing Optimal and Sub-optimal Immunostaining

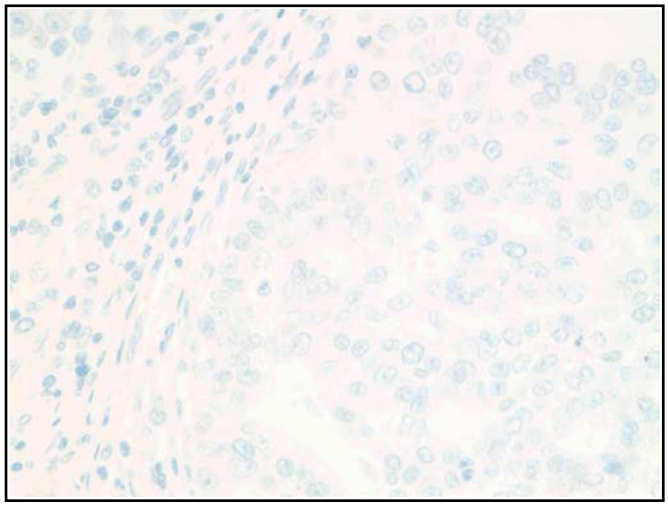


Fig 1. Expected HER2 negative 'Sample A' from UK NEQAS distributed gastric tumour. Stained with the Ventana 4B5 Pathway on the Benchmark ULTRA, CC1 36 minutes.

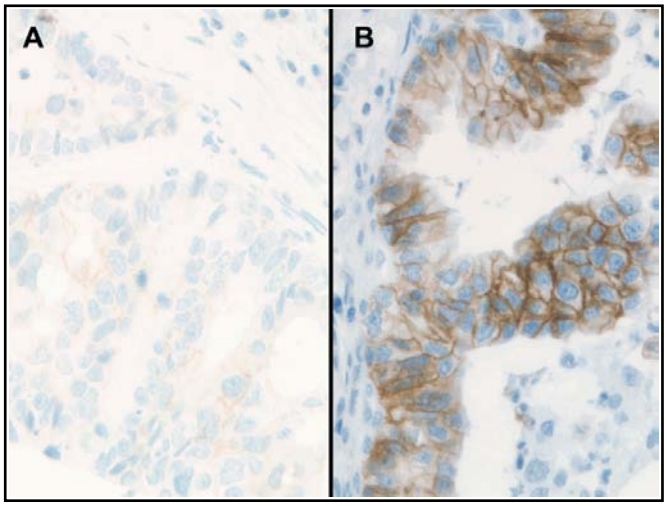


Fig 2. UK NEQAS distributed 1+ gastric tumour (sample B) (A) Expected level of staining. Stained using same method in fig 1. (B) Excessive membrane staining, which was interpreted as 2+. Stained using LabVision SP3 clone on a Dako Autostainer link 48 with PT link retrieval.

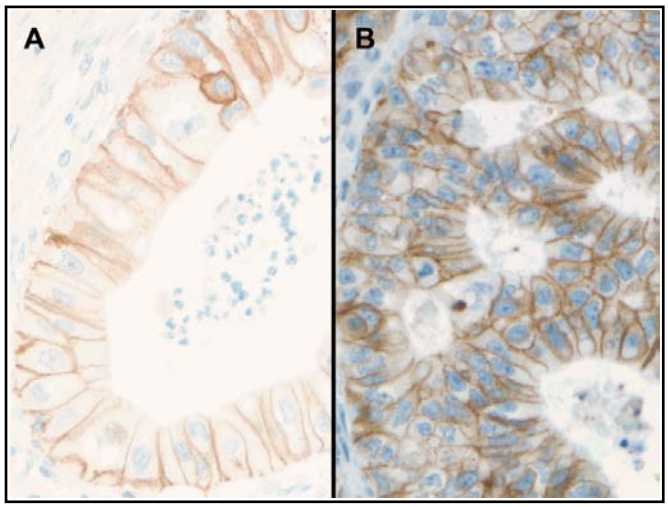


Fig 3. UK NEQAS distributed 2+ gastric tumour (Sample C) (A) Expected level of 2+ membrane staining. Stained with the same method in fig 1. (B) Excessive membrane staining, which was interpreted as 3+. Stained using LabVision SP3 clone on a Dako Autostainer link 48 with PT link retrieval.

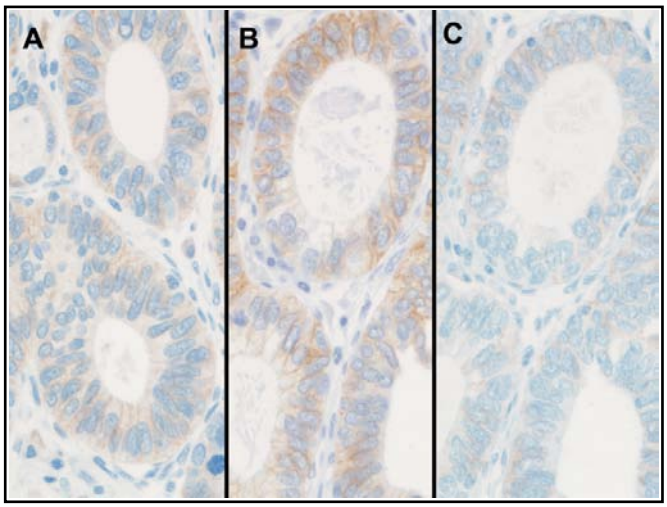


Fig 4. (A-C) Three examples of weak staining from UK NEQAS distributed 2+ gastric tumour (Sample C) (Compare with Fig 3A). Samples stained with (A) Leica Oracle CB11, (B) Dako Herceptest and (C) Ventana Pathway 4B5.

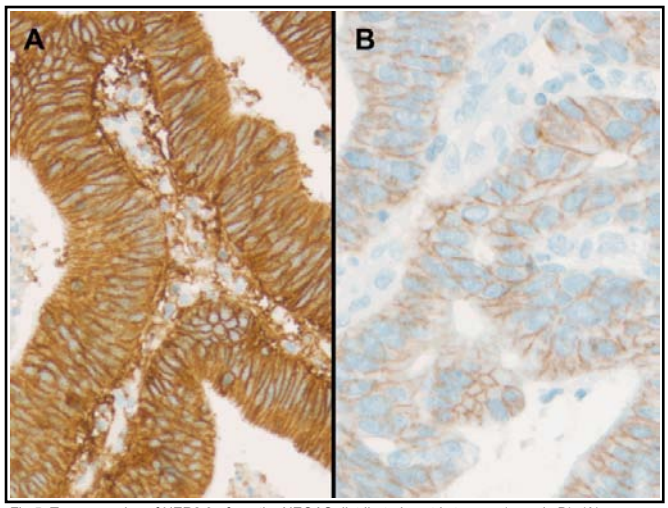


Fig 5. Two examples of HER2 3+ from the NEQAS distributed gastric tumour (sample D). (A) Expected level of complete intense membrane staining. (B) Staining much weaker than expected. Both examples stained with Ventana Pathway 4B5 following recommended protocols. Assessors could not pinpoint why there was such as difference in staining.

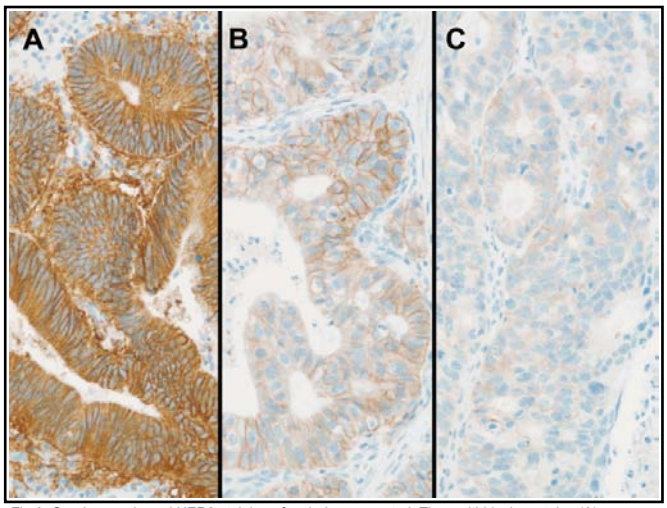
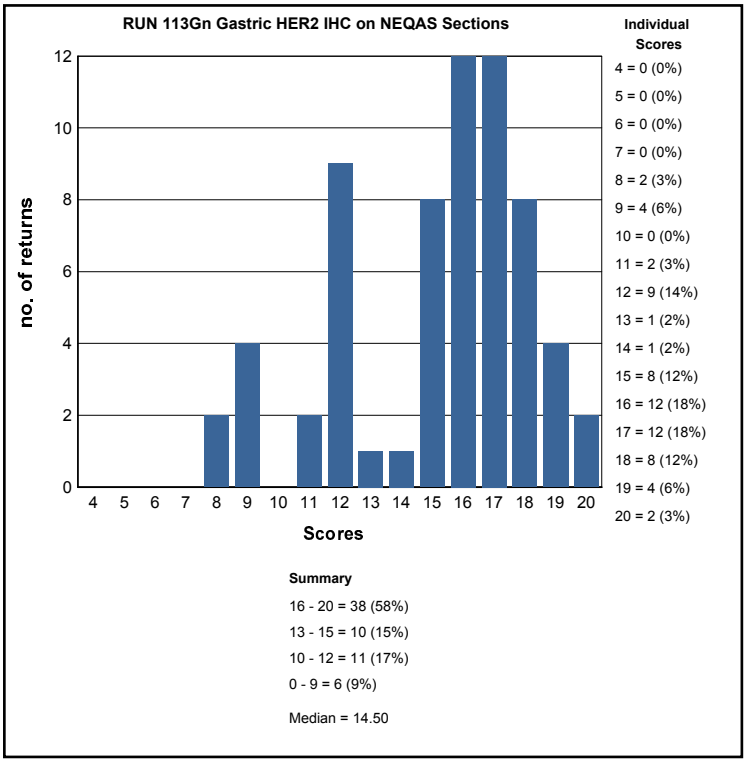
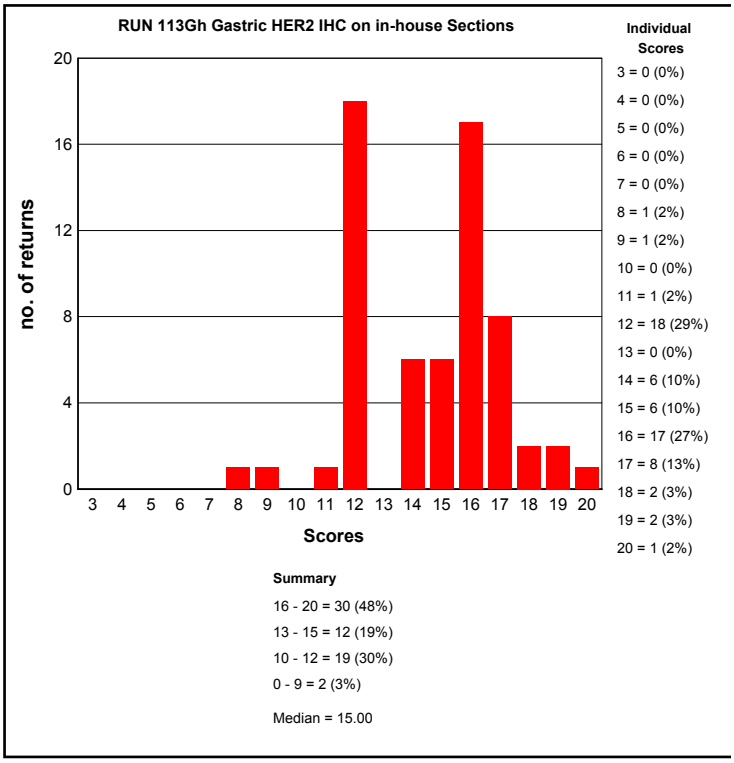


Fig 6. Good example and HER2 staining of an in-house control. The multi-block contains (A) 3+, (B) 2+, (C) 1+ and (D) negative expressing gastric tumours.

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: 113

Primary Antibody	N	%
Dako Link HercepTest SK001 (poly)	5	20
Labvision / Neomarkers RM-9103 (SP3)	1	0
Leica Oracle HER2 Bond IHC (CB11)	1	0
Ventana Confirm 790-4493 (4B5)	17	76
Ventana Confirm 790/800-2996 (4B5)	1	100
Ventana Pathway 790-100 (4B5)	4	50
Ventana Pathway 790-2991 (4B5)	35	86

Gastric HER2 ICC Run: 113

Automation	N	%
Dako Autostainer Link 48	4	0
Dako Autostainer Plus Link	2	50
Leica Bond-III	1	0
Ventana Benchmark GX	2	100
Ventana Benchmark ULTRA	26	85
Ventana Benchmark XT	28	79

Gastric HER2 ICC Run: 113

Heat Mediated Retrieval	N	%
Dako PTLink	6	17
Leica ER1 25 mins	1	0
Ventana CC1 16mins	1	100
Ventana CC1 20mins	1	100
Ventana CC1 24mins	3	67
Ventana CC1 32mins	6	83
Ventana CC1 36mins	11	82
Ventana CC1 40mins	1	100
Ventana CC1 56mins	1	100
Ventana CC1 64mins	4	75
Ventana CC1 mild	23	78
Ventana CC1 standard	5	100
Ventana CC2 36mins	1	0

Gastric HER2 ICC Run: 113

Detection	N	%
AS PER KIT	6	33
Dako EnVision FLEX (K8000/10)	1	0
Dako HerCep Test Autor (SK001)	2	50
Ventana iView system (760-091)	3	100
Ventana OptiView Kit (760-700)	5	80
Ventana UltraView Kit (760-500)	46	78

Gastric HER2 ICC Run: 113		
Enzyme Retrieval	**N**	**%**
AS PER KIT	3	67
NOT APPLICABLE	28	64

Gastric HER2 ICC Run: 113		
Chromogen	**N**	**%**
AS PER KIT	12	58
Dako FLEX DAB	3	33
Ventana DAB	4	75
Ventana iview	1	100
Ventana Ultraview DAB	44	80

BEST METHODS

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

Gastric HER2 IHC - Method 1

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

Primary Antibody:	Ventana Pathway 790-2991 (4B5) Dilution 1: RTU
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 20mins
EAR:	
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

Gastric HER2 IHC - Method 3

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

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

Gastric HER2 IHC - Method 4

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

Primary Antibody:	Ventana Confirm 790-4493 (4B5) , 24 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 mild
EAR:	
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

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	Gold Standard	Second Antibody
Antigens Assessed:	Cyclin D1	CD5
Tissue Sections circulated:	Reactive tonsil and mantle cell lymphoma in lymph node.	Reactive tonsil and mantle cell lymphoma in lymph node.
Number of Registered Participants:	223	
Number of Participants this Run	211 (95%)	

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 & 5):

- 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 & 6):

- 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: CD5

CD5 is a 67-kDa transmembrane glycoprotein, involved in B- and T-cell receptor signal transduction (Taylor). Normal cell types which are positive for this antigen include thymocytes, the majority of T-cells and a small number of B-cells (B-1 lymphocytes). This marker is useful in a variety of diagnostic situations (Taylor; Bishop; Dabbs):

B-cell Lymphomas

- Positive in the vast majority (>90%) of B-cell chronic lymphocytic leukaemia and B-cell small lymphocytic lymphomas
- Positive in the majority of mantel cell lymphomas
- Negative in almost all other low grade B-cell lymphomas e.g. follicular lymphoma

T-cell Lymphomas

- Positive in most (approximately 85%) of T-cell acute lymphoblastic leukaemia and lymphoblastic lymphomas
- Distinguishes T-cell lymphoma (CD5 +ve) from extra nodal T/NK cell lymphoma (CD5 -ve)

Thymic Carcinoma

- Positive in most thymic carcinomas (60-100%), distinguishing thymic carcinomas from pulmonary carcinomas which are usually CD5 –ve.

Features of Optimal Immunostaining (Figs 7, 8, 10 & 11)

- Strong, predominantly membranous staining of the majority of the T-cells in the inter-follicular area of the tonsil
- Moderate staining of the scattered B-cells in the mantle zone of the tonsil
- Strong, predominantly membranous staining of the tumour cells of the MCL
- Darker staining of any normal scattered T-cells within the MCL
- Clean background

Features of Suboptimal Immunostaining (Figs 9 & 12)

- Weak staining of cells expected to stain, especially T-cells in the inter-follicular areas.
- Poor localisation of staining to cell membranes.
- Disruption of normal cellular detail, due to excessive or incorrect antigen retrieval.

References

1. Taylor CR, Burns J. The demonstration of plasma cells and other immunoglobulin-containing cells in formalin-fixed, paraffin-embedded tissues using peroxidise-labelled antibody. J Clin Pathol 19 74; 27: 14-20.
2. Bishop PW. Immunohistochemistry vade mecum. *http://www.e-i mmunohistochemistry.info/*
3. Dabbs DJ. Immunohistology of metastatic carcinomas of unknown primary. In: Dabbs DJ (ed) Diagnostic immunohistochemistry (end ed). 2006.

Assessment Summary:

Cyclin D1:

205 laboratories submitted their slides for the Cyclin D1 assessment. The results were similar to the last assessment (Run 112), but slightly lower than the first Cyclin D1 assessment of the year (Run 110), shown in the table below:

Cyclin D1 Pass Rates:				
	Run 110	**Run 111**	**Run 112**	**Run 13**
Acceptable	85% (N=177)	68% (N=143)	77% (N=160)	79% (N=161)
Borderline	13% (N=27)	19% (N=40)	16% (N=34)	14% (N=28)
Unacceptable	2% (N=5)	13% (N=27)	6% (N=13)	8% (N=16)

Similarly to previous results, weak staining was the main reason for marks being deducted and therefore receiving a borderline pass or in 16 cases, where there was very little staining at all, the labs received a failed score. This was not particularly attributable to a particular antibody clone, but mostly due to an inappropriate dilution or antigen retrieval protocol. All of the participating labs also submitted their in-house control sections, cut and placed on the same slide as the Neqas tissue. The pass rates for the in-house tissues were slightly higher than the Neqas samples, with only 5 labs failing

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the assessment, and again, this was due to weak or diffuse staining of the antibody.

A variety of antibodies and automated platforms were used in the assessment. However, overall the most popular antibody clone was the SP4 from various sources, but mostly the LabVision/Thermo Scientific SP4 antibody. This was used by 62 participants and showed an acceptable pass rate of 69%. The Ventana rabbit monoclonal SP4-R antibody was also popular, used by 44 participants, and showed an acceptable pass rate of 95%. There was also an increase in the number of labs using the Dako EP12 clone: 42 participants submitted slides stained with this antibody, which showed an acceptable pass rate of 83%.

CD5

211 laboratories submitted their slides for the CD5 assessment. The pass rates were very good with 96% of participants achieving an acceptable pass rate (scores of 13≥20/20) . Participating labs for this assessment run apart from 1 lab also submitted their in-house material. These results showed very similar pass rates to the Neqas sections (summarised in the table below):

CD5 Pass Rates Run 113:		
	Neqas Section	In-House
Acceptable	96% (N=205)	97% (N=204)
Borderline	2% (N=5)	1% (N=3)
Unacceptable	2% (N=4)	1% (N=3)

It was noted that the same labs that failed on their in-house sample also failed on the Neqas distributed section. For 2 of the labs this was due to very weak staining, with many cells expected to stain not staining at all. 1 lab showed excessive background and non-specific staining, caused by excessive antigen retrieval. It was interesting to see that only 1 lab is now using the Dako CD5/54/F6 clone, and unfortunately the section failed the assessment due to weak and insufficient staining. On previous CD5 assessments it was highlighted that this particular clone was not performing too well: Some slides were showing little or no staining on the Neqas distributed mantle cell lymphoma tissue, and a lack of staining in the B cells in the mantle zone of the Neqas distributed tonsil. Conversely, the Dako 4C7 antibody is performing well. This was used by 24 participants in the current assessment, and showed a pass rate of 97%. The most popular CD5 choice was the Novocastra/Leica 4C7 clone, used by 103 participants, and showed a pass rate of 99%. The antibody was used on a variety of automated staining platforms and at a range of dilutions. Nearly all the labs used tonsil as their in house control. It was encouraging to see that some labs had also used a multi-block of both tonsil and a mantle cell lymphoma.

Selected Images showing Optimal and Sub-optimal Immunostaining

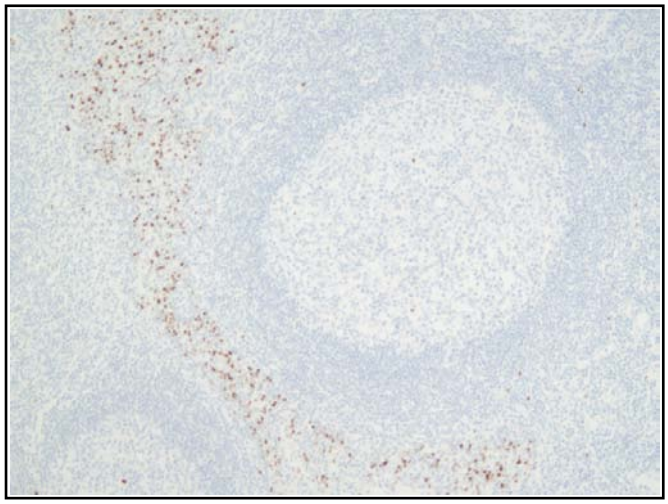


Fig 1. Optimal demonstration of Cyclin D1 in the UK NEQAS reactive tonsil. There is moderate to strong nuclear staining while the background remains clean. Stained with the LabVision/Thermo Scientific SP4 antibody, 1:50, on the Ventana ULTRA (CC1 40 mins) with OptiView detection.

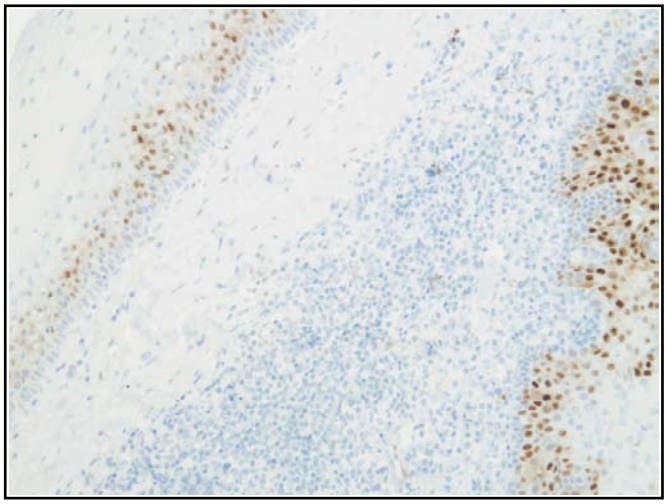


Fig 2. Good demonstration of Cyclin D1 on the UK NEQAS distributed reactive tonsil, showing moderate to strong nuclear staining of the suprabasal squamous epithelial cells. Section stained with the Dako EP12 RTU antibody, on the Dako Autostainer link 48 with PT link high pH retrieval for 20 minutes.

Fig 3. Unacceptable demonstration of Cyclin D1 on the UK NEQAS distributed tonsil section. Staining is weak and diffuse with non-specific background. Stained using the Dako DCS-6 antibody, 1:100, on the Leica Bond III with ER2 retrieval for 20 minutes in high pH buffer.

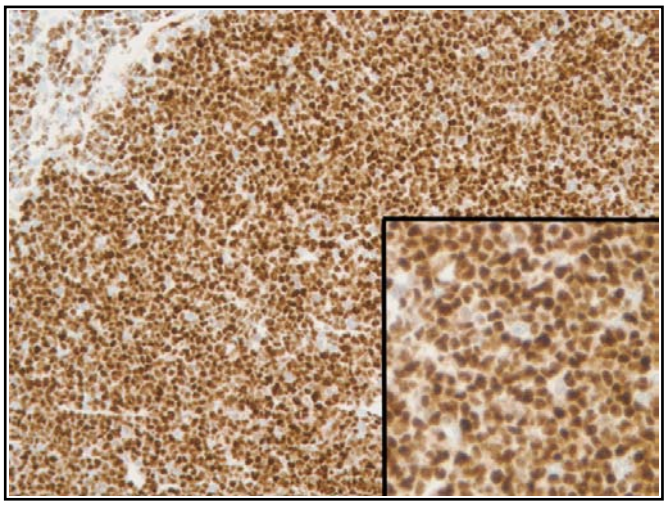


Fig 4. Optimal staining for Cyclin D1 on the UK NEQAS distributed mantel cell lymphoma (shown to better advantage in the high power insert). Virtually all of the tumour cells show strong nuclear staining. Same protocol as Fig 1.

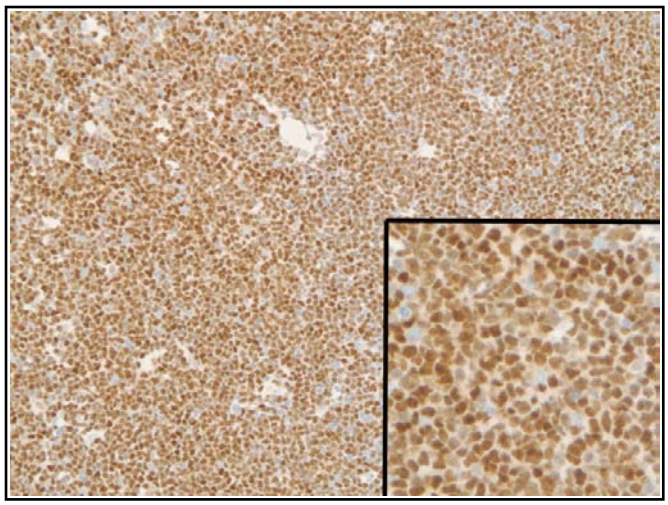


Fig 5. Good demonstration of Cyclin D1 on the UK NEQAS distributed mantel cell lymphoma (MCL). The example shows moderate to strong distinct nuclear staining of the tumour cells. Stained using the Ventana SP4-R pre-diluted antibody on the Benchmark ULTRA (CC1 64 mins.) and OptiView detection.

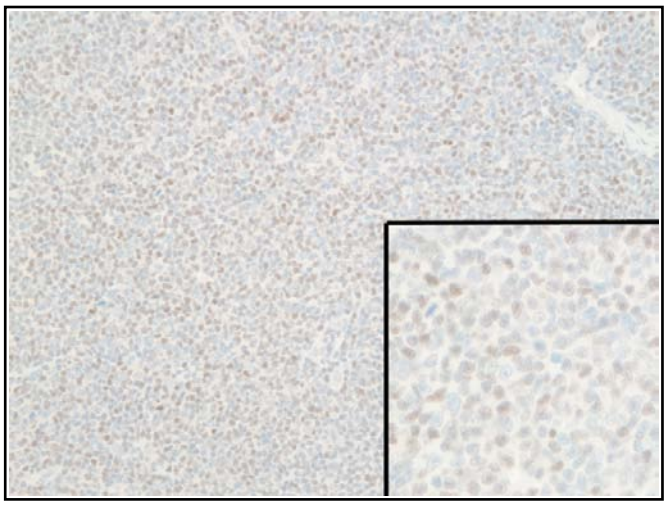


Fig 6. Poor demonstration of Cyclin D1 on the UK NEQAS distributed mantel cell lymphoma sample (compare to Figs 4 & 5): The staining is weak with many of the cells expected to stain not demonstrated. Stained using the Labvision antibody (1:25) on the Leica BondMax with ER2 20 minutes.

Selected Images showing Optimal and Sub-optimal Immunostaining

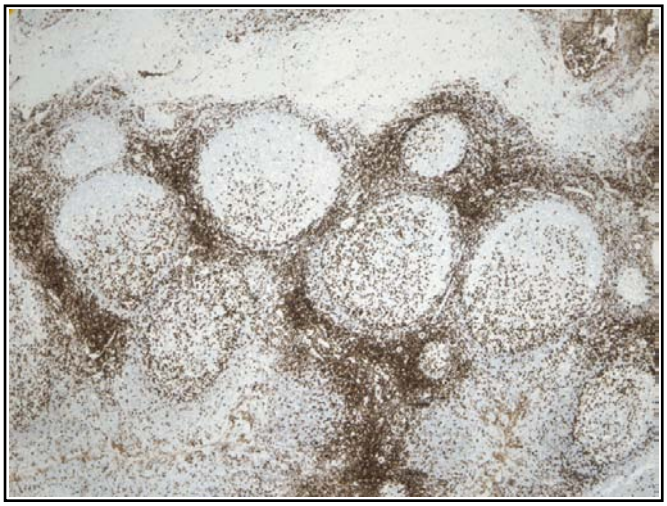


Fig 7. Good example of CD5 staining on the UK NEQAS reactive tonsil. Even at low power it is clear to see the strong staining of the T-cells and also the B-cells in the mantle zone. Section stained with the Leica/Novocastra 4C7 antibody,1:100, on the Leica Bond III, ER2 retrieval for 30 minutes.

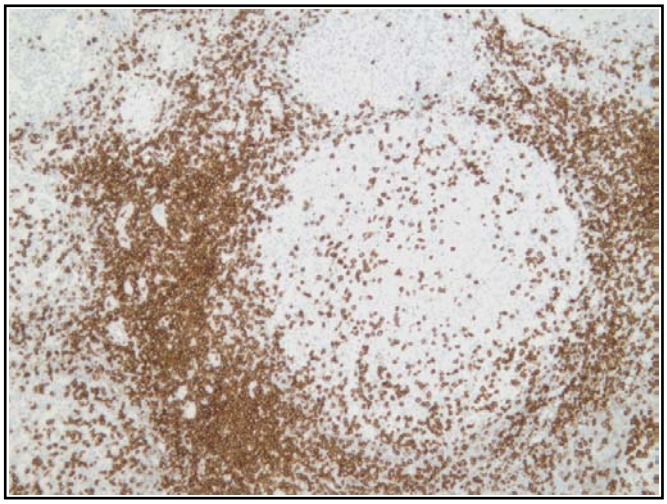


Fig 8. Optimal demonstration of CD5 in the UK NEQAS reactive tonsil. All the T-cells of the inter-follicular areas and scattered T-cells in the germinal centres show strong predominantly membranous staining. Stained with the Ventana SP19 pre-diluted antibody on the Benchmark XT with OptiView detection kit.

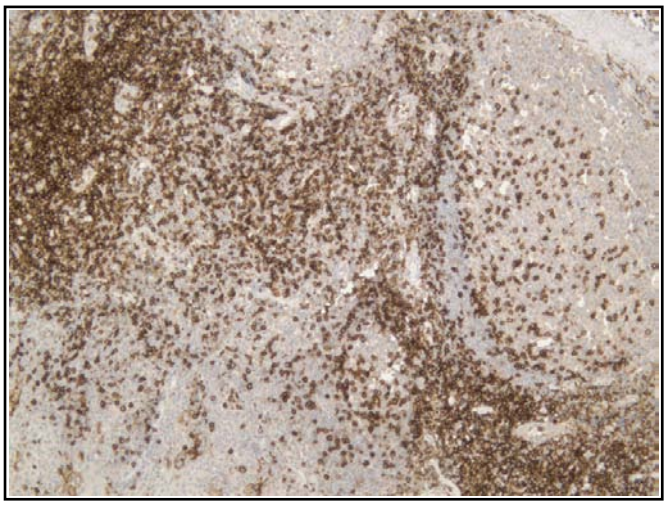


Fig 9. Sub-optimal demonstration of CD5 in the UK NEQAS reactive tonsil. Although all of the expected B and T-cells are staining, there is also background staining. However, this was not considered to interfere with the diagnosis. Stained using the Leica/Novocastra 4C7 antibody, on the BondMax with ER2 retrieval for 20 minutes.

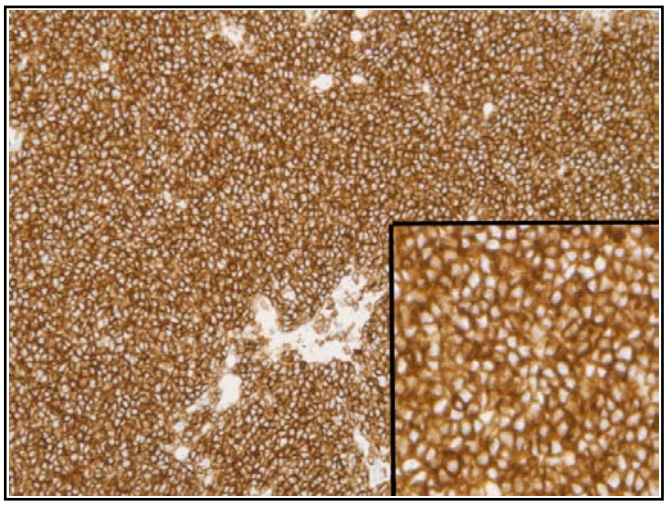


Fig 10. Good demonstration of CD5 in the UK NEQAS distributed mantle cell lymphoma. Both the neoplastic cells and scattered normal T-cells show strong membranous staining (shown to better advantage in the high power insert). Same protocol as Fig 8.

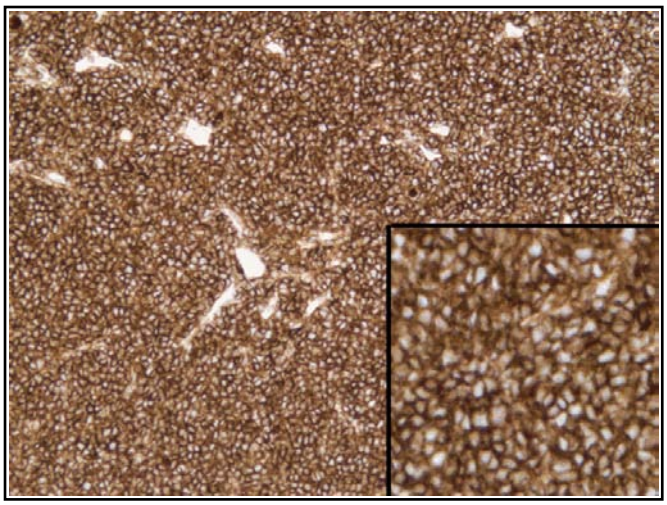


Fig 11. Optimal demonstration of CD5 in the UK NEQAS distributed mantle cell lymphoma, showing strong membranous staining of tumour cells. Same protocol as Fig 7.

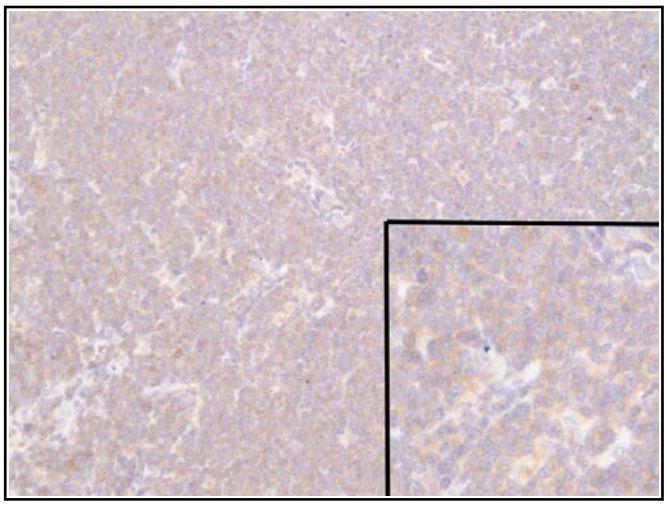
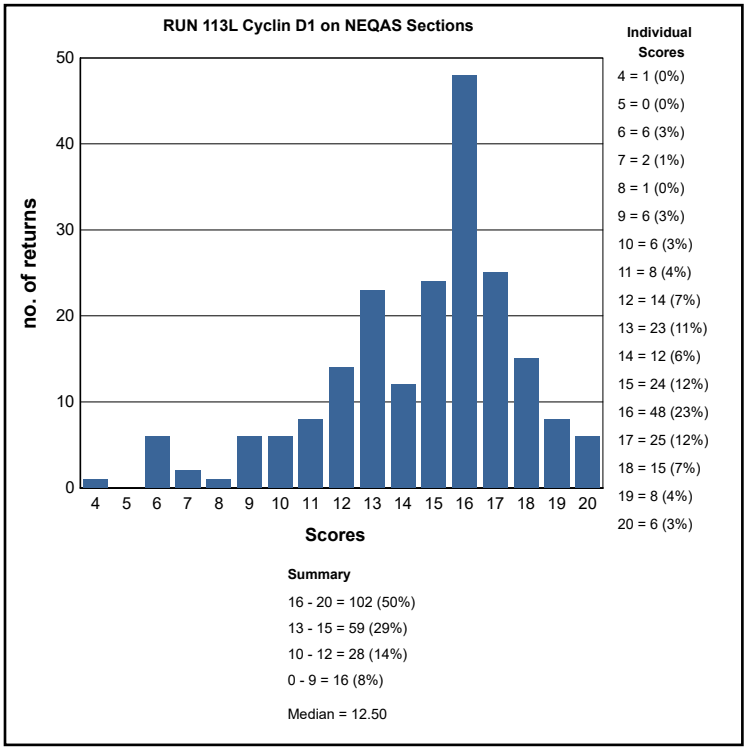
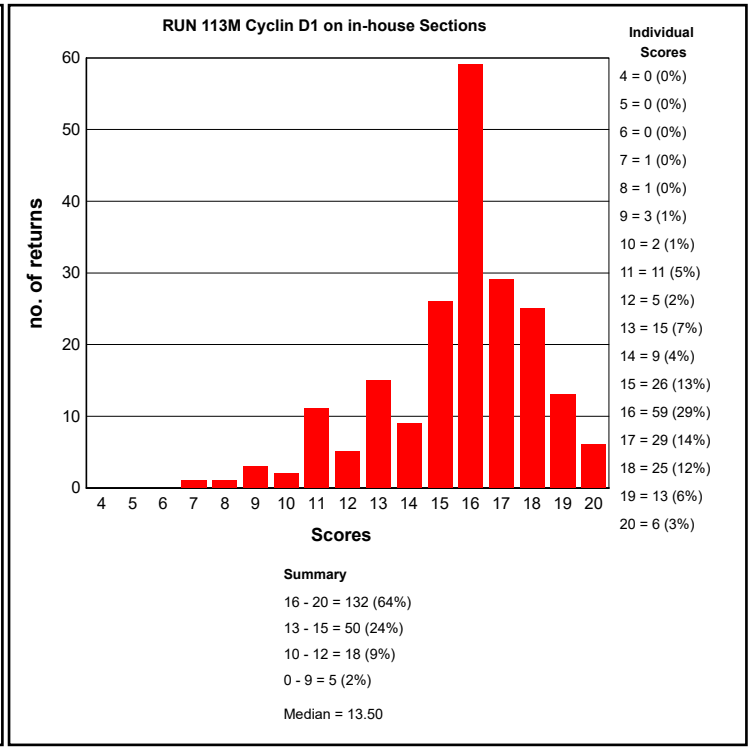
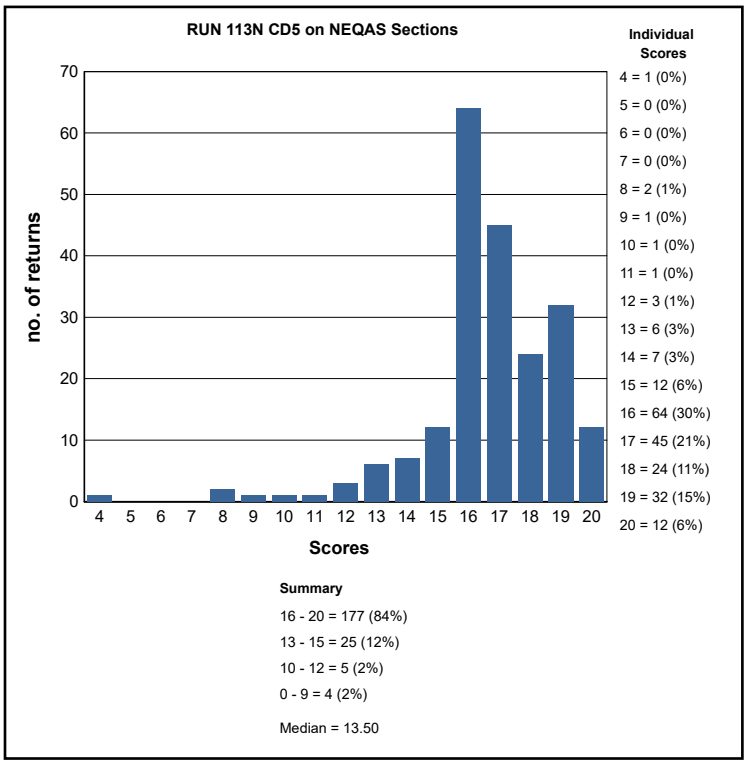


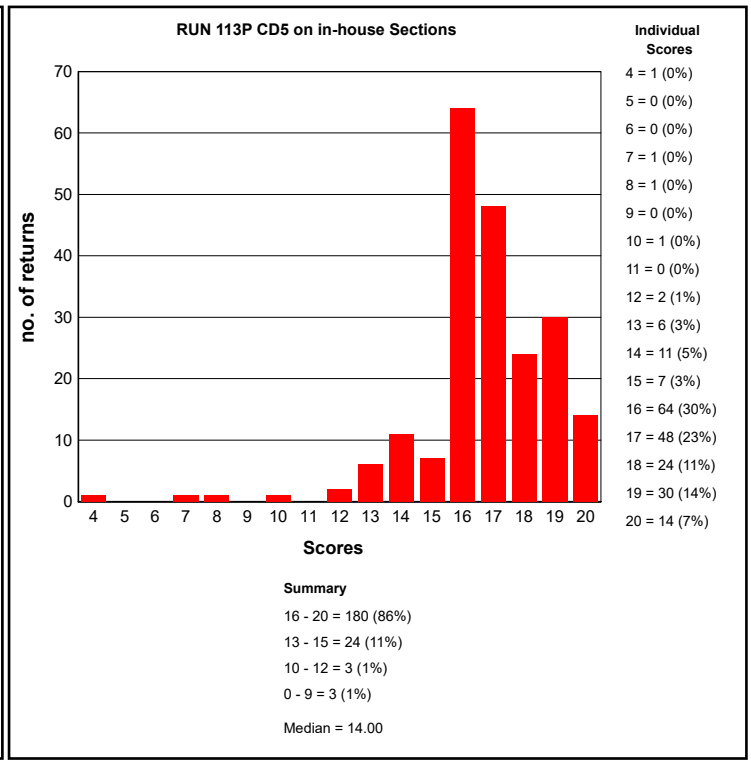
Fig 12. Poor demonstration of CD5 in the UK NEQAS mantle cell lymphoma: The staining is weak and diffuse (compare to Figs 10 & 11). Stained using the Gennova AP10123C antibody, 1:100 on the Ventana ULTRA with CC1 antigen retrieval for 52 minutes. Same protocol as Fig 1.

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: 113

Primary Antibody : Cyclin D1

Antibody Details	N	%
AbCam SP4 16663	3	100
Cell Marque (RBT14) CRC011	1	100
Cell Marque (SP4) 241-R	9	100
Dako (DCS-6) M7155	1	0
Dako (EP12) M3642	20	75
Dako (SP4) M3635	1	100
Dako FLEX RTU (SP4) IR152	1	100
Dako RTU (EP12) IR083/IS083	22	91
Lab Vision/Thermo Sci MS 210P (DCS-6)	1	100
LabVision/Thermo Sci (SP4) RM-9104	62	69
Leica/Novo (P2D11F11) NCL-Cyclin D1-GM-CE	6	0
Leica/Novo RTU (P2D11F11) NCL-RTU-CyclinD1	1	100
Menapath (SP4) MP-307	2	50
NeoMarkers/Thermo Sci (EPR2241IHC) RM-2113	6	67
Neomarkers/Thermo Sci MS 210 PO (DC5-6)	1	100
Other	13	54
Vector rbm VP-RM03 (SP4)	1	100
Ventana (SP4-R) 790-4508	44	95
Ventana 250-2723 (P2D11F11)	1	100
Ventana rbm 760-4282 (SP4)	3	100

Lymphoma Run: 113

Primary Antibody : CD5

Antibody Details	N	%
Biogenex MU430-UC (4C7)	1	100
Cell Marque 205M/S-x (4C7)	3	67
Dako IR082 RTU FLEX Link (4C7)	11	91
Dako IS082 RTU Auto Plus (4C7)	2	100
Dako M3641 (4C7)	11	100
Dako M7194 (CD5/54/F6)	1	0
Gennova AP10123C	1	0
Labvision RM-9119 (SP19)	3	100
Leica Bond RTU PA0168 (4C7)	14	93
NeoMarkers MS-393-S (4C7)	2	100
Novocastra Bond PA0168 (4C7)	8	100
Novocastra NCL-CD5-4C7 (4C7)	30	97
Novocastra NCL-L- CD5-4C7 (4C7)	63	97
Novocastra RTU-CD5-4C7 (4C7)	2	100
Other	4	75
Spring Bio. M3190 (SP19)	1	100
Vector VP-C322 (4C7)	4	100
Ventana 760-4280 (SP19)	4	100
Ventana CD5 790-4451 (SP19)	42	100

Lymphoma Run: 113

Heat Mediated Retrieval	CD5 N	CD5 %	Cyclin D1 N	Cyclin D1 %
Biocare Decloaking Chamber	1	100	0	0
Dako Omnis	4	100	6	83
Dako PTLink	24	96	21	95
Lab vision PT Module	2	100	2	100
Leica ER1 10 mins	1	100	0	0
Leica ER1 20 mins	5	100	0	0
Leica ER1 30 mins	7	100	2	0
Leica ER1 40 mins	0	0	1	0
Leica ER2 20 mins	43	93	27	56
Leica ER2 30 mins	14	100	23	39
Leica ER2 40 mins	0	0	13	77
Microwave	2	100	2	0
None	0	0	1	100
Other	0	0	2	100
Pressure Cooker	2	100	2	100
Pressure Cooker in Microwave Oven	0	0	1	100
Steamer	0	0	1	0
Ventana CC1 20mins	0	0	1	100
Ventana CC1 24mins	2	50	3	100
Ventana CC1 32mins	14	100	14	93
Ventana CC1 36mins	2	100	2	50
Ventana CC1 40mins	12	100	6	100
Ventana CC1 48mins	5	100	5	100
Ventana CC1 52mins	2	50	1	100
Ventana CC1 56mins	6	100	4	100
Ventana CC1 64mins	17	100	25	92
Ventana CC1 76mins	3	100	2	100
Ventana CC1 88mins	1	100	0	0
Ventana CC1 92mins	1	100	2	50
Ventana CC1 extended	2	100	4	100
Ventana CC1 mild	2	100	5	100
Ventana CC1 standard	32	91	22	82
Ventana CC2 64mins	0	0	1	0
Ventana CC2 92mins	0	0	1	100
Water bath 95-98 OC	1	100	1	100

Lymphoma Run: 113

Enzyme Mediated Retrieval	CD5 N	CD5 %	Cyclin D1 N	Cyclin D1 %
AS PER KIT	2	100	2	100
NOT APPLICABLE	114	97	110	79
Ventana Protease	0	0	1	100

Lymphoma Run: 113	CD5		Cyclin D1	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	11	100	14	86
BioGenex SS Polymer (QD 420-YIKE)	0	0	1	100
BioGenex SSM-link (LP000-UL)	1	100	0	0
Dako EnVision FLEX (K8000/10)	5	80	5	80
Dako EnVision FLEX+ (K8002/12)	15	100	13	92
Dako Envision HRP/DAB (K5007)	1	100	2	100
Dako Envision+ HRP mouse K4004/5/6/7	2	100	0	0
Dako Envision+ HRP rabbit K4008/9/10/11	0	0	2	100
Leica Bond Polymer Define (DS9713)	0	0	1	0
Leica Bond Polymer Refine (DS9800)	65	95	61	56
MenaPath X-Cell Plus (MP-XCP)	1	100	0	0
None	0	0	1	100
NOT APPLICABLE	1	100	2	100
Other	9	100	8	50
Ventana iView system (760-091)	3	100	4	75
Ventana OptiView (760-700) + Amp. (7/860-099)	2	50	1	100
Ventana OptiView Kit (760-700)	41	95	41	98
Ventana UltraView Kit (760-500)	48	96	47	83

Lymphoma Run: 113	CD5		Cyclin D1	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	1	100	1	100
Dako Autostainer Link 48	22	95	19	95
Dako Autostainer plus	2	100	1	100
Dako Autostainer Plus Link	2	100	3	100
Dako Omnis	5	100	6	83
LabVision Autostainer	2	100	3	67
Leica Bond Max	30	93	28	46
Leica Bond-III	41	98	41	59
Menarini - Intellipath FLX	1	100	0	0
None (Manual)	2	100	2	50
Shandon Sequenza	0	0	1	0
Ventana Benchmark GX	2	50	1	100
Ventana Benchmark ULTRA	61	95	66	91
Ventana Benchmark XT	36	97	32	88

Lymphoma Run: 113	CD5		Cyclin D1	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	24	96	28	86
BioGenex Liquid DAB (HK153-5K)	1	100	1	100
Dako EnVision Plus kits	2	100	3	100
Dako FLEX DAB	23	96	21	86
Dako REAL EnVision K5007 DAB	1	100	2	100
Dako REAL K5001 DAB	0	0	1	0
Leica Bond Polymer Refine kit (DS9800)	64	95	61	54
menapath xcell kit DAB (MP-860)	1	100	0	0
NOT APPLICABLE	0	0	2	100
Other	13	92	14	86
Ventana DAB	23	96	23	96
Ventana iview	1	100	1	0
Ventana Ultraview DAB	55	96	48	85

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

Primary Antibody: Dako (EP12) M3642 , 40 Mins, 21 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, PH: 9
EAR:
Chromogen: Dako FLEX DAB, 21 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection: Dako Envision+ HRP rabbit K4008/9/10/11 , 15 Mins, 21 ºC Prediluted

Cyclin D1 - Method 2

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

Primary Antibody: Dako (EP12) M3642 , 32 Mins, 37 ºC Dilution 1: 50
Automation: Ventana Benchmark ULTRA
Method: Ventana Optiview
Main Buffer: Ventana reaction buffer (950-300), PH: 7.6
HMAR: Ventana CC1 32mins
EAR:
Chromogen: Ventana DAB, 37 ºC., Time 1: 8 Mins, Time 2: 8 Mins
Detection: Ventana OptiView Kit (760-700) , 32 Mins, 37 ºC Prediluted

Cyclin D1 - Method 3

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

Primary Antibody:	LabVision/Thermo Sci (SP4) RM-9104 , 40 Mins, 37 ºC Dilution 1: 50
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.49
HMAR:	Ventana CC1 40mins
EAR:	NOT APPLICABLE
Chromogen:	Other, 37 ºC., Time 1: 8 Mins, Time 2: 4 Mins
Detection:	Ventana OptiView Kit (760-700) Prediluted

Cyclin D1 - Method 4

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

Primary Antibody:	Ventana (SP4-R) 790-4508 , 44 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 48mins, Buffer: Ultra CC1 (Cat 950-224), PH: 8
EAR:	
Chromogen:	Other, PH: 7, 37 ºC., Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) Prediluted

BEST METHODS - Secondary Antibody

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

CD5 - Method 1

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

Primary Antibody:	Ventana CD5 790-4451 (SP19) , 20 Mins, RT ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 76mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB, Time 1: 8 Mins, Time 2: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, RT ºC Prediluted

CD5 - Method 2

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

Primary Antibody:	Dako IR082 RTU FLEX Link (4C7) , 20 Mins Prediluted
Automation:	Dako Omnis
Method:	Dako FLEX kit
Main Buffer:	AS PER KIT
HMAR:	Dako Omnis, Buffer: Dako high pH TRS
EAR:	
Chromogen:	Dako FLEX DAB, Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Other , 20 Mins Prediluted

CD5 - Method 3

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

Primary Antibody:	Leica Bond RTU PA0168 (4C7) , 15 Mins, 20 ºC Prediluted
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800), 20 ºC., Time 1: 8 Mins, Time 2: 8 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 10 Mins, 20 ºC Prediluted

CD5 - Method 4

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

Primary Antibody:	Novocastra Bond PA0168 (4C7) , 15 Mins, Ambient ºC
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800), Time 2: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, Ambient ºC

Neil Bilbe

	Gold Standard	Second Antibody
Antigens Assessed:	Synaptophysin	IDH-1
Tissue Sections circulated:	Oligodendroglioma & cerebellum	Oligodendroglioma
Number of Registered Participants:	65	
Number of Participants this Run	58 (89%)	

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: IDH1

IDH1 belongs to two distinct subclasses. The protein is the NADP(+)-dependent isocitrate dehydrogenase found in the cytoplasm and peroxisomes.

Originally, mutations in IDH1 were detected in an integrated genomic analysis of human glioblastoma multiforme. Since then it has become clear that mutations in IDH1 and its homologue IDH2 are among the most frequent mutations in diffuse gliomas, including diffuse astrocytoma, and secondary glioblastoma. Mutations in IDH1 are often the first hit in the development of diffuse gliomas, suggesting IDH1 mutations as key events in the formation of these brain tumors. Glioblastomas with a wild-type IDH1 gene have a median overall survival of only 1 year, whereas IDH1-mutated glioblastoma patients have a median overall survival of over 2 years. The high frequency and distribution of the IDH1 R132H mutation among specific brain tumour entities allow the highly sensitive and specific discrimination of various tumours by immunohistochemistry, such as anaplastic astrocytoma from primary glioblastoma or diffuse astrocytoma WHO grade II from pilocytic astrocytoma or ependymoma. Noteworthy is the discrimination of the infiltrating edge of tumours with IDH1 mutation from reactive gliosis.

N.B. IDH1 has also been shown in human acute myeloid leukaemia.

Features of Optimal Immunostaining

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

Features of Suboptimal Immunostaining

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

References:

1. Capper D, Zentgraf H, Balss J, Hartmann C, von Deimling A. Monoclonal antibody specific for IDH1 H132R mutation. Acta Neuropathol. 118(5): 599-601, 2009
2. Capper D, Weissert S, Balss J, Habel A, Meyer J, Jäger D, Ackermann U, Tessmer C, Korshunov A, Zentgraf H, Hartmann C, von Deimling A. Characterization of R132H mutation-specific IDH1 antibody binding in brain tumors. Brain Pathol. 20(1): 245-254, 2010
3. Andrulis M, Capper D, Luft, T, Hartmann C, Zentgraf H, von Deimling A. Detection of isocitrate dehydrogenase 1 mutation R132H in myelodysplastic syndrome by mutation-specific antibody and direct sequencing. Leuk Res. 34(8):1091-1093, 2010
4. Andrulis M, Capper D, Meyer J, Penzel R, Hartmann C, Zentgraf H, von Deimling, A. IDH1 R132H mutation is a rare event in MPN as determinded by a mutation specific antibody. Haematologica 95(10):1797-1798, 2010.
5 Capper D, Sahm F, Hartmann C, Meyermann R, von Deimling A, Schittenhelm J. Application of mutant IDH1 antibody to differentiate diffuse glioma from nonneoplastic central nervous system lesions and therapy-induced changes. Am J Surg Pathol. 34(8):1199-1204, 2010
See also the IDH-1 presentation given at our Participants Meeting 2014 www.ukneqasiccish.org/wp/wp-content/uploads/2016/04/idh1_talk_2014.pdf

Assessment Summary:

65 labs are currently registered for neuropathology. Seven participants did not return *any* slides. The 58 labs that submitted, returned both their NEQAS (**G**) and in-house (**H**) synaptophysin slides. 17 (29%) participants had declared that they did not stock an IDH-1 marker (**J & K**). Ten of these submitted the alternative antibody given by NEQAS: Ki67/MIB-1; the remaining 7 labs did not send any 2nd antigen slides. This left a total of 218 slides for assessment. Breakdown: **G=58, H=58, J=51, K=51 = 218**

Only three labs (5%) did not place their in-house control on the NEQAS slides, of which only one appeared to be due to section adhesion. There appeared to be a high percentage of labs using neurological material for in-house, although this could be higher still.

Synaptophysin (**G&H**)

There were 6 failed slides for the Gold (**G&H**). Five of these were on the NEQAS section (9%) with a single in-house slide also failing (2%). Four of the failed NEQAS (**G**) slides were due to weak staining of the tumour; the other failed to stain the cerebellum, probably due to too dilute a primary: Novocastra NCL-SYNAP-299 at 1:1000 using the Dako Omnis system. The four that had weak staining of the tumour all had different protocols. Two employed a RTU Ventana marker (Confirm SP-11 and MRQ-40) both with CC1, on an Optiview and Benchmark XT respectively; another the Dako SY-38 clone, with ER2 on the Bond Max; and the remaining lab also used the Novocastra, at 1:100, CC1, on Benchmark XT, with acceptable cerebellum staining.

IDH-1 (**J&K**)

This was the first time IDH-1 had been requested. Out of the 51 labs submitting the 2nd antigen, 41 (80%) used IDH-1, and 10 (20%) the alternative antibody of MIB-1. Of the 38 labs who entered IDH-1 protocols, 37 (97%) employed the Dionova monoclonal (clone H09); the other lab (3%) used the Abcam ab135658 (polyclonal), supplied by Milipore. The remaining few labs did not enter any IDH-1 protocols.

The Abcam polyclonal (1:100) user failed due to weak demonstration, allied with some background staining. Their data shows HMAR using TRIS buffer at pH9 (citrate buffer at pH6 is recommended though).

The 7 labs who obtained sub-optimal results with the H09 clone (all weak staining of the tumour) used a variety of methods; antibody dilution ranged from 1:20 (datasheet recommendation) to 1:1000; pre-treatments were: PT link low pH (2), CC1 mild (2), ER2 (1), Dako Omnis (1), and a water bath at 95 °C (1). Only one automation was used more than once, the Dako Autostainer Link 48. Therefore it is difficult to deduce any concrete trends or suggestions from the data.

Conversely, if we look at the best performing labs: There were 5 participants who scored at least 19/20 for their NEQAS IDH-1 slides (**J**). The average dilution of the antibody was 1:65 (1:130 for *all* IDH-1 users), used for an average of 36 minutes, retrieved at high pH (Dako PT Link, Leica ER2, Omnis), on a Dako or Leica platform.

Selected Images showing Optimal and Sub-optimal Immunostaining

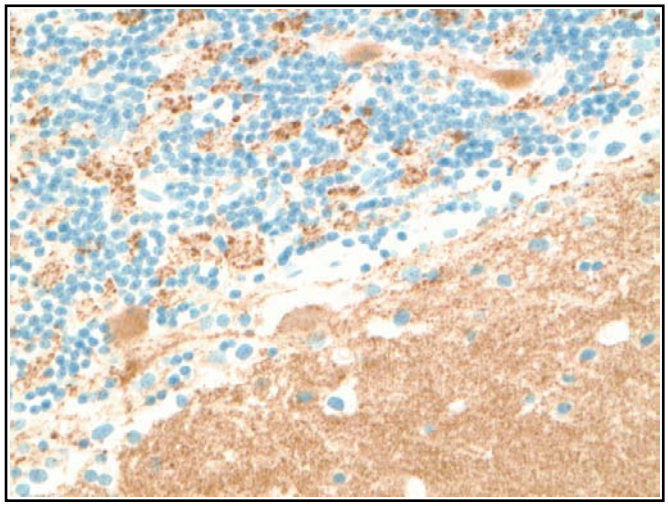


Fig 1. Sub-optimal demonstration of synaptophysin on NEQAS cerebellum section. Staining is quite weak, and was assessed as borderline. Stained using Ventana CONFIRM SP-11, using the Ventana CC1 52 mins, Ventana UltraView Kit, on the Ventana Benchmark ULTRA.

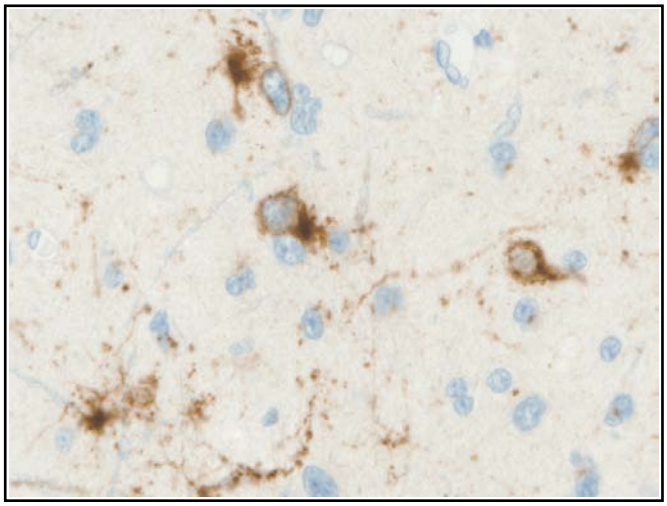


Fig 2. Good, albeit slightly weak, demonstration of synaptophysin on NEQAS glioma section (compare with Fig 3). Primary was the RTU DAK-SYNAP clone, 10 mins, with the Dako Omnis Target Retrieval Solution Low, detected with the prediluted Dako EnVision FLEX kit on the Dako Omnis.

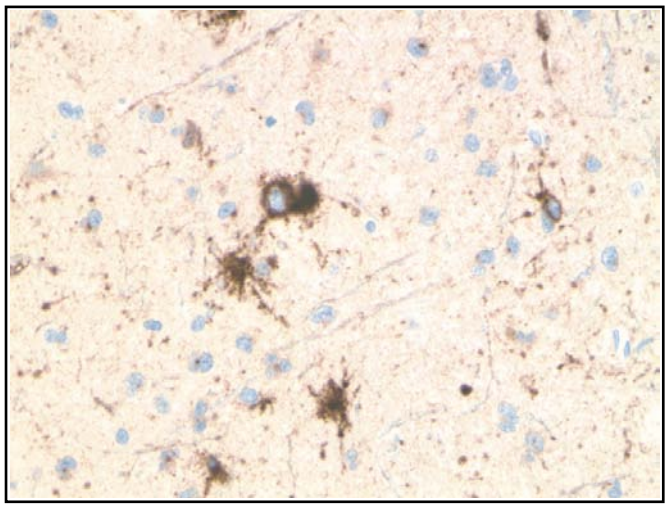


Fig 3. Optimal demonstration of synaptophysin on NEQAS glioma section. Staining intensity is excellent, and the counterstain level is ideal. Dako DAK-SYNAP clone (no dilution given), pretreated using Leica ER1 20 mins, using the Leica Bond Polymer Refine (DS9800) kit, on a Leica Bond-III.

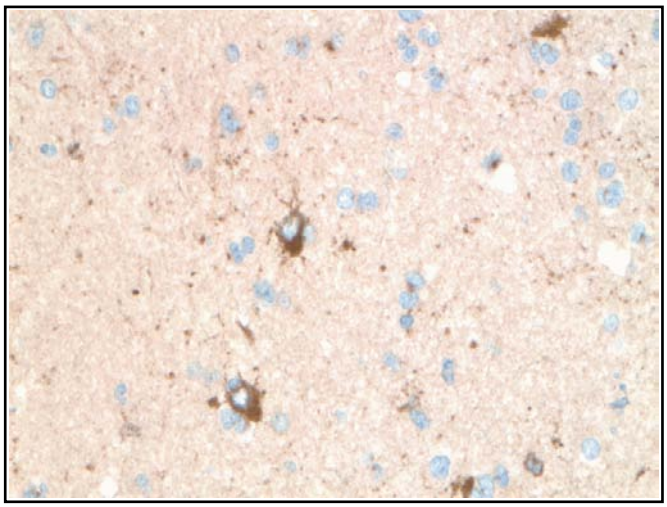


Fig 4. Sub-optimal demonstration of synaptophysin on a NEQAS section. Some staining of the tumour is missing. This slide was assessed as borderline. Ventana 760-4595 (MRQ-40), prediluted, 16 mins, with the Ventana CC1 32mins, a Ventana OptiView Kit kit, on the Benchmark ULTRA.

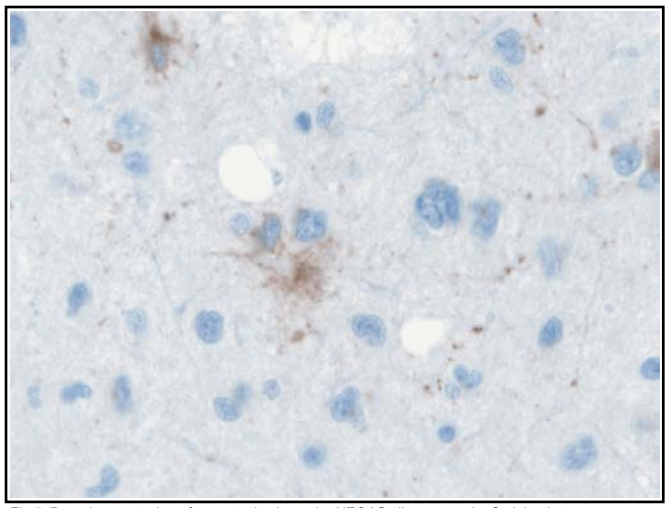


Fig 5. Poor demonstration of synaptophysin on the NEQAS glioma sample. Staining is too weak for reliable diagnostic use, the counterstain is heavy. Slide was failed by the assessors. Novocastra NCL-L-SYNAP-299, 1:100, Ventana CC1, 32mins, Ventana UltraView Kit, on a Benchmark XT.

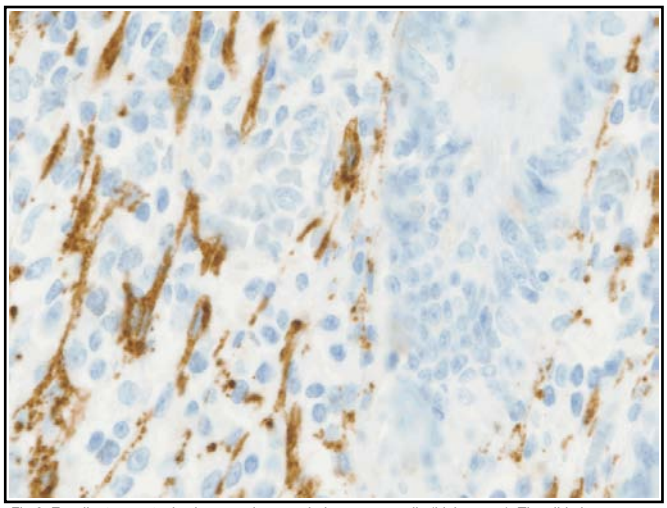


Fig 6. Excellent synaptophysin example on an in-house appendix (high power). The slide is clean, staining is precise, the counterstain good. RTU Dako DAK-SYNAP clone, 20 mins, with a Dako PTLink, pH9, 20 mins, with a RTU Dako EnVision FLEX kit, on the Dako Autostainer Link 48.

Selected Images showing Optimal and Sub-optimal Immunostaining

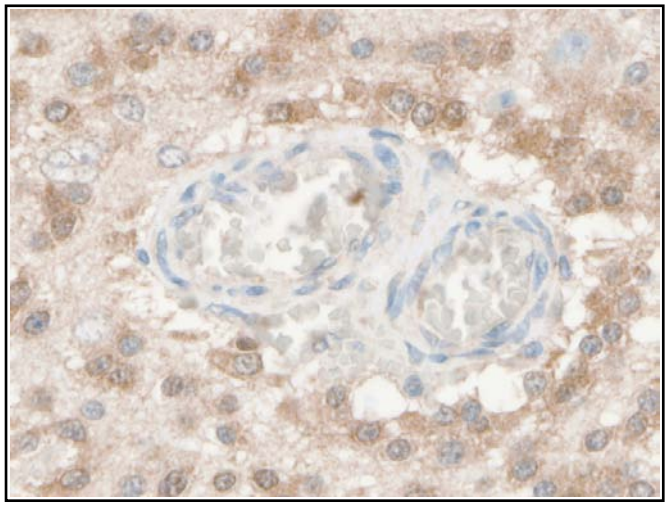


Fig 7. Sub-optimal demonstration of IDH-1 on the NEQAS oligodendroglioma. Staining is diffuse, and there is also some background staining; although the slide did pass the assessment. Dionova H09 clone, 1:10, with a Leica ER1, 30 mins, a Leica BondMAx Refine KIT, on the Leica Bond-III.

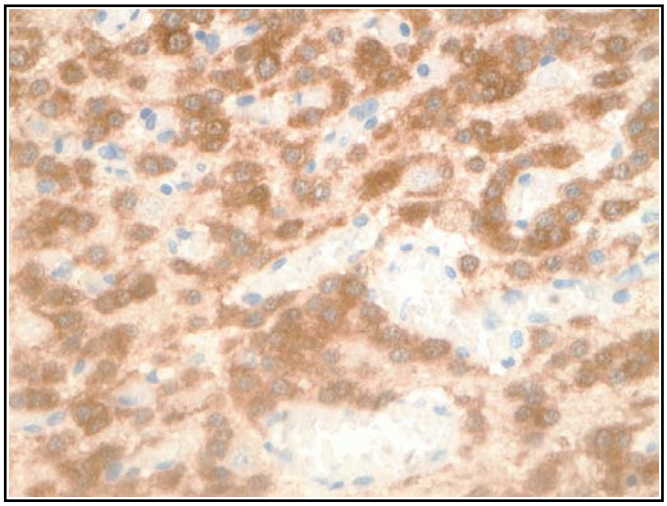


Fig 8. Optimal demonstration of IDH-1 on NEQAS glioma tissue. The tumour is nicely stained against a clean background. Dionova H09 clone, 1:20, 15 mins, at RT, with Leica ER2 20 mins, Leica Bond Polymer Refine kit, on a Leica Bond-III.

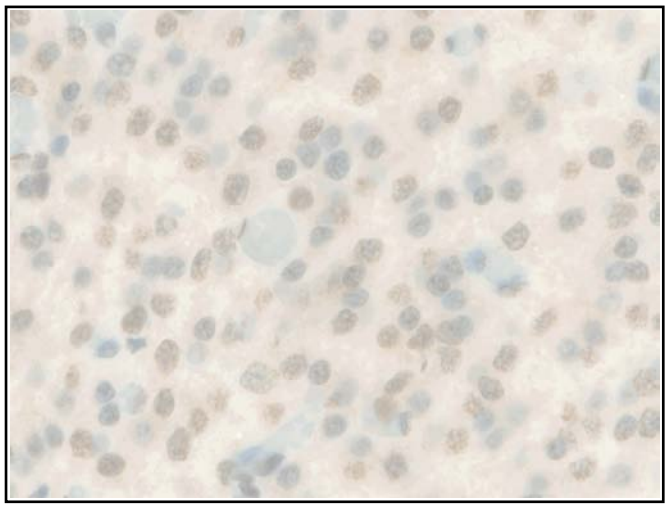


Fig 9. Sub-optimal IDH-1 demonstration on NEQAS glioma tissue. Although some tumour is stained it is far too weak for safe diagnostic use. The slide failed. Abcam ab135658, polyclonal, (sole user), 1:100, 20 mins, a high pH Dako PTLink, the RTU Dako EnVision FLEX kit, on Dako Autostainer Link 48.

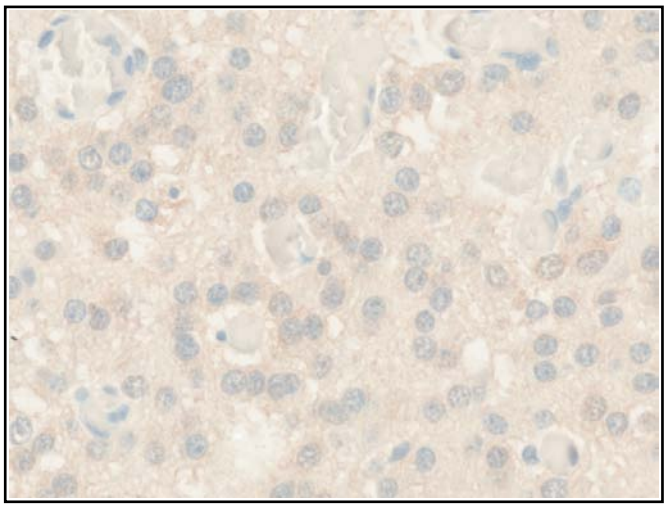


Fig 10. Poor IDH-1 demonstration on the NEQAS glioma. There is only a background wash of 'colour' and it is difficult to discern positive from negative components. (Compare with Fig 8). Dionova (H09) at 1:50, with Leica ER2 40 mins, Leica BondMAx Refine KIT, on a Leica Bond Max platform.

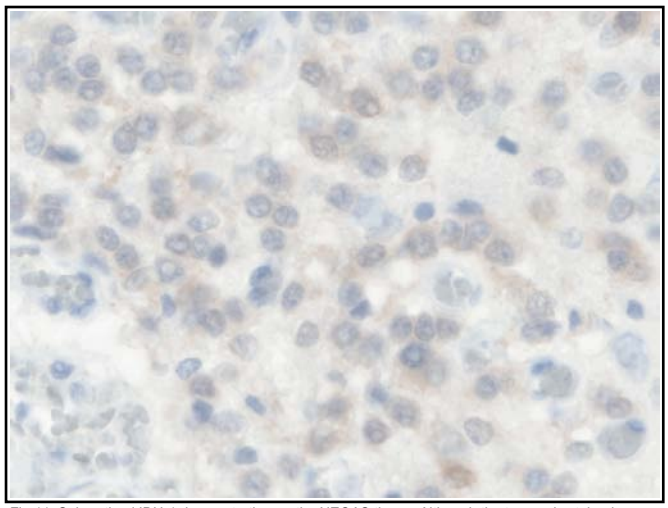


Fig 11. Sub-optimal IDH-1 demonstration on the NEQAS tissue. Although the tumour is stained it is too weak. The slide was therefore scored as borderline. Dionova (H09), 1:200, 30 mins, in a Biocare Decloaking Chamber, a RTU Biocare polymer (M4U534) detection kit, on a Menarini - Intellipath FLX

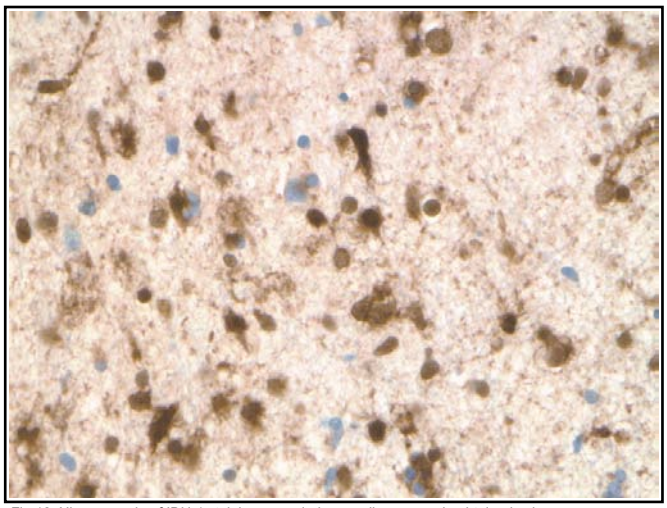
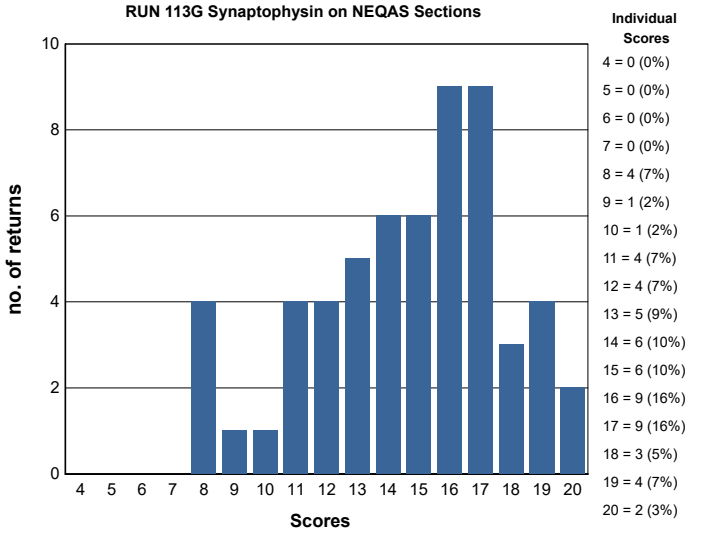
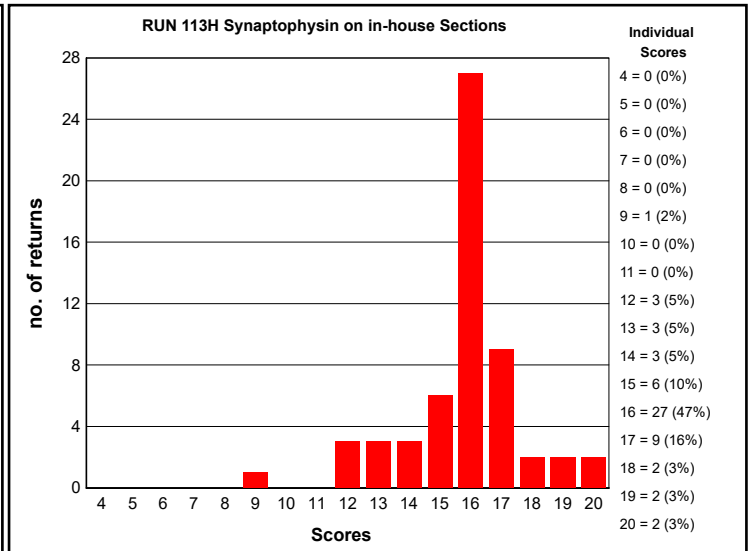
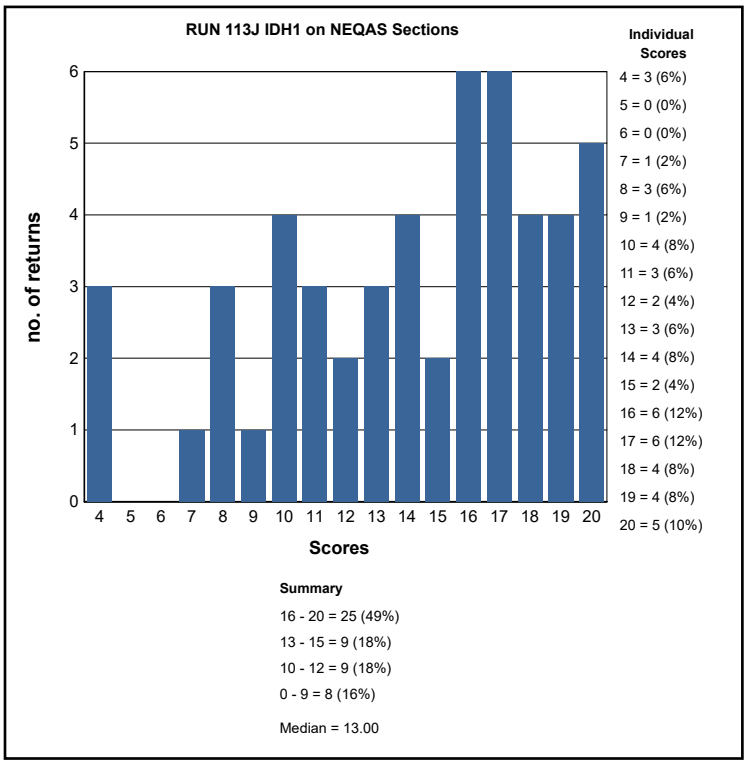


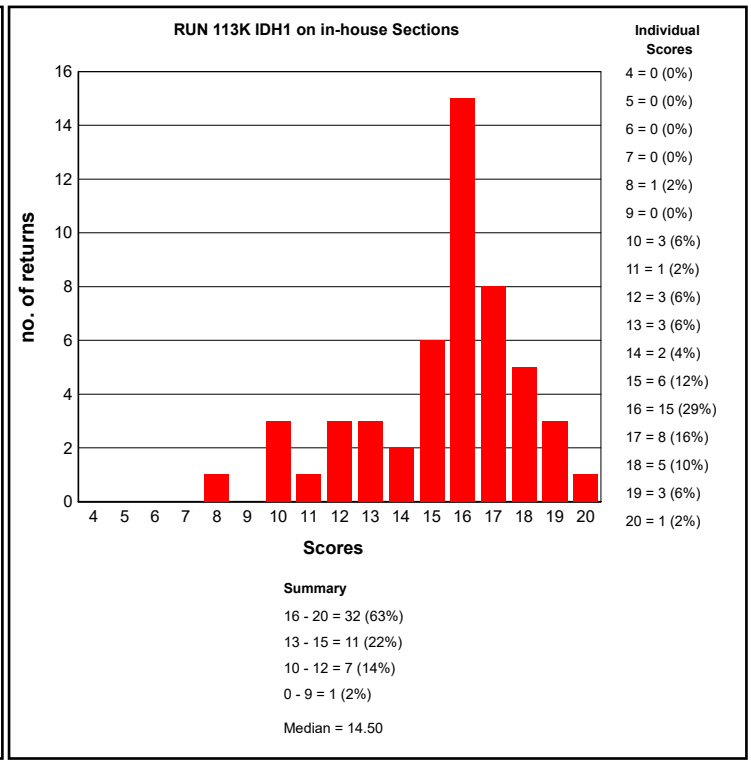
Fig 12. Nice example of IDH-1 staining on an in-house glioma sample obtained using an ultrasonic aspirator. Dionova (H09), diluted to the recommended 1:20, with Leica ER1 for 30 mins, with the Leica Bond Polymer Refine kit, on a Leica Bond-III.

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: 113

Primary Antibody : Synaptophysin

Antibody Details	N	%
BioGenex AM 6 (clone SNP)	2	50
Biogenex MU 363 UC	1	100
Cell Marque 336R-94/95/96/97 (MRQ-40)	1	100
DAKO FLEX IR776 (SY38)	2	100
Dako M0776 (clone SY38)	3	67
NeoMarkers SP11	1	0
Novocastra Bond RTU PA0299 (rb poly)	3	67
Novocastra NCL-L-SYNAP-299 (27G12)	9	89
Novocastra NCL-SYNAP-299 (27G12)	9	89
Other	18	83
Ventana 760-4595 (MRQ-40)	4	50
Ventana CONFIRM 790-4407 (SP-11)	5	40

Neuropathology Run: 113

Primary Antibody : IDH1

Antibody Details	N	%
Abcam ab135658 (Polyclonal)	1	0
Dionova 1DH1R32H (H09)	37	62

Neuropathology Run: 113

Heat Mediated Retrieval	IDH1 N	IDH1 %	Synaptophysin N	Synaptophysin %
_Leica BondMax ER2	0	0	1	0
Biocare Decloaking Chamber	1	0	1	100
Dako Omnis	2	50	3	67
Dako PTLink	8	38	11	91
Leica ER1 20 mins	0	0	3	100
Leica ER1 30 mins	4	100	4	100
Leica ER2 20 mins	6	100	8	75
Leica ER2 30 mins	5	80	3	67
Leica ER2 40 mins	1	0	0	0
None	0	0	1	100
Other	1	0	1	100
Ventana CC1 24mins	1	0	1	0
Ventana CC1 32mins	3	67	3	33
Ventana CC1 40mins	0	0	1	0
Ventana CC1 48mins	1	100	0	0
Ventana CC1 52mins	1	100	1	0
Ventana CC1 56mins	1	100	1	100
Ventana CC1 64mins	2	50	7	86
Ventana CC1 mild	1	100	2	50
Ventana CC1 standard	2	100	5	80
Ventana CC2 44mins	1	0	0	0
Ventana CC2 64mins	1	0	0	0
Water bath 95-98 OC	1	0	1	100

Neuropathology Run: 113

Enzyme Mediated Retrieval	IDH1 N	IDH1 %	Synaptophysin N	Synaptophysin %
AS PER KIT	0	0	2	100
NOT APPLICABLE	18	50	36	75
Other	0	0	1	100

Neuropathology Run: 113	IDH1		Synaptophysin	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	6	67	8	100
Biocare polymer (M4U534)	1	0	1	100
Dako EnVision FLEX (K8000/10)	4	75	4	100
Dako EnVision FLEX+ (K8002/12)	3	0	6	67
Dako Envision HRP/DAB (K5007)	0	0	1	100
Leica Bond Polymer Refine (DS9800)	12	92	18	78
None	1	0	0	0
Other	2	0	1	100
Ventana OptiView Kit (760-700)	6	50	7	43
Ventana UltraView Kit (760-500)	7	71	12	67

Neuropathology Run: 113	IDH1		Synaptophysin	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	8	50	11	100
Dako EnVision Plus kits	2	50	2	100
Dako FLEX DAB	6	33	8	75
Dako REAL EnVision K5007 DAB	0	0	1	100
Leica Bond Polymer Refine kit (DS9800)	13	92	18	78
Other	5	20	4	0
Ventana DAB	3	100	2	100
Ventana Ultraview DAB	7	71	12	67

Neuropathology Run: 113	IDH1		Synaptophysin	
Automation	**N**	**%**	**N**	**%**
Dako Autostainer Link 48	8	38	12	92
Dako Autostainer Plus Link	1	0	0	0
Dako Omnis	2	50	3	67
Leica Bond Max	6	83	7	43
Leica Bond-III	10	90	13	100
Menarini - Intellipath FLX	1	0	1	100
None (Manual)	0	0	1	100
Ventana Benchmark ULTRA	9	56	13	69
Ventana Benchmark XT	6	83	8	50

BEST METHODS - Gold Standard Antibody

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

Synaptophysin - Method 1

Participant scored 18/20 (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 17/20 (In House slide) using this method.

Primary Antibody: Novocastra NCL-SYNAP-299 (27G12) , 32 Mins, 37 ºC Dilution 1: 20
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)

Synaptophysin - Method 3

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

Primary Antibody:	Novocastra NCL-SYNAP-299 (27G12) Dilution 1: 40
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 30 mins
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	AS PER KIT

Synaptophysin - Method 4

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

Primary Antibody:	Ventana CONFIRM 790-4407 (SP-11) , 72 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins, Buffer: 0
EAR:	
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700) Prediluted

BEST METHODS - Secondary Antibody

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

IDH1 - Method 1

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

Primary Antibody:	Dionova 1DH1R32H (H09) , 15 Mins, 25 ºC Dilution 1: 1/50
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

IDH1 - Method 2

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

Primary Antibody:	Dionova 1DH1R32H (H09) , 15 Mins, R/T ºC Dilution 1: 20
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)

IDH1 - Method 3

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

Primary Antibody:	Dionova 1DH1R32H (H09) , 20 Mins, 32 ºC Dilution 1: 75
Automation:	Dako Omnis
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis, Buffer: Target Retrieval Solution High
EAR:	
Chromogen:	Dako FLEX DAB, 32 ºC., Time 1: 5 Mins
Detection:	Dako EnVision FLEX (K8000/10) , 20 Mins, 32 ºC Prediluted

IDH1 - Method 4

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

Primary Antibody:	Dionova 1DH1R32H (H09) , 30 Mins, ROOM ºC Dilution 1: 1/80
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer, PH: 7.6
HMAR:	Dako PTLink, Buffer: Dako EnVision retrieval sln high pH
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, ROOM ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako EnVision FLEX (K8000/10) , 30 Mins, ROOM ºC Prediluted

Neil Bilbe and Irena Srebotnik Kirbis

	Gold Standard	Second Antibody
Antigens Assessed:	Melanoma markers	Calretinin
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:	83 - Cell block 58 (70%), Cytospin 25 (30%)	
Number of Participants this Run	76 (92%)	

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 cartilagenous 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: Calretinin

Calretinin is a 29 kDa calcium-binding protein involved in calcium signalling. It is found abundantly in neurons and the thymus, and is associated with the kinetocore during the cell cycle. Outside the nervous system, calretinin is found in a number of cells with varying expression, including mesothelial cells, steroid producing cells, testicular cells, ovarian surface epithelium, some neuroendocrine cells, breast glands, and hair follicle cells. Diagnostically, calretinin is used as a positive marker for both benign mesothelium and in malignant mesothelioma (Saydan *et al*). It's use in the identification of mesothelioma in cytological preparations (Doglioni *et al*), and in the differential diagnosis between malignant mesothelioma and adenocarcinoma in FFPE cell blocks of cytological fluids, washings and aspirates (Wiezorekd and Krane) have been described. Calretinin can also be used to help differentiate lung tumours (Marchevsky), and also to distinguish between different types of brain tumour, i.e. neuronal rather than glial differentiation (Leong *et al*).

Features of Optimal Immunostaining
- Strong cytoplasmic staining of the mesothelial cells.
- Clean background with no non-specific staining of other cell types not expected to stain.
- Adequate nuclear counterstain.

Features of Sub-optimal Immunostaining
- Weak or absent staining in the mesothelial cells.
- Uneven staining.
- Excessive background staining.
- Excessive non-specific staining of cell types or components not expected to satin.
- Inadequate nuclear counterstain.

References

1. Saydan N, et al. Expression of calretinin in human mesothelioma cell lines and cell cycle analysis by flow cytometry. Anticancer Res 21 (1A): 181-8.
2. Doglioni C, et al. Calretinin: a novel immunocytochemical marker for mesothelioma. Am J Surg pathol 1996; 20: 1037-1046.
3. Wieczorek TJ, et a.. Diagnostic utility of calretinin immunohistochemical marker for mesothelioma. Am J Surg pathol 1996; 20: 1037-1046.
4. Marchevsky AM. Application of immunohistochemistry to the diagnosis of malignant mesothelioma. Arch Pathol Lab Med. 132 (3): 397-401.
5. Leong A, et al. Manual of Diagnostic Cytology (2nd ed.) Greenwich Medical Media Ltd. pp. 45-46.

References (cell blocks in cytology)

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

Assessment Summary:

At the start of the run there were 83 registered participants, with 76 labs submitting at the time of assessment. 7 participants did not return any slides at all, and another 5 labs failed to send in at least one of their slides/sections. This resulted in a total of 296 slides for the assessors to evaluate.

For this run we reverted to the choice of 3 melanoma markers as the Gold antigen (**R**), rather than **S100 only** in Run 112.

With some labs not returning either their NEQAS or in-house slides, for one or other antigen, the breakdown of returns was:
R=75, S=74, T=74, U=73 = 296

The overall pass rate was 91% (268 slides), borderline 6% (19), with a failure rate of 3% (9).

An average score for the quality of immunocytochemistry across all four types of slides (R, S, T, U) obtained in this run was 16/20.

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

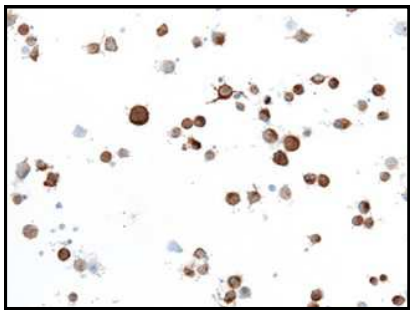


Fig 1: HMB45 on cytospin prepared from 113 R

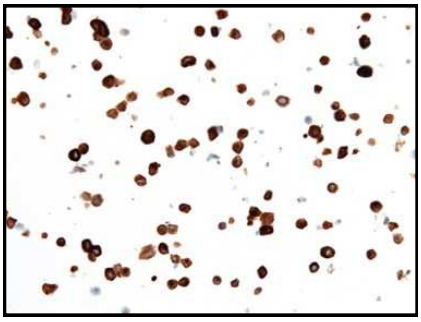


Fig 2: Melan A on cell block prepared from 113 R

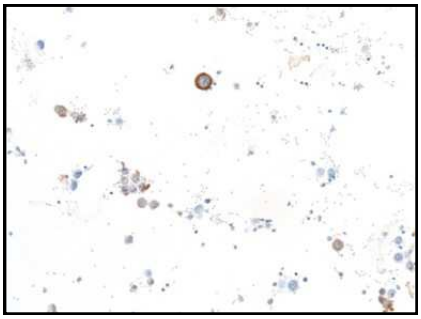


Fig 3: Calretinin on cytospin prepared from 113 T

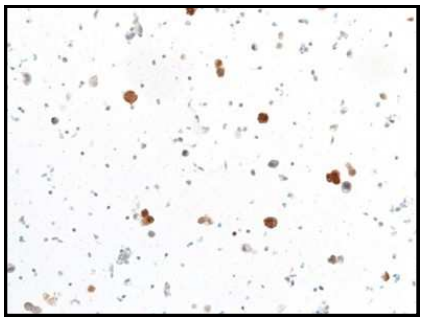


Fig 4: Calretinin on cell block prepared from 113 T

Assessment outcomes:

Apart from the number of borderline Calretinin slides (**T**), results were consistent. No significant difference in sub-optimal outcomes between cell blocks (10%) and cytospins (12%) on NEQAS slides (**R & T**) were seen in relation to overall totals of 58 CB labs and 25 CS labs.

Melanoma markers (R & S)

Four slides failed the assessment; two (3%) on the NEQAS slides (**R**), both on cell block sections (see Fig 4), and two (3%) in-house controls (**S**), one cell block and a cytospin.

The NEQAS slides were stained using 1) S100: Novocastra NCL-L-S100p, 1:400, 24 mins, no RT, on a Benchmark XT; and 2) Melan A: Ventana RTU (MART-1) 790-2990, 32 mins, CC1 64 mins, on a Benchmark ULTRA. Assessors commented that the staining was weak and uneven (S100); and antigen not demonstrated (Melan A). The lack of retrieval for the S100 slide would help to explain the findings, and also as we reported in Run 112, S100 does not perform as well as either HMB45 or Melan A.
Of more concern is the Melan A result. This antibody has performed well in the previous two runs where melanoma markers have been requested (110, 111) with overall pass rates of 100% and 92% respectively, and 90% for this run (113). The participant has also performed respectfully, but there has been a decline in performance from Run 110 (17/20), Run 111 (13/20) and now Run 113 (4/20). The protocol has remained unchanged, which would appear to indicate a drop off in reagent/antibody selectivity; but the lab has entered a new batch number for each run, so deterioration of a single batch is not responsible.
Seven labs used S100 (9%), three on a CB, and four on a CS. The average score for these seven slides (**R**) was 14/20 (Run 112 where only S100 was used the score was similar: 13.5), for all NEQAS melanoma slides (**R**) the score was 16/20, and for HMB45 and Melan A only slides 17/20. This reinforces the findings reported in Run 112.

The two failed in-house melanoma slides (**S**) were Dako monoclonal HMB45, 1:50, 30 mins, ER1 10 mins, on a Bond III on a cytospin from a lymph node, which had non-specific staining and background due to the use of retrieval (not recommended on CS); and Dako Melan A, 1:100, 28 mins, CC1 std, on a Benchmark XT on a cell block from an ascites. The sample was considered as a poor choice of control (fail).

Calretinin (T & U)

Five slides failed the assessment (3%), three on the NEQAS slides (4%), and two on the in-house samples (3%). One participant failed both their NEQAS (**T**) and in-house Calretinin slides (**U**), both of which were on a CB (pleural effusion control sample); Novocastra clone CAL6, no dilution given, CC1 8 mins, on a Benchmark ULTRA; the other NEQAS CB slide (**T**) used a Cell Marque polyclonal, 1:200, 30 mins, ER1 10 mins, on a Bond Max. The participant who failed their NEQAS slide (**T**) on a CS (see Fig 10), employed the Novocastra 5A5 clone, 1:100, 30 mins, no RT, on a Dako Autostainer. All three slides had very weak and/or no staining as comments.

For the other failed in-house slide (**U**) the lab submitted a smear; Invitrogen polyclonal, 1:200, 20 mins, ER1 10 mins, manual method. Staining was too weak and this was not a suitable control sample.

As commented on above, the main finding from the assessment was the relatively high number of borderline results on the NEQAS Calretinin (**T**) slides. 11/74 (14%) submitted, received a score of between 10 –12. The main comments were weak staining, uneven staining, with a few slides also having excessive background staining.

For those receiving borderline scores for their 2nd antigen (**T**), seven different primary antibodies/clones were used from five different suppliers. The most common of these numerically was the Dako DAK-Calret 1 clone, used by four labs, and the Invitrogen DC8 clone used by two labs, although statistically this represents 24% (4/17) and 33% (2/6) of users. A single user who received a borderline mark employed each of the following markers: the Cell Marque (100% borderline scores), Dako RTU DAK-Calret 1 clone (17%), Novocastra NCL-CALRET 5A5 clone (20%), Novocastra/Leica CAL6 clone (13%), and also the Ventana SP65 clone (16%).

Overview of Assessment Results Run 113 (cell block and cytospin figures for the NEQAS borderline and failed slides only)

Slide	Marker	Pass rate	Borderline		Fail	
			Cell Block	Cytospin	Cell Block	Cytospin
R (NEQAS)	Melanoma	71 (95%)	1 (1%)	1 (1%)	2 (3%)	0 (0%)
S (In-House)	Melanoma	69 (93%)	3 (4%)		2 (3%)	
T (NEQAS)	Calretinin	60 (81%)	7 (9%)	4 (5%)	2 (3%)	1 (1%)
U (In-House)	Calretinin	68 (93%)	3 (4%)		2 (3%)	
Total (Average)		268 (91%)	19 (6%)		9 (3%)	

Selected Images showing Optimal and Sub-optimal Immunostaining

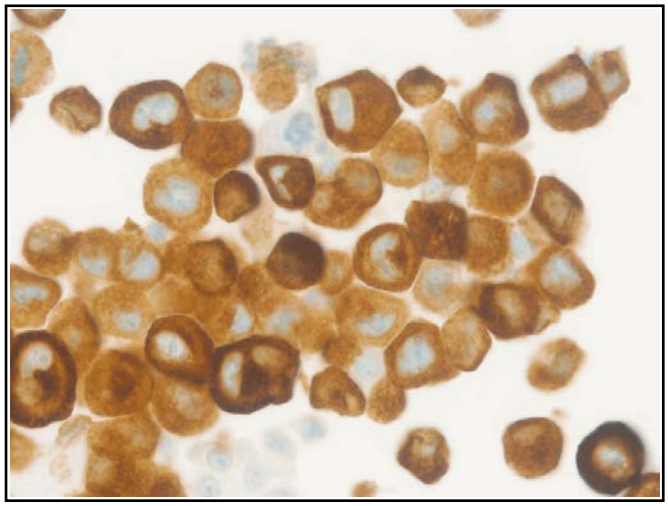


Fig 1. Optimal staining on a NEQAS CB section. Melanoma cells are nicely demonstrated against a clean background, and good counterstain intensity. Prediluted Ventana Melan A (MART-1), 16 mins, at RT, with a Ventana CC1 standard, and the Ventana UltraView Kit, on the Benchmark ULTRA.

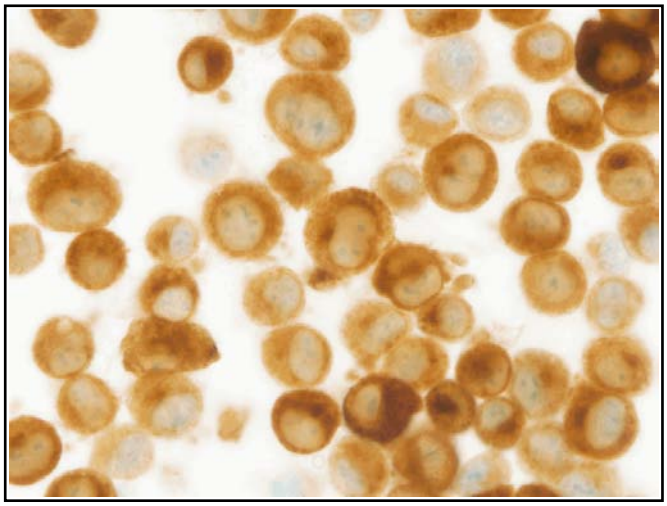


Fig 2. Sub-optimal melanoma staining on a NEQAS CS sample. The staining is slightly weak, and the haematoxylin pale. Novocastra/Leica NCL-MELAN A (Melan A), without retrieval, detected with the Ventana UltraView Kit, on the Ventana Benchmark ULTRA.

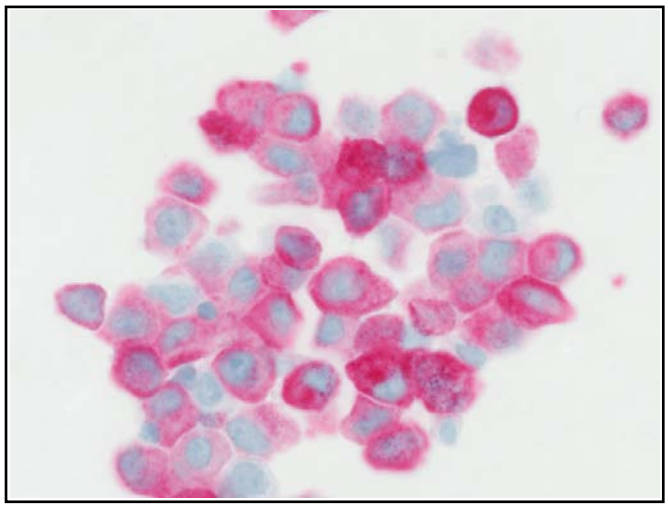


Fig 3. Excellent demonstration of melanoma cells on a NEQAS CB. Staining is crisp with little or no bleeding or background hue. Novocastra/Leica NCL-MELAN A (Melan A), 1:25, 56 mins, Ventana CC1 64 mins, UltraView Kit, and Ventana Enhanced Alk. Phos. Red Detection Kit, again on an ULTRA.

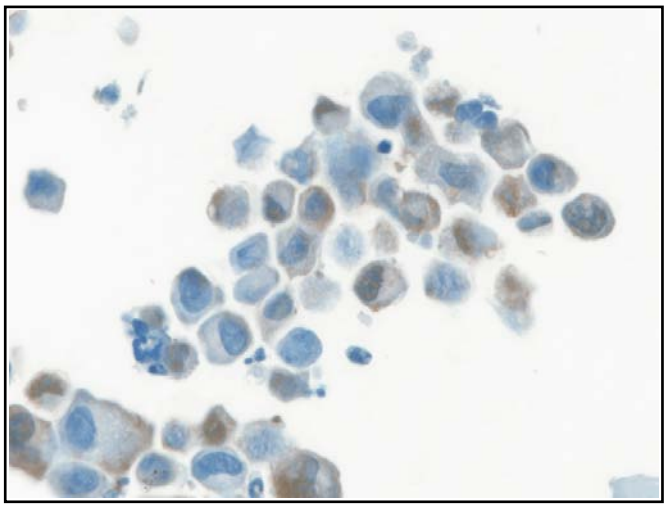


Fig 4. Poor demonstration of melanoma cell on a NEQAS CB. Staining is weak and uneven, allied with a heavy counterstain. The slide failed. Novocastra/Leica NCL-S100p (S100), 1:400, 24 mins, stated no retrieval used, detected with Ventana UltraView Kit (760-500) on a Ventana Benchmark XT.

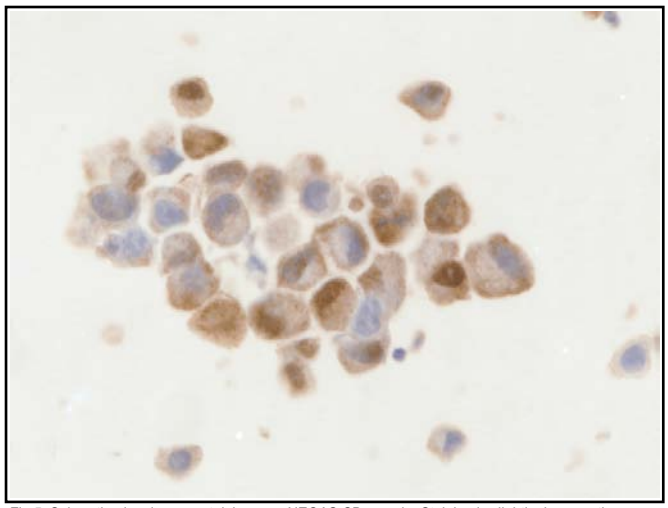


Fig 5. Sub-optimal melanoma staining on a NEQAS CB sample. Staining is slightly down on the expected level and assessed as borderline pass. Dako Z0311 (S100), 1:400, 8 mins, Ventana CC1 8 mins, with a RTU Ventana OptiView Kit (760-700), on the Ventana Benchmark ULTRA.

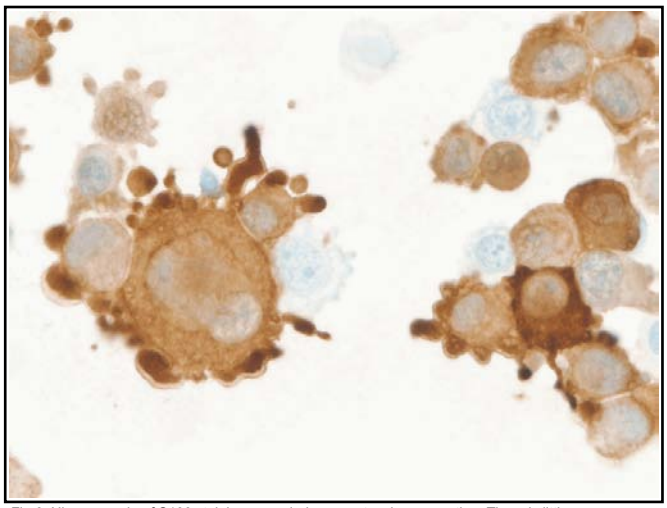


Fig 6. Nice example of S100 staining on an in-house cytospin preparation. There is little or no background or non-specific staining. Dako Z0311 (S100), 1:5000, no HMAR, using a Ventana iView Kit, and the Ventana Benchmark XT.

Selected Images showing Optimal and Sub-optimal Immunostaining

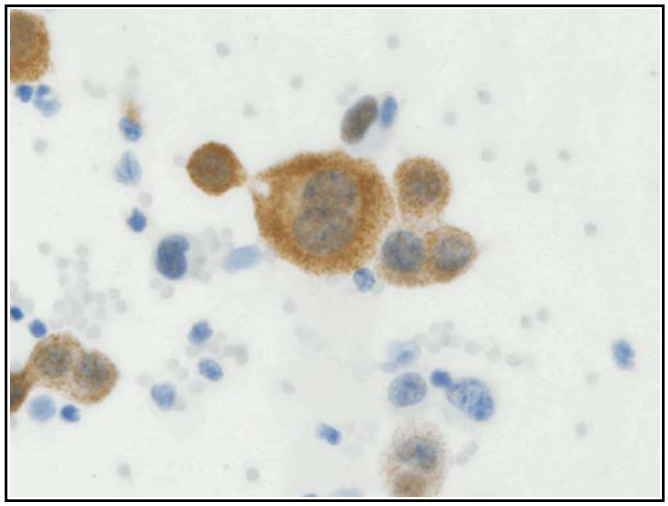


Fig 7. Good demonstration of calretinin on a NEQAS CS sample. The counterstain is a little heavy, otherwise a nice slide. Novocastra/Leica NCL-L-CALRET-566 (CAL6), 1:200, no HMAR, Leica Bond Polymer Refine kit, on a Leica Bond-III.

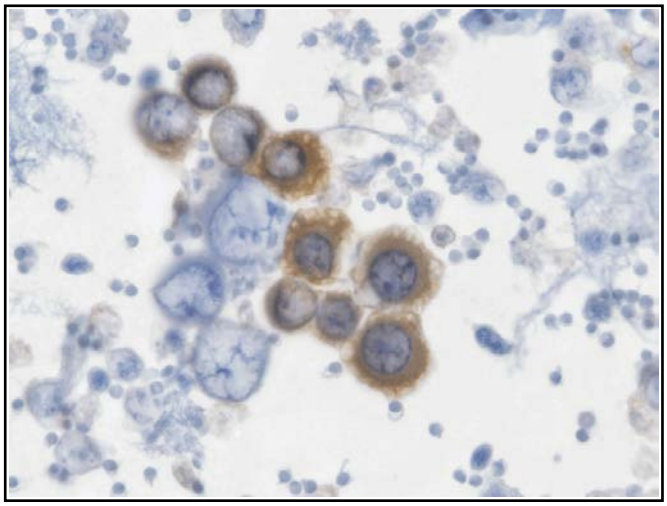


Fig 8. Sub-optimal calretinin staining on a NEQAS sample. Staining is uneven, the counterstain too heavy, and retrieval has been used on this cytospin. Invitrogen 18-0211 (DC8), 1:50, 20 mins, Ventana CC1 mild 30 mins, RTU Ventana UltraView Kit, on a Ventana Benchmark XT.

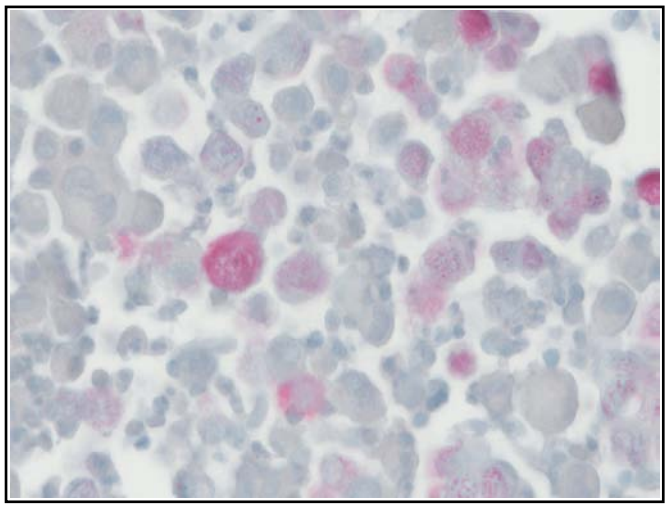


Fig 9. Sub-optimal calretinin staining on a NEQAS CB sample. Some carcinoma cells are stained, but only weakly, and the counterstain hue is poor. Low borderline score. RTU Ventana 790-4467 (SP65), 32 mins, Ventana CC1 16 mins, RTU Ventana UltraView Kit, on the Ventana Benchmark ULTRA.

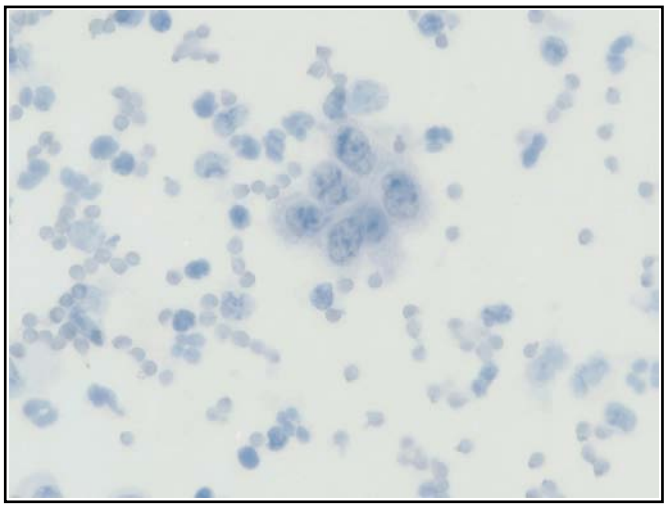


Fig 10. Poor demonstration of calretinin on a NEQAS CS slide. Staining is very weak or absent, and therefore failed the assessment. Novocastra NCL-CALRET (5A5), 1:100, 30 mins, no HMAR, prediluted Dako Envision HRP/DAB (K5007) kit, on a Dako Autostainer.

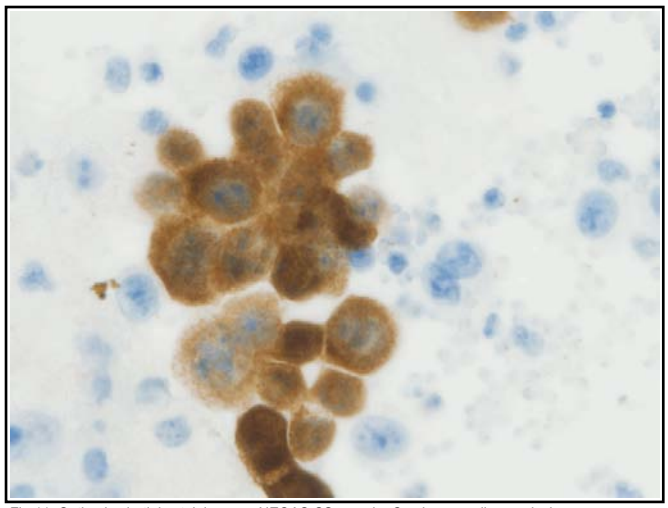


Fig 11. Optimal calretinin staining on a NEQAS CS sample. Carcinoma cells are nicely demonstrated. Dako M7245 (DAK-Calret 1), 1:100, 30 mins, no evidence of any retrieval, with a Leica BondMAx Refine KIT, on the Leica Bond-III.

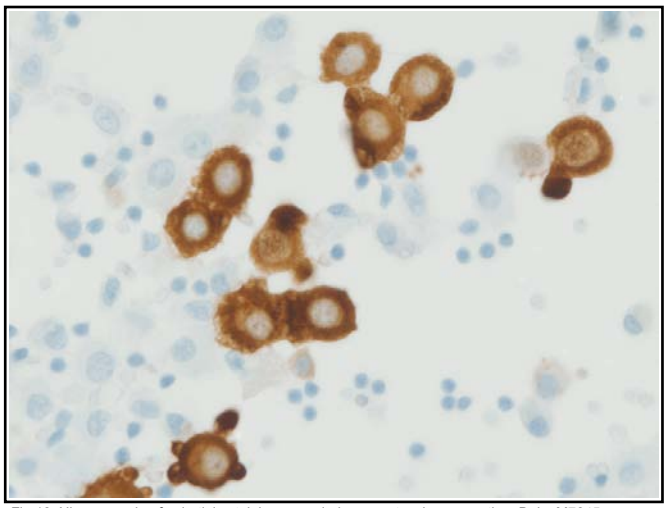
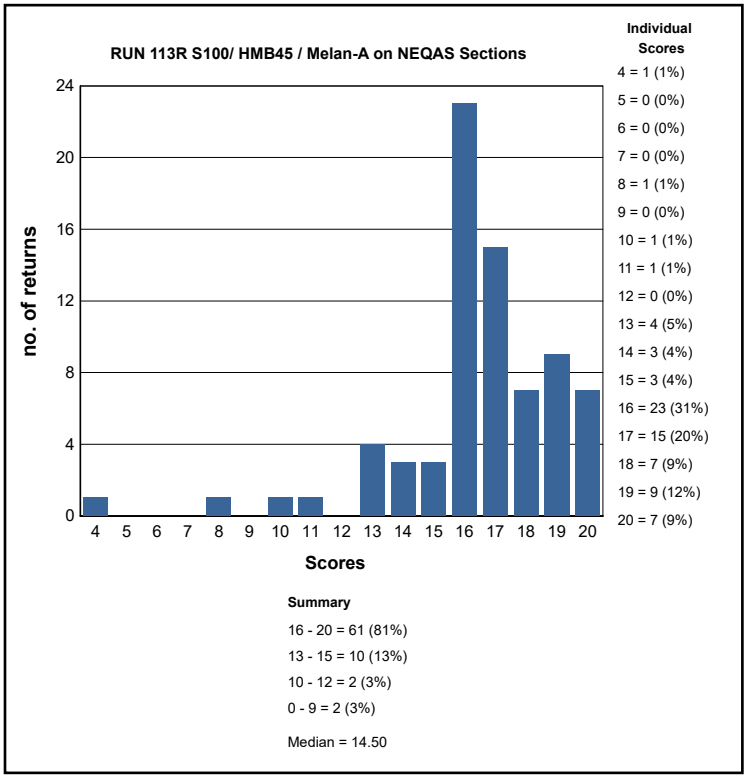
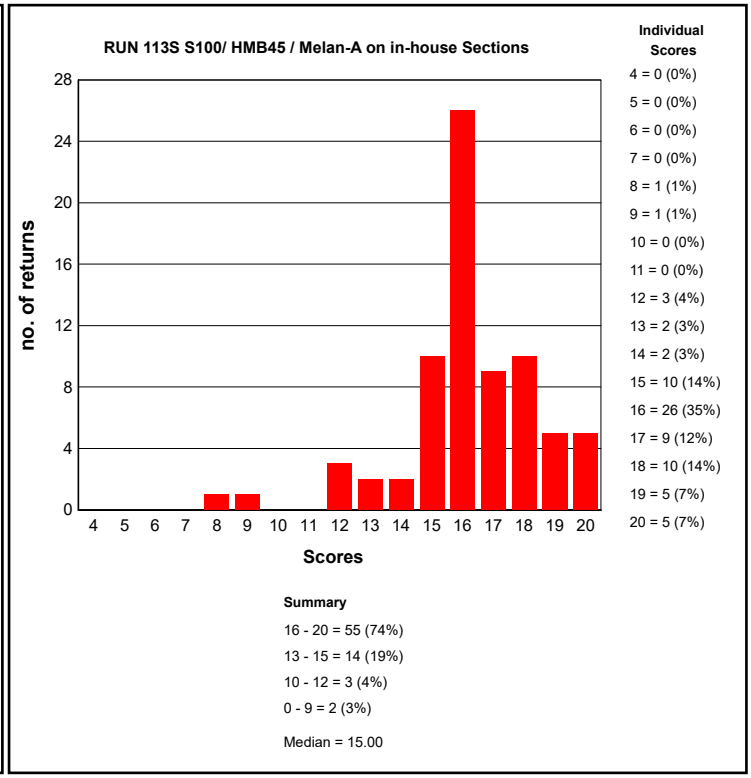
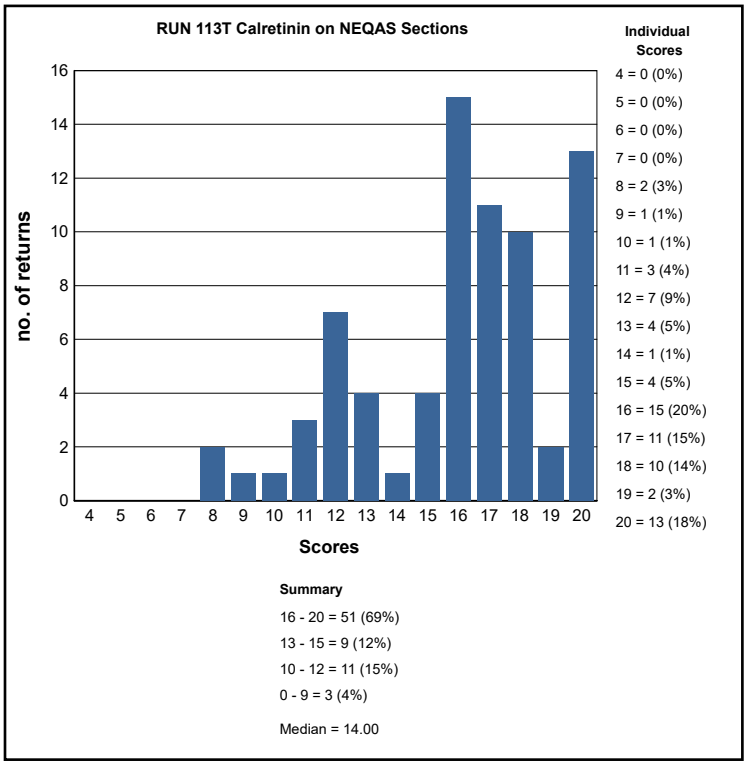


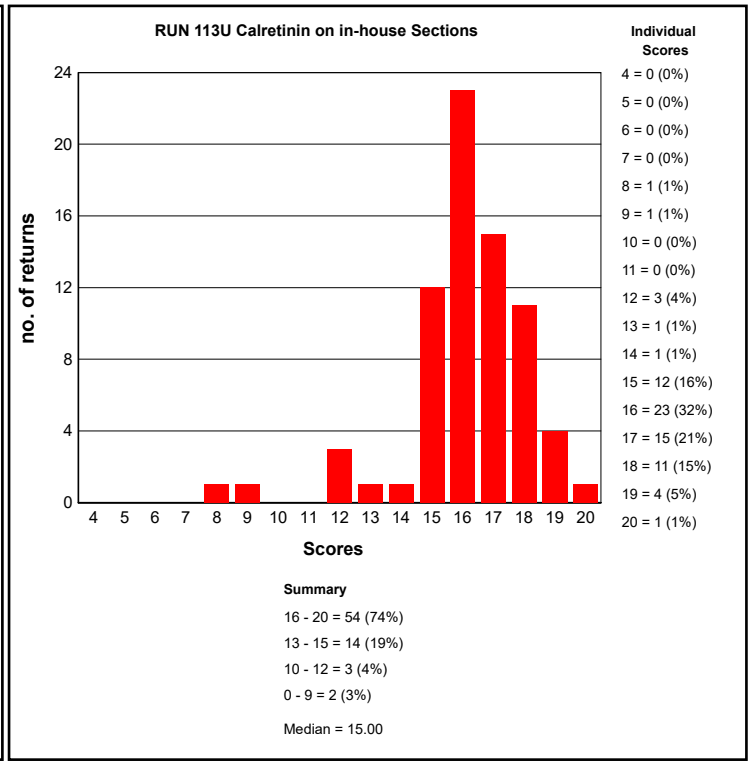
Fig 12. Nice example of calretinin staining on an in-house cytospin preparation. Dako M7245 (DAK-Calret 1), 1:40, no retrieval employed, with a Ventana iView Kit on a Ventana Benchmark XT.

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: 113

Primary Antibody : S100/ HMB45 / Melan-A

Antibody Details	N	%
A. Menarini MU 001 UC (HMB45)	1	100
Biogenex Melan A (MART-1) (A103) AM361/MU361	2	100
Cell Marque CMA710 (HMB45)	1	100
Dako FLEX RTU Melan A (A103) IR633	6	100
Dako M0634 (HMB45)	12	100
Dako M7196 (A103 Melan A)	17	100
Dako Z0311 (S100)	6	83
Novocastra/Leica NCL-MELAN A (Melan A)	12	100
Novocastra/Leica NCL-RTU-MelanA(A103) PA0233	3	100
Novocastra/Leica NCL-S100p (S100)	1	0
Novocastra/Leica PA00027 RTU (HMB45)	1	0
Other	2	100
Ventana 790-4360 (HMB45) Confirm melanosome	1	100
Ventana Melan A (MART-1) 790-2990	10	90

Cytology Run: 113

Primary Antibody : Calretinin

Antibody Details	N	%
Cell Marque - CMC757/758/759	1	0
Cell Marque 232A-74/75/76/77/78 (R Poly)	2	50
Dako IR627 RTU (DAK-Calret 1)	1	100
Dako IS627 RTU FLEX (DAK-Calret 1)	6	83
Dako M7245 (DAK-Calret 1)	17	76
Invitrogen18-0211 (DC8)	6	67
Novocastra NCL-CALRET (5A5)	5	60
Novocastra/Leica NCL-L-CALRET-566 (CAL6)	8	75
Novocastra/Leica PA0346 RTU (CAL6)	1	100
Novocastra/Leica RTU-CALRET (5A5)	1	100
Thermo Fisher/Labvision RM-9113 (SP13)	1	100
Ventana 250 270	1	100
Ventana 790-4467 (SP65)	16	94
Zymed/Invitrogen 18 0211(DC8)	7	100

Cytology Run: 113

Primary Antibody : S100/ HMB45 / Melan-A

Antigen Retrieval	N	%
YES	27	36
NO	49	64
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	27	
Not Specified	0	

Cytology Run: 113

Primary Antibody : Calretinin

Antigen Retrieval	N	%
YES	27	36
NO	49	64
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	27	
Not Specified	0	

Cytology Run: 113

Heat Mediated Retrieval

Cytology Run: 113

Heat Mediated Retrieval

Cytology Run: 113

Enzyme Mediated Retrieval

Cytology Run: 113

Enzyme Mediated Retrieval

Cytology Run: 113

Detection	Calretinin		S100/ HMB45 / Melan-A	
	N	%	N	%
AS PER KIT	7	86	8	88
BioGenex SS Polymer (QD 430-XAKE)	1	100	0	0
Dako EnVision FLEX (K8000/10)	1	100	1	100
Dako EnVision FLEX+ (K8002/12)	8	88	8	100
Dako Envision HRP/DAB (K5007)	1	0	1	100
LabVision UltraVision LP HRP (TS 125 HD)	1	0	0	0
Leica Bond Polymer Refine (DS9800)	20	80	21	95
None	1	100	1	100
Other	2	100	2	100
Ventana iView system (760-091)	3	100	4	100
Ventana OptiView Kit (760-700)	8	75	10	90
Ventana UltraView Kit (760-500)	18	67	17	94

Cytology Run: 113

Automation	Calretinin		S100/ HMB45 /	
	N	%	N	%
BioGenex GenoMX 6000i	1	100	0	0
Dako Autostainer	1	0	1	100
Dako Autostainer Link 48	9	89	9	100
Dako Autostainer Plus Link	1	100	2	100
Dako Omnis	2	50	2	100
Leica Bond Max	10	80	10	90
Leica Bond-III	12	92	13	100
None (Manual)	2	50	0	0
Ventana Benchmark GX	3	100	3	100
Ventana Benchmark ULTRA	19	74	21	90
Ventana Benchmark XT	15	73	14	93

Cytology Run: 113

Chromogen	Calretinin		S100/ HMB45 /	
	N	%	N	%
AS PER KIT	9	100	9	100
BioGenex Liquid DAB (HK153-5K)	1	100	0	0
DAKO DAB+	0	0	1	100
Dako DAB+ Liquid (K3468)	1	0	0	0
Dako EnVision Plus kits	1	100	0	0
Dako FLEX DAB	8	88	9	100
Leica Bond Polymer Refine kit (DS9800)	20	80	21	95
Other	6	50	5	80
Ventana DAB	8	88	5	100
Ventana Enhanced Alk. Phos. Red Detection Kit	0	0	3	100
Ventana iview	2	50	4	100
Ventana Ultraview DAB	19	74	18	89

BEST METHODS - Gold Standard Antibody

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

S100/ HMB45 / Melan-A - Method 1

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

Primary Antibody:	Dako FLEX RTU Melan A (A103) IR633 , 20 Mins, 25 ºC Prediluted
Automation:	Dako Autostainer Plus Link
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: high
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 25 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 20 Mins, 25 ºC Prediluted

S100/ HMB45 / Melan-A - Method 2

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

Primary Antibody:	Novocastra/Leica NCL-MELAN A (Melan A) , 56 Mins, RT ºC Dilution 1: 25
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Enhanced Alk. Phos. Red Detection Kit
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana Enhanced Alk. Phos. Red Detection Kit
Detection:	Ventana UltraView Kit (760-500)

S100/ HMB45 / Melan-A - Method 3

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

Primary Antibody:	Dako M0634 (HMB45) , 30 Mins, rt ºC Dilution 1: 150
Automation:	Dako Autostainer
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, rt ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 15 Mins, rt ºC Prediluted

S100/ HMB45 / Melan-A - Method 4

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

Primary Antibody:	Dako M0634 (HMB45) , 15 Mins, 20 ºC Dilution 1: 100
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590), PH: 7.6
HMAR:	None
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), 20 ºC., Time 1: 10 Mins, Time 2: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, 20 ºC Prediluted

BEST METHODS - Secondary Antibody

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

Calretinin - Method 1

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

Primary Antibody:	Zymed/Invitrogen 18 0211(DC8) , 30 Mins, 23 ºC Dilution 1: 1:120
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer, PH: 7.6
HMAR:	Dako PTLink, Buffer: ENVSION TR SOLUTION HIGH pH, PH: 9
EAR:	
Chromogen:	Dako FLEX DAB, 23 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 35 Mins, 23 ºC Prediluted

Calretinin - Method 2

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

Primary Antibody:	Novocastra/Leica NCL-L-CALRET-566 (CAL6) , 15 Mins, 20 ºC Dilution 1: 100
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER1 20 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

Calretinin - Method 3

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

Primary Antibody:	Dako IR627 RTU (DAK-Calret 1) , 20 Mins, 32 ºC Prediluted
Automation:	Dako Omnis
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis, Buffer: Env FLEX TRS High pH
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 32 ºC., Time 1: 5 Mins
Detection:	Dako EnVision FLEX (K8000/10) , 20 Mins, 32 ºC Prediluted

Calretinin - Method 4

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

Primary Antibody:	Ventana 790-4467 (SP65) , 20 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.6
HMAR:	Ventana CC1 standard
EAR:	
Chromogen:	Ventana Ultraview DAB, 37 ºC., Time 1: 8 Mins, Time 2: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC

The Alimentary Tract Module: GIST Run 113

Suzanne Parry

	First Antibody	Second Antibody
Antigens Assessed:	CD117	S100
Tissue Sections circulated:	GIST, Desmoid and normal appendix.	
Number of Registered Participants:	118	
Number of Participants this Run	114 (97%)	

Introduction

Gold Standard: CD117

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

Features of Optimal Immunostaining: **(See Figs 1& 3)**

- 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
- Good localisation of CD117 to cells of the GIST (Fig 3)
- No staining of the desmoid tumour

Features of Sub-optimal Immunostaining: **(See Figs 2, 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 2 & 5)
- Staining of the desmoid tumour (Fig 5)

Second Antibody: S100

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

Features of Optimal Immunostaining: (see Figs 7-9)

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

Features of Sub-optimal Immunostaining: (see Figs 10-12)

- Weak and/or patchy staining of the nerve bundles, fibres and macrophages in the appendix (Fig 10)
- Weak and/or patchy staining of the nerve elements in the desmoid tumour section
- Excessive background or non specific staining (Figs 11 & 12)
- Staining of the GIST
- Staining of the desmoid tumour

References

1. **Cordless et al.,** Biology of Gastrointestinal Stromal Tumours. J Clin Oncol 2004, 22(18): 3813-3825.
2. **Blay et al.,** Consensus meeting for the management of gastrointestinal stromal tumors. Report of the GIST Consensus Conference of 20-21 March 2004, under the auspices of ESMO. Ann Oncol 2005 6: 566-578.
3. **Parfitt JR, Rodriguez-Justo M, Feakins R, Novelli MR** (2008) Gastrointestinal Kaposi's sarcoma: CD117 expression and the potential for misdiagnosis as gastrointestinal stromal tumour. Histopathology 2008, 52: 816-23.
4. **Novelli M, Rossi S, Rodriguez-Justo M, Taniere P, Seddon B, Toffolatti L, Sartor C, Hogendoorn PC, Sciot R, Van Glabbeke M, Verweij J, Blay JY, Hohenberger P, Flanagan A, Dei Tos AP.** DOG1 and CD117 are the antibodies of choice in the diagnosis of gastrointestinal stromal tumours. Histopathology 2010, 57(2):259-270.
5. **Nakajima T, Watanabe S, Sato Y et al.** An immunoperoxidase study of S-100 protein distribution in normal and neoplastic tissue. Am J Surg pathol 1982; 6: 715-726.

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 S100) using their routine protocol. Sequential sections from the same tissue block were used for both the requested antibodies. The assessment was carried out around a multi-header microscope with each slide being assessed by 4 independent assessors and a microscope lead. All 3 samples on the slide were viewed, and then a final overall score out of 5 was given by each individual assessor. These four scores were then combined to give an overall score out of 20.

Assessment Summary:

CD117

Results from the CD117 assessment showed good pass rates, and very similar to the previous Runs carried out in the Neqas assessment year. Results are summarised in the table below:

CD117 Pass Rates:				
	Run 110	Run 111	Run 112	Run 13
Acceptable	82% (N=92)	78% (N=88)	83% (N=93)	89% (N=101)
Borderline	15% (N=17)	17% (N=19)	13% (N=14)	8% (N=9)
Unacceptable	3% (N=3)	5% (N=6)	4% (N=5)	3% (N=4)

Only 4 labs failed the assessment and this was due to very weak staining, particularly in the GIST, and therefore all assessors agreed that these assays could potentially lead to a false negative result. One of these labs is using the Dako CD117 clone with no pre-treatment, which could explain the weak staining. It is recommended on the commercial datasheet that antigen retrieval is used with this particular antibody. All participating labs apart from 1 also submitted their in-house control tissue for assessment. These showed similar pass rates to the Neqas tissue with 90% of labs receiving an acceptable pass. Most labs are now using composite control blocks including a normal appendix and gastric tissue for their in-house tissues. Similarly to previous CD117 assessments, the Dako polyclonal antibody remains the most popular choice used by 82% of participants, and showed an acceptable pass rate of 88%.

S100

S100 was chosen as the second antibody for this assessment. The Neqas distributed GIST was known to be negative for S100, and similarly to the Neqas desmoid tissue, the GIST acted as a good control to highlight any non-specific staining. The pass rates were also very good, with 88% achieving an acceptable pass, 11% a borderline, and only 2 labs (2%) failed the assessment. The reason for failure was due to very weak staining of the appendix nerve bundles and fibres. The in-house control slide assessment showed even better pass rates, with 96% of labs receiving an acceptable-excellent score, 4 labs received a borderline, and only 1 lab (1%) failed on their in-house tissue, due to very weak staining. The borderline labs were marked down either to non-specific staining of some cells or poor tissue quality.

Selected Images showing Optimal and Sub-optimal Immunostaining

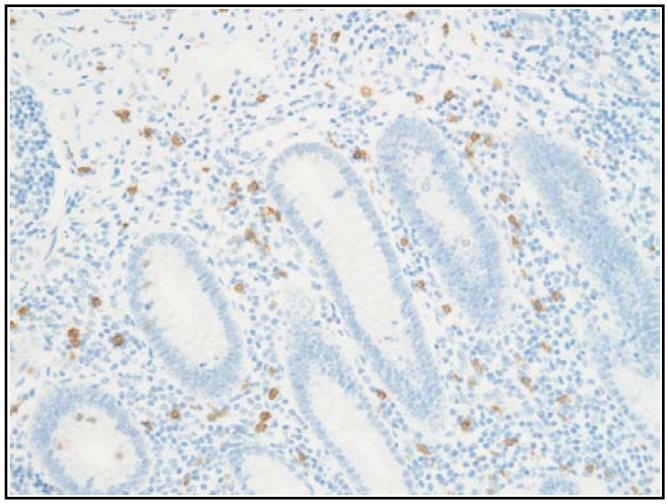


Fig 1. Good demonstration of CD117 in the UK NEQAS distributed appendix. The mast cells show distinct membranous staining, while the background remains clean. Stained with the Epitomics EP10 (1:100) on a Leica Bond Max, with Leica ER1 retrieval for 30 mins and Bond Polymer Refine Detection kit.

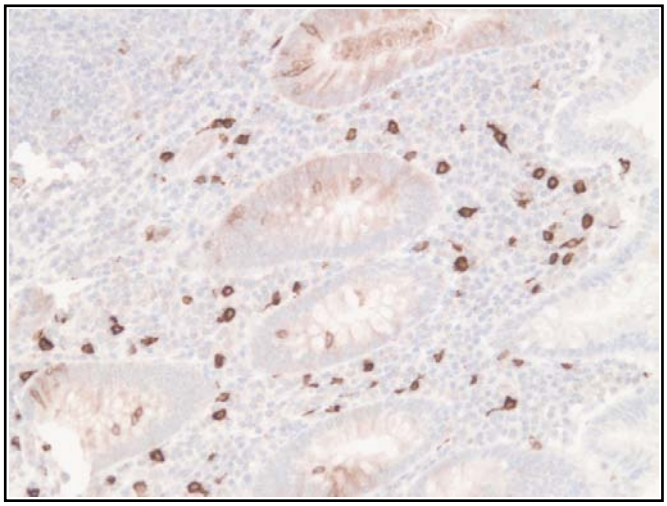


Fig 2. Sub-optimal demonstration of CD117 in the UK NEQAS distributed appendix. Although mast cells are staining as expected, there is also non-specific staining of crypts and lymphocytes. Stained with the Dako A4502 (rb poly) (1:200) on a Leica Bond III. Heat retrieval details not provided.

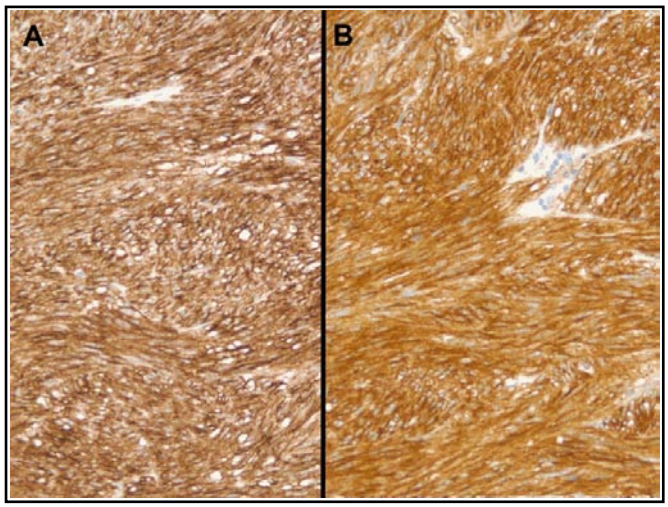


Fig 3. (A & B) Good example of CD117 staining of the UK NEQAS distributed GIST, showing strong cytoplasmic and membranous staining in the tumour cells. (A) Stained using same protocol as in fig 2. (B) Stained using same protocol as in fig 1.

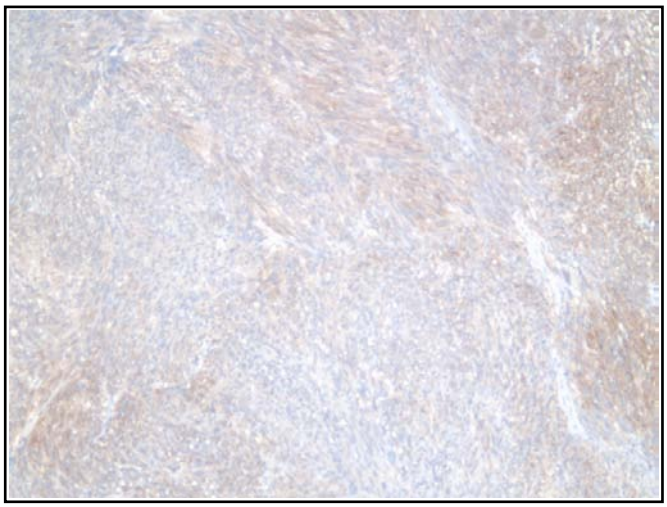


Fig 4. Poor CD117 staining in the UK NEQAS distributed GIST section (compare to Fig 3). The staining is very weak and patchy. Stained with the Dako polyclonal antibody (1:100), on a Ventana Benchmark XT but with no antigen retrieval, with a Ventana Ultraview DAB detection.

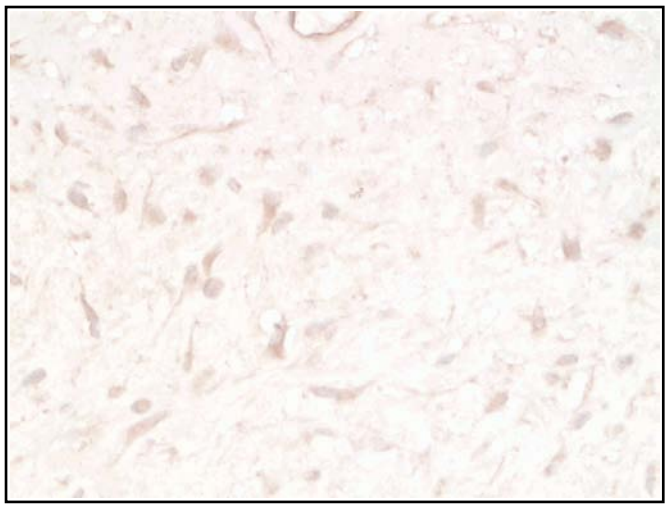


Fig 5. Sub-optimal CD117 staining in the UK NEQAS distributed desmoid tumour. The example shows excessive background and non-specific staining. The slide was stained with the Dako A4502 (rb poly) (1:200) on a Leica Bond III. Heat retrieval details not provided.

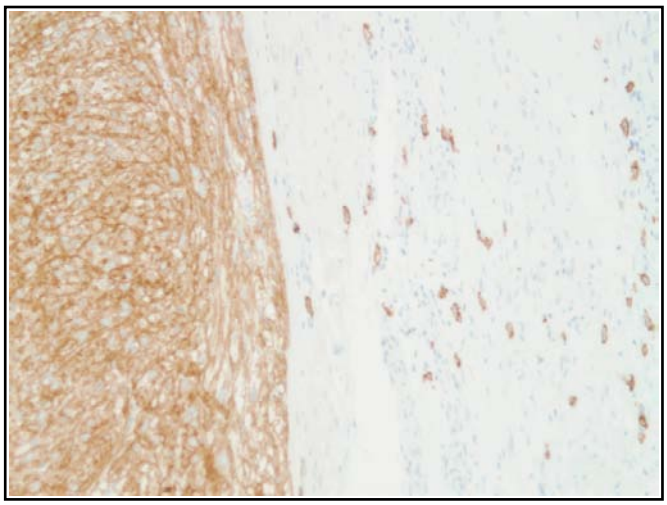


Fig 6. Good in-house control for submitted for assessment of CD117: The section showed good strong staining of the GIST and surrounding muscle layer mast cells and Cells of Cajal. Stained with Leica/Novocastra NCL-CD117 (T595) (1:200) on a BioGenex GenoMX 6000i with A. Menarini Liquid Stable DAB kit.

Selected Images showing Optimal and Sub-optimal Immunostaining

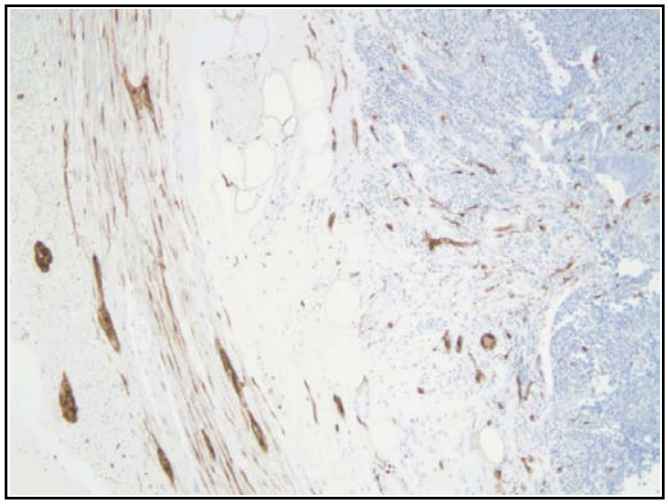


Fig 7. Optimal demonstration of S-100 in the UK NEQAS distributed appendix. There is strong distinct staining of macrophages and nerve fibres in the lamina propria, and ganglion and nerve fibres in the muscularis propria. Section stained with the Dako polyclonal antibody (1:1000) on a Leica Bond III, no antigen retrieval and Leica Bond Polymer Refine detection.

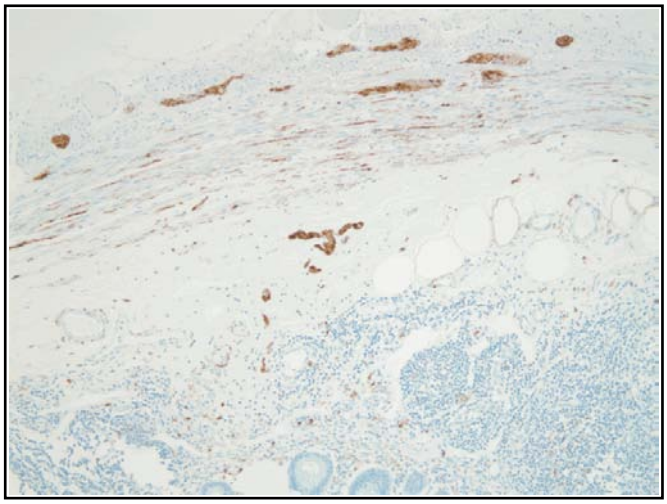


Fig 8. Optimal demonstration of S-100 in the UK NEQAS distributed appendix.Strong distinct staining of macrophages and nerve fibres in the lamina propria, and ganglion and nerve fibres in the muscularis propria. Section stained with the Ventana 790 2914 (4C4.9 clone, prediluted) on a Ventana Benchmark ULTRA, no antigen retrieval and Ventana Ultraview detection.

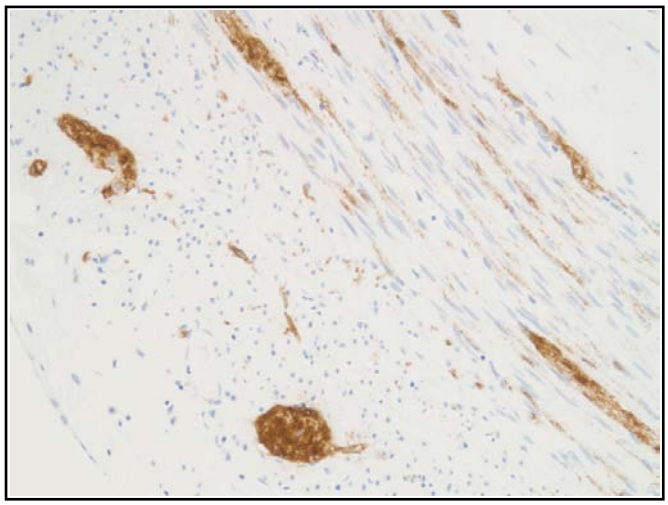


Fig 9. High power magnification of S-100 in the UK NEQAS distributed appendix. Strong distinct staining of macrophages and nerve fibres in the lamina propria, and ganglion and nerve fibres in the muscularis propria. Section stained with the Dako Z0311 (S100 poly, 1:2000) on a Ventana Benchmark ULTRA, Ventana Protease 1 retrieval and Ventana UltraView detection.

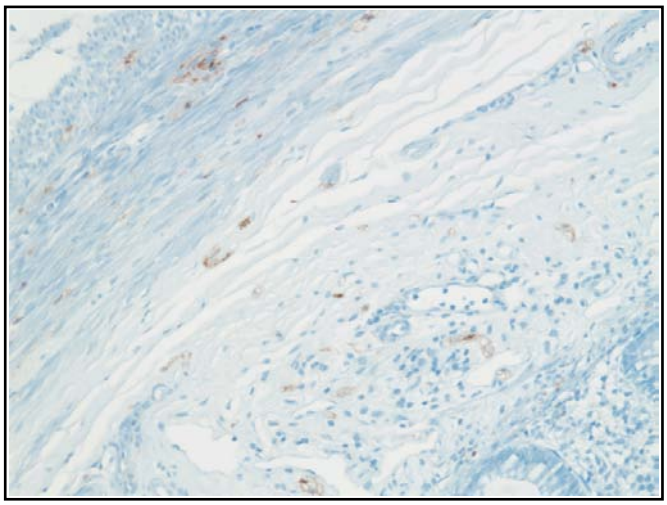


Fig 10. Suboptimal demonstration of S-100 in the UK NEQAS distributed appendix (compare with Figs 7-9). The staining is weak with fewer macrophages, nerve fibres and ganglions demonstrated than expected. No antibody or staining details provided.

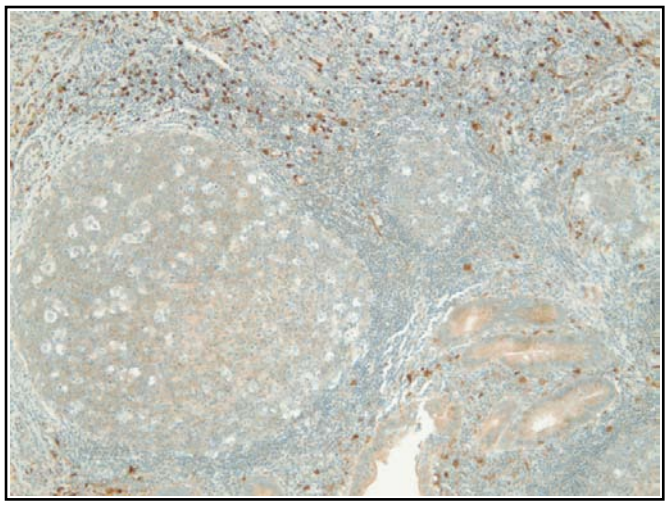


Fig 11. Suboptimal S-100 staining in the UK NEQAS distributed appendix (compare with Figs 7-9) demonstrating excessive background staining, possibly due to excessive enzyme retrieval. Section stained with the Dako Z0311 (S100 poly, 1: 2000) on a Ventana Benchmark ULTRA, with Ventana Protease 1 retrieval and Ventana UltraView detection.

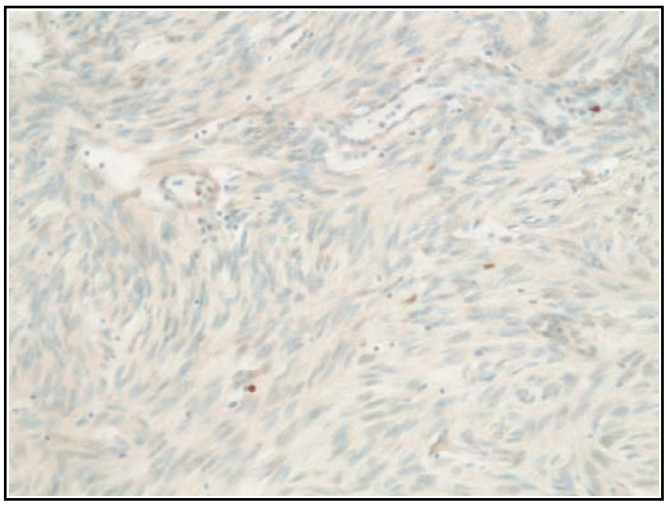
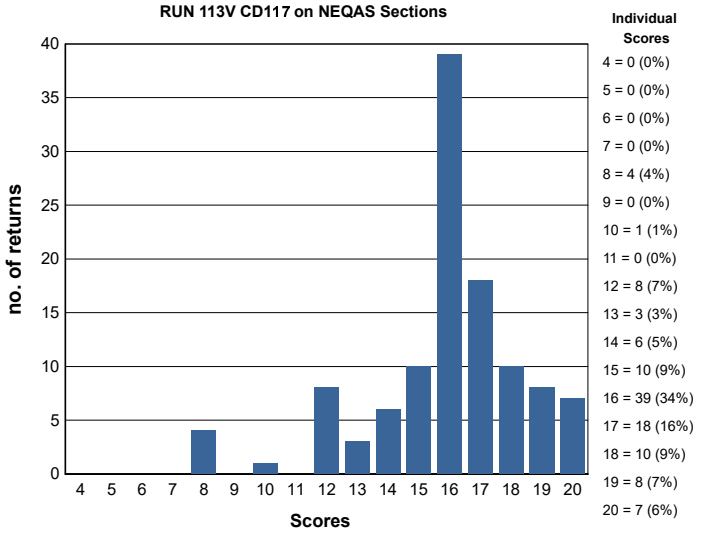
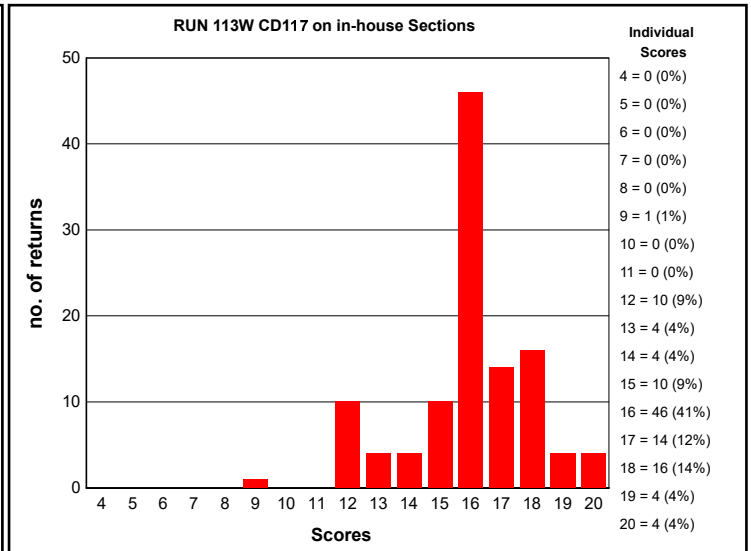
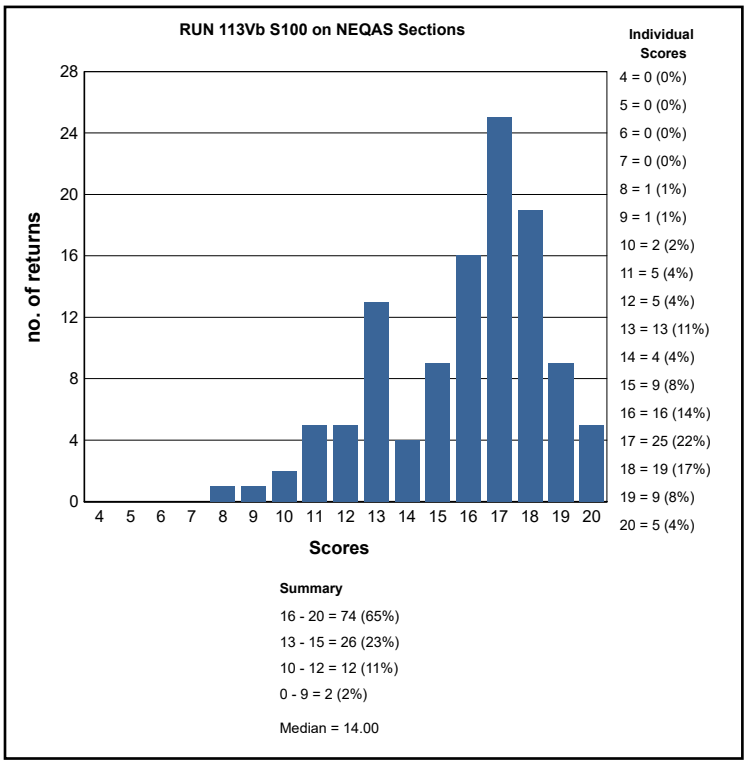


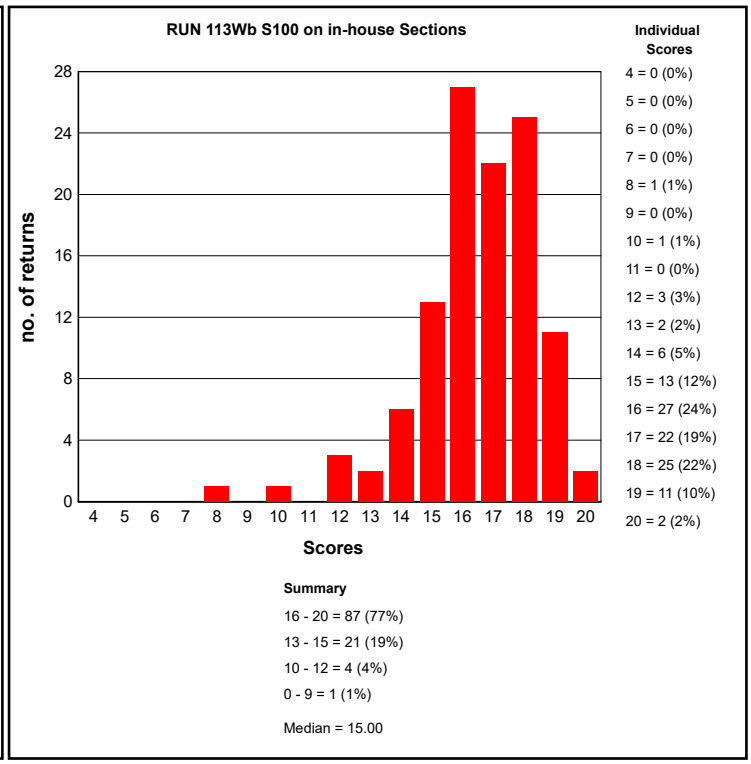
Fig 12. Suboptimal staining of S-100 in the UK NEQAS distributed desmoid section. Although the expected nerve elements are strongly demonstrated, the section also shows inappropriate non-specific and background staining. Stained using same method in Fig 11.

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: 113

Primary Antibody : CD117

Antibody Details	N	%
Cell Marque 117R/S-xx (YR145)	3	100
Dako A4502 (rb poly)	93	88
Epitomics AC-0029 (EP10)	1	100
Leica RTU (EP10) PA0007	2	100
Leica/Novocastra NCL-CD117 (T595)	1	100
Other	1	100
Ventana 790-2939 (rb poly)	1	0
Ventana 790-2951 (9.7)	11	91

Alimentary Tract Pathology Run: 113

Primary Antibody : S100

Antibody Details	N	%
Biogenex MU058-UC	2	100
Cell Marque CMA 716 (S100)	1	100
Dako Omnis GA504 RTU Flex (polyclonal)	1	100
Dako IR504 RTU FLEX (polyclonal)	2	100
Dako IS504 RTU Plus (polyclonal)	2	100
Dako Z0311 (S100 poly)	60	90
Diagnostic Biosystem Medite RP035 (S100 poly)	1	100
Leica/Novocastra NCL--L-S100p (polyclonal)	15	87
Leica/Novocastra PA0900 RTU (polyclonal)	6	67
Other	1	0
Ventana 760 2523 (S100 poly)	7	100
Ventana 790 2914	14	93

Alimentary Tract Pathology Run: 113

Heat Mediated Retrieval	CD117 N	CD117 %	S100 N	S100 %
Dako Omnis	0	0	1	100
Dako PTLink	14	71	12	100
Lab vision PT Module	1	100	0	0
Leica ER1 10 mins	1	100	2	100
Leica ER1 20 mins	4	100	0	0
Leica ER1 30 mins	4	100	2	100
Leica ER2 10 mins	1	100	0	0
Leica ER2 20 mins	18	89	5	100
Leica ER2 30 mins	8	100	0	0
None	3	67	38	82
Other	0	0	1	100
Pressure Cooker	1	100	1	100
Pressure Cooker in Microwave Oven	0	0	1	100
Ventana CC1 16mins	0	0	5	100
Ventana CC1 24mins	0	0	2	100
Ventana CC1 32mins	9	100	2	100
Ventana CC1 36mins	5	40	2	100
Ventana CC1 40mins	2	100	0	0
Ventana CC1 48mins	3	100	0	0
Ventana CC1 52mins	1	100	0	0
Ventana CC1 56mins	4	100	0	0
Ventana CC1 64mins	13	85	0	0
Ventana CC1 88mins	1	100	0	0
Ventana CC1 8mins	0	0	9	100
Ventana CC1 extended	0	0	1	100
Ventana CC1 mild	7	100	9	100
Ventana CC1 standard	12	92	4	100
Ventana CC2 8mins	0	0	1	0

Alimentary Tract Pathology Run: 113

Enzyme Mediated Retrieval	CD117 N	CD117 %	S100 N	S100 %
AS PER KIT	2	100	4	100
NOT APPLICABLE	71	87	32	100
Other	1	100	6	83
VBS Bond Enzyme 1	0	0	16	56
VBS Bond Enzyme 2	0	0	1	0
Ventana Protease	0	0	1	100
Ventana Protease 1 (760-2018)	0	0	14	93

Alimentary Tract Pathology Run: 113	CD117		S100	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	8	75	10	100
BioGenex SS Polymer (QD 430-XAKE)	1	100	0	0
Dako EnVision FLEX (K8000/10)	1	0	1	100
Dako EnVision FLEX+ (K8002/12)	7	86	4	100
Dako Envision HRP/DAB (K5007)	0	0	1	100
Dako Envision+ HRP mouse K4004/5/6/7	0	0	1	100
Dako Envision+ HRP rabbit K4008/9/10/11	1	0	0	0
Leica Bond Polymer Refine (DS9800)	36	94	32	78
None	0	0	1	100
NOT APPLICABLE	1	100	1	100
Other	3	100	5	80
Ventana iView system (760-091)	2	50	3	100
Ventana OptiView (760-700) + Amp. (7/860-099)	0	0	1	100
Ventana OptiView Kit (760-700)	24	96	18	89
Ventana UltraView Kit (760-500)	28	86	27	96

Alimentary Tract Pathology Run: 113	CD117		S100	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	1	100	0	0
Dako Autostainer Link 48	12	67	9	100
Dako Autostainer plus	1	100	0	0
Dako Autostainer Plus Link	2	100	1	100
Dako Omnis	0	0	1	100
LabVision Autostainer	0	0	1	100
Leica Bond Max	11	100	12	75
Leica Bond X	0	0	1	100
Leica Bond-III	27	93	21	76
Ventana Benchmark GX	1	100	1	100
Ventana Benchmark ULTRA	35	89	33	91
Ventana Benchmark XT	23	87	21	100

Alimentary Tract Pathology Run: 113	CD117		S100	
Chromogen	**N**	**%**	**N**	**%**
A. Menarini Liquid Stable DAB kit	1	100	0	0
AS PER KIT	18	94	20	95
BioGenex Liquid DAB (HK153-5K)	0	0	1	100
Dako EnVision Plus kits	1	100	0	0
Dako FLEX DAB	9	67	7	100
Dako REAL EnVision K5007 DAB	0	0	1	100
Leica Bond Polymer Refine kit (DS9800)	33	94	32	78
Other	7	86	5	80
Ventana DAB	11	100	9	89
Ventana Enhanced Alk. Phos. Red Detection Kit	0	0	3	100
Ventana iview	2	50	3	100
Ventana Ultraview DAB	31	84	30	93

BEST METHODS - Gold Standard Antibody

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

CD117 - Method 1

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

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

CD117 - Method 2

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

Primary Antibody: Epitomics AC-0029 (EP10) , 60 Mins, amb ºC Dilution 1: 100
Automation: Leica Bond Max
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER1 30 mins
EAR:
Chromogen: Leica Bond Polymer Refine kit (DS9800), amb ºC., Time 2: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 15 Mins, amb ºC Prediluted

CD117 - Method 3

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

Primary Antibody:	Dako A4502 (rb poly) , 30 Mins, ROOM ºC Dilution 1: 1/300
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer, PH: 7.6
HMAR:	Dako PTLink, Buffer: DAKO ENVISION RETRIEVAL SLN HIGH pH, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, ROOM ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 30 Mins, ROOM ºC Prediluted

CD117 - Method 4

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

BEST METHODS - Secondary Antibody

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

S100 - Method 1

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

Primary Antibody:	Ventana 790 2914 , 32 Mins, rt ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	None
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB, RT ºC., Time 1: 8 Mins, Time 2: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, rt ºC Prediluted

S100 - Method 2

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

Primary Antibody:	Dako Z0311 (S100 poly) , 20 Mins Dilution 1: 1000
Automation:	Leica Bond-III
Method:	AS PER KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	None
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins

S100 - Method 3

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

Primary Antibody:	Leica/Novocastra NCL--L-S100p (polyclonal) , 30 Mins, RT ºC Prediluted
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	None
EAR:	Other, RT ºC. Digestion Time NEQAS: 10 Mins. In-House: 10 Mins
Chromogen:	Leica Bond Polymer Refine kit (DS9800), RT ºC., Time 1: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, RT ºC Prediluted

S100 - Method 4

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

Primary Antibody:	Dako Z0311 (S100 poly) Dilution 1: 2000
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	None
EAR:	Ventana Protease 1 (760-2018)
Chromogen:	AS PER KIT
Detection:	Ventana OptiView Kit (760-700)

Lynch Syndrome/HNPCC Run 113

Keith Miller and Suzanne Parry

	Gold Standard	Second Antibody
Antigens Assessed:	MSH2	MSH6
Tissue Sections circulated:	Positive tumour, negative tumour and normal appendix	
Number of Registered Participants:	86	
Number of Participants This Run:	80 (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 **MSH2** (1st Antibody) and **MSH6** (2nd antibody) using their routine protocols. The sections comprised of normal appendix and two colonic tumours (one with loss of MMR protein and one with no loss of MMR). Assessment of the stained slides was carried out around a multi-header microscope, with each slide being assessed by 4 independent assessors and a microscope lead. All samples on the slide were viewed, and then a final overall score out of 5 was given by each individual assessor. These four scores were then combined to give an overall score out of 20.

Features of Optimal Immunostaining: (Figs 1, 3, 5, 7, 9 &10)
Appendix: (Figs 1 & 2)
Strong nuclear staining of the epithelium at the base and lower third-half of the crypts with fading of nuclear staining intensity in the middle and upper thirds of the crypts, to negative or weak staining of epithelial nuclei at the luminal surface.

- Strong staining of lymphoid follicles.

Tumour without loss of MMR protein: (Figs 3 & 9)
Strong nuclear staining in the tumour cells.

- Strong nuclear staining in the lymphocytes and stromal cells.

Tumour with loss of MMR protein: (Figs 5 & 10)

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

Features of Suboptimal Immunostaining: (Figs 2, 4, 6 8 & 11)
Appendix: (Figs 2 & 8)

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

Tumour without loss of MMR protein: (Figs 4 & 11A)

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

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

- 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 on the Neqas distributed samples for both MSH2 and MSH6 were very similar to the last time these markers were assessed (as shown in the table below):

Neqas MMR Pass Rates:				
	MSH2		MSH6	
	Run 111	Run 113	Run 111	Run 113
Acceptable	67% (N=51)	73% (N=58)	68% (N=53)	69% (N=55)
Borderline	25% (N=19)	18% (N=14)	21% (N=16)	19% (N=15)
Unacceptable	8% (N=6)	9% (N=8)	11% (N=9)	12% (N=10)

Similarly to the previous run, the main reason for lower scores or failure was due to weak staining. False positive staining in the tumour known to be negative was also seen in several sections, and therefore these labs failed the assessment. One of these labs is using the Ventana Optiview detection kit with an extra amplification step in their protocol and CC1 antigen retrieval for 64 minutes. The appendix section of the same slide also showed very granular staining, and this is thought to be linked to the Optiview kit, as this was seen on several other slides using the Optiview detection kit. The added amplification step on this particular slide, along with the highly sensitive detection and lengthy antigen retrieval was probably the cause of the false positive staining. Excessive background seen on several slides was also thought to be due to excessive antigen retrieval.

The Ventana G219-1129 clone was the most popular **MSH2** antibody; used by 39% of participants and showed an acceptable pass rate of 77%. The Dako FE11 clone was also popular; used by 31% of participants and showed an acceptable pass rate of 84%. While the Ventana clone is only being used on the Ventana automated system, the Dako antibody is performing well on all of the commercial company automated platforms. Both the Ventana clone 44 and the Dako EP49 clone were the most popular choices of **MSH6** antibodies used in this assessment. They showed acceptable pass rates of 79% and 77% respectfully.

The **in-house** control sections showed slightly higher pass rates than the Neqas material (summarised in the table below):

In-House Run 113 Pass Rates:		
	MSH2	MSH6
Acceptable	76% (N=60)	79% (N=63)
Borderline	14% (N=11)	14% (N=11)
Unacceptable	6% (N=5)	8% (N=6)

Most laboratories submitted appendix alongside a tumour of known mismatch marker expression. This appendix is ideal to not only demonstrate specific nuclear staining, but also highlight the expected gradation of intensity of epithelial staining down the crypts, and whether a positive expressing tumour or a negative tumour are used as controls, the section should show some stromal cells to act as a positive internal control for the section. Both positive and negative tumour controls were used by some laboratories, mostly constructed as a TMA or small punches. This is ideal to help gauge the assay and to highlight any weak staining, or if there were any false positive in the negative tumour.

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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

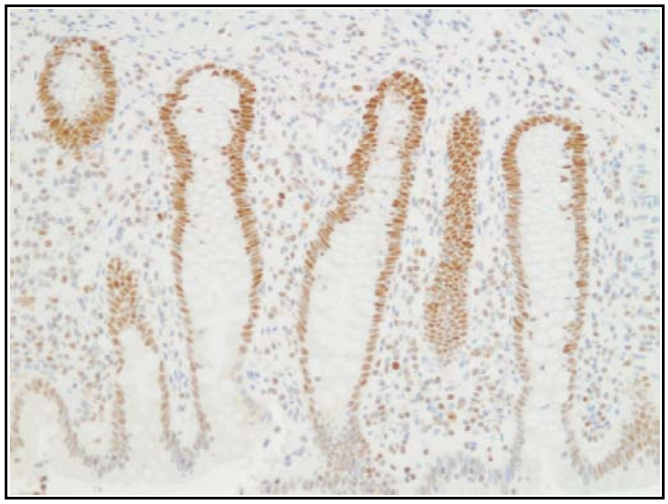


Fig 1. Optimal demonstration of MSH2 in the UK NEQAS distributed appendix. Strong MSH2 staining of the epithelial and stromal cells, with fading of staining intensity towards the luminal surface of the epithelial crypts. Stained with the Dako FLEX RTU IR085 (FE11), on a Dako Autostainer Link 48, with high pH and Dako FLEX DAB.

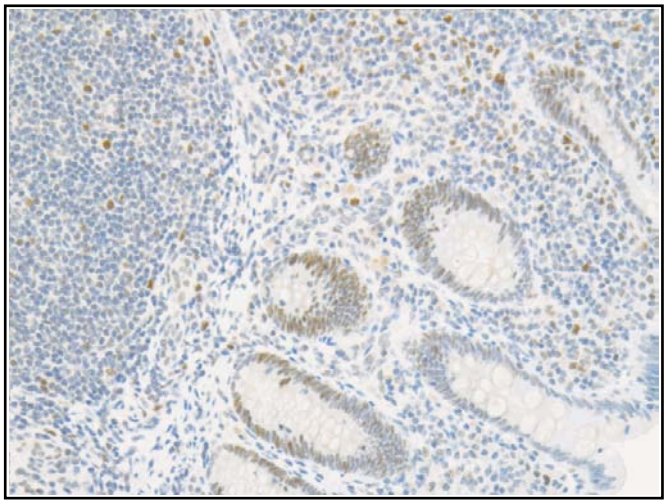


Fig 2. Weak demonstration of MSH2 on the UK NEQAS distributed appendix. Compared to fig1, the epithelial and stromal cells are more weakly stained. Stained with Calbiochem NA26/T (GB12, 1:50), on a Leica Bond III, using Leica ER2 retreieval for 30 mins, with Leica Bond Polymer Refine kit.

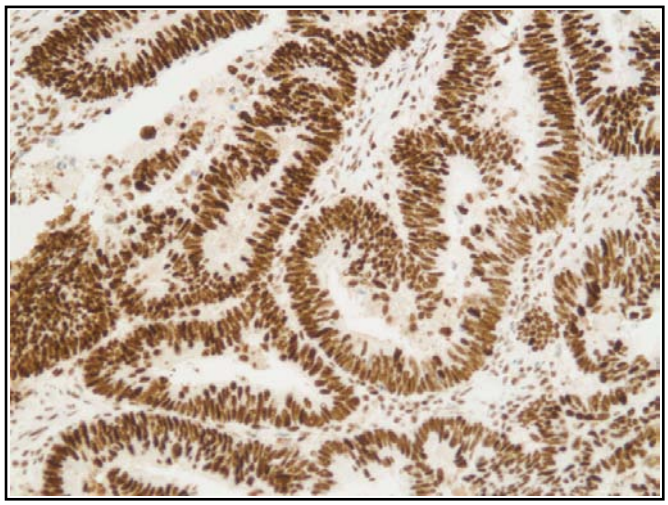


Fig 3. Optimal demonstration of MSH2 on the UK NEQAS distributed colonic tumour demonstrating strong nuclear staining of virtually all tumour cells, with staining in lymphocytes and stromal cells. Stained with the Dako FLEX RTU IR085 (FE11), on a Dako Autostainer Link 48, with high pH and Dako FLEX DAB.

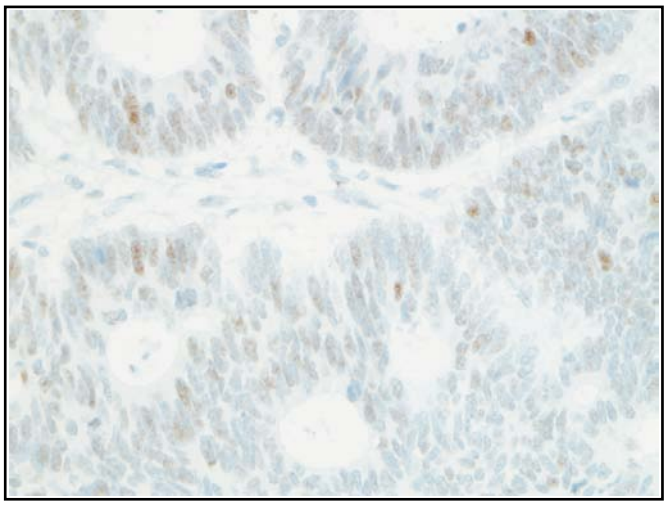


Fig 4. Weak demonstration of MSH2 on the UK NEQAS distributed MSH2 positive colonic tumour (compare with fig 3). The antibody incubation time was only 8 minutes which could be a reason for the very weak staining. Stained with the Dako FLEX RTU IR085 (FE11), on a Ventana Benchmark ULTRA, Ventana CC1 64mins, and Ventana OptiView detection.

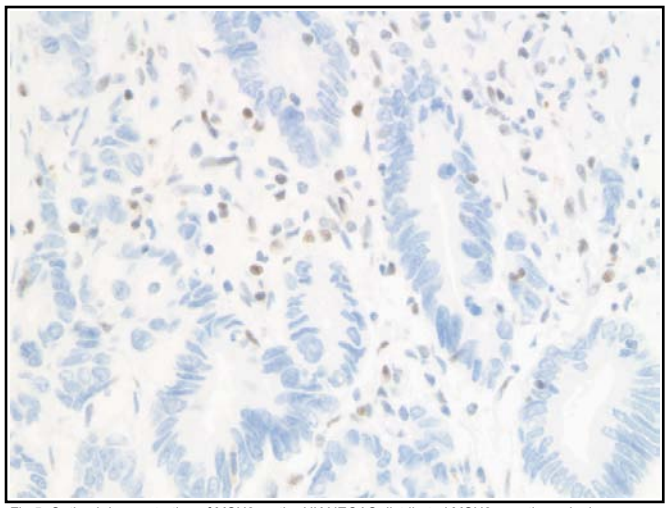


Fig 5. Optimal demonstration of MSH2 on the UK NEQAS distributed MSH2 negative colonic tumour showing only the intratumoral lymphocytes and stromal cells staining positive. Stained with the Dako FLEX RTU IR085 (FE11), on a Dako Autostainer Link 48, with high pH and Dako FLEX DAB.

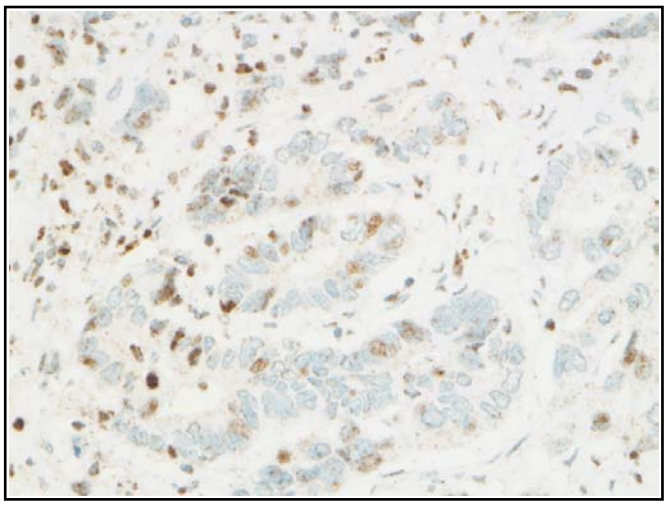


Fig 6. False-positive demonstration of MSH2 on the UK NEQAS distributed MSH2 negative colonic tumour (compare to Fig 5). Stained with the Dako FLEX RTU IR085 (FE11), on a Ventana Benchmark ULTRA, Ventana CC1 64mins, and Ventana OptiView + Optiview Amplification. 'OptiView + Optiview Amplification' is not recommended.

Selected Images showing Optimal and Sub-optimal Immunostaining

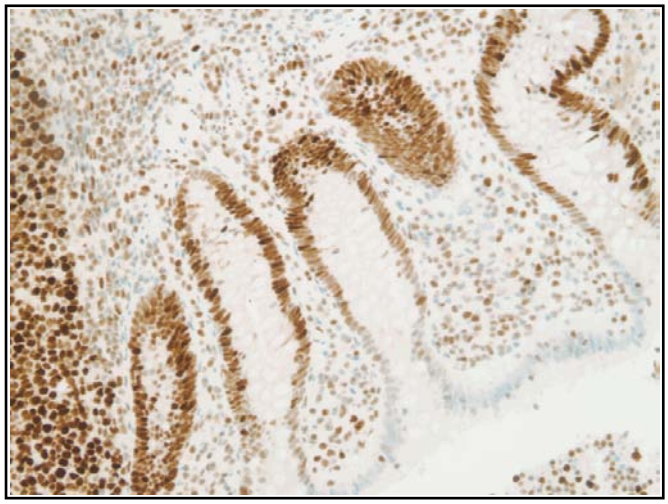


Fig 7. Optimal demonstration of MSH6 in the UK NEQAS distributed appendix. Strong MSH6 staining of the epithelial and stromal cells, with fading of staining intensity towards the luminal surface of the epithelial crypts. Stained with the Dako M3646 (EP49), on a Ventana Benchmark ULTRA, CC1 64mins retrieval, and Ventana OptiView detection.

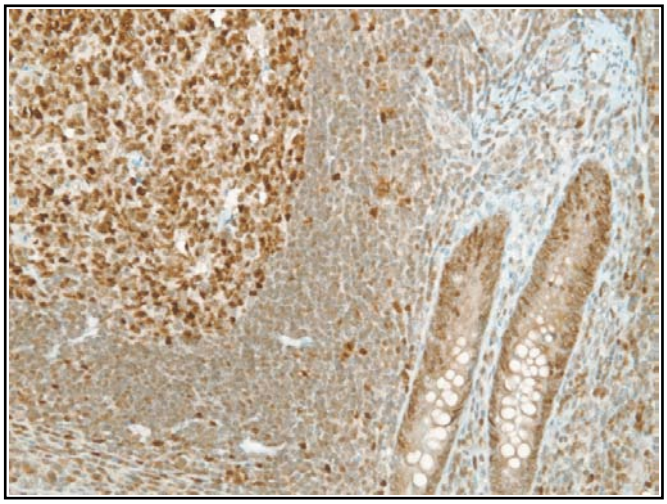


Fig 8. Suboptimal demonstration of MSH6 in the UK NEQAS distributed appendix. There is excessive non-specific and background staining. Stained with the Leica/Novocastra NCL-L-MSH6 (PU29, 1:100) on a Ventana Benchmark ULTRA, CC1 64mins retrieval, and Ventana UltraView detection.

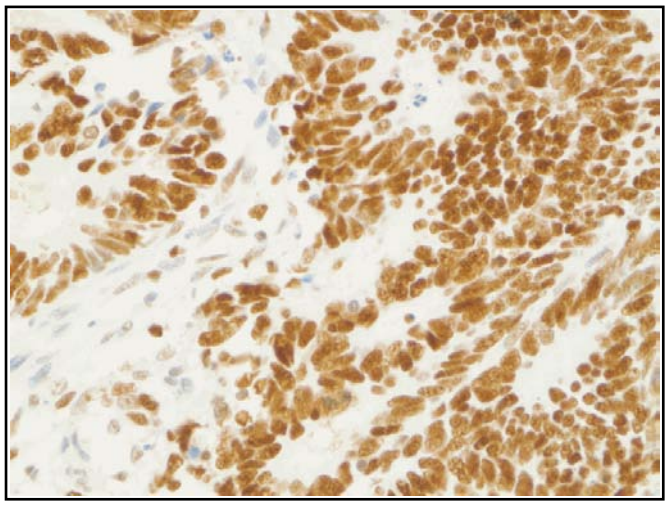


Fig 9. Optimal demonstration of MSH6 on the UK NEQAS distributed colonic tumour demonstrating strong nuclear staining of virtually all tumour cells, with staining in lymphocytes and stromal cells. Stained with the Dako FLEX RTU IR086 (EP49) for 15 minutes, on a Leica Bond Max (Leica ER1 30 mins), with Leica Bond Polymer Refine detection.

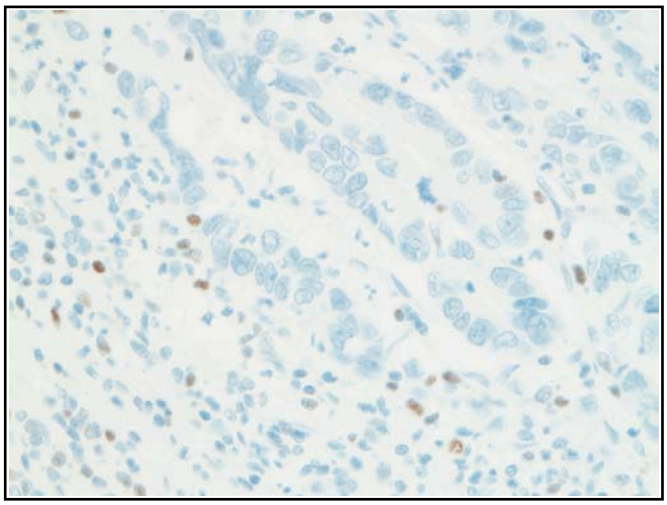


Fig 10. Optimal demonstration of MSH6 on the UK NEQAS distributed MSH6 negative colonic tumour showing intratumoral lymphocytes and stromal cell staining. Stained with the Abcam AB92471 (EPR3945), dilution not provided, on a Ventana Benchmark XT with CC1 64 minutes retrieval. Detection system not provided.

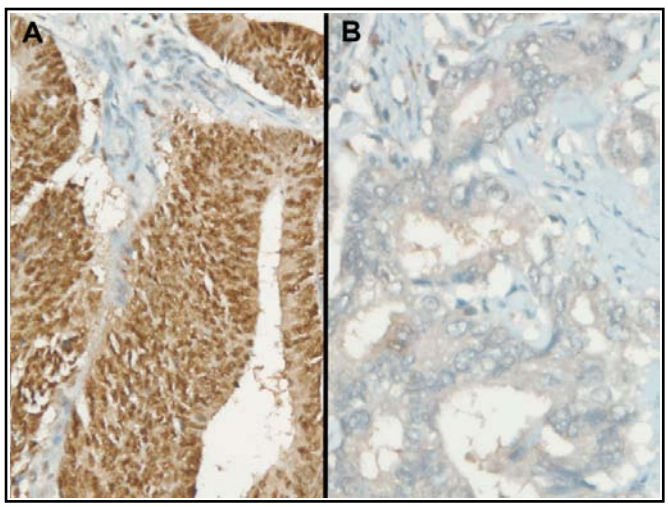


Fig 11. Suboptimal MSH6 on the UK NEQAS (A) positive and (B) negative colonic tumours. There is morphological damage and non-specific staining. Stained with the Leica/Novocastra NCL-L-MSH6 (PU29, 1:100 for 32 mins) on a Ventana Benchmark ULTRA (CC1 64 mins) with Ventana UltraView detection.

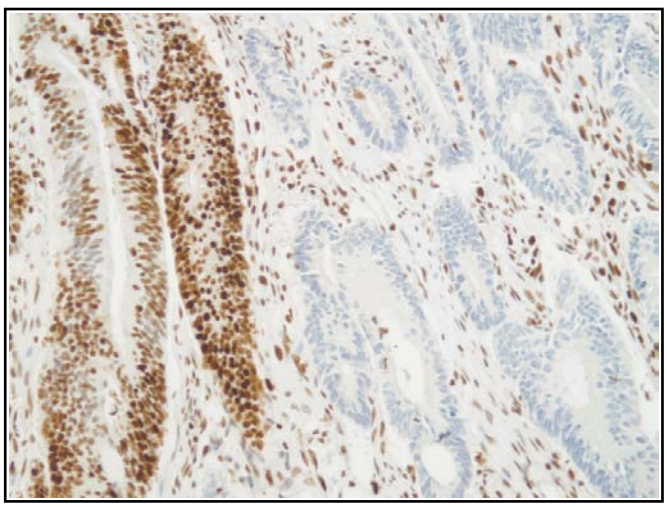
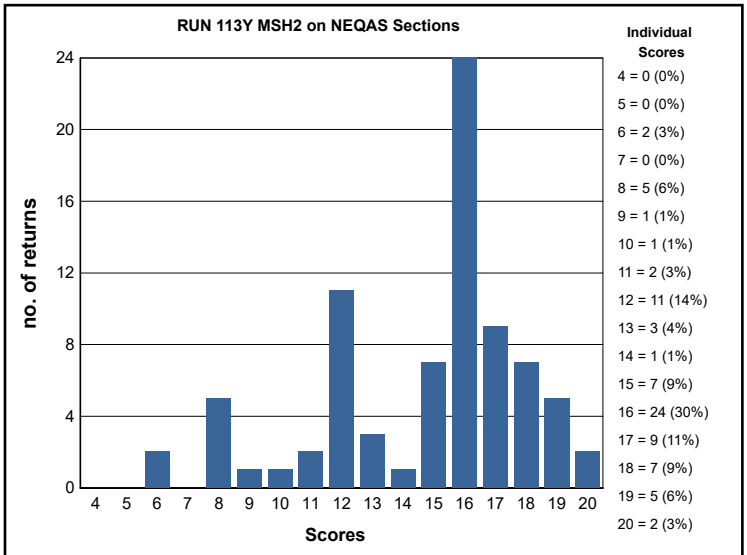
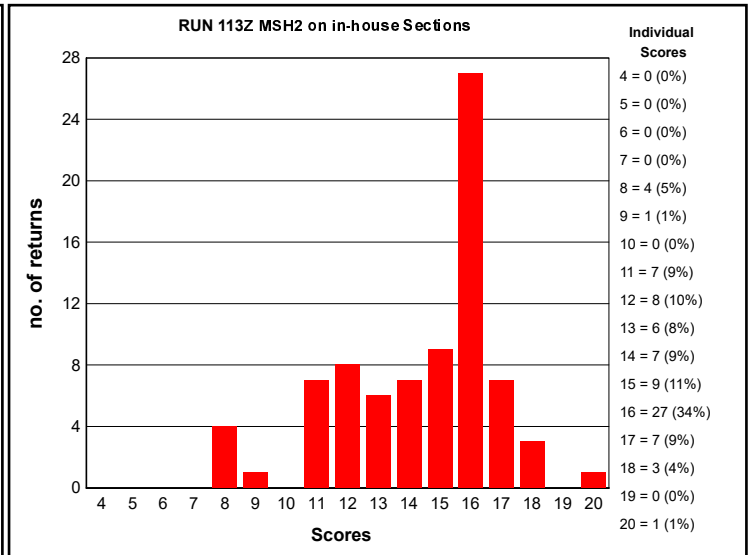
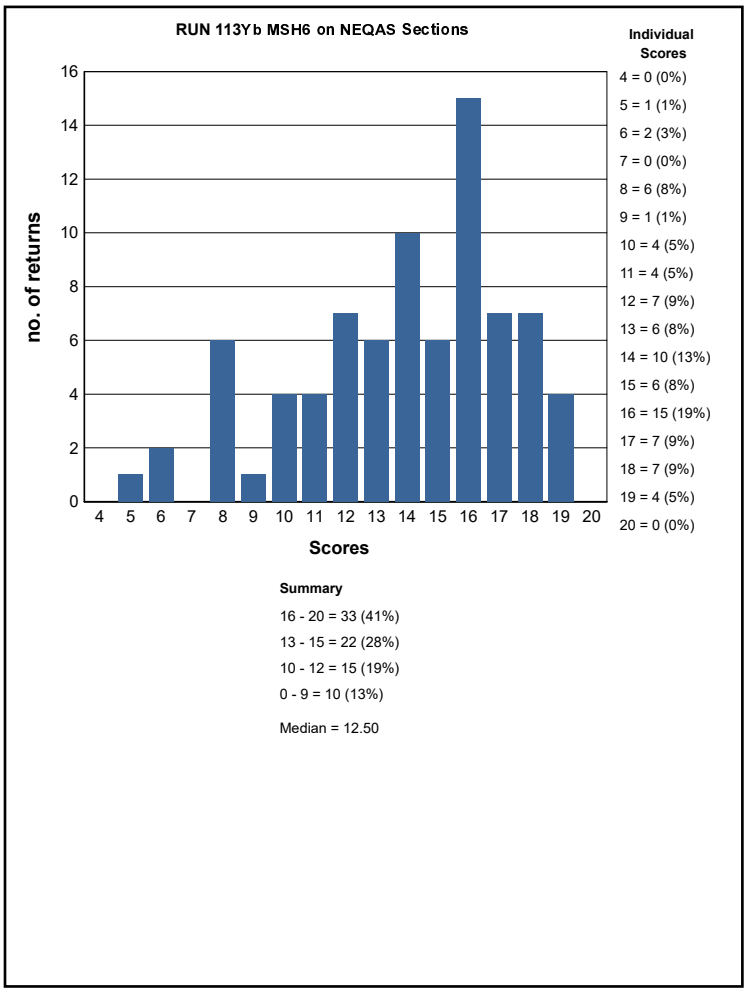


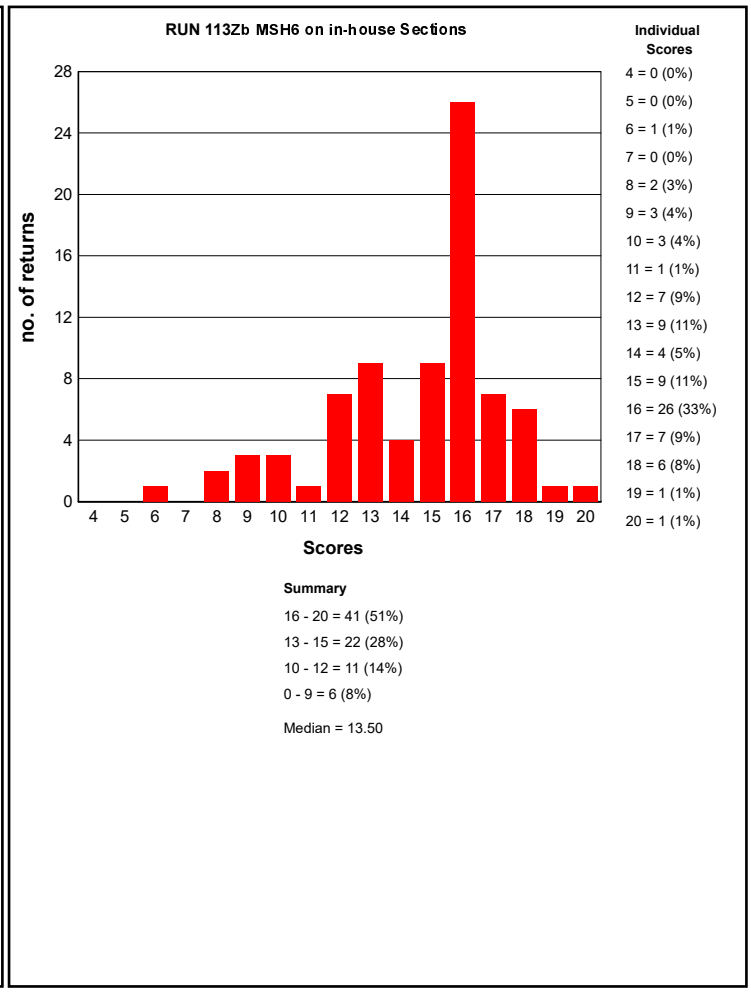
Fig 12. Good demonstration of in-house MSH6 on a heterogeneous tumour showing areas positive and negative MSH6 staining. Stained with the Dako M3646 (EP49, 1:50 for 60 mins) antibody, on a Dako Autostainer plus with Dako PTLink retrieval (no time provided) and Dako FLEX detection.

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: 113

Primary Antibody : MSH2

Antibody Details	N	%
BD Bio/Pharmingen 556349 (G219-1129)	4	50
Calbiochem NA26/T (GB12)	1	0
Calbiochem NA27/T (FE11)	1	100
Cell Marque CMAx/Cx (G219-1129)	7	43
Dako FLEX RTU IR085 (FE11)	14	86
Dako M3639 (FE11)	11	82
Epitomics AC-0211 RED2	1	100
Invitrogen 33-7900 (FE11)	2	50
Leica/Novocastra NCL-MSH2 (25D12)	7	57
Ventana 760-4265 (G219-1129)	31	77

Primary Antibody : MSH6

Antibody Details	N	%
Abcam AB92471 (EPR3945)	2	100
BD T. Labs/BioSci/Pharmingen 610918 (44)	6	17
BD T. Labs/BioSci/Pharmingen 610919 (44/MSH6)	5	40
Cell Marque 287M-14/15/16 (44)	1	100
Cell Marque 287R-24/25/26 (SP93)	2	100
Cell Marque 287R-27/28 RTU (SP93)	1	0
Dako FLEX RTU IR086 (EP49)	14	86
Dako M3646 (EP49)	18	67
Epitomics AC-0047 (EP49)	2	100
Invitrogen RTU 08-1374 (44)	1	0
Leica/Novocastra NCL-L-MSH6 (PU29)	1	0
Other	2	50
Ventana CONFIRM 790-4455 (44)	24	79

HNPCC Run: 113

Heat Mediated Retrieval	MSH2 N	MSH2 %	MSH6 N	MSH6 %
Biocare Decloaking Chamber	1	100	1	0
Dako Omnis	1	0	1	0
Dako PTLink	8	88	8	75
Lab vision PT Module	1	0	1	0
Leica ER1 20 mins	2	50	1	0
Leica ER1 30 mins	1	100	3	33
Leica ER2 20 mins	13	69	9	78
Leica ER2 30 mins	5	80	8	38
Leica ER2 40 mins	5	60	4	50
Ventana CC1 24mins	0	0	1	100
Ventana CC1 32mins	9	67	5	100
Ventana CC1 36mins	1	0	1	100
Ventana CC1 40mins	2	50	3	67
Ventana CC1 44mins	1	100	0	0
Ventana CC1 48mins	6	100	3	67
Ventana CC1 56mins	2	50	3	100
Ventana CC1 64mins	13	77	13	69
Ventana CC1 72mins	0	0	3	100
Ventana CC1 80mins	1	0	2	50
Ventana CC1 88mins	1	100	2	100
Ventana CC1 92mins	2	50	2	100
Ventana CC1 mild	2	100	0	0
Ventana CC1 standard	1	100	2	50
Ventana CC2 92mins	0	0	2	100

HNPCC Run: 113

Enzyme Mediated Retrieval	MSH2 N	MSH2 %	MSH6 N	MSH6 %
AS PER KIT	2	50	2	50
NOT APPLICABLE	40	70	39	72

HNPCC Run: 113	MSH2		MSH6	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	6	83	6	83
Biocare polymer (M4U534)	1	100	1	0
Dako EnVision FLEX (K8000/10)	0	0	1	100
Dako EnVision FLEX+ (K8002/12)	6	83	5	60
Leica Bond Polymer Refine (DS9800)	24	71	24	54
Other	2	0	2	0
Ventana iView system (760-091)	1	100	0	0
Ventana OptiView (760-700) + Amp. (7/860-099)	5	40	4	50
Ventana OptiView Kit (760-700)	28	82	27	89
Ventana UltraView Kit (760-500)	5	40	7	57

HNPCC Run: 113	MSH2		MSH6	
Automation	**N**	**%**	**N**	**%**
Dako Autostainer Link 48	6	100	6	83
Dako Autostainer plus	2	0	2	0
Dako Autostainer Plus Link	1	100	1	100
Dako Omnis	1	0	1	0
Leica Bond Max	10	50	8	38
Leica Bond-III	16	81	17	59
Menarini - Intellipath FLX	1	100	1	0
Ventana Benchmark GX	1	100	1	100
Ventana Benchmark ULTRA	28	71	29	79
Ventana Benchmark XT	13	77	13	85

HNPCC Run: 113	MSH2		MSH6	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	20	80	21	76
Dako EnVision Plus kits	1	100	1	100
Dako FLEX DAB	5	80	5	60
Leica Bond Polymer Refine kit (DS9800)	24	75	23	57
Other	8	63	8	88
Ventana DAB	14	57	12	75
Ventana Ultraview DAB	7	71	9	56

BEST METHODS - Gold Standard Antibody

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

MSH2 - Method 1

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

Primary Antibody: Dako M3639 (FE11) , 15 Mins, RT ºC Dilution 1: 50
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 20 mins, PH: 9
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

MSH2 - Method 2

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

Primary Antibody: Dako FLEX RTU IR085 (FE11) , 20 Mins, 25 ºC Prediluted
Automation: Dako Autostainer Link 48
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako PTLink, Buffer: high
EAR: NOT APPLICABLE
Chromogen: Dako FLEX DAB, 25 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection: Dako EnVision FLEX+ (K8002/12) , 15 Mins, 25 ºC Prediluted

MSH2 - Method 3

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

Primary Antibody:	Dako M3639 (FE11) Dilution 1: 15
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 40 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

MSH2 - Method 4

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

Primary Antibody:	Ventana 760-4265 (G219-1129) , 32 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins, Buffer: 0
EAR:	
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700)

BEST METHODS - Secondary Antibody

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

MSH6 - Method 1

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

Primary Antibody:	Dako M3646 (EP49) , 15 Mins, RT ºC Dilution 1: 50
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins, Buffer: 0, PH: 9
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

MSH6 - Method 2

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

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

MSH6 - Method 3

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

Primary Antibody:	Dako FLEX RTU IR086 (EP49) , 20 Mins, 21 ºC Prediluted
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	AS PER KIT
HMAR:	Dako PTLink, Buffer: DAKO HIGH pH TRS, PH: 9
EAR:	
Chromogen:	AS PER KIT, 21 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	AS PER KIT , 20 Mins, 21 ºC Prediluted

MSH6 - Method 4

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

Primary Antibody:	Cell Marque 287R-24/25/26 (SP93) , 60 Mins, 37 ºC Dilution 1: 200
Automation:	Ventana Benchmark XT
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 88mins, Buffer: CC1, PH: 9
EAR:	
Chromogen:	AS PER KIT, 37 ºC., Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 37 ºC Prediluted

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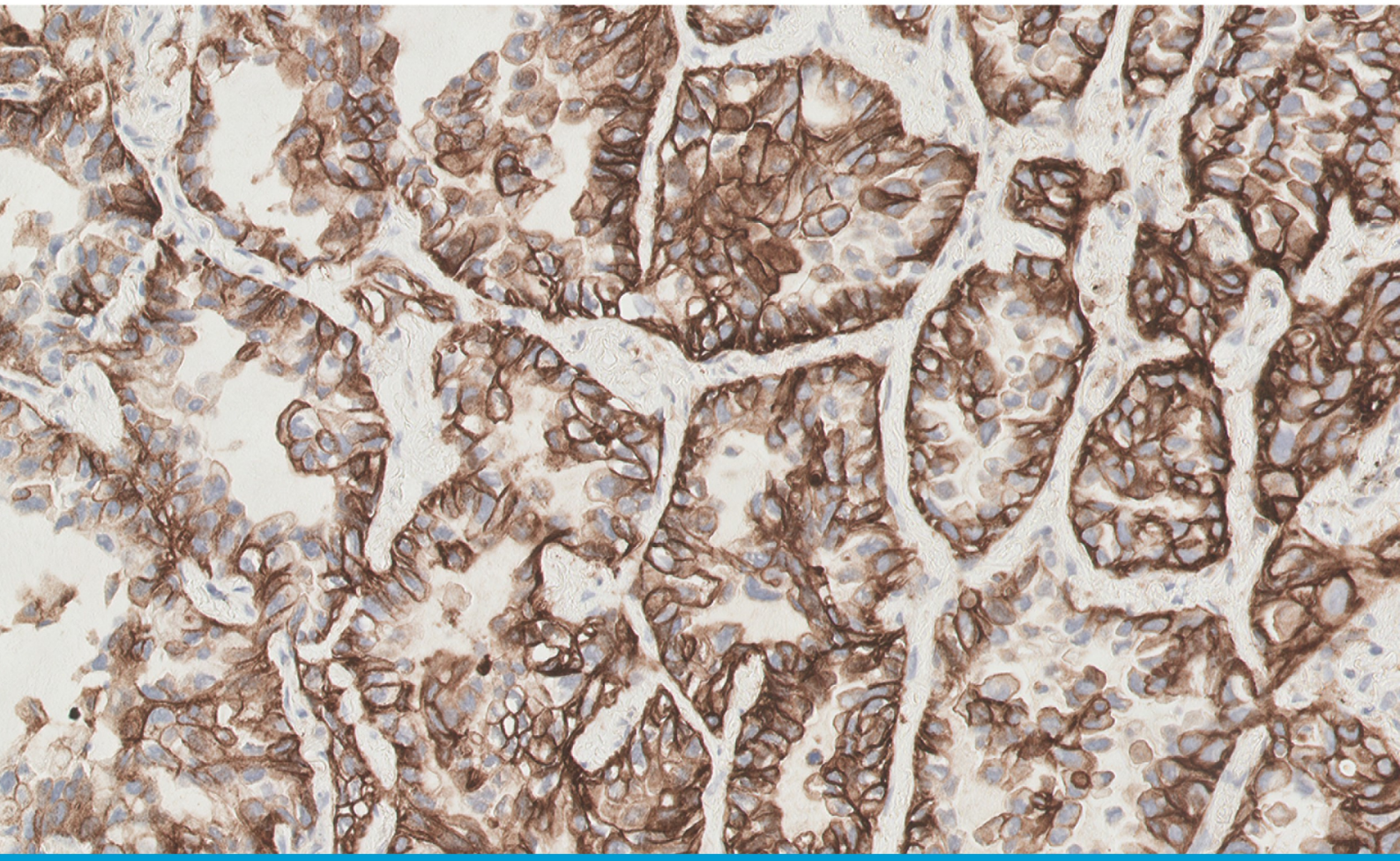


50249 2016JUN09

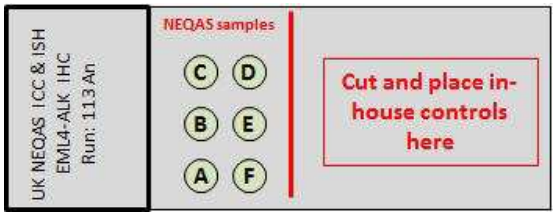
Agilent Technologies

Non-Small Cell Lung Carcinoma (NSCLC) ALK IHC EQA Run 113

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

Antibody Assessed:	ALK
Sections Circulated:	Composite slide consisting of lung adenocarcinoma, normal lung and appendix samples (see table below)
Number of Registered Participants:	56
Number of Participants This Run:	47 (84%)

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



Sample code	Sample	IHC status (Roche D5F3)
A	Appendix	+ve in ganglion cells
B	NSCLC adenocarcinoma	-ve
C	NSCLC adenocarcinoma	+ve
D	Normal lung	-ve
E	Cell line: Adenocarcinoma	+ve
F	Cell line: 50% isogenic + 50% adenocarcinoma	Approximately 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

Assessment Criteria

Same slide NEQAS & In-house controls

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

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

Interpretation criteria incorporating staining intensity

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

Assessment scoring guidelines

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

Assessment interpretation:

Score	Interpretation
16-20/20:	**Excellent:** Samples of very good staining quality and show the expected level of staining
13-15/20:	**Acceptable:** Some slight technical issues noted by some of the assessors, but overall the staining is suitable for interpretation.
10-12/20:	**Borderline:** Overall the staining is clinically relevant but technical improvements can be made.
4-9/20:	**Unacceptable:** Overall the samples are of unacceptable quality for clinical interpretation and technical improvements need to be made. Marks may have been deducted due to: - False positive/negative ALK IHC membrane staining - Excessive cytoplasmic staining - Non-specific or background staining
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.	

Non-Small Cell Lung Carcinoma (NSCLC) ALK IHC EQA Run113

Assessment Results & Discussion

Features Of Acceptable Staining: (Figs 1, 2, 3, 5A, 6 & 8)

- Moderate to strong cytoplasmic staining of the ganglion cells in the appendix
- Moderate to strong granular staining of the positive tumour sample (C)
- Moderate to strong granular staining of the positive tumour cell line samples (E and F). The ALK negative cells within the mixed cell line (sample F) should remain unstained.
- No staining in the negative tumour sample (B)
- No staining in the normal lung sample (D)
- No background or inappropriately localised staining

Features Of Suboptimal or Unacceptable Staining: (Figs 4, 5B, 7, 9 &10)

- Weak or no staining of the ganglion cells in the appendix
- False negative or absence of staining of known ALK positive tumour cells
- Non-specific staining, including background and excessive Tyramide staining

Pass Rates

There was an increase in the number of participating labs compared to the previous assessment; from 40 to 47 labs. The pass rates were also very encouraging, with over 80% of labs achieving an excellent pass on the Neqas distributed tissue, and no labs failed at all on either the Neqas material or the in-house tissue. The results are summarised in the table below:

NSCLC ALK Pass Rates Run 113:		
	Neqas Section	In-House
Excellent	81% (N=38)	39% (N=18)
Acceptable	13% (N=6)	13% (N=6)
Borderline	6% (N=3)	48% (N=22)
Unacceptable	0% (N=0)	0% (N=0)

3 labs received a borderline pass on the Neqas material; 2 of these labs were marked down as they showed no staining of the ganglion cells and axons in the appendix tissue, although the other samples on the slides showed the correct positive or negative results. Another lab that received a borderline score showed non-specific staining in the Neqas samples.

The main antibody clones used include the D5F3, ALK1 and 5A4. The following points highlight the main findings:

- The Roche D5F3 was the main antibody used accounting for 72% (n=34) of users, and this antibody showed an acceptable-excellent pass rate of 97%. Although this antibody is intended to be stained on the Ventana Benchmark XT or the Benchmark GX, several labs also stained with this antibody on the Ventana ULTRA platform and achieved acceptable-excellent results.
- The Leica Biosystems 5A4 clone was used by 4participants and this showed an acceptable pass rate of 75%. This was used in the concentrated or pre-diluted form, and both of which were shown to work well on the Leica Bond and Dako autostainers. 1 laboratory using the Novocastra/Leica antibody on the Ventana Benchmark received a borderline score of 12/20. This submission was scored down for weak staining. The assessors suggested that the lab's protocol is further optimised and re-validated.
- The cell signalling D5F3 clone was used by 3 participants; all were stained on the Ventana Benchmark XT and showed an excellent standard of staining.
- The Dako ALK1 was used by 1 participant. This slide received a borderline of 12/20 due to weak staining. Please note: The Dako ALK1 is not recommended by Dako for NSCLC staining.

In-house Control Results

Of the 47 participants taking part in this assessment, 46 (98%) also submitted in-house controls with the majority placing their samples alongside the NEQAS sample. The pass rates showed a lower "excellent" pass rate of 39% (N=18), and in parallel more labs achieved a borderline score. Again, there were no failed slides.

Participating labs are provided with instructions on the recommendations set out by UK NEQAS ICC for the choice of in-house controls. (See recommendations below). Any labs that do not provide this composite control tissue is scored a maximum borderline mark of 12/20.

ALK IHC Control Recommendation

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

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

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

- Commercially available control material (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.

Tissues Used In The run 113 Assessment:

There were numerous numbers and tissue types submitted for in-house controls and a breakdown of the data is shown in the table summary below:

No. Participants (%)	In-House Tissue composition
10 (21%)	NSCLC (+ve) & NSCLC (-ve)
2 (4%)	Cell line (+ve) & Cell line (-ve)
10 (21%)	NSCLC (+ve) & NSCLC (-ve) & appendix
3 (6%)	NSCLC (+ve)
1 (2%)	NSCLC (-ve)
8 (17%)	Appendix
2 (4%)	NSCLC (+ve) & Appendix
1 (2%)	NSCLC (+ve) & Appendix & Lymphoma (ALCL)
1 (2%)	NSCLC (+ve) & Appendix & tonsil
1 (2%)	NSCLC (+ve) & Lung SCC
1 (2%)	NSCLC (-ve) & Appendix
1 (2%)	Appendix & Tonsil & Pancreas & Liver
1 (2%)	Lymph Node
1 (2%)	Lymphoma (ALCL) (+ve) & Lymphoma (ALCL) (-ve)
1 (2%)	No in-house returned
3 (6%)	In-house details not provided

- 44 labs (46%) submitted the UK NEQAS recommended composite control, which included both positive and negative ALK IHC NSCLCs, several of these also included an appendix section

- Although many other labs also submitted composite controls, they did not include both positive and negative ALK NSCLC tumours, with many opting for a postivie tumour and appendix.
- 13 (27%) of participants submitted only a single tissue control, which mainly consisted of an appendix. 3 labs submitted a positive tumour, 1 lab only a negative tumour, and 1 lab submitted a lymph node: A single ALK positive sample or ALK negative sample is not able to provide information on possible false-positive results or false-negative results (respectively).

Lymphoma is not recommended as a control in the lung setting. It was encouraging to see that less labs submitted this as their in-house control this run compared to previous NSCLC ALK assessments. For example, only 2 labs (4%) submitted lymphoma samples alone in the current run. A further 1 lab submitted lymphoma alongside a positive NSCLC and appendix. In the previous run 112 assessment , 6 participants (15%) submitted lymphoma as part of their in-house control. Lymphoma control can lead to potential false-negative results in the lung setting.

Scoring Systems Employed by Participants

A straightforward '+ve/-ve' interpretation scoring system was used by 76% of participants, with 24% using an intensity based method (3+,2+,1+ and Neg.) (see summary table below):

		Scoring Methods Used	
Antibody (clone)	**n =**	**+ve / -ve**	**3+,2+,1+, neg.**
Ventana/Roche (D5F3)	34	97% (N=33)	3% (N=1)
Novocastra NCL-ALK (5A4)	4	50% (N=2)	50% (N=2)
Cell Signalling Tech. (D5F3)	3	33% (N=1)	66% (N=2)
Dako M7195 (ALK1)	1	100% (N=1)	-
Abcam ab17127 (5A4)	1	100% (N=1)	-
	Overall	**76%**	**24%**

As previously indicated the scoring method appears to be detection system related. In summary:

- **Roche:** 97% (n=33) indicated that they used a simple '+ve/-ve' scoring criteria.
- **Novocastra (5A4 clone):** 2 out of 4 (50%) participants used a '+ve/-ve' method and the other 2 (50%) used an intensity based method.
- **Cell Signalling Technologies:** 1 out of the 3 (33%) participants used a '+ve/-ve' method and the other 2 (66%) used an intensity based method.
- **Dako:** 100% (n=1) used a +ve/-ve scoring criteria
- **Abcam:** 100% (n=1) used a +ve/-ve scoring criteria

As indicated in the introduction, although there is evidence that ALK IHC along with intensity could be used as an affective screening tool,[13,14] there is also further evidence that the variability in intensity is due to the choice of detection system. For example, the Roche tyramide detection system provides a more clear cut distinction between a positive or negative tumour.

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1. Jemal A, Bray F, Center MM, et al. Global cancer statistics. CA Cancer J Clin 2011;61:69–90
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3. Camidge DR, Christensen J, Bang YJ. Addressing right drug-right target-right patient in phase I studies to accelerate bench to clinical benefit time: ALK gene rearrangements and the development of PF-02341066 in NSCLC. J Thorac Oncol 2010;5:S233.
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10. Takeuchi K, Choi YL, Soda M, et al. Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. Clin Cancer Res 2008;14:6618–6624.
11. Paik JH, Choe G, Kim H, et al. Screening of anaplastic lymphoma kinase rearrangement by immunohistochemistry in nonsmall cell lung cancer: correlation with fluorescence in situ hybridization. J ThoracOncol 2011;6:466-472.
12. Savic S, Diebold J, Zimmermann AK, Jochum W, Baschiera B, Grieshaber S, Tornillo L, Bisig B, Kerr K, Bubendorf L. Screening for ALK in non-small cell lung carcinomas: 5A4 and D5F3 antibodies perform equally well, but combined use with FISH is recommended.. Lung Cancer. 2015 May (ahead of print).
13. Fiona H. Blackhall, Solange Peters, Lukas Bubendorf et al., (2014) Prevalence and Clinical Outcomes for Patients With ALK-Positive Resected Stage I to III Adenocarcinoma: Results From the European Thoracic Oncology Platform Lungscape Project. J.Clin. Oncol 32 (25): 2780 - 2788.
14. Georg Hutarew, Cornelia Hauser-Kronberger, Felix Strasser et al., (2014) Immunohistochemistry as a screening tool for ALK rearrangement in NSCLC: evaluation of five different ALK antibody clones and ALK FISH. histopahology 65:398-407.

Acknowledgements

We are grateful for Novartis Pharmaceuticals UK Limited for providing an educational grant which was used to help setup the NSCLC ALK EQA module. Novartis are not privy to any data/results until they are publically available.

Note: 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).

Selected Images showing Optimal and Sub-optimal Immunostaining

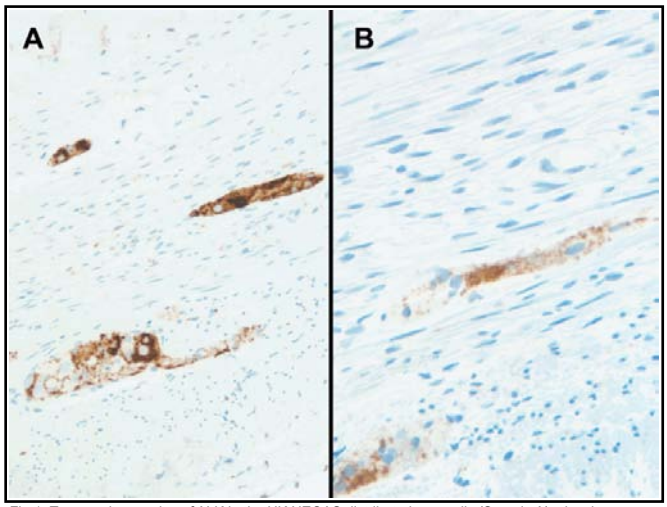


Fig 1. Two good examples of ALK in the UK NEQAS distributed appendix (Sample A), showing expected positive staining of ganglion cells and axons. (A) Ventana D5F3 assay with recommended protocols (B) 5A4 clone carried out on a Ventana with OptiView detection.

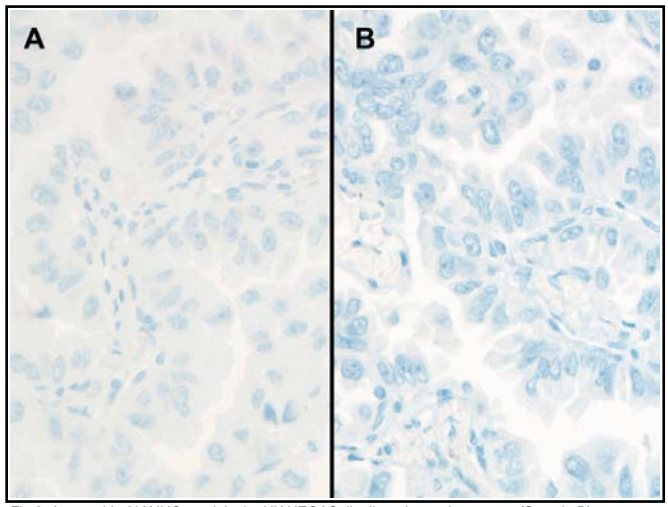


Fig 2. Acceptable ALK IHC result in the UK NEQAS distributed negative tumour (Sample B). Same protocols for (A) and (B) as shown in Fig 1.

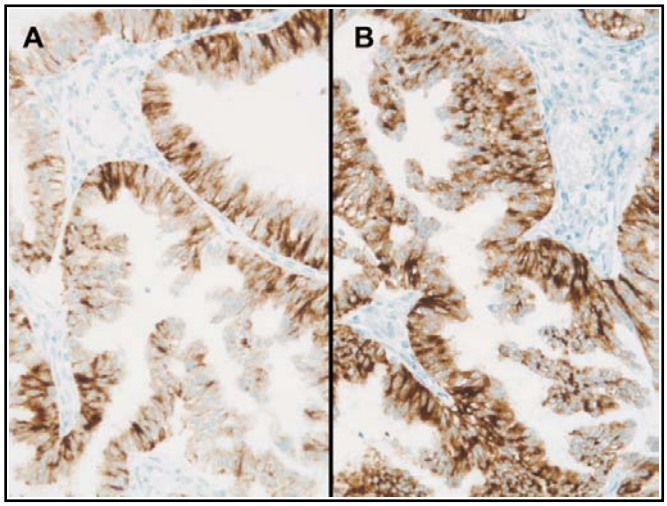


Fig 3. Good examples of ALK IHC positivity in the UK NEQAS distributed positive tumour (sample C). (A & B) The section shows strong membranous and cytoplasmic staining of the neoplastic cells. (A) Cell signalling D5F3 and (B) 5A4. Both stained on a Ventana XT with OptiView detection.

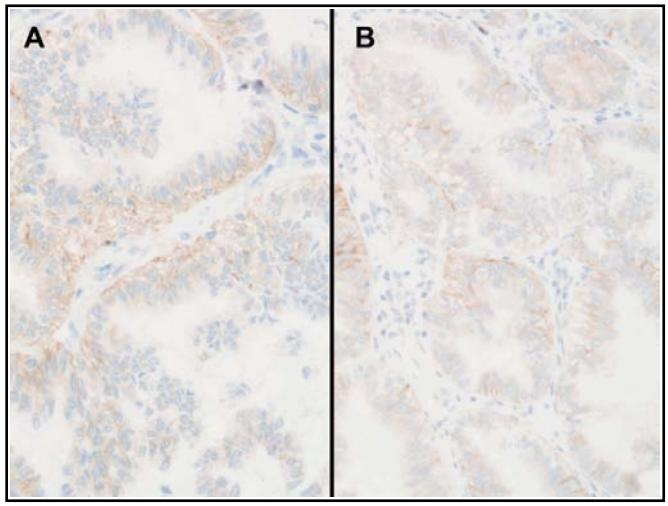


Fig 4. Sub-optimal ALK staining on the UK NEQAS distributed tumour (samples C) (compare with Fig 3). Staining is much weaker than expected. (A) Dako ALK1 clone on a Dako Autostainer link 48 with Envision detection (B) Novocastra 5A4 clone, stained on a Ventana Benchmark ULTRA, CC1 64 mins with OptiView detection.

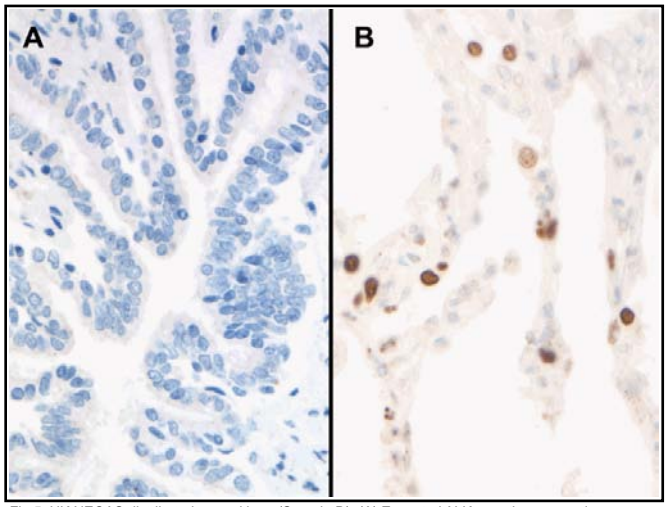


Fig 5. UK NEQAS distributed normal lung (Sample D). (A) Expected ALK negative expression (B) Non-specific nuclear staining. (B) Stained using the Ventana D5F3 assay so assessment team were unsure why the non-specific staining was present.

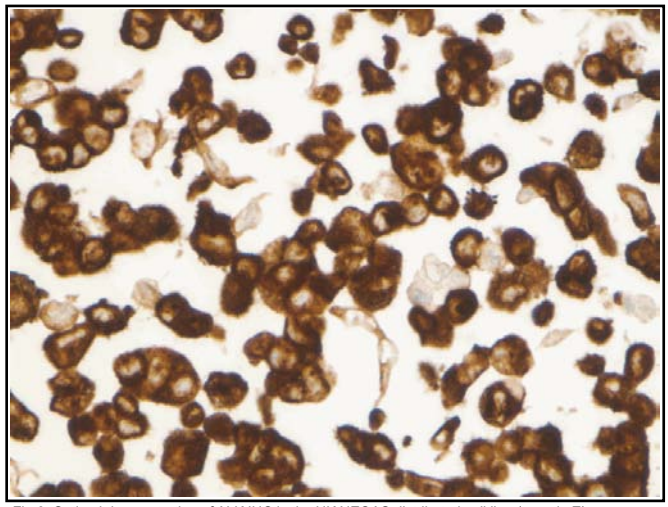


Fig 6. Optimal demonstration of ALK IHC in the UK NEQAS distributed cell line (sample E). There is good strong membranous staining of all tumour cells. Stained using Ventana D5F3 assay with recommended protocols.

Selected Images showing Optimal and Sub-optimal Immunostaining

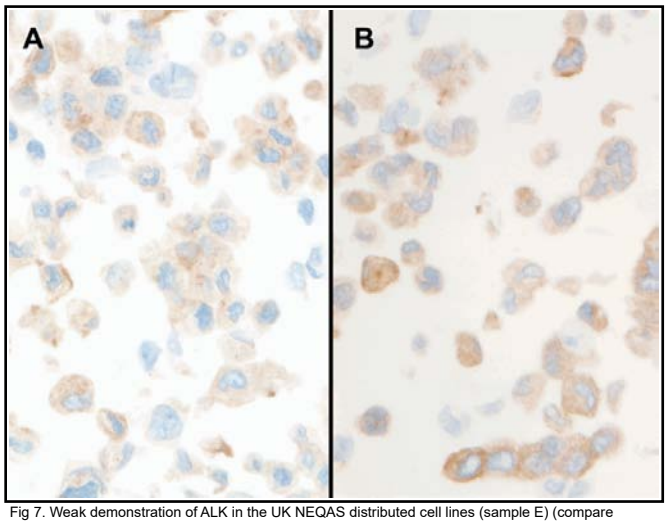


Fig 7. Weak demonstration of ALK in the UK NEQAS distributed cell lines (sample E) (compare with fig 6). Samples stained with (A) Dako ALK1 clone on a Dako Autostainer link 48 with Envision detection (B) Novocastra 5A4 clone, stained on a Ventana Benchmark ULTRA, CC1 64 mins with OptiView detection.

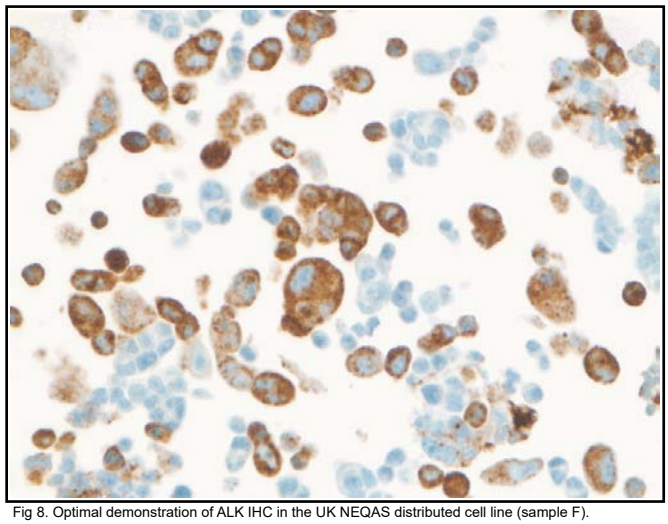


Fig 8. Optimal demonstration of ALK IHC in the UK NEQAS distributed cell line (sample F). This sample was a mixture of ALK positive and negative cell lines. Stained using the 5A4 clone on a Ventana XT, CC1 for 40 mins and OptiView detection.

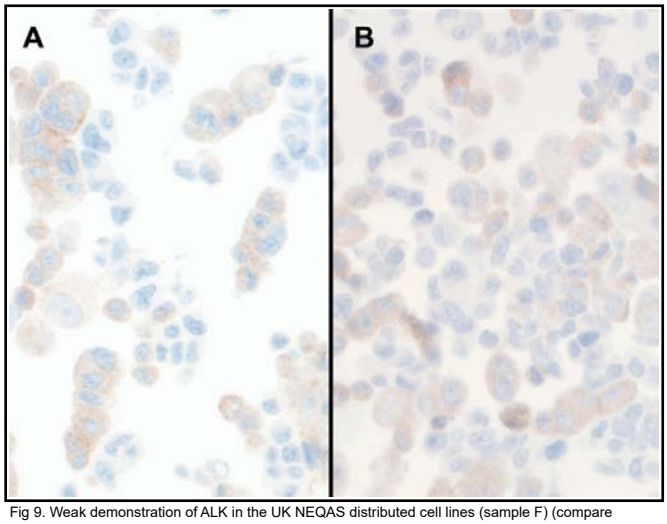


Fig 9. Weak demonstration of ALK in the UK NEQAS distributed cell lines (sample F) (compare with fig 8). Samples stained with (A) Dako ALK1 clone on a Dako Autostainer link 48 with Envision detection (B) Novocastra 5A4 clone, stained on a Ventana Benchmark ULTRA, CC1 64 mins with OptiView detection.

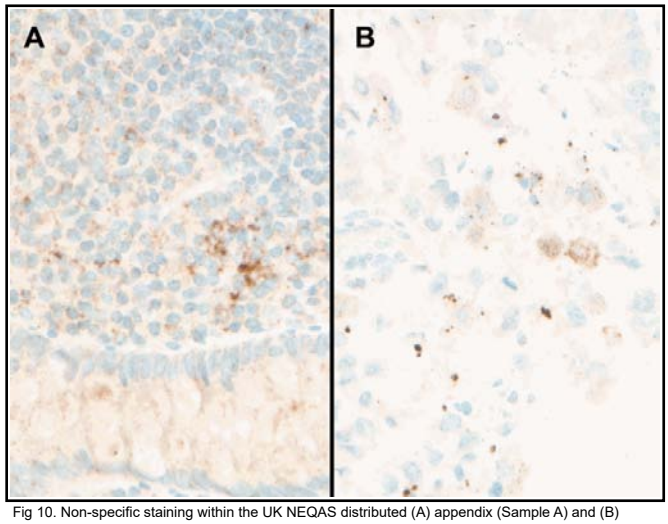


Fig 10. Non-specific staining within the UK NEQAS distributed (A) appendix (Sample A) and (B) negative tumour (sample B). Assessors were unsure why there was such non-specific staining as the sample was stained using the Ventana D5F3 assay with recommended protocols.

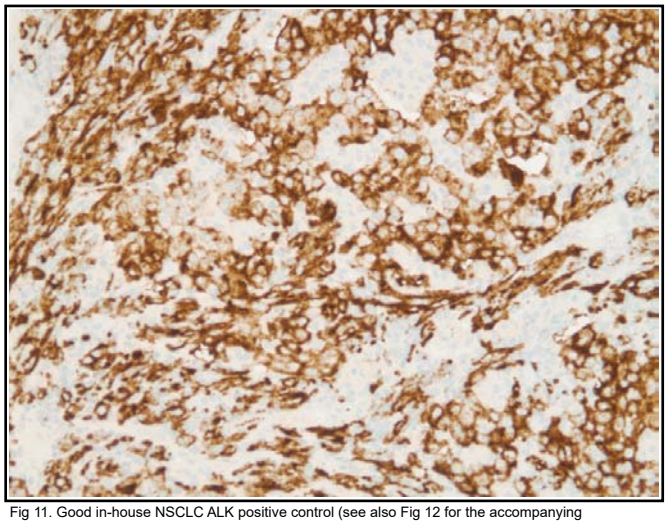


Fig 11. Good in-house NSCLC ALK positive control (see also Fig 12 for the accompanying negative tumour and appendix). Stained using the Ventana D5F3 assay on a Benchmark Ultra following recommended protocols.

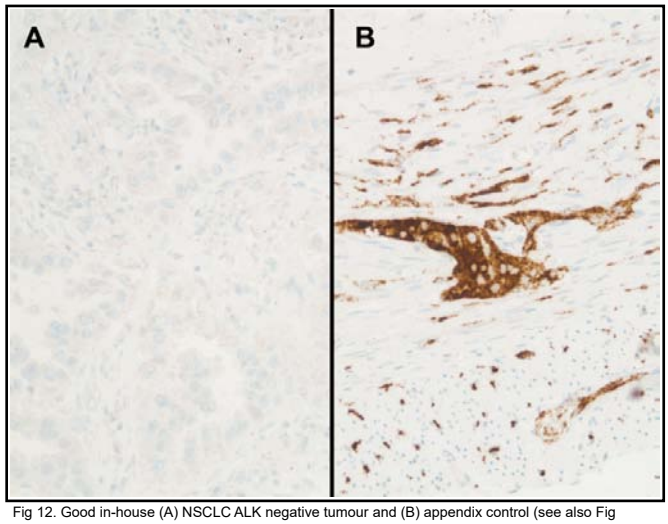
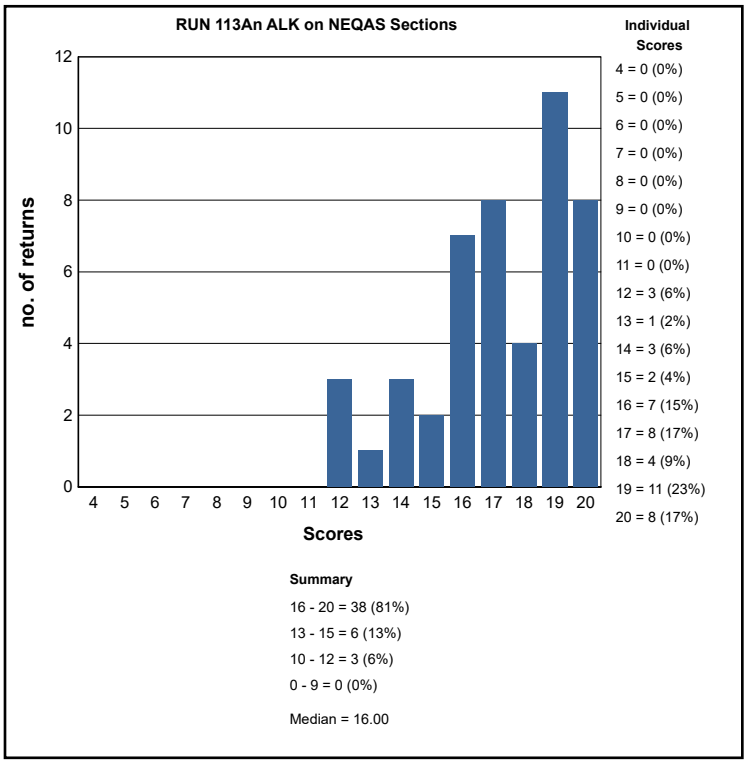
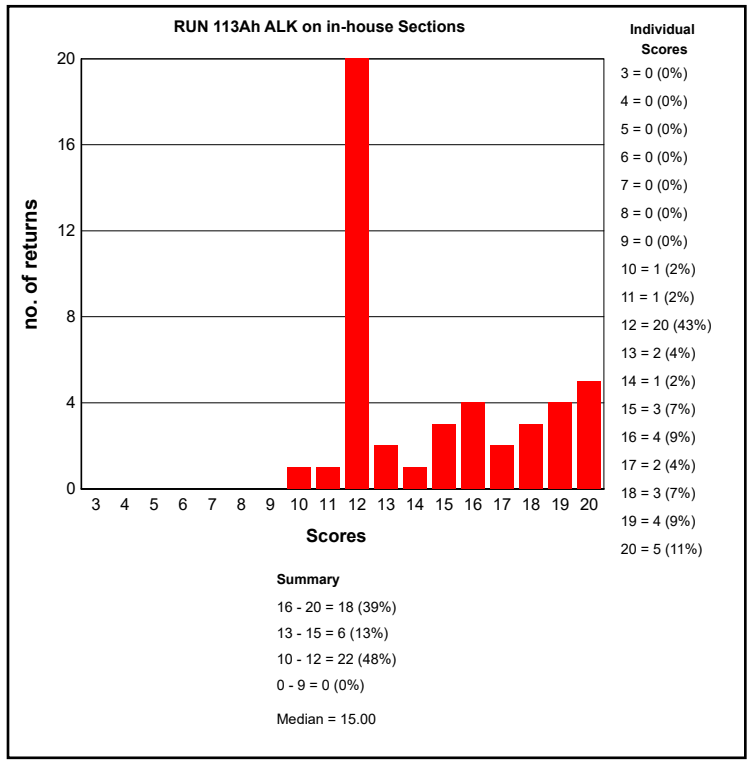


Fig 12. Good in-house (A) NSCLC ALK negative tumour and (B) appendix control (see also Fig 11 for the accompanying ALK positive tumour). Stained using the Ventana D5F3 assay on a Benchmark Ultra following recommended protocols.

GRAPHICAL REPRESENTATION OF PASS RATES





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

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

ALK NSCLC Run: 113

Primary Antibody	N	%
Abcam (5A4)	1	100
Cell Sign. Tech. (D5F3)	3	100
Dako M7195 (ALK1)	1	0
Novocastra NCL-ALK (5A4)	4	75
Ventana/Roche (D5F3)	34	97

ALK NSCLC Run: 113

Automation	N	%
Dako Autostainer Link 48	3	67
Leica Bond Max	1	100
Leica Bond-III	1	100
Ventana Benchmark GX	2	100
Ventana Benchmark ULTRA	10	90
Ventana Benchmark XT	26	96

ALK NSCLC Run: 113

Heat Mediated Retrieval	N	%
Dako PTLink	2	100
Leica ER2 20 mins	1	100
Leica ER2 30 mins	1	100
None	1	0
Ventana CC1 32mins	1	100
Ventana CC1 40mins	1	100
Ventana CC1 56mins	1	100
Ventana CC1 64mins	1	0
Ventana CC1 88mins	1	100
Ventana CC1 92mins	28	96
Ventana CC1 extended	3	100
Ventana CC1 standard	2	100

ALK NSCLC Run: 113

Detection	N	%
AS PER KIT	1	100
Dako EnVision FLEX (K8000/10)	1	100
Leica Bond Polymer AP Red Detection (DS9305)	1	100
Leica Bond Polymer Refine (DS9800)	1	100
Other	1	0
Ventana OptiView (760-700) + Amp. (7/860-099)	21	95
Ventana OptiView Kit (760-700)	16	94

ALK NSCLC Run: 113

Enzyme Retrieval	N	%
NOT APPLICABLE	15	87
Ventana Protease	2	100

ALK NSCLC Run: 113

Chromogen	N	%
AS PER KIT	16	100
Dako DAB K3468	1	100
Dako FLEX DAB	2	50
Leica Bond Polymer Refine kit (DS9800)	1	100
Other	1	100
Ventana DAB	20	95
Ventana Ultraview DAB	2	50

BEST METHODS

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

ALK - Method 1

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

Primary Antibody:	Ventana/Roche (D5F3) , 16 Mins Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	
Main Buffer:	
HMAR:	Ventana CC1 92mins
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099)

ALK - Method 2

Participant scored 17/20 (UK NEQAS Slide) and 13/20 (In House slide) using this method.

Primary Antibody:	Novocastra NCL-ALK (5A4) , 15 Mins Dilution 1: 1:25
Automation:	Leica Bond Max
Method:	
Main Buffer:	
HMAR:	Leica ER2 30 mins
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	Leica Bond Polymer AP Red Detection (DS9305) , 30 Mins

ALK - Method 3

Participant scored 19/20 (UK NEQAS Slide) and 12/20 (In House slide) using this method.

Primary Antibody:	Cell Sign. Tech. (D5F3)
Automation:	Ventana Benchmark XT
Method:	
Main Buffer:	
HMAR:	Ventana CC1 standard
EAR:	Ventana Protease
Chromogen:	Ventana DAB
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099)

The HER2 ISH Module - Interpretive Run 42

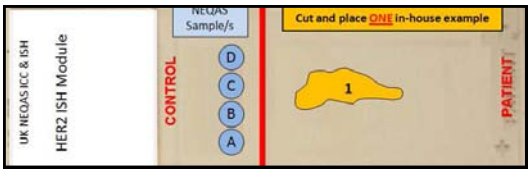
Merdol Ibrahim and Suzanne Parry

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	141 (81%)

Tissue Expression levels: The summary table below gives details of the HER2 phenotype and genotype of the breast carcinoma tissues circulated

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



Introduction

Breast cancer HER2 status should be determined in all newly diagnosed and recurrent breast cancers. In-situ hybridization (ISH), using either fluorescent (FISH) or brightfield chromogenic methods are the recognised techniques to determine the amplification status of these breast cancers. Most of these cases will have been put forward for ISH as a reflux test after being diagnosed as IHC 2+ equivocal by IHC, but in some instances ISH is used as an upfront method of testing to determine those patients that will go forward for Trastuzumab (Herceptin) therapy.

Recommendations

There are several recommendations regarding HER2 ISH testing including those by CAP (USA) and UK. 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.

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 scheme) 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 is also open to non-UK laboratories as an aid to monitoring and improving performance: At present the scheme has participating laboratories in over 28 countries.

Assessment Procedure

The assessment of slides utilises a statistical method in order to provide more concise information with regards to inter-observer variability in enumerating HER2 copy, chromosome 17 and overall ratios (See table 1: **Statistical Approach to the Scoring System**).

Assessment Results

Only 1 participant used a copy number alone method in the current assessment (run 42). This lab is using the Ventana SISH assay and received an excellent standard score of 12/12. The rest of the participating labs are using the dual probe ratio method. A full breakdown of the ratio method pass rates is shown in Table 2. 54% of labs are using the FISH technique and 46% are using a CISH method (Table 2), which was similar to the previous Run (41). The Pathvysion Vysis kit still remains the most popular FISH method, and this was used by 30% of labs (Table 2). The Ventana DDISH was the most favoured chromogenic ISH technique, which was used by 33% of labs (accounting for 72% of all CISH methods).

Overall the acceptable pass rate, irrespective of method, was 68% (≥30/36). A further 22% received a borderline pass (24-29/36), and 14 labs (10%) failed the assessment (scores of <24/36). The acceptable pass rate for the FISH users was 70% compared to 65% for those labs using the chromogenic method.

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. Walker RA, Bartlett JM, Dowsett M, Ellis IO, Hanby AM, Jasani B, Miller K. Pinder SE. (2008) HER2 testing in the UK: further update recommendations. J Clin Pathol;61818-824.
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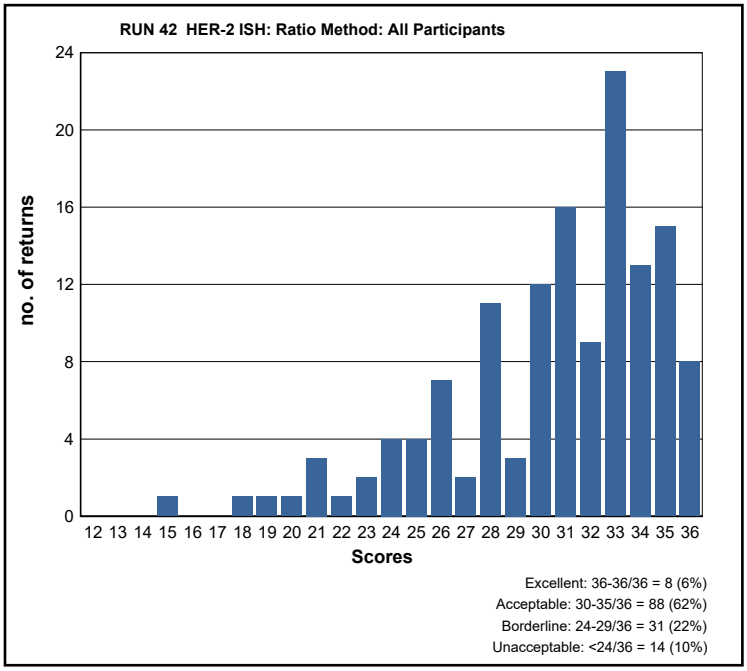
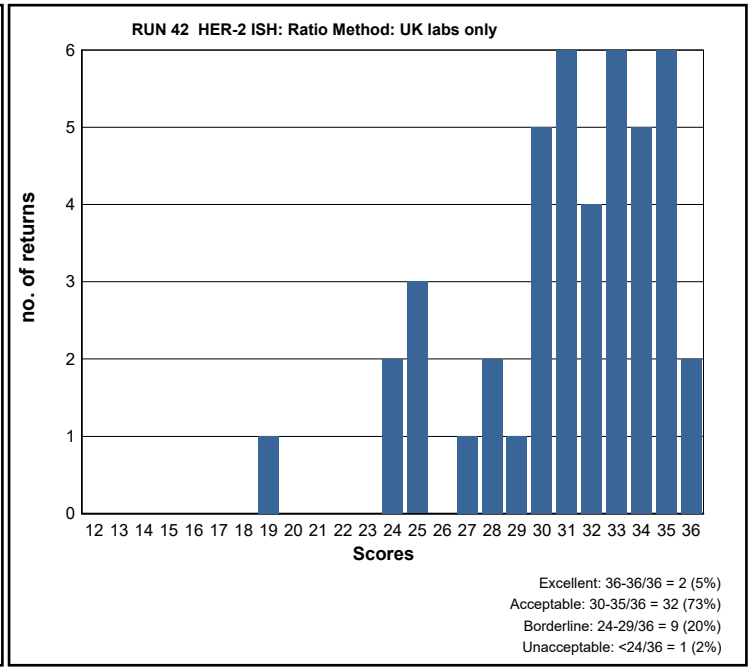
4. Rakha EA, Pinder SE, Bartlett JM, Ibrahim M et al. et al., (2014) Updated UK Recommendations for HER2 assessment in breast cancer. J Clin Pathol. 68 (2):93-99.

GRAPHICAL REPRESENTATION OF PASS RATES (ratio methods only)





METHODS USED and PASS RATES

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

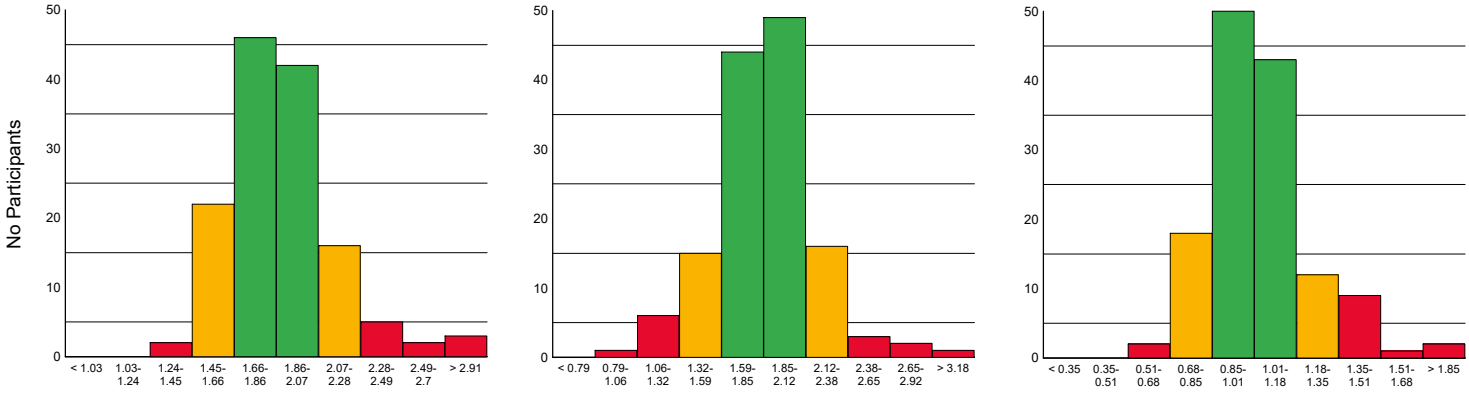
Ratio Method	N	% Pass (score ≥ 30/36)
Ratio: Dako DuoCISH	1	0%
Ratio: Dako IQFISH pharmDX	10	70%
Ratio: Dako Pharm Dx	2	50%
Ratio: In house FISH	1	100%
Ratio: Kreatech Probes	4	75%
Ratio: Leica HER2 FISH TA9217	6	83%
Ratio: Other - CISH	1	0%
Ratio: Other - FISH	6	67%
Ratio: Pathvysion Vysis Kit	41	76%
Ratio: Ventana BDISH 800-098/505	8	50%
Ratio: Ventana DDISH (780/800-4422)	46	67%
Ratio: Ventana Inform Silver ISH	5	60%
Ratio: Zytovision ZytoDot 2C	1	0%
Ratio: Zytovision ZytoLight	7	86%

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

FREQUENCY HISTOGRAMS

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

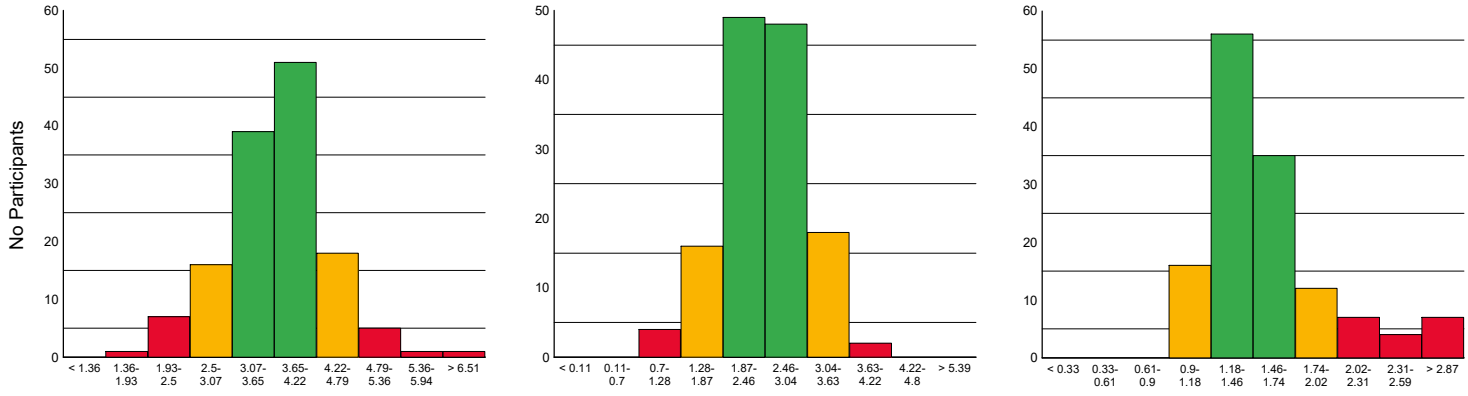
Green = mean +/- 1 Stdev Amber = mean +/- 2 Stdev Red = mean ≥ +/- 3 stdev



Sample A: Av. HER2 copy

Sample A: Av. Chr17

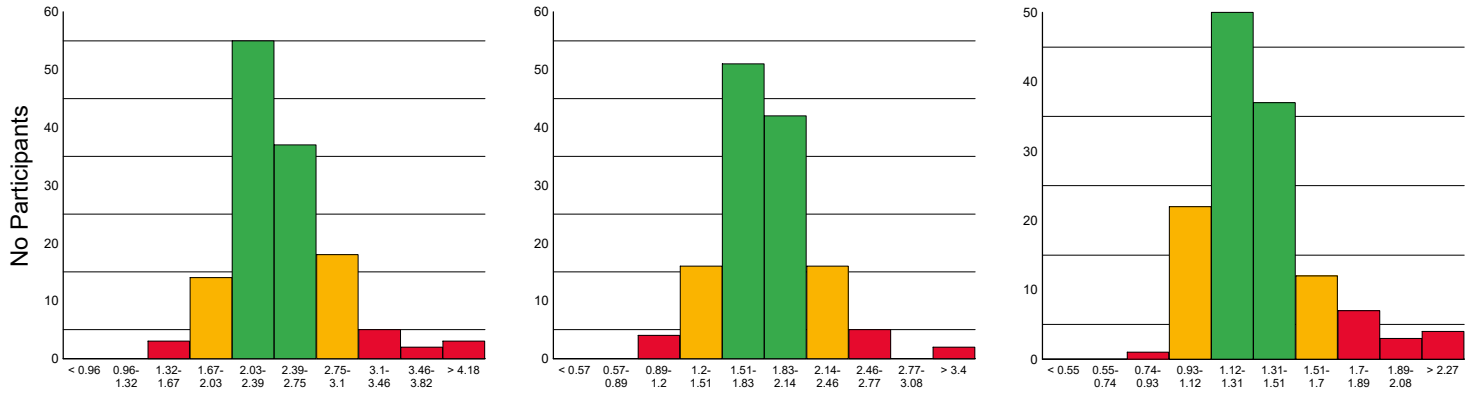
Sample A: Ratio



Sample B: Av. HER2 copy

Sample B: Av. Chr17

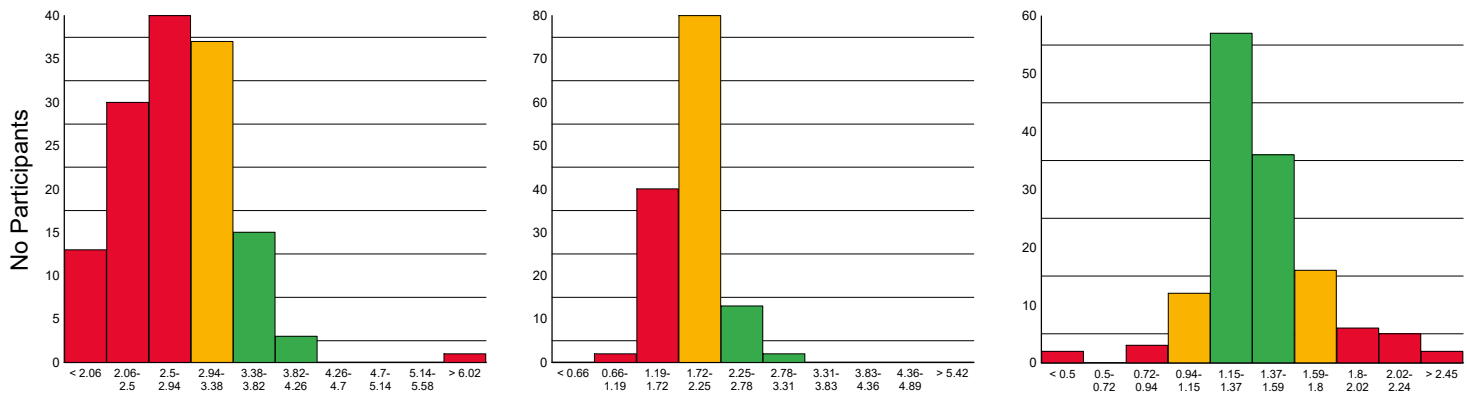
Sample B: Ratio



Sample C: Av. HER2 copy

Sample C: Av. Chr17

Sample C: Ratio



Sample D: Av. HER2 copy

Sample D: Av. Chr17

Sample D: Ratio

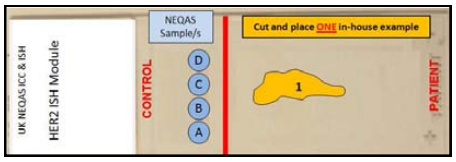
The HER2 ISH Module - Technical (Pilot) Run 42

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	134 (77%) (71 Fluorescent and 63 Chromogenic)

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

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



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

Assessment Procedure

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

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

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.
A summary of the assessment scoring criteria and it's interpretation is shown in the table on the next page

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

In-house 'example/s' are requested for this module and participants will be required to place them on the distributed NEQAS slides. Please note that only 1 in-house sample is required.

Results Summary

CISH Results:

Selected images Figs 7-12 show examples of the acceptable and unacceptable levels of staining of a CISH method. The overall results from the current CISH technical assessment (Run42) are similar to what has been seen with the previous assessments throughout the Neqas year. Results are summarised in the table below:

CISH Pass Rates:				
	Run 39	**Run 40**	**Run 41**	**Run 42**
Acceptable	50% (N=25)	54% (N=33)	48% (N=33)	46% (N=29)
Borderline	38% (N=19)	21% (N=13)	28% (N=19)	21% (N=13)
Unacceptable	12% (N=6)	25% (N=15)	24% (N=17)	33% (N=21)

It was disappointing to see that 21 labs (33%) failed the assessment, which was an increase from the beginning of the assessment year (Run 38). 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. Some failures were due to very weak or no HER2 signals. The borderline passes were mainly marked down due to weak, but still readable signals; again, mostly due to weak Cen17 signals. These observational results refer mainly to the Ventana BDISH or DDISH methods, which were used by most of the labs (86%) who submitted brightfield ISH slides for this technical assessment. The Dako DuoCISH method was used by 1 lab, and this slide achieved a borderline pass and was marked down due to weak Cen17 in some of the cores and also excessive counterstain. The Zytovision ZytoDot 2C method was used by 2 labs, and both of these slides failed the assessment: One slide showed morphology damage, caused by excessive pre-treatment. The other ZytoDot 2C user had no HER2 signals.

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

Scoring Interpretation	
Acceptable Individual Assessor 4-5/5 or Overall score ≥13/20	The NEQAS distributed samples and/or in-house tissue show a good/very good standard of staining and are suitable for interpretive analyses.
Borderline Individual Assessor 3/5 or Overall Score = 10-12/20	The NEQAS distributed samples and/or in-house tissue is borderline interpretable with 2 out of the 4 assessors indicating that improvements can be made with respect for example: Excessive / Weak Nuclear staining Level of probe hybridisation Excessive background staining **Also see assessor comments on your report**
Unacceptable Individual Assessor 1-2/5 or Overall score ≤9/20 =	The NEQAS distributed samples and/or in-house tissue is unacceptable for interpretation with at least 3 out of the 4 assessors indicating that there are hybridisation issues, which could be due: Excessive / Weak Nuclear staining Poor probe hybridisation Missing Her2 copy no. / CEN 17 Excessive background staining **Also see assessor comments on your report**
Score = 0	Slide Not submitted for assessment

FISH Results

Images of acceptable and unacceptable levels of staining are illustrated in figures 1-6. Overall the current FISH technical results (Run 42) were an improvement on the previous assessments carried out throughout the Neqas year. The results are summarised in the table below:

	FISH Pass Rates:			
	Run 39	**Run 40**	**Run 41**	**Run 42**
Acceptable	62% (N=38)	75% (N=54)	71% (N=52)	79% (N=56)
Borderline	13% (N=8)	6% (N=4)	8% (N=6)	13% (N=9)
Unacceptable	25% (N=15)	19% (N=14)	21% (N=15)	8% (N=6)

The main reason for unacceptable results was due to weak or no signals. We cannot be sure if this fading or total loss of signal may be due to slides not being sealed or stored correctly. However, UK Neqas does provide recommendations for this to try and prevent any loss of signal.

The Pathvysion Vysis kit still remains the most popular FISH method, used by 51% of labs for this assessment, and showed an acceptable pass rate of 67%. This was a great improvement from the previous Run (41), where only 32% achieved an acceptable pass using this method. The Dako IQFISH kit was the next popular choice of method, used by 15% (N=11) of FISH submissions, and this showed an acceptable pass rate of 91%. A variety of other assays were used, including the 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. In particular, the UK NEQAS data indicates that there has been a definite move towards the use of the Ventana DDISH method. 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.

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

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

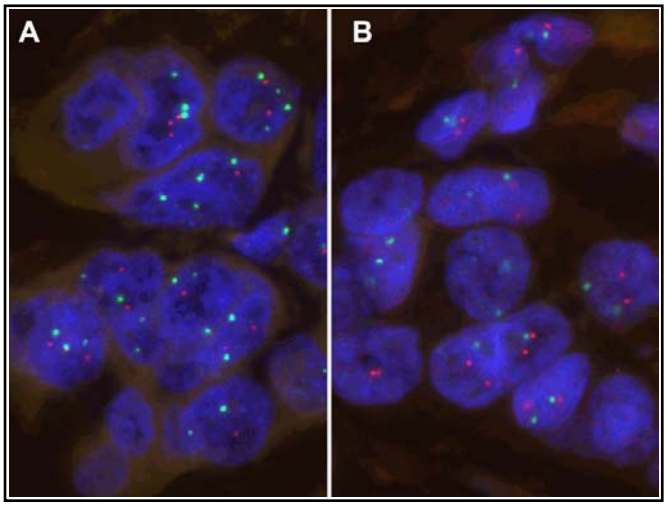


Fig 1. Acceptable FISH examples from UK NEQAS distributed 'non-amplified' case (sample A), stained using, (A) Leica Leica TA927 and (B) Dako IQFISH. Both samples shown without DAPI, and demonstrate distinct HER2 (red) and Chr17 (green) signals.

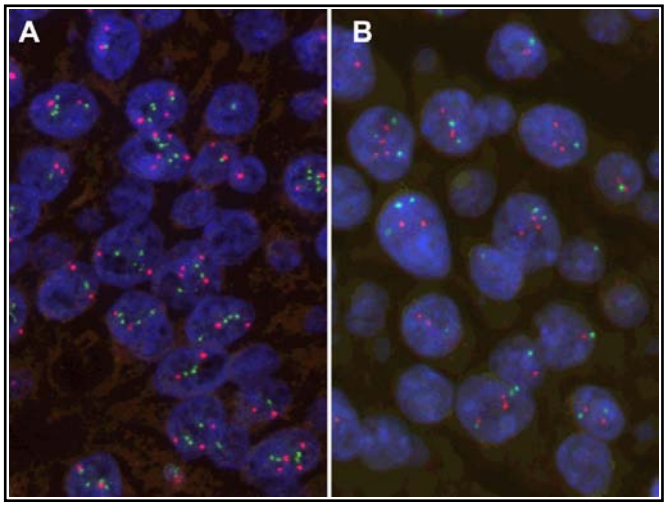


Fig 2. Acceptable FISH examples from UK NEQAS distributed 'non-amplified' case (sample B), stained using, (A) Zytovision ZytoLight. Note that HER2 signals are green and Chr17 signals are red. (B) Dako IQFISH. HER2 signals (red) and Chr17 signals (green). Average signals for this sample were 3.65 for HER2 and 2.46 for Chr 17.

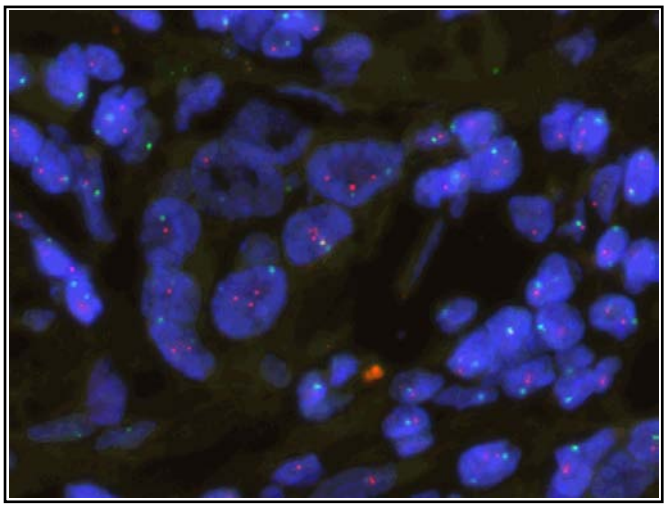


Fig 3. Acceptable FISH examples from UK NEQAS distributed 'non-amplified' case (sample C). Sample stained using Dako IQFISH, with distinct HER2 (red) and Chr17 (green) signals. Average signals for this sample were 2.39 for HER2 and 1.83 for Chr 17.

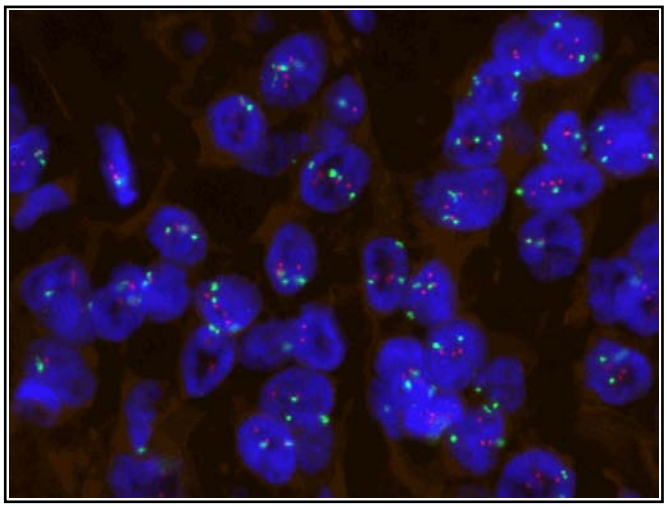


Fig 4. Acceptable FISH examples from UK NEQAS distributed 'non-amplified' case (sample D). Sample stained using Pathvysion Vysis Kit, with distinct HER2 (red) and Chr17 (green) signals. Average signals for this sample were 3.82 for HER2 and 2.78 for Chr 17.

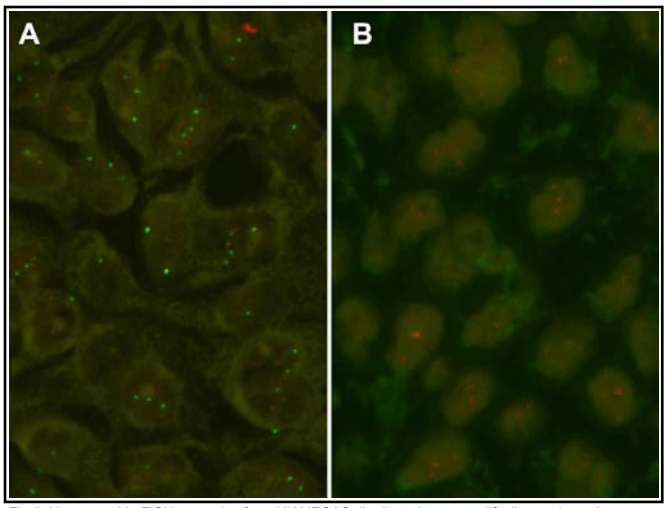


Fig 5. Unacceptable FISH examples from UK NEQAS distributed 'non-amplified' case (sample D). Note DAPI is not shown. (A) Very weak/absent HER2 (green) signal. (B) Very weak/absent Chr17 (red) signal. Both samples stained using the Pathvysion Vysis Kit.

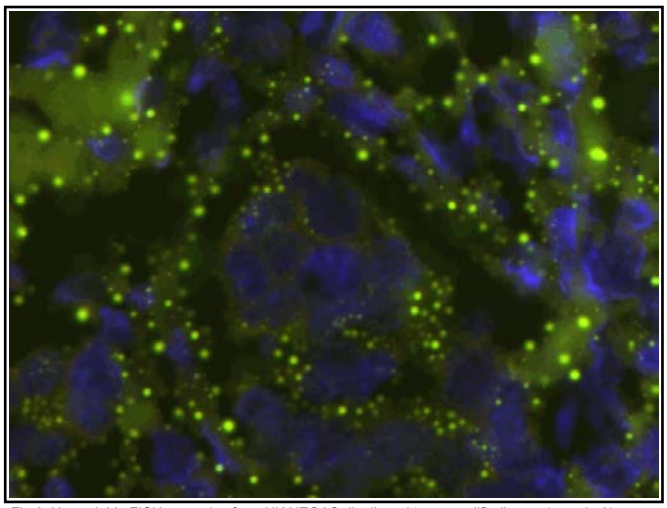


Fig 6. Unreadable FISH examples from UK NEQAS distributed 'non-amplified' case (sample A). The cause of this is possibly due to objective oil seeping under the coverslip. Participants are advised to seal the coverslip to the slide using clear varnish prior to counting and sending back slides for technical assessment.

Selected Images showing Optimal and Sub-optimal Immunostaining

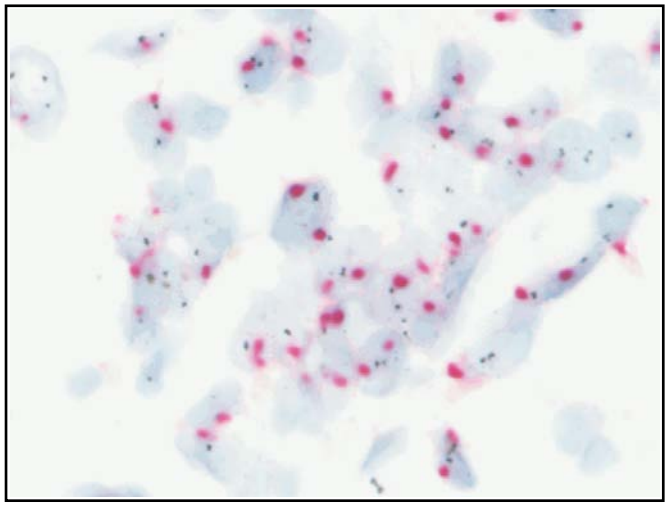


Fig 7. Acceptable Ventana DDISH in the UK NEQAS 'non-amplified' tumour (sample A). Both the HER2 (black) and Chr17 (red) signals are distinguishable with the expected level of copies per cell.

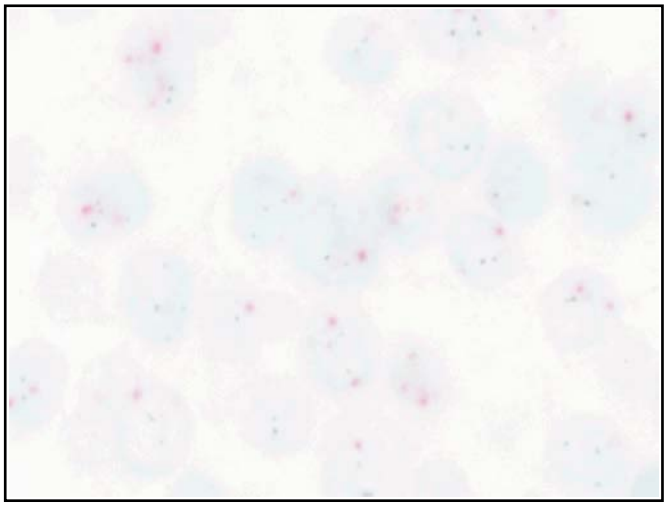


Fig 8. Unacceptable level of staining using Ventana DDISH in UK NEQAS 'sample B'. Overall both the HER2 (black) and Chr17 (red) signals are very weaker and sample was deemed to be unreliable for interpretation.

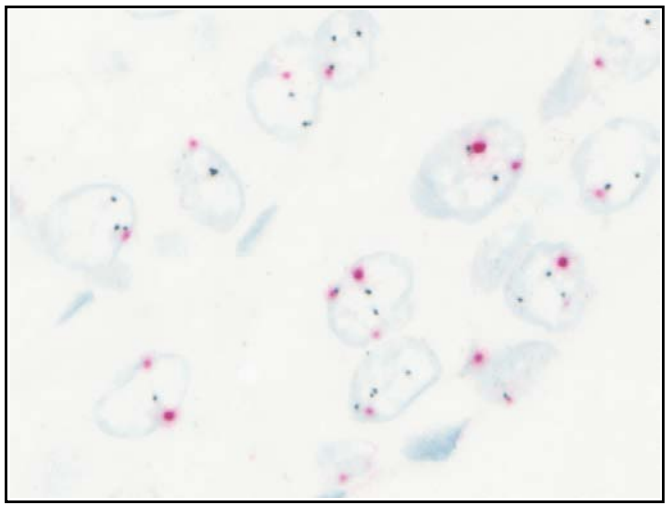


Fig 9. Acceptable Ventana DDISH in the UK NEQAS 'non-amplified' tumour (sample C). Both the HER2 (black) and Chr17 (red) signals are distinguishable with the expected level of copies per cell.

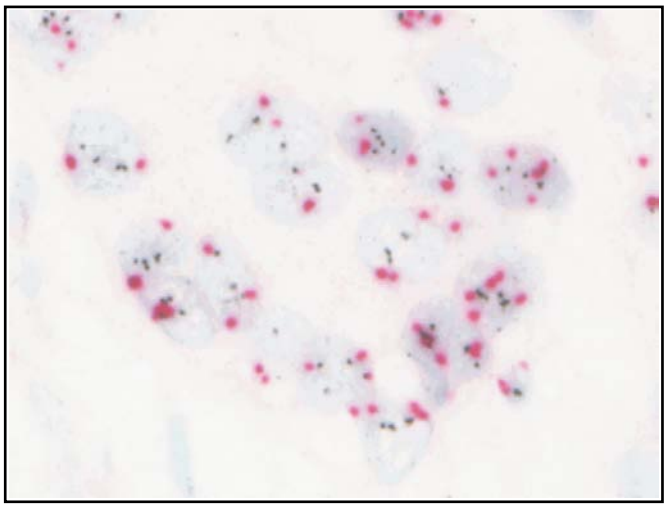


Fig 10. Acceptable Ventana DDISH in the UK NEQAS 'non-amplified' tumour (sample D). Both the HER2 (black) and Chr17 (red) signals are distinguishable with the expected level of copies per cell

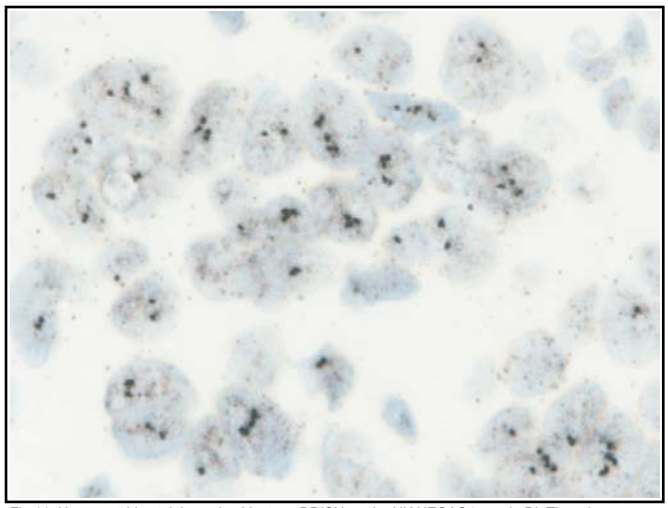


Fig 11. Unacceptable staining using Ventana DDISH on the UK NEQAS 'sample D'. There is excessive silver chromogen deposit (black) and lack of Chr17 (red) making this sample clinically unreadable.

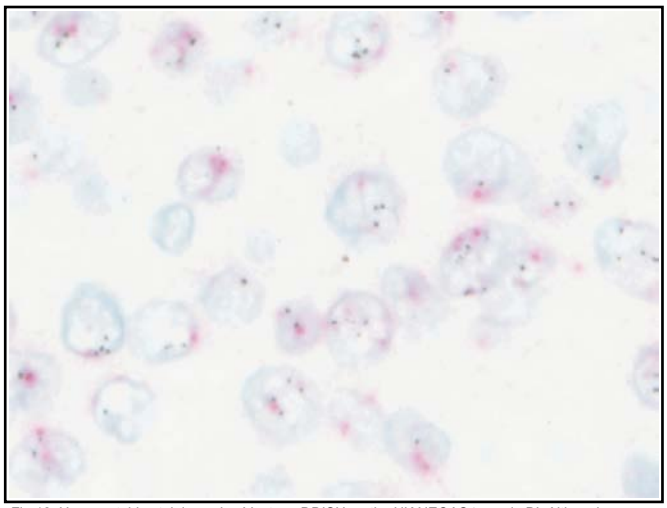
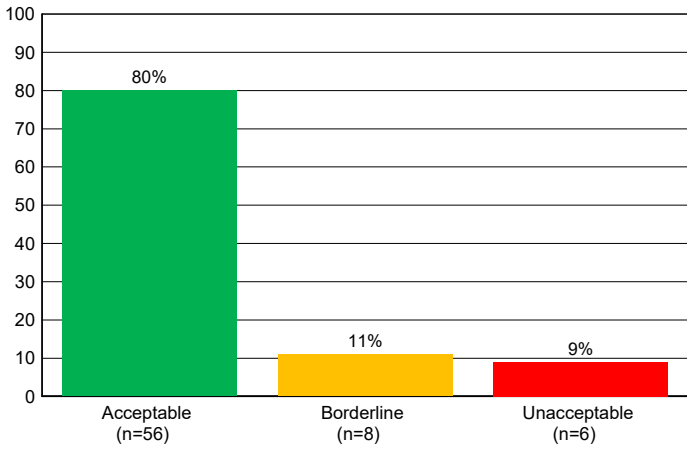


Fig 12. Unacceptable staining using Ventana DDISH on the UK NEQAS 'sample D'. Although HER2 copies (black) are present, the Chr17 signal has leeched making it unreadable. As this is a two probe system, this slide did not pass the assessment.

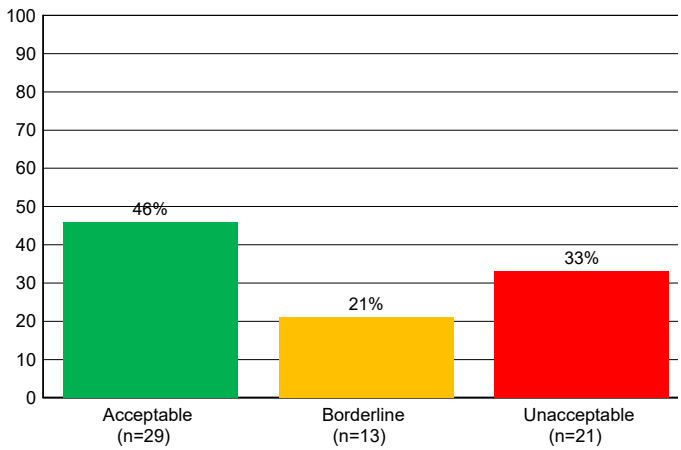
Technical ISH: Pass Rates and Methods

Overall Pass Rates

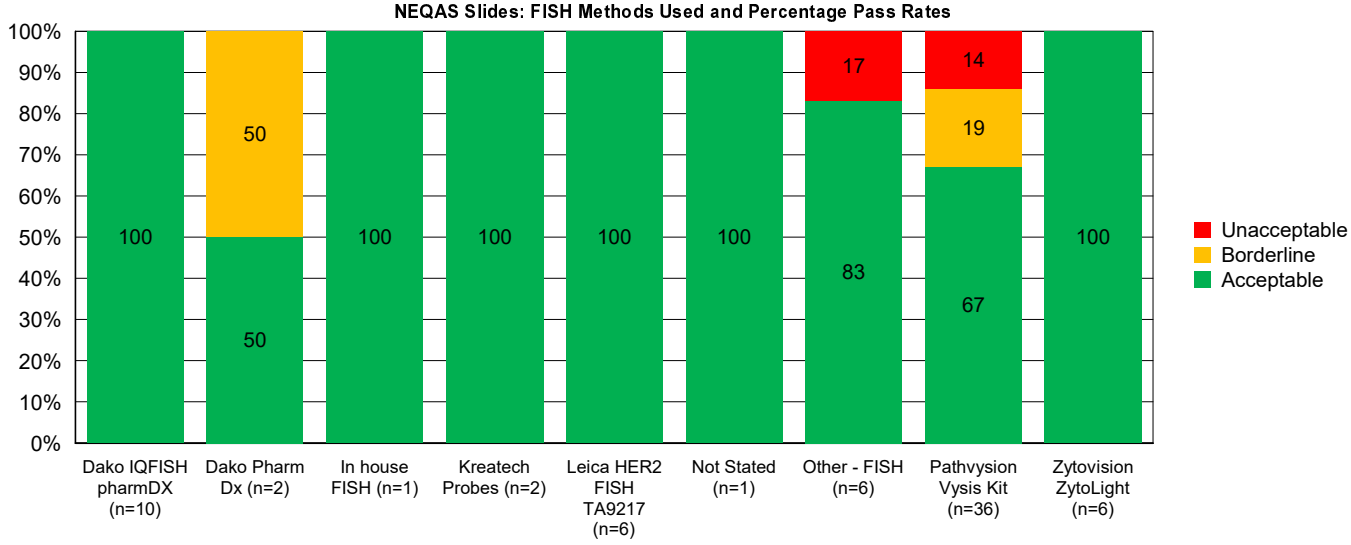
FISH NEQAS slide (n=70)



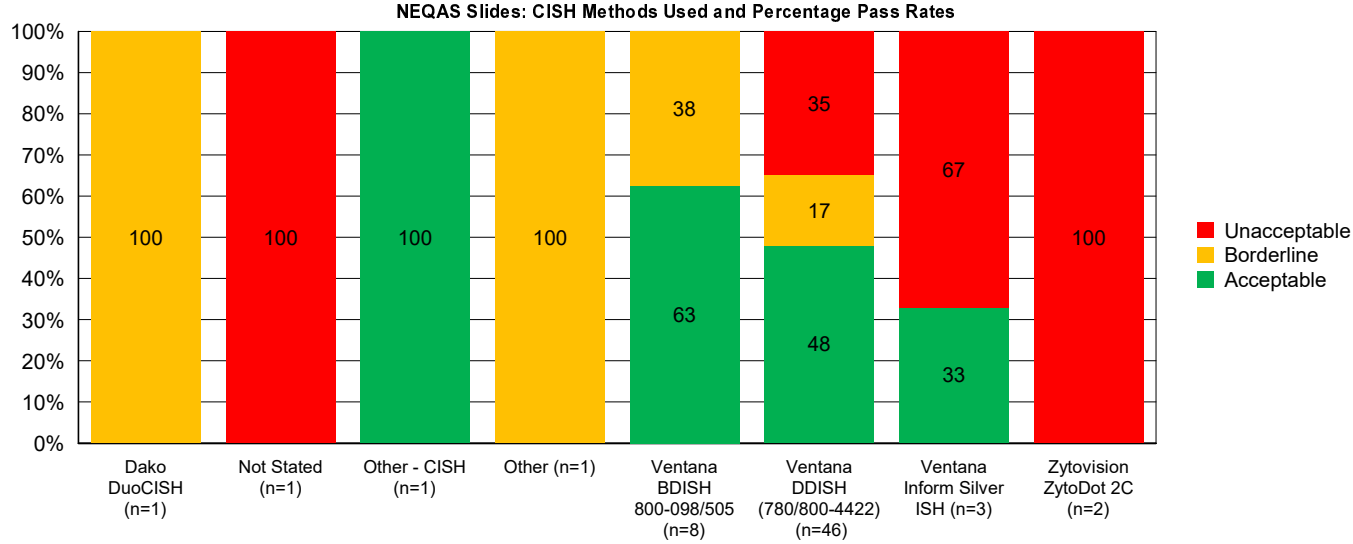
CISH NEQAS Slide (n=63)



NEQAS Slides: FISH Methods Used and Percentage Pass Rates



NEQAS Slides: CISH Methods Used and Percentage Pass Rates



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