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Immunocytochemistry

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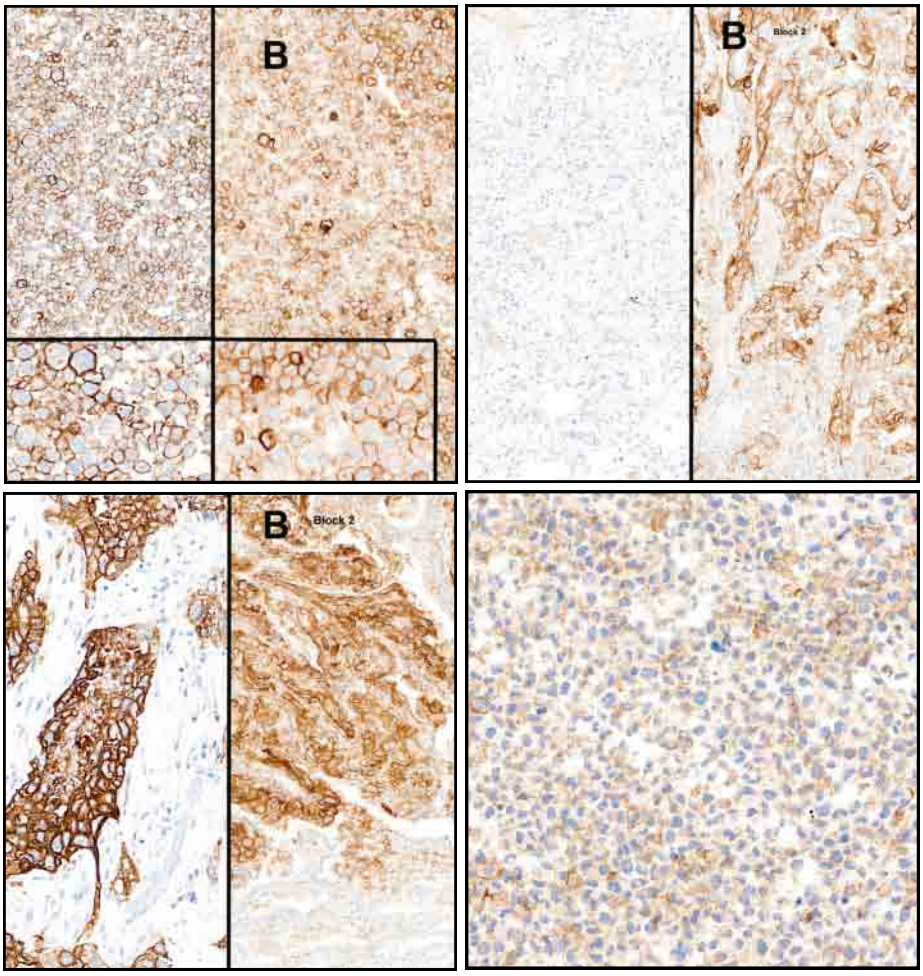
Assessment Dates: 5th January — 18th January 2017



Cover Photo: Taken from the UK NEQAS ICC & ISH PD-L1 Pre-Pilot :

Top Left: Optimal staining of the NEQAS strongly positive cell line (Core A)
Top Right: Optimal staining of the NEQAS tumour (Core G) Block 1 is tumour negative, Block 2 (Image B) is moderate to strongly positive for PD-L1
Bottom Left: Excellent demonstration of the NEQAS positive tumour (Core H)
Bottom Right: Weak demonstration of the strongly positive cell line (Core A)

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UK NEQAS ICC has over 100 Assessors (scientists and pathologists) including the UK, Australia, Canada, Denmark, Germany, Hungary, Ireland, Portugal, Slovenia, South Africa, Sweden and Switzerland.
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Journal layout and design prepared by UK NEQAS ICC & ISH

TITLE: UK NEQAS ICC & ISH Pre-Pilot Meeting for PD-L1 Immunohistochemistry in Non-Small Cell Lung Carcinoma

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Introduction

Lung cancer is the most common cancer in the world and the leading cause of cancer deaths worldwide (Globocan, 2017) with non-small cell lung carcinoma (NSCLC) representing approximately 85% of lung cancer diagnoses (Cancer ResearchUK, 2014). Recent approaches to NSCLC management has focused on targeting immune checkpoint inhibitors which has shown promising responses in patients with NSCLC and other tumour types (Brahmer et al., 2015, Garon et al., 2015). The main focus of immune checkpoint inhibitors has been centered on programmed cell death 1 (PD-1, B7-H1, CD274) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). These are found on the surface of cytotoxic T-cell lymphocytes and have been identified as two main immune checkpoints which are activated by tumours to suppress anti-tumour T-cell responses (Planchard et al., 2016). The programmed cell death/programmed cell death-ligand (PD-1/PD-L1) signaling pathway has been identified as a crucial regulatory pathway of T-cell exhaustion in cancer (Jiang et al., 2015).

In the therapeutic setting, blocking the immune checkpoint inhibition by PD-1 and PD-L1 monoclonal antibodies may lead to re-activation of T-lymphocytes allowing them to maintain their function of facilitating tumour cell death (Brahmer and Pardoll, 2013). Evidence by several clinical trials suggests response by patients with NSCLC may be predicted by positive expression of PD-L1 on tumour cells and associated immune cells anti-CTLA-4, (Borghaei et al., 2015, Brahmer et al., 2015). Assessment of PD-L1 status by immunohistochemistry (IHC) analysis is currently the approved method for identifying potential responders to PD-1/PD-L1 therapies.

PD-L1 for NSCLC is the most recent biomarker to be introduced to the repertoire of modules offered by the UK National External Quality Assessment Scheme for Immunocytochemistry and In Situ Hybridisation (UK NEQAS ICC & ISH). PD-L1 is unique to other biomarkers in that at least four different therapies have been developed or are in the development phase targeting PD-1/PD-L1, and have been clinically validated with four different PD-L1 assays to determine patient eligibility and likelihood of response to their respective therapies. Different cut-off thresholds for each of these assay therapies were determined on the basis of the predictive value and clinical data during co-development of the therapy and companion diagnostic (Hirsch et al., 2017) (Table 1). All of these assays are interpreted by determining the percentage of tumour showing PD-L1 expression, known as the tumour proportion score (TPS). Some assays also include the assessment of associated immune cells, known as the IC score.

PD-L1 Diagnostic Assays	Staining platform	Host and binding site	PD-1/PD-L1 Approved therapy	Positivity cut-off threshold for PD-1/PD-L1 treatment
Dako 22C3 pharmDx assay	Dako Autostainer Link 48	Mouse/ Extracellular	Pembroluzimab (Keytruda□, Merck)	>1% TPS (2nd line therapy) >50% TPS (1st line therapy)
Dako 28-8 pharmDx assay	Dako Autostainer Link 48	Rabbit/ extracellular	Nivolumab (Opdivo□, MSD)	>1%, >5% and >10 TPS*
Ventana SP263 assay	Ventana Benchmark ULTRA	Rabbit /Intracellular	Pembroluzimab (Keytruda□, BMS),	>1% TPS (2nd line therapy) >50% TPS (1st line therapy)
			Nivolumab (Opdivo□, MSD)	>1%, >5% and >10 TPS (2nd line therapy)*
Ventana SP142 assay	Ventana Benchmark ULTRA	Rabbit/ intracellular	Atezolizumab (Tencentriq□, ROCHE)	>50% TPS or >10% IC**
PD-L1 standalone antibodies□□□				
E1L3N (CST)	N/A	Rabbit/ intracellular	N/A	N/A
28-8 (Abcam)	N/A	Rabbit/ extracellular	N/A	N/A
SP142 (SB)	N/A	Rabbit/ Intracellular	N/A	N/A
22C3 (Dako)	N/A	Mouse/ Extracellular	N/A	N/A

Table 1: PD-L1 assays approved for PD-1/PD-L1 therapies for NSCLC. Abbreviations: TPS: tumour proportion score; CST: Cell Signaling Technologies; SB: Spring Bioscience. * May be associated with enhanced survival from Nivolumab, ** May be associated with enhanced survival from Atezolizumab, ***Not validated for clinical use

Nivolumab (OPDIVO®, Bristol-Myers Squibb) has been approved worldwide for use in patients with previously treated metastatic NSCLC. Pembrolizumab (KEYTRUDA®, Merck) has been approved worldwide in this setting as well as for patients with untreated NSCLC. Atezolizumab (TECENTRIQ®, Genetech) is approved by the Food and Drug Administration (FDA) organisation for use in the second line setting for NSCLC in the US only. These therapies in addition to Durvalumab (IMFINZI™, AstraZeneca) have also been approved for other tumour types such as melanoma and urothelial carcinoma. Pembrolizumab is currently the only PD-1/PD-L1 therapy

for which patients are selected based on their PD-L1 IHC expression status, using the approved companion diagnostic test (CDx); the Dako 22C3 PharmDx (Dako, Denmark). In early May, 2017, the Ventana SP263 Assay (Ventana, Tuscon) was given the European Conformity (CE) mark of approval as a companion diagnostic test for Pembrolizumab. This has the potential to significantly increase access to the use of approved PD-L1 assays for laboratories who do not have access to the Dako automated staining instruments, specifically the Autostainer link 48 required for the Dako 22C3 PD-L1 IHC assay.

Clinical trials have reported that PD-L1 expression detected by specific optimised PD-L1 assays may be associated with an enhanced response to PD-1/PD-L1 therapies, and have been approved by the FDA and/or CE marked as complementary diagnostic tests, and are available for use in the UK, Europe and elsewhere. A complementary diagnostic test is not a mandatory requirement for patient selection to a specific therapy, rather an optional test which provides additional information to the oncologist and clinicians on the likelihood of the disease responding to the treatment.

Assessment Procedure

Ahead of the pre-pilot NSCLC PD-L1 IHC assessment, an advisory group meeting was held. This consisted of an expert panel of pathologists and scientists trained for interpretation of PD-L1. The meeting provided a full appraisal on the best way to establish a rigid PD-L1 IHC NSCLC EQA module, in order to identify and assist laboratories who may require technical assistance and provide them with information as to the most robust reliable methods and guidelines.

Unstained formalin-fixed-paraffin-embedded (FFPE) sections from two different multi-blocks (blocks 1 and 2) were circulated (Figures 1 & 2). Each participant received slides for staining from either block 1 or 2, which consisted of a combination of cell lines and NSCLC tissue to stain for the PD-L1 IHC assessment. Participants were also asked to cut their own in-house PD-L1 control material on the same slide as the UK NEQAS distributed sections and stain with their routine PD-L1 method which they employ within their laboratory. Participants were also asked to submit their methodology and return their slides for peer review and assessment by the pre-pilot assessment panel.

The assessment team initially discussed the format for the assessment and whether to **a)** score each assay independently using the threshold cut-offs devised by the manufacturing companies; **b)** score the intensity of the staining as weak, moderate or strong; **c)** to score each individual TMA on the percentage of tumour cells (TCs) showing PD-L1 expression; and **d)** whether the PD-L1 positive immune cells (ICs) should be scored.

A consensus was reached to score each individual core based on the tumour proportion score (TPS), which is the percentage of TCs with PD-L1 positivity regardless of intensity. ICs would not be included in the scoring, apart from when assessing the Ventana/Roche SP142 assay, where a separate assessment of IC is part of the scoring guideline as an indicator of Atezolizumab treatment inclusion.

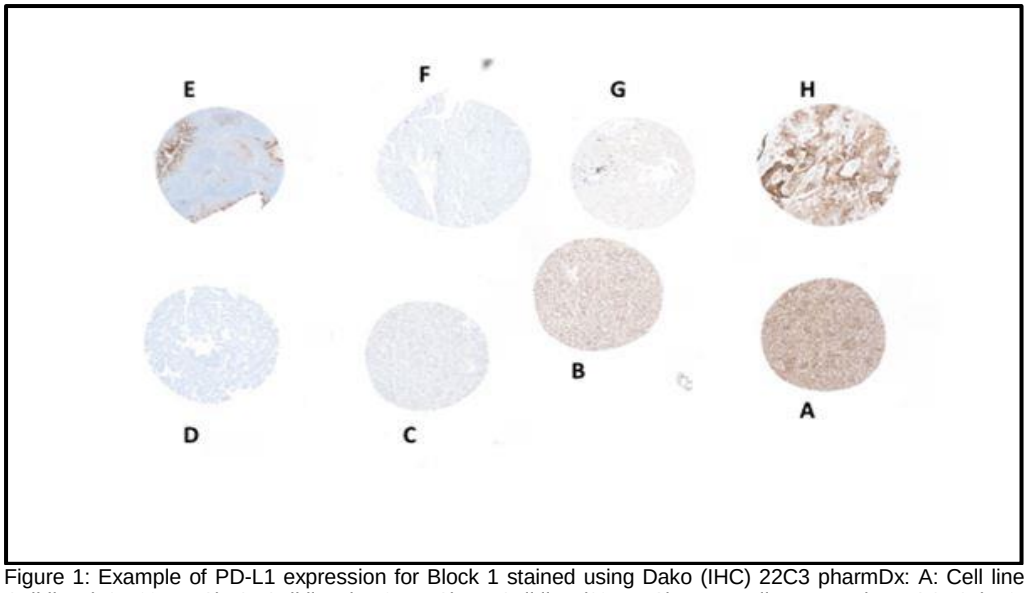

Figure 1: Example of PD-L1 expression for Block 1 stained using Dako (IHC) 22C3 pharmDx: A: Cell line (80-100%TPS); B: Cell line (50-79% TPS); C: Cell line (1-4% TPS); D: Cell line (0% TPS); E: Tonsil; F: Negative NSCLC (<1% TPS); G: Negative NSCLC (<1% TPS); H: Positive NSCLC (80-100% TPS).

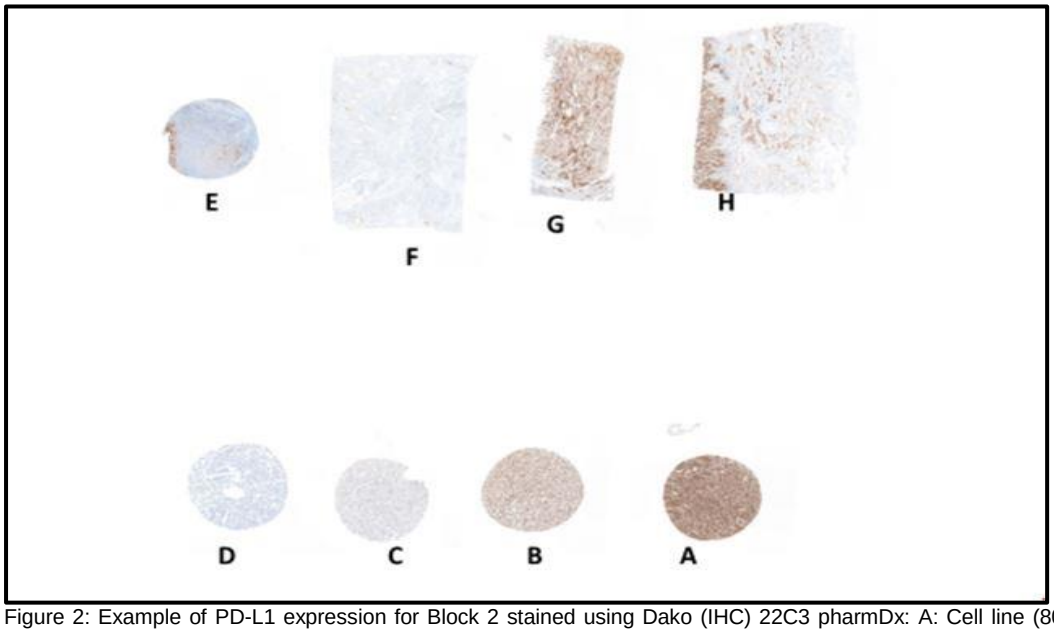

Figure 2: Example of PD-L1 expression for Block 2 stained using Dako (IHC) 22C3 pharmDx: A: Cell line (80-100%TPS); B: Cell line (50-79% TPS); C: Cell line (1-4% TPS); D: Cell line (0% TPS); E: Tonsil; F: Negative NSCLC (<1% TPS); G: Positive NSCLC (80-100% TPS); H: Positive NSCLC (80-100% TPS).

The panel decided the best way to record the TPS was to place each TMA and the participants' in-house control tissue into BIN categories, as outlined in Table 2 below. These BIN cut-off ranges would ensure that the positivity thresholds for each PD-L1 assay would be incorporated. An overall consensus score on technical merit out of 5 was then provided. (4-5/5 Acceptable; 3/5 Borderline; 1-2/5 Unacceptable).

TPS (%)	**<1% (negative)**	**1-5%**	**5-9%**	**10-24%**	**25-49%**	**50-79%**	**80-100%**
BIN	1	2	3	4	5	6	7

Table 2: TPS BIN categories, Abbreviation: TPS, tumour proportion score

Each block and at every 25th serial level, sections were stained by the manufacturers of the Dako/Agilent 22C3 and 28.8 and the Ventana/Roche SP263 and SP142 approved PD-L1 assays. These stained sections were used as the 'Gold standards', and the overall percentage of stained cells for each core of these sections determined the parameters for providing an overall score for each of the participant's slides. The two Dako/Agilent clones (22C3 and 28.8) and the Ventana/Roche SP263 clone showed similar levels of PD-L1 expression, and as expected the Ventana/Roche SP142 clone showed slightly lower levels of PD-L1 expression in the TCs. These are referred to in Tables 3 & 4.

The assessment panel was divided into two separate groups and the slides were reviewed by both groups. Each team of assessors consisted of at least one PD-L1 specialist pathologist who had been trained in the interpretation of all PD-L1 assays. Other assessors included pathologists, biomedical scientists and clinical scientists also trained with the various assays.

Sample Code	Sample	Expected TPS (BIN category) Staining pattern
A (Block 1/2)	Cell line (High)	80-100% Strong membrane staining
B (Block 1/2)	Cell line (Medium)	50-79% Moderate to strong membrane staining
C (Block 1/2)	Cell line (Low)	1-4% Weak membrane staining
D (Block 1/2)	Cell line (negative)	<1% No staining present
E (Block 1/2)	Tonsil	Strong membrane staining in the intra epithelium Moderate punctate staining in germinal centres (Scored as acceptable, borderline or unacceptable)
F (Block 1/2)	Negative NSCLC	<1% Weak in TCs, strong membrane staining in ICs and macrophages
G (Block 1)	Negative NSCLC	0 No tumour present
G (Block 2)	Positive NSCLC	80-100% Moderate-strong membrane staining (heterogenous) in TCs and ICs
7H (Block 1/2)	Positive NSCLC	80-100% Strong membrane staining in TCs and ICs Non-specific staining in fibroblasts

Table 3: Expected PD-L1 expression levels in the UK NEQAS ICC & ISH samples for the approved IHC assays SP263 (Roche), 28.8 and 22C3 (Dako Agilent) . Abbreviations: NSCLC; Non-small cell lung cancer; TC: tumour cell; IC: immune cells.

The slides in each group were assessed around a multi-header microscope of consultant histopathology grade (lenses of planapo quality). A UK NEQAS ICC & ISH scientist was present with each team of assessors to observe, provide support with protocols and to record the overall scores.
Slides were initially scanned on lower power using a x4 objective lens. Assessment of positive staining was then performed at x10 and x20. The x40 objective lens was used only when a more detailed study was required, but was not necessary to derive the result for the TPS. For each sample core the TPS represented a consensus of all observers.

Sample Code	**Sample**	**Expected TPS (BIN category)** **Staining pattern**	**Expected ICs (BIN catergory)**
A (Block 1/2)	Cell line (High)	80-100% Strong membrane staining	
B (Block 1/2)	Cell line (Medium)	10-24% Moderate membrane staining	
C (Block 1/2)	Cell line (Low)	<1% No staining present	
D (Block 1/2)	Cell line (negative)	0% No staining present	
E (Block 1/2)	Tonsil	Strong membrane staining in the intra epithelium Moderate punctate staining in germinal centres (Scored as acceptable, borderline or unacceptable)	
F (Block 1/2)	Negative NSCLC	<1% Weak in TCs, strong membrane staining in ICs and macrophages	<1%
G (Block 1)	Negative NSCLC	0 No tumour present	5%
G (Block 2)	Positive NSCLC	80-100% Moderate-strong membrane staining (heterogenous) in TCs and ICs	5%
H (Block 1/2)	Positive NSCLC	80-100% Strong membrane staining in TCs and ICs Non-specific staining in fibroblasts	1-4%

Table 4: Expected PD-L1 expression levels in the UK NEQAS ICC & ISH samples for the approved IHC SP142 (Roche) assay. Abbreviations: NSCLC; Non-small cell lung cancer; TC: tumour cell; IC: immune cells.

The UK NEQAS ICC & ISH distributed tonsil section (sample E) was assessed as either acceptable (A), borderline (B) or unacceptable (U). Unacceptable or borderline results were mostly due to weak staining. All of the other 7 UK NEQAS samples were assessed with an interpretive percentage BIN score value. In addition, the Ventana/Roche SP142 clone was also provided with an IC percentage score as outlined in Table 4.

In addition to the interpretative score given for each of the eight UK NEQAS ICC & ISH cores, an overall mark out of 5 was given in respect of the expression of PD-L1 within the determined parameters set by the Gold Standards relevant to the PD-L1 clone. Morphological appearance and other technical issues were also taken into consideration when providing the overall mark and assessor feedback. The scoring criteria is described in Table 5.

Score	PD-L1 Demonstration
4-5	Good/Excellent demonstration of PD-L1
3	Acceptable demonstration – slightly weak/strong staining; some of the required components may be missing or there may be non-specific/inappropriate staining present
1-2	Failure to demonstrate the required PD-L1 components

Table 5: Description of results for technical merit for NEQAS and In-house PD-L1 stain

RESULTS

A breakdown of pass rates and methodologies on the NEQAS distributed samples are summarised in Table 6 and Figure 3.

The two most common approved assays used were the Dako 22C3 PharmDx and the Ventana SP263 assay. Only 1 in 7 participants (14%) and 2 in 13 participants (15%) respectively received an unacceptable score. Conversely, 11 participants used the Dako 22C3 as a concentrate or RTU as an LDT with less than 50% (5/11) achieving and acceptable or borderline result. Three participants used the Ventana SP142 assay, with all achieving an acceptable score. Two laboratories employed the SP142 (Spring Bioscience) in diluted form which both yielded unacceptable, weaker than expected results. Six additional laboratories employed LDT methods with other PD-L1 antibody clones. One LDT received an acceptable score using the CST E1L3N PD-L1 clone, optimised at a dilution of 1/100 on the Leica Bond III instrument. Laboratories with borderline (n=3) or unacceptable (n=2) scores predominantly showed weak, diffuse staining and/or damaged morphology. These LDT PD-L1 clones included the Abcam 28-8 PD-L1 clone (n=1), another 28-8 clone (vendor unspecified) (n=1), BioCare CAL10 PD-L1 clone (n=2) and the CST E1L3N (n=2).

Overall, the pass rates show that participants using the PD-L1 approved assays achieved higher results than laboratories using LDT methods (see figure 4 below):

PD-L1 Assay/ Primary Antibody	Automation	Detection Kit	Acceptable	Borderline	Unacceptable	Total n = 43
Dako/Agilent 22C3 PharmDx Assay	Dako Autostainer Link 48	Dako Envision FLEX +	5 (72%)	1 (14%)	1 (14%)	n = 7
Dako/Agilent 22C3 mAb Concentrate	Dako Autostainer	Dako Envision	-	1 (50%)	1 (50%)	n = 2
	Leica BondMaX	Leica Bond Polymer Refine	-	-	1 (100%)	n = 1
	Ventana Benchmark Ultra	Ventana Optiview	1 (17%)	4 (66%)	1 (17%)	n = 6
	Manual Stain	Dako REAL Envision	-	1 (50%)	1 (50%)	n = 2
Dako/Agilent 28-8 PharmDx Assay	Dako Autostainer Link 48	Dako Envision FLEX +		1		n = 1
Ventana/Roche SP263 Assay	Ventana Benchmark	Ventana Optiview	9 (70%)	2 (15%)	2 (15%)	n = 13
Ventana/Roche SP142 Assay	Ventana Benchmark	Ventana Optiview	3 (100%)	-	-	n = 3
Spring Bioscience SP142 mAb Concentrate	Ventana Benchmark Ultra	Ventana Optiview	1 (100%)	-	-	n = 1
	Ventana Benchmark XT	Ventana Ultraview	1 (100%)	-	-	n = 1
Abcam 28-8 mAb Concentrate	Ventana Benchmark XT	Ventana Ultraview	-	-	1 (100%)	n = 1
28-8 Supplier not specified	Not specified	Not specified		1 (100%)		n = 1
Biocare CAL10 mAb Concentrate	Ventana Benchmark Ultra	Ventana Ultraview	-	1 (100%)	-	n = 1
	Leica Bond III	Leica Bond Polymer Refine	-	1 (100%)	-	n = 1
Cell Signaling Technologies mAb E1L3N Concentrate	Ventana Benchmark Ultra	Ventana Ultraview			1 (100%)	n = 1
	Leica Bond III	Leica Bond Polymer Refine	1 (100%)	-	-	n = 1

Table 6: Breakdown of pass rates into methodologies employed. Acceptable (A): Score 4-5/5; Borderline (B): Score 3/5; Unacceptable (U): Score 1-2/5.

It was noted that although some participants using the Ventana clone assays stated they used the manufactures' approved method, not all participants were using the correct detection system: Some participants used the UltraView detection kit which is not the recommended approved method, since it yields slighter weaker staining. Participants should be aware that the approved Ventana assays requires the OptiView detection kit for labelling and amplification. If there is any confusion, participants should contact their Ventana representative to confirm the approved Ventana SP142 and SP263 assay methods.

The groups concluded that the most variation in staining amongst the PD-L1 assays was seen in the cell lines, indicating difficulties in obtaining optimal sensitivity. Weaker or stronger than expected cell line expression and damaged morphology were the main reasons for an unacceptable score. Diffuse and weaker than expected staining in the NEQAS tonsil suggested problems with attaining the required specific-

ity for the test, particularly with those employing their own LDTs. The NSCLC tumour tissue appeared to be more robust, with the least variation in staining observed.

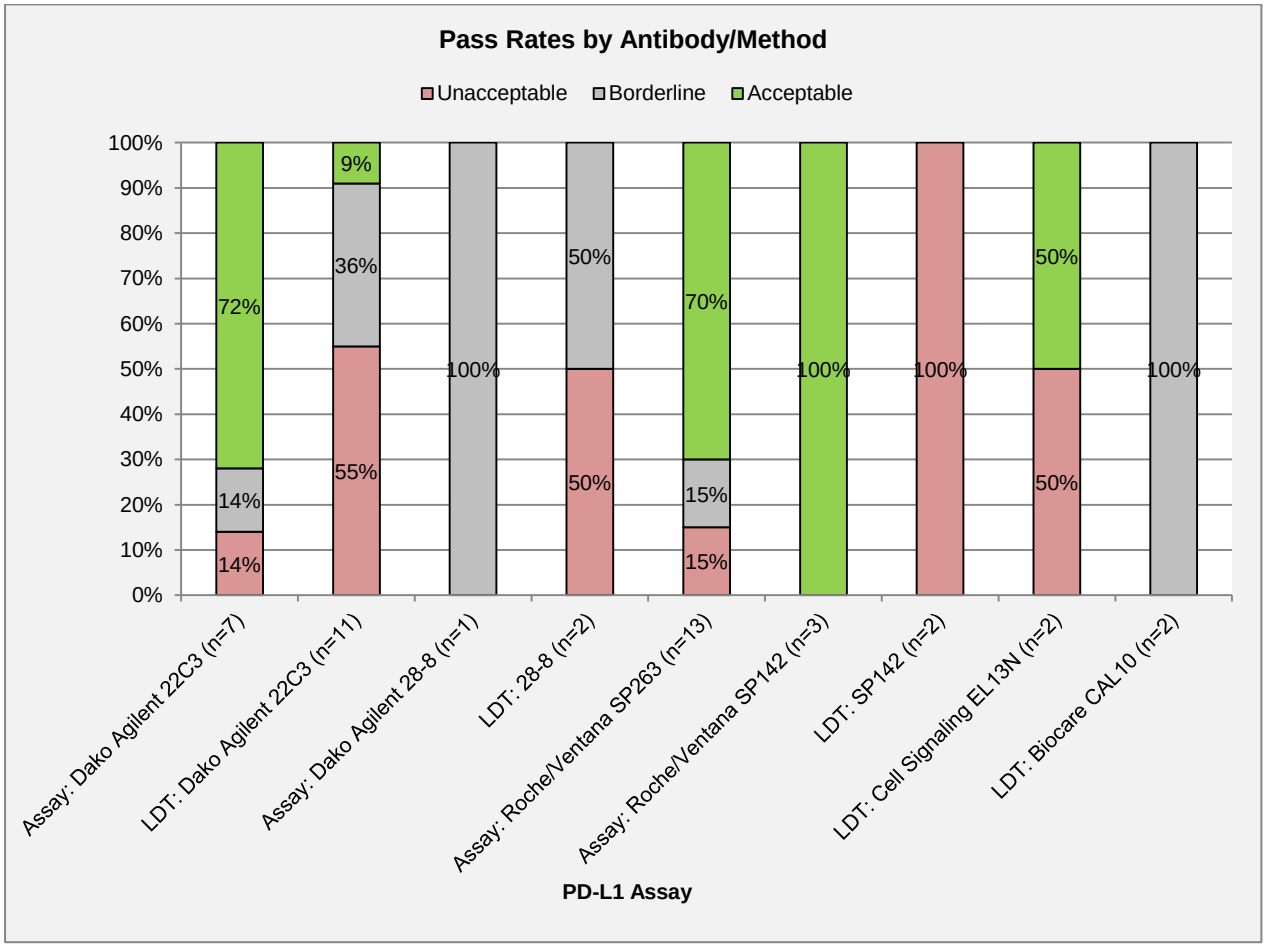

Figure 3: PD-L1 EQA Pre-pilot results overall score summary

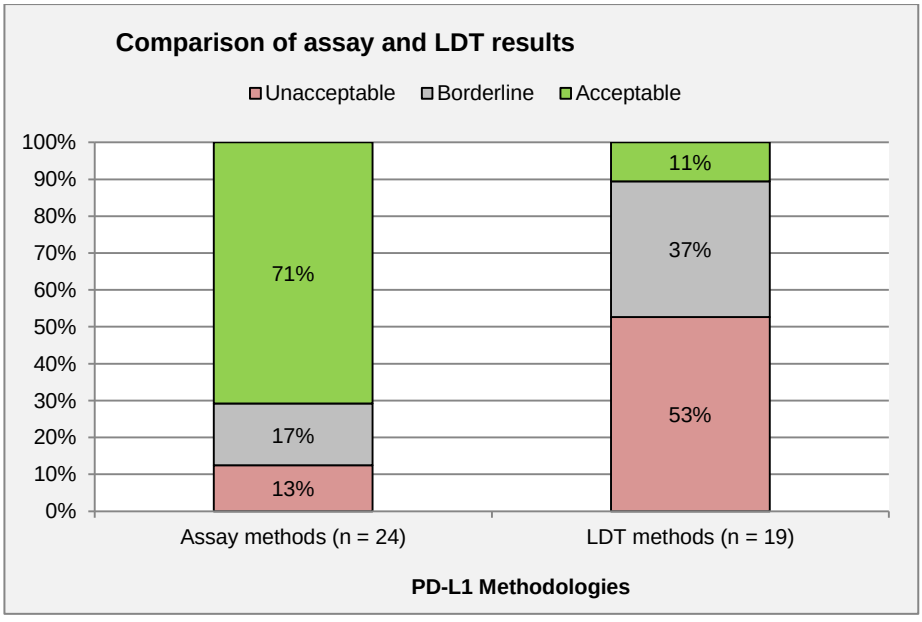

Figure 4: PD-L1 EQA Pre-pilot results comparing assays with LDT score

Images Showing Examples of the Expected PD-L1 Expression and Sub-Optimal Demonstration in the UK Neqas Samples

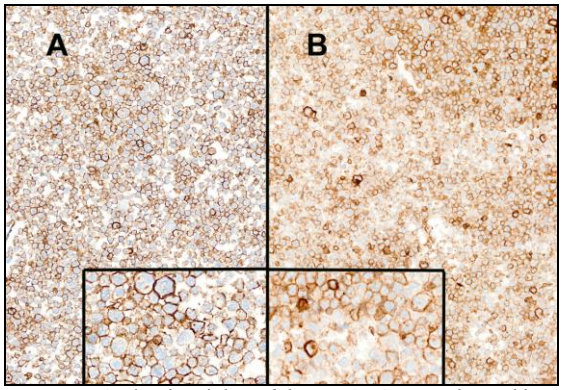

Image 1: Optimal staining of the NEQAS strongly positive cell line (Core A). There is strong membrane staining as expected in 80-100% of the cells. A: Dako 22C3 PharmDx FDA approved assay. B: Ventana SP263 FDA approved assay.

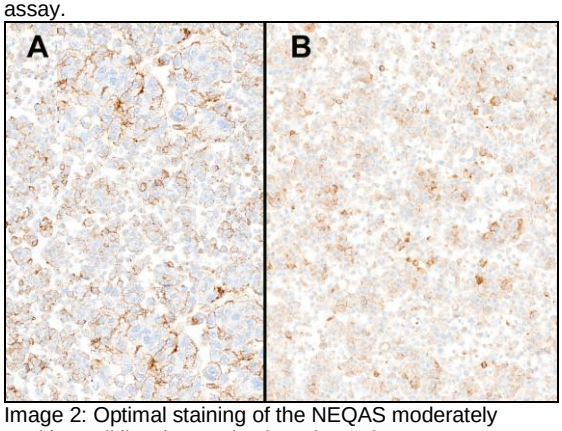

Image 2: Optimal staining of the NEQAS moderately positive cell line (Core B). There is moderate to strong membrane staining in 50-79% of nuclei. A: Dako 22C3 PharmDx FDA approved assay. B: Ventana SP263 FDA approved assay.

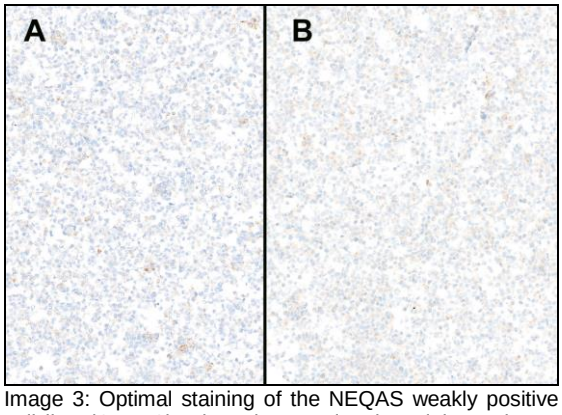

Image 3: Optimal staining of the NEQAS weakly positive cell line (Core C). There is occasional partial membrane staining in the expected 1-4% of tumour nuclei. There is a minimal amount of non-specific cytoplasmic staining present in Image 3B. A: Dako 22C3 PharmDx FDA approved assay. B: Ventana SP263 FDA approved assay.

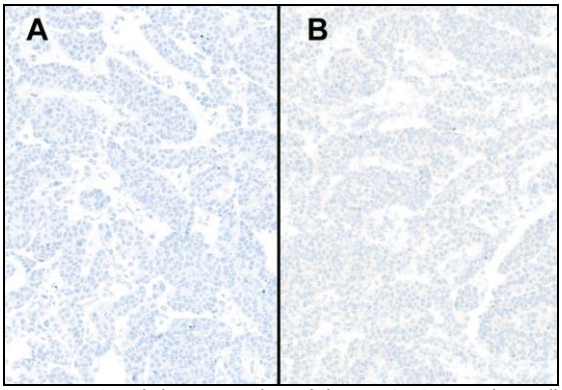

Image 4: Good demonstration of the NEQAS negative cell line (Core D). There is a weak cytoplasmic blush present in figure 4B. A: Dako 22C3 PharmDx FDA approved assay. B: Ventana SP263 FDA approved assay.

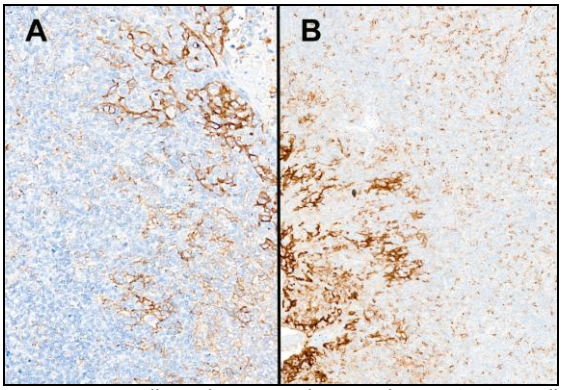

Image 5: Excellent demonstration on the NEQAS tonsil (Core E). There is strong membrane staining in the intra-epithelium and strong to moderate punctate staining in the T-lymphocytes. A: (Block 1) Dako 22C3 PharmDx FDA approved assay. B: (Block 2) Ventana SP263 FDA assay.

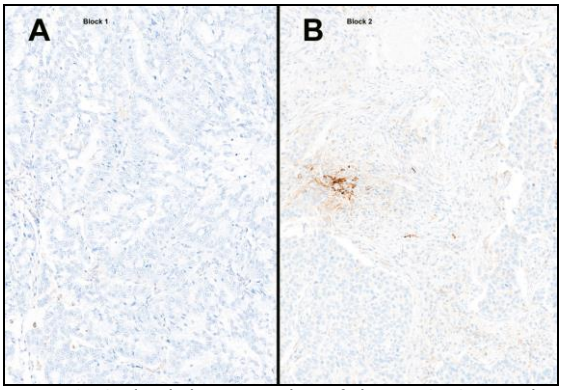

Image 6: Optimal demonstration of the NEQAS negative tumour (Core F). Image 6B (block 2) demonstrates PD-L1 staining of the immune cells within the tumour-stromal interface. A: (Block 1) Dako 22C3 PharmDx FDA approved assay. B: (Block 2) Ventana SP263 FDA approved assay.

Images Showing Examples of the Expected PD-L1 Expression and Sub-Optimal Demonstration in the UK Neqas Samples

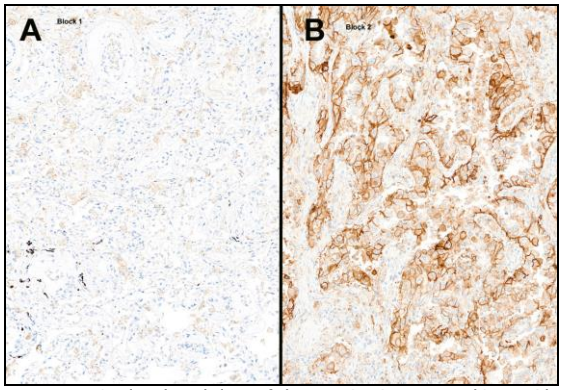

Image 7: Optimal staining of the NEQAS tumour (Core G). Block 1 (7A), is a tumour negative for PD-L1 expression with moderate membrane staining of the tumour macrophages. Block 2 (7B) shows moderate to strong positive expression for PD-L1 in 80-100% of tumour cells. A: Dako 22C3 PharmDx FDA approved assay. B: Ventana SP263 FDA approved assay.

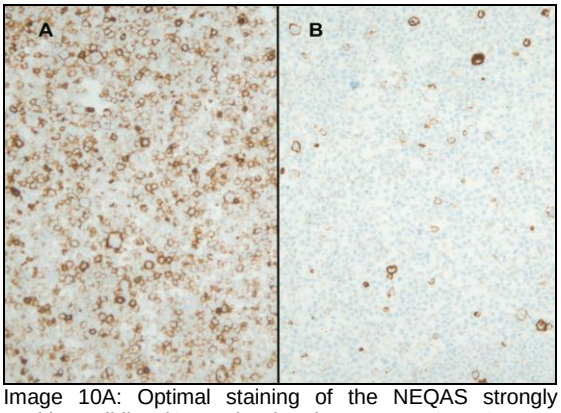

Image 10A: Optimal staining of the NEQAS strongly positive cell line (Core A) using the Ventana SP142 assay in 80-100% of tumour cells. Method: Ventana SP142 FDA approved assay. Figure 10B. Weak demonstration of the Core A using the Ventana SP142 concentrate showing only 5-9% PD-L1 expression. Method: SP142 clone (1:30), Ventana Benchmark XT (CC1 Extended; UltraView)

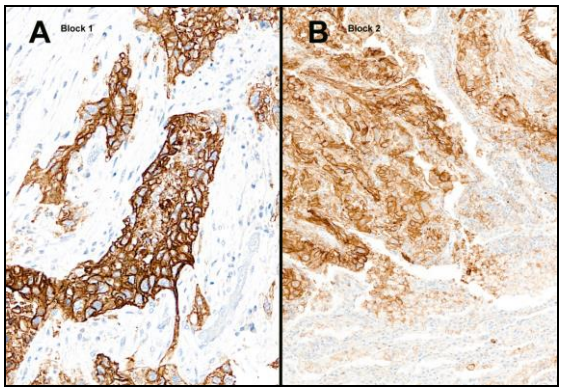

Image 8: Excellent demonstration of the NEQAS positive tumour (Core H). The tumour cells in both Block 1 and Block 2 (Images 8A & 8B respectively) show strong membrane staining in the 80-100%. A: Dako 22C3 PharmDx FDA approved assay. B: Ventana SP263 FDA approved assay.

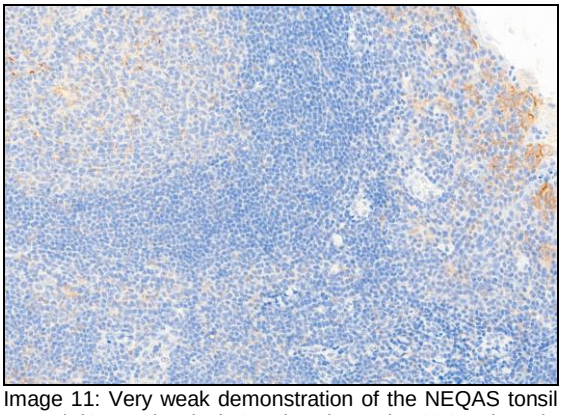

Image 11: Very weak demonstration of the NEQAS tonsil control (Core E), Block 2 using the Dako 22C3 clone in concentrate form. The staining in the intra-epithelium is weak and the lymphocyte staining is weak and patchy also. Method: Dako 22C3 (1/25); BondmaX, (ER2 20'; Bond Polymer Refine)

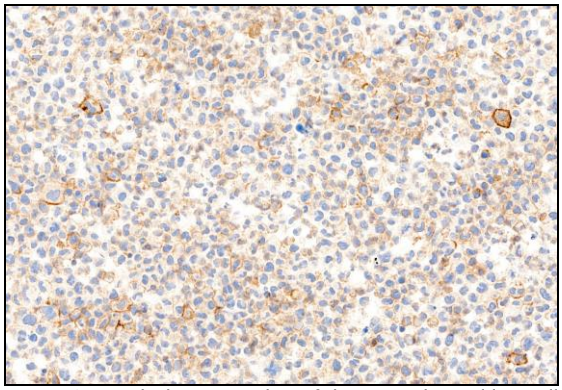

Image 9: Weak demonstration of the strongly positive cell line (Core A) using the Dako 22C3 concentrate. The staining is patchy and diffuse and many positive cells are lacking any membrane staining. The PD-L1 expression has dropped into the 50-79% Bin. (Method: Image 11).

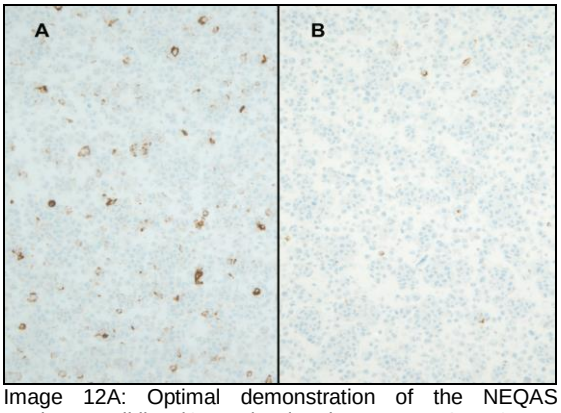

Image 12A: Optimal demonstration of the NEQAS moderate cell line (Core B) using the Ventana SP142 FDA approved assay. Expected expression level is 10-24%. Image 12B: Inappropriate demonstration of Core B with staining in 1-4% of tumour. Method: As in Image 10B.

Images Showing Examples of the Expected PD-L1 Expression and Sub-Optimal Demonstration in the UK Neqas Samples

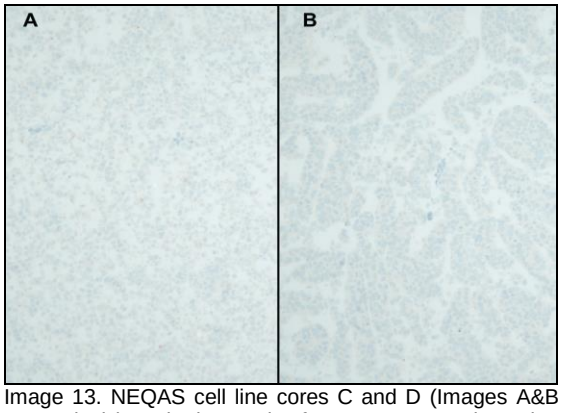

Image 13. NEQAS cell line cores C and D (Images A&B respectively) are both negative for PD-L1 expression using the Ventana SP142 clone. Method: Ventana SP142 FDA approved assay.

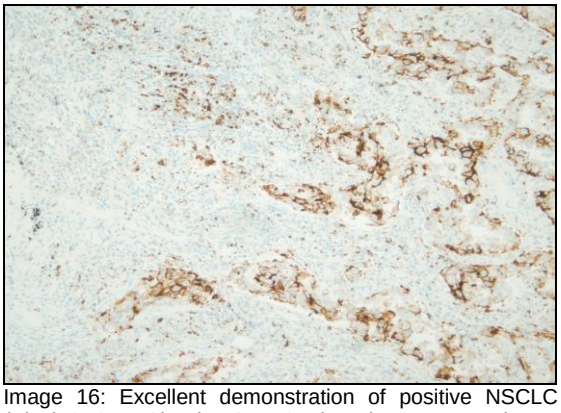

Image 16: Excellent demonstration of positive NSCLC (Block 2, Core G) using SP142. There is strong membrane staining in the tumour and punctate staining of the tumour associated lymphocytes. Method: Ventana SP142 assay.

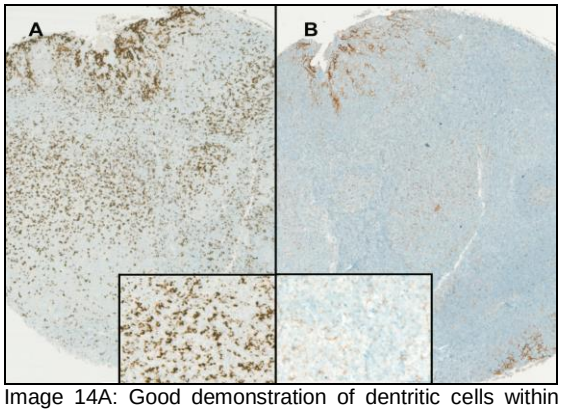

Image 14A: Good demonstration of dentritic cells within the epithelium of the tonsil (Core E). There is strong punctate staining within the germinal centres and T-cells of the paracortical areas. Method: SP142 Identical to Img. 13. Image 14B: Very weak demonstration of the NEQAS tonsil. There is some staining within the tonsil epithelium, however the germinal centres and paracortical areas show weak demonstration of PD-L1. Method: As in Image 10B.

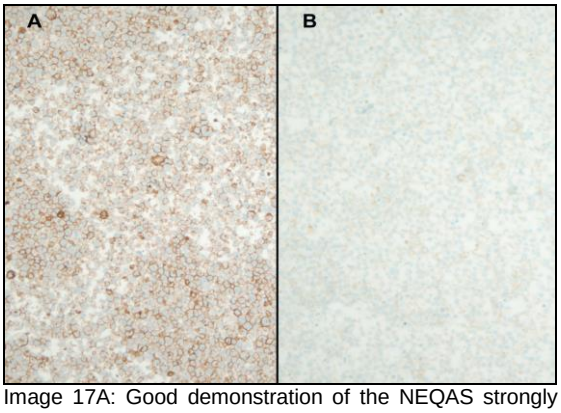

Image 17A: Good demonstration of the NEQAS strongly positive cell line (Core A) using an LDT with staining in 80-100% of tumour cells. Method: CST E1L3N (1/100), Leica Bond III (HMAR: ER2 20'; Detection: Bond Refine kit). Image 17B: Weak demonstration of the NEQAS core A with only 50-79% positive tumour cells using an LDT. Method: Abcam 28.8 (1:50); Ventana Benchmark (HMAR: CC1 Standard; Detection: UltraView).

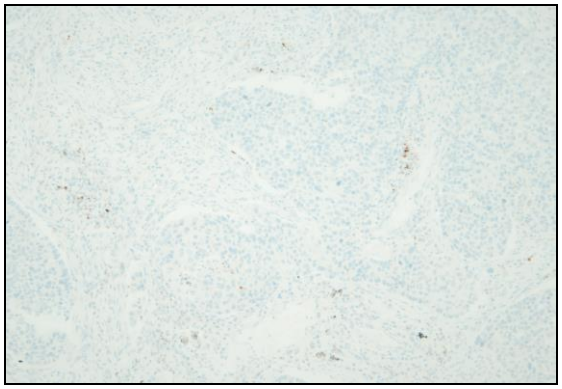

Image 15: Good demonstration of the NEQAS negative tumour (Core F, Block 2) using the Ventana SP142 assay. The tumour is negative, but the immune cells in the intratumoural stroma display a distinct punctate staining.

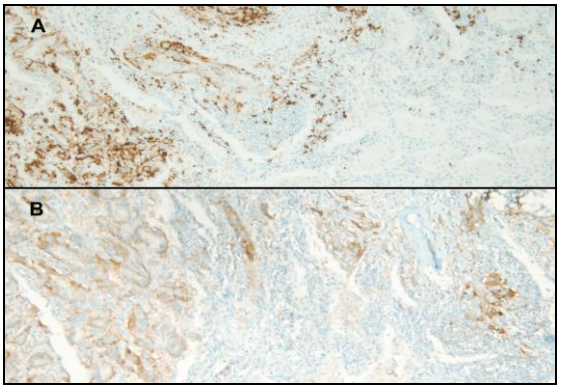

Image 18A: Optimal staining of the positive NSCLC (Block 2, Core H) using SP142 assay. In Image 18B, the staining is weak and diffuse and there is minimal staining of the ICs. Method as in Image 10B.

Images Showing Examples of the Expected PD-L1 Expression and Sub-Optimal Demonstration in the UK Neqas Samples

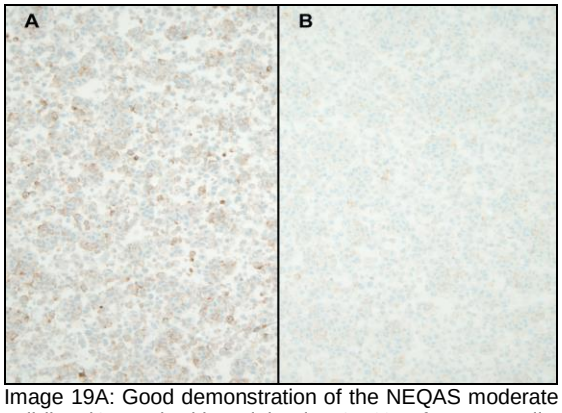

Image 19A: Good demonstration of the NEQAS moderate cell line (Core B) with staining in 50-79% of tumour cells. LDT Method: As in Image 17A. Image 19B: Weak demonstration of Core B using the LDT method outlined in Image 17B.

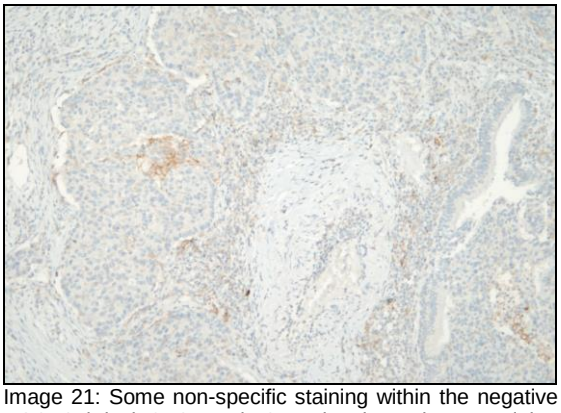

Image 21: Some non-specific staining within the negative NSCLC (Block 2, Core F). Occasional membrane staining may lead to a false-positive diagnosis. Clone: CST E1L3N. Methods not supplied.

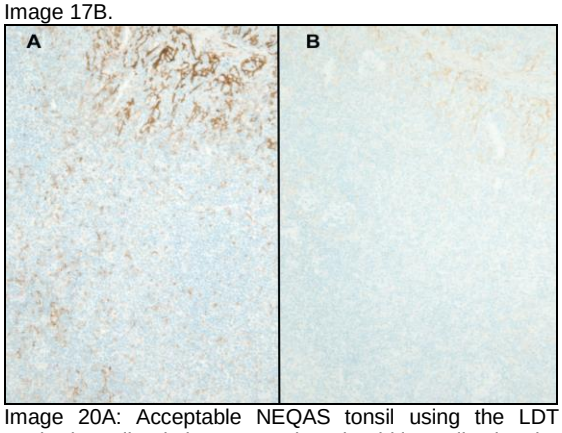

Image 20A: Acceptable NEQAS tonsil using the LDT method outlined in 17A. The dendritic cells in the epithelium are well defined and there is punctate staining within the paracortical and germinal areas. Image 20B: Staining is weak within the epithelium and there is barely perceptible staining within the germinal centres. LDT Method as in Image 17B.

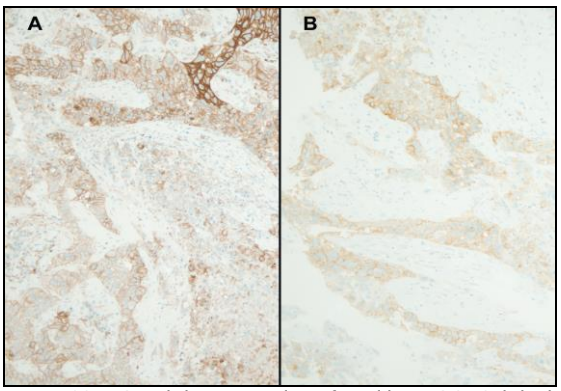

Image 22A: Good demonstration of positive NSCLC (Block 2, Core H). LDT Method as described in Image 17A. Image 22B shows weaker demonstration of the antigen, however the percentage of tumour cells staining is still within the 80-100% range. LDT Method as in Image 17B.

DISCUSSION

An important issue was raised concerning the range of PD-L1 assays being used. At the time of the pre-pilot meeting, only the Dako 22C3 PharmDx assay had been clinically validated for Pembrolizumab which is the only PD-L1/PD-1 treatment approved by the EMA and NICE for NSCLC first and second-line treatment. It was acknowledged that not all UK and European laboratories have access to the Dako Link 48 IHC platform which supports the Dako 22C3 PharmDx assay and therefore, some laboratories have resolved in usin alternative tests. Other options available were the Dako 28-8 PharmDx, Ventana SP263, Ventana SP142 or an LDT. This raised the issue of how the interchanging of the assays by the laboratories may lead to misclassification of PD-L1 status. The cell lines are a useful tool to assess the sensitivity of the assay and control for any changes in assay its dynamic range however there was consensus that a mixture of cell lines, NSCLC tissue (close to the clinical cut-off threshold) and tonsil was ideal to address sensitivity, specificity of the chosen assay/LDT. Laboratories should also note whether the pathologist interpreting the PD-L1 slides has been trained specifically for the PD-L1 assay they are interpreting.

The panel appreciated that it is not possible for every laboratory to offer Companion Diagnostic testing using the approved test for each of the approved drugs since not all laboratories have access to all the staining platforms required by the assay manufacturers and the cost involved in providing each platform to each lab would be prohibitive. As more information emerges about the comparability of the PD-L1 assays as well as LDTs (Scheel et al., 2016, Hirsch et al., 2017), a number of laboratories are opting for the more affordable route of LDTs. Those wishing to provide a service for PD-L1 testing outside the approved assays/platforms have or are developing their own ‘in-house’ methods for PD-L1 expression (LDT) should note that extreme care must be taken to validate rigorously each LDT against the approved assay in the context of the therapy to be given to the patient (by this we mean taking into consideration thresholds for positivity and scoring methods that may include assessment of IC). Additionally, ongoing verification must continue to ensure laboratories can detect any shifts in the sensitivity of their LDTs. It is important to note that laboratories should identify the therapy patients may be receiving dependent on the result of the test they are using in order for their submitted NEQAS slide to be cross referenced against the PD-L1 assay clinically validated to that drug.

A suggestion to participants for an optimal in-house control material is a control which shows a range of expression levels of PD-L1 within NSCLC. This can be demonstrated as either one piece of NSCLC tissue with heterogeneous expression or multiple pieces. A tonsil should also be included for technical demonstration to portray varying levels of PD-L1 in normal tissue. Many laboratories included a piece of tonsil for their own in-house control, which in many cases demonstrated weaker staining than the NEQAS submitted tonsil. This appeared to be due to poor fixation of participants’ own tonsil material. Some laboratories included placenta as their only in-house control material. Product data sheets supplied by PD-L1 assay manufacturers do recommend placenta as a control, however, this is not ideal as placenta contains a high antigenic PD-L1 load with no intermediate levels of expression and

therefore cannot detect drops and peaks in sensitivity levels. Technical staff and pathologists should be aware of the expected staining patterns exhibited within tonsil with respect to the PD-L1 assay they are using (Tables 3 & 4).
Internal positive controls should not be accepted as an adequate stand-alone control as these often contain high levels of antigen concentration. An optimal external control is one that expresses low levels of the antigen in question to monitor the assay's sensitivity (Tsao et al., 2017).

CONCLUSION

The pre-pilot PD-L1 IHC meeting was successful in establishing assessment guidelines for PD-L1 assessment in NSCLC. The findings suggested that the use of a clinically validated PD-L1 IHC assay performs better during assessment than adopting an LDT. However, devising and validating an optimal method against the clinical assay associated with the PD-1/PD-L1 therapy offered and continual verification of the test can produce the expected results. An optimal in-house control for participants would include a dynamic range of PD-L1 expression on NSCLC in addition to a sample of tonsil. The Pilot PD-L1 IHC NSCLC module will be offered with the next upcoming routine modules.

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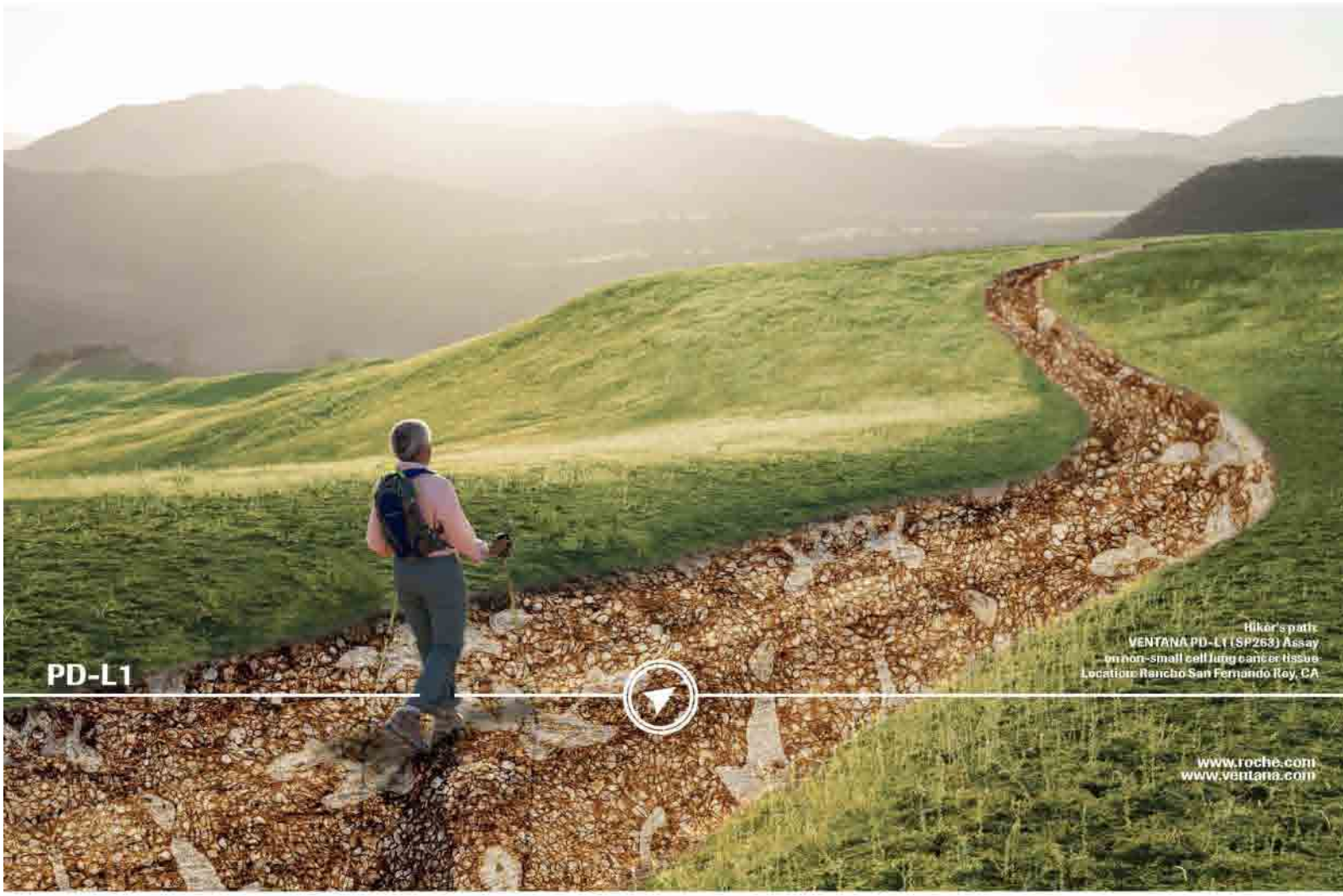

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Registration number 571546. Date of Preparation: November 2016. Material No: 08176230001

Gavin Rock and Suzanne Parry

	Gold Standard	Second Antibody
Antigens Assessed:	Ki-67	E-Cadherin
Tissue Sections Circulated:	Two Breast Carcinoma Sections and Normal Tonsil	Lobular and Ductal Breast Carcinoma Sections
Number of Registered Participants:	317	
Number of Participants this Run	308 (97%)	

Introduction

Gold Standard: Ki-67

Ki-67 is a 345-395 kDa nuclear protein involved in the maintenance and up regulation during the cell division cycle. Ki-67 is present in the cell nuclei in all actively proliferating cells during late G1, S, M and G2 stages of the cell cycle and mitosis, but is not expressed in cells during the resting phase G0 (non-cycling cells). The Ki-67 antigen is used to measure the growth fraction of a given cell population (Ki-67 labelling index), i.e. the percentage of cells staining positive for Ki-67. The Ki-67 labelling index (LI) is used to assess the course of cancer in various solid tumours: It is known to correlate with tumour grade, survival and recurrence. For example, in the lymphoma setting the percentage nuclear staining with Ki-67 is used as a prognostic marker: Tumour grade is closely associated with the percentage of Ki-67 stained nuclei in non-Hodgkin's lymphoma (Brown et al, Hall et al.); with <20% Ki-67 expression seen in low grade lymphomas and >20% Ki-67 expression associated with high grade lymphomas. Furthermore, low grade lymphomas with a labelling index >5% have a worse prognosis than those with an index of <5%. In glial tumours the Ki-67 proliferative index can also be used to aid differentiation between benign (LI <10%) and malignant (LI >10%) lesions. Low grade astrocytomas have a labelling index of 0-5%, while higher grade anaplastic and glioblastomas usually have an index >10% (Torp). In the breast cancer setting, the Ki-67 proliferative index is used as both a predictive and prognostic marker (Dowsett et al).

Features of Optimal Immunostaining:

Tonsil: (Figs 1 & 2)

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

Breast Tumour A: (Fig 3)

- Moderate to strong staining of 10% of tumour cells.
- Clean background with no non-specific staining in the stroma or cell types not expected to stain.

Breast Tumour B: (Fig 4)

- Intense and well-localised nuclear staining in approximately 70% of tumour cells.

Features of Sub-Optimal Immunostaining: (Figs 2 & 5)

- Weak, uneven, partially missing staining of relevant cells.
- Fewer tumour cells staining than expected in the breast.

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Second Antigen: E-Cadherin

E-Cadherin is a calcium-dependent cell-cell adhesion molecule with an important role in all epithelial cell behaviour, tissue formation (Pecina-Slaus, 2003). Studies have shown loss of E-Cadherin either partially or completely is involved with epithelial tumour initiation and progression through mutation, epigenetic signalling and endocytosis (Roy and Berx, 2008). Loss of cell-cell adhesion may arise in cellular detachment leading to tumour metastasis (pathologyoutlines.com). Identifying altered or loss of expression of E-cadherin is particularly useful in differentiating between ductal (E-Cadherin positive) and lobular (E-Cadherin negative) breast carcinomas (Singhai et al, 2011). It can also aid in distinguishing adenocarcinoma from mesothelioma (visabl.com) and is positive in oesophageal adenocarcinoma, squamous carcinoma and transitional cell carcinoma of the bladder (DAKO). Normal staining pattern for E-cadherin is membranous.

Features of Optimal Immunostaining (Figs 7, 9a & 11):

- Moderate to strong membranous staining of all the breast tumour epithelial cells in the breast ductal carcinoma.
- No staining of the breast lobular carcinoma.
- Moderate to strong staining of the benign glands in the breast tumour samples.
- Clean background with no non-specific staining

Features of Sub-Optimal Immunostaining: (Figs 8 & 10)

- Weak, uneven or no staining of tumour epithelial cells in the breast ductal carcinoma.
- Non-specific staining of cell types or components not expected to stain, including staining of the breast lobular carcinoma.
- Weak or no staining of the benign glands in the breast tumour samples.
- Excessive background staining.

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Assessment Summary

Ki67

308 laboratories submitted slides for the Ki67 assessment, and all but 3 submitted their in-house control sections for this run. The results show a slight decrease in acceptable results from Run 115, as shown in the table below:

Ki-67 Pass Rates : NEQAS section		
	Run 115	Run 116
Acceptable	78% (N=237)	74% (N=230)
Borderline	12% (N=37)	14% (N=43)
Unacceptable	10% (N=32)	11% (N=35)

The reason for failure was either due to very weak, lower expression and percentage of cells staining than expected, and in some cases, no staining particularly in the breast samples.

The most popular clone used in this run was Dako M7240 (clone MIB1) used by 140 participants. This showed a pass rate of 65%. Another popular choice was the Ventana 790-429 -86 (clone 30-9), used by 71 participants with a 97% acceptable pass rate. The overall acceptable pass rate of the in-house submissions was 91% compared to the UK NEQAS of 74%. The discordance between the Neqas and in-house results may in part be due to the fact that many laboratories are only using appendix as their in-house positive control, which is most often very strongly stained. The UK Neqas ICC & ISH scheme recommends that best practice is to use a multi –tissue block in-house control, to include normal tissue as well as a known positive tumour. However, laboratories were not penalised for only submitting a single in-house control tissue.

E-Cadherin

299 laboratories submitted slides for the E-Cadherin assessment, and only 1 of these participants did not submit their in-house control. Of those that participated, 221 laboratories (73%) achieved an acceptable score. Results on the participants' in-house samples showed a much higher acceptable pass rate at 91%. See summary of results in the table below:

E-Cadherin Pass Rates Run 116		
	NEQAS	IN-HOUSE
Acceptable	73% (N=221)	91% (N=271)
Borderline	18% (N=53)	6% (N=18)
Unacceptable	8% (N=25)	3% (N=9)

The most common E-cadherin antibody was the Dako M3612 (Clone NCH-38) used by 84 participants, but this only received an acceptable pass rate of 54%. Laboratories using this antibody mostly loss marks due to weak staining. In some cases this was due to an inappropriate antibody dilution or antigen retrieval technique. This Dako clone was also popular in many other forms, such as the RTU and for specific use on the Dako Omnis platform. Overall, this Dako clone was used by 114 participants, and showed an acceptable pass rate of 82%. Other popular antibodies employed were the Leica E-CAD-L-CE (Clone 36B5) and the Ventana 790-4497 (Clone 36) with pass rates of 79% and 90% respectively. 32 participants did not stock the E-cadherin antibody and therefore were given the option to stain with the epithelial membrane antigen (EMA) as an alternative. Most laboratories that stained with EMA received an acceptable pass mark. 16 laboratories did not provide their methodology details.

Selected Images showing Optimal and Sub-optimal Immunostaining

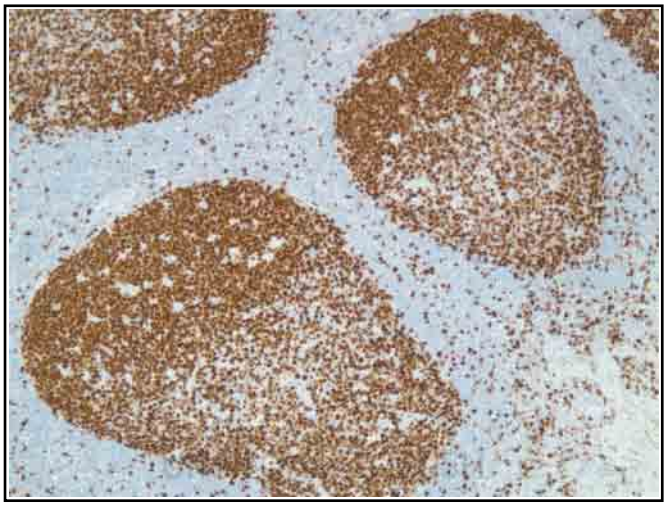


Fig 1. Optimal demonstration of Ki-67 in the UK NEQAS distributed tonsil. The example shows intense and well-localised staining of the germinal centre B-cells while the background remains unstained and clean. Stained with the Dako MIB-1 clone, 1:100, on the Dako autostainer with pre-treatment in the PT Link with high pH retrieval buffer.

Fig 2. Sub-optimal demonstration of Ki-67 in the UK NEQAS distributed tonsil (compare to Fig 1). Thestaining is weak with fewer cells demonstrated than is expected. The corresponding breast tumours in this section had no staining. Stained with the Dako MIB-1 clone, 1:150, on the Leica BondMax with ER2 retrieval for 30 minutes.

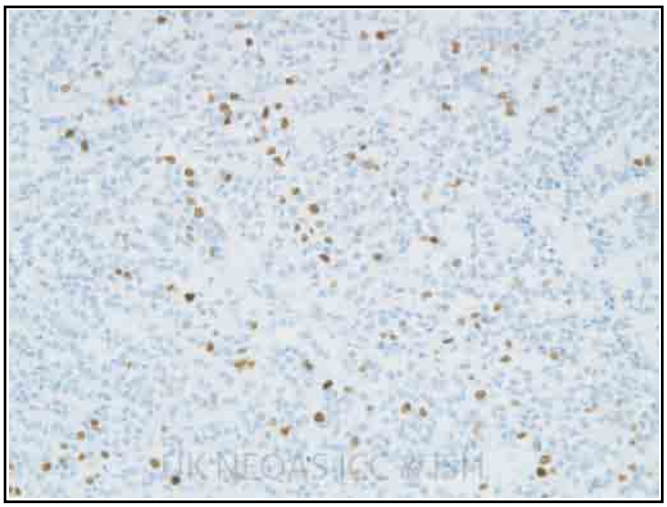


Fig 3. Optimal staining of Ki-67 in the UK NEQAS low expressing breast tumour. As expected the section shows moderate to strong distinct nuclear staining in 10% of the tumour cells. (Same protocol as Fig 1).

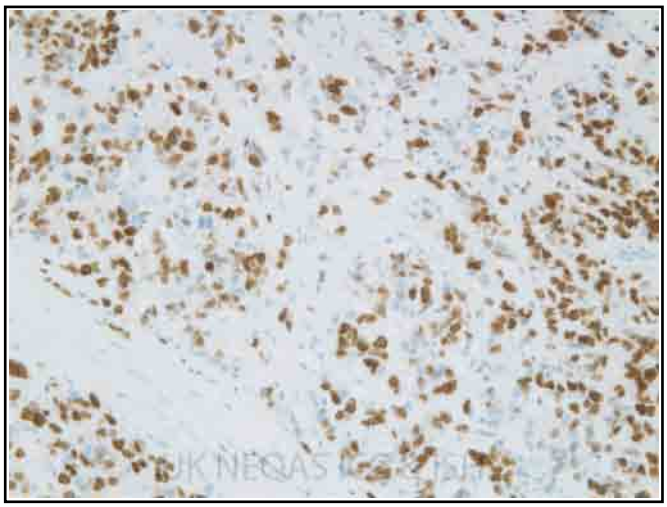


Fig 4. Optimal demonstration of Ki-67 in the UK NEQAS high expressing breast tumour. The example shows the expected level of staining, with 70% of tumour nuclei showing moderate to strong distinct nuclear staining. (Same protocol as Figs 1&3).

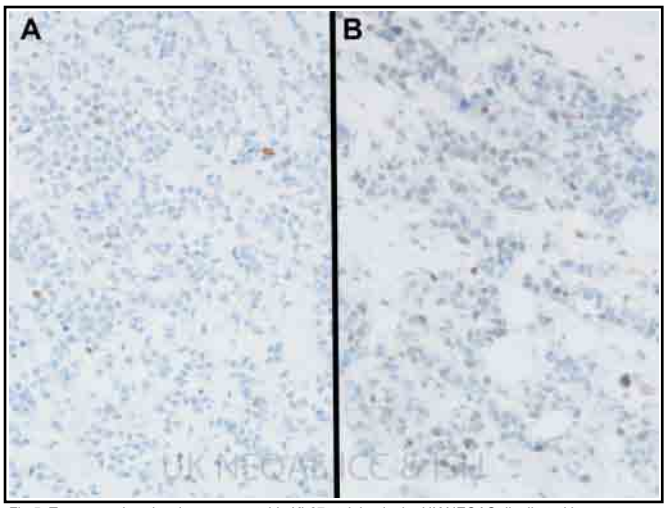


Fig 5. Two examples showing unacceptable Ki-67 staining in the UK NEQAS distributed breast samples (compare to Figs 3&4). Both the low expressing tumour (A) and the high expressing tumour (B) show weak staining and a much lower percentage of positive staining cells than expected. (A): Dako Omnis; (B): Leica BondMax, both with the Dako MIB-1 clone.

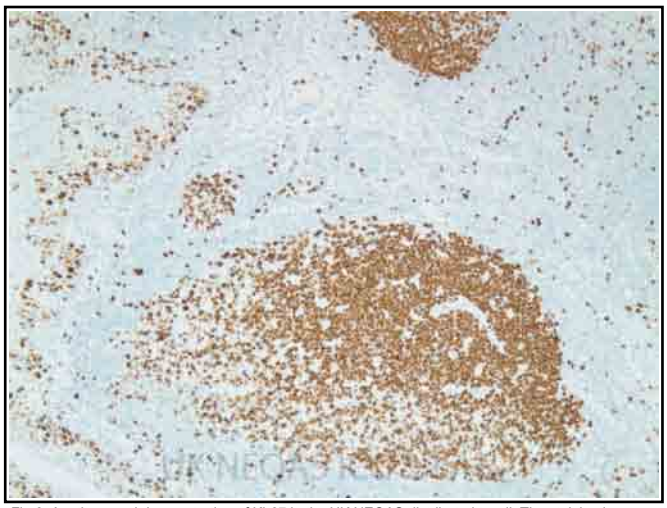


Fig 6. Another good demonstration of Ki-67 in the UK NEQAS distributed tonsil. The staining is intense and well-localised in the germinal centre B-cells and also seen in the supra-basal squamous epithelium. Stained with the Ventana 30-9 pre-diluted antibody on the Ventana Benchmark XT using a CC1 standard protocol.

Selected Images showing Optimal and Sub-optimal Immunostaining

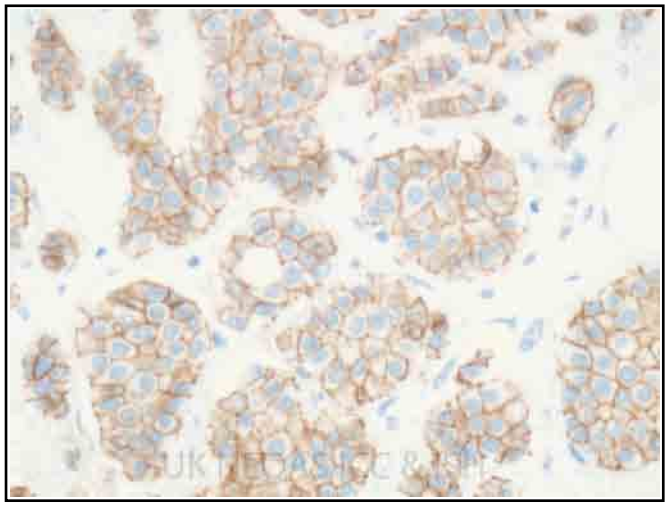


Fig 7. Optimal demonstration of E-Cadherin staining in the UK NEQAS breast ductal carcinoma sample. Most of the tumour cells show strong distinct membranous staining while the background remains clean. Section stained with the Ventana pre-diluted clone 36 antibody on the Benchmark XT with CC1 standard antigen retrieval.

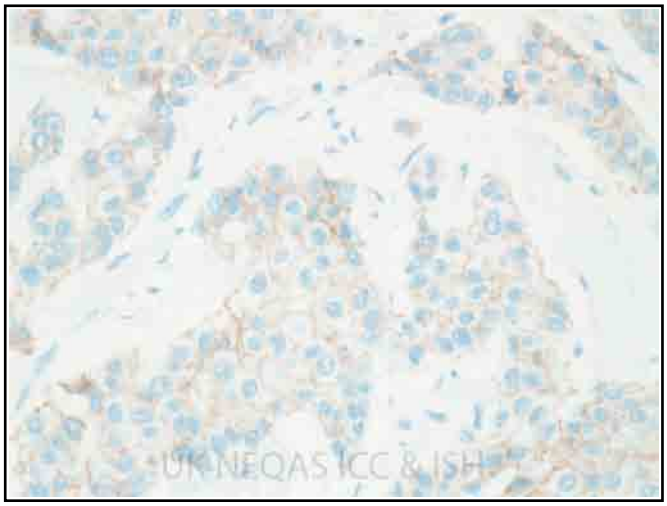


Fig 8. Sub-optimal staining of E-Cadherin in the UK NEQAS breast ductal carcinoma sample. The staining is weak, with some of the tumour cells expected to stain not being demonstrated (compare to Fig 7). Stained with Ventana pre-diluted clone 36 antibody on the Benchmark ULTRA with CC1 mild antigen retrieval.

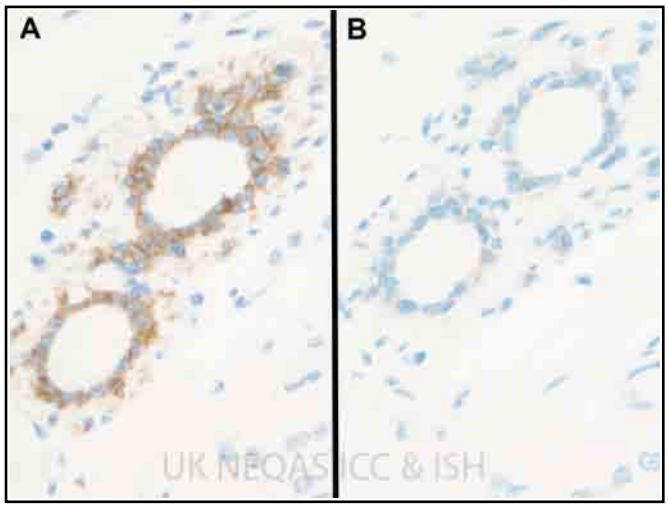


Fig 9. Staining of the benign gland in the UK NEQAS breast lobular carcinoma sample. As expected, the breast lobular carcinoma was negative for E-Cadherin. However, the benign gland within the sample should be positive for E-Cadherin (as seen in section A). Example B shows sub-optimal demonstration with virtually no staining of the benign duct.

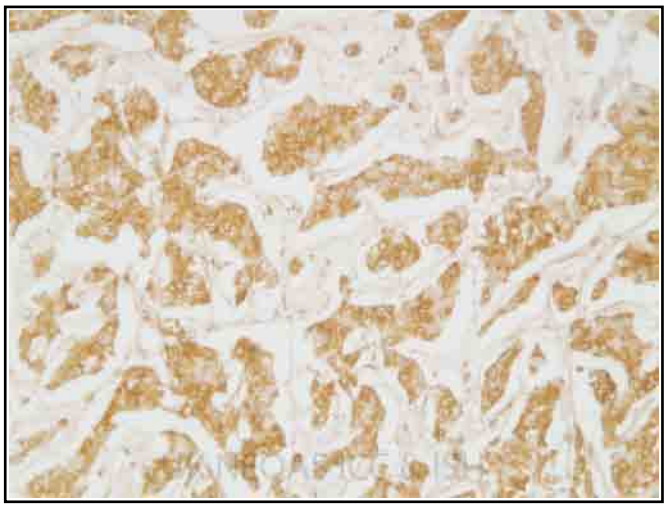


Fig 10. Sub-optimal demonstration of E-Cadherin in the UK NEQAS breast ductal carcinoma sample. Although the tumour is staining as expected, the sample also shows excessive background staining. This is most likely due to inappropriate pre-treatment. Stained with the Menarini EP700Y clone with pre-treatment in the microwave with low pH retrieval buffer.

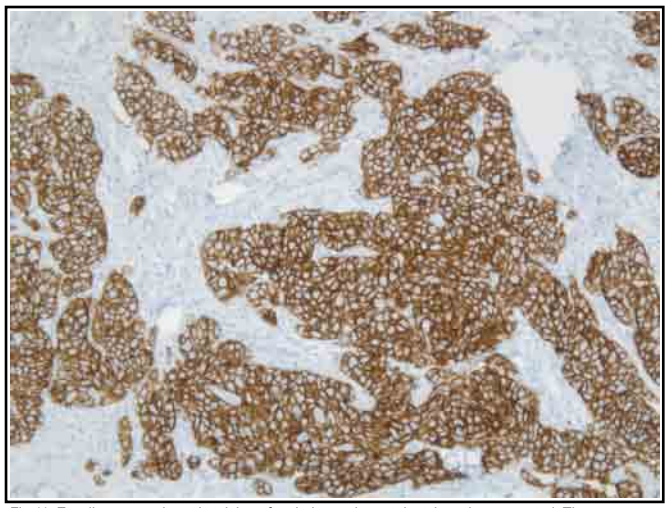
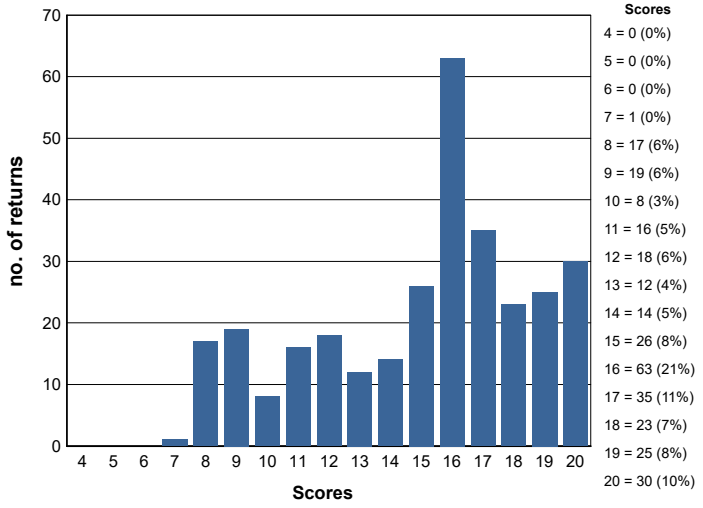


Fig 11. Excellent example and staining of an in-house breast ductal carcinoma control. The membranous staining is strong, while the background remains clean. Stained with the Dako NCH-38 antibody, 1:50 on the Ventana ULTRA, CC1 for 40 Minutes and Optiview detection.

Fig 12. Another good example of a E-Cadherin staining on a participants in house appendix control, showing strong membranous staining of E-Cadherin in the epithelial cells. Stained with the Ventana 36 clone on the ULTRA, CC1 for 64 minutes.

GRAPHICAL REPRESENTATION OF PASS RATES

RUN 116A Ki-67 / MIB1 on NEQAS Sections



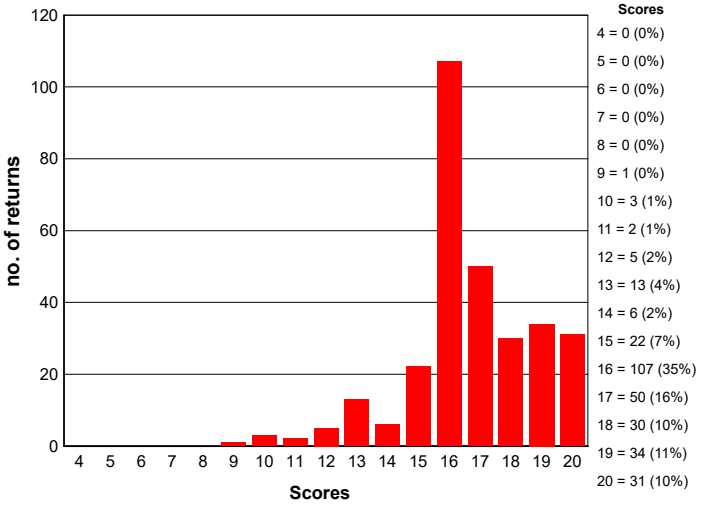
Individual Scores
4 = 0 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 1 (0%)
8 = 17 (6%)
9 = 19 (6%)
10 = 8 (3%)
11 = 16 (5%)
12 = 18 (6%)
13 = 12 (4%)
14 = 14 (5%)
15 = 26 (8%)
16 = 63 (21%)
17 = 35 (11%)
18 = 23 (7%)
19 = 25 (8%)
20 = 30 (10%)

Summary

16 - 20 = 176 (57%)
13 - 15 = 52 (17%)
10 - 12 = 42 (14%)
0 - 9 = 37 (12%)

Median = 13.50

RUN 116B Ki-67 / MIB1 on in-house Sections



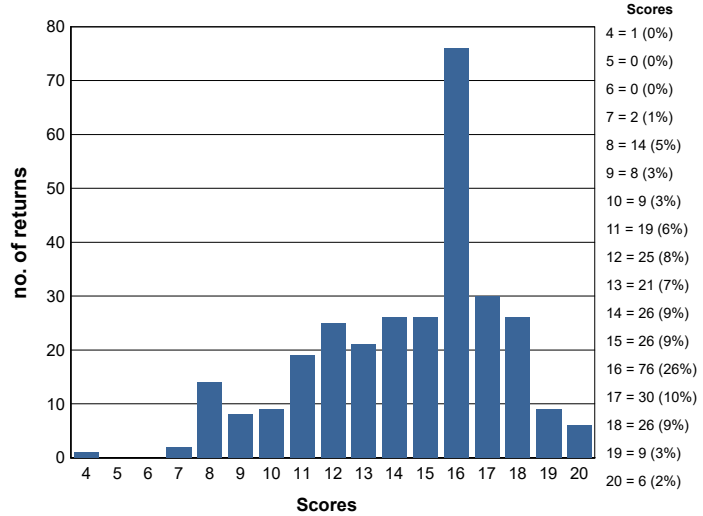
Individual Scores
4 = 0 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 0 (0%)
8 = 0 (0%)
9 = 1 (0%)
10 = 3 (1%)
11 = 2 (1%)
12 = 5 (2%)
13 = 13 (4%)
14 = 6 (2%)
15 = 22 (7%)
16 = 107 (35%)
17 = 50 (16%)
18 = 30 (10%)
19 = 34 (11%)
20 = 31 (10%)

Summary

16 - 20 = 252 (83%)
13 - 15 = 41 (13%)
10 - 12 = 10 (3%)
0 - 9 = 1 (0%)

Median = 14.50

RUN 116C E-Cadherin on NEQAS Sections



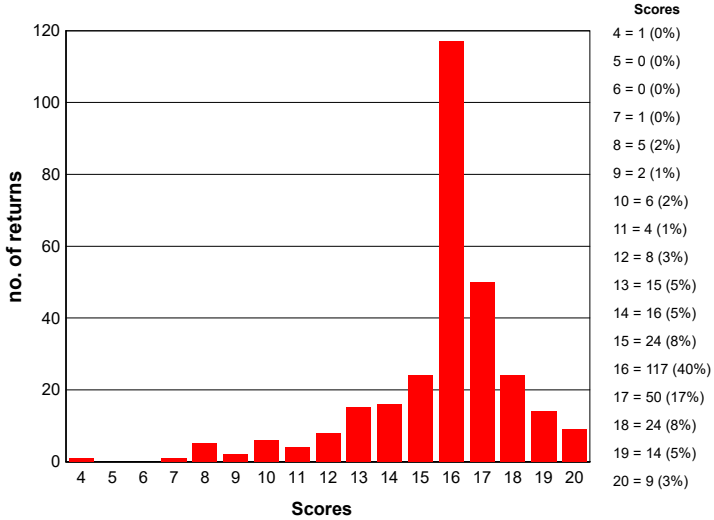
Individual Scores
4 = 1 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 2 (1%)
8 = 14 (5%)
9 = 8 (3%)
10 = 9 (3%)
11 = 19 (6%)
12 = 25 (8%)
13 = 21 (7%)
14 = 26 (9%)
15 = 26 (9%)
16 = 76 (26%)
17 = 30 (10%)
18 = 26 (9%)
19 = 9 (3%)
20 = 6 (2%)

Summary

16 - 20 = 147 (49%)
13 - 15 = 73 (24%)
10 - 12 = 53 (18%)
0 - 9 = 25 (8%)

Median = 13.00

RUN 116D E-Cadherin on in-house Sections



Individual Scores
4 = 1 (0%)
5 = 0 (0%)
6 = 0 (0%)
7 = 1 (0%)
8 = 5 (2%)
9 = 2 (1%)
10 = 6 (2%)
11 = 4 (1%)
12 = 8 (3%)
13 = 15 (5%)
14 = 16 (5%)
15 = 24 (8%)
16 = 117 (40%)
17 = 50 (17%)
18 = 24 (8%)
19 = 14 (5%)
20 = 9 (3%)

Summary

16 - 20 = 214 (72%)
13 - 15 = 55 (19%)
10 - 12 = 18 (6%)
0 - 9 = 9 (3%)

Median = 13.00

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

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

General Pathology Run: 116

Primary Antibody : Ki-67 / MIB1

Antibody Details	N	%
Dako M7240 (clone MIB1)	139	65
Dako N1574 (clone Ki67)	1	100
NeoMarkers RM 9106 (clone SP6)	3	100
Novocastra NCL-Ki67 (clone MM1)	3	67
Ventana 760-2910	3	67
Other	8	75
Dako IR/IS626 (clone MIB-1)	21	71
Leica RTU PA0410 (MM1)	4	25
Leica PA0118 (MM1)	5	40
Leica PA0230 (K2)	29	93
Ventana 790-4286 (clone30-9)	71	97
Cell Marque 275R-14/15/16/17/18 (SP6)	5	60
Dako Omnis GA626 (MIB-1)	11	27

General Pathology Run: 116

Primary Antibody : E-Cadherin

Antibody Details	N	%
Cell Marque AC-0003 (Clone EP6)	1	0
Dako M3612 (Clone NCH-38)	84	54
Leica/Novocastra E-CAD-L-CE (Clone 36B5)	43	70
Leica/Novocastra PA0387 (Clone 36B5) Bond RTU	23	87
ThermoFisher 33-4000 (Clone 4A2C7)	2	100
Ventana 790-4497 (Clone 36)	50	90
Other	48	77
Dako Omnis GA059 (Clone NCH-38)	10	80
Dako IR059 (Clone NCH-38)	15	93
Dako IS059 Flex (Clone NCH-38)	5	100

General Pathology Run: 116

Heat Mediated Retrieval	E-Cadherin N	E-Cadherin %	Ki-67 / MIB1 N	Ki-67 / MIB1 %
Biocare Decloaking Chamber	1	100	2	100
Dako Omnis	12	92	14	29
Dako PTLink	31	58	33	79
Lab vision PT Module	3	100	3	100
Leica ER1 10 mins	1	100	0	0
Leica ER1 20 mins	13	38	5	40
Leica ER1 30 mins	6	83	0	0
Leica ER2 10 mins	1	100	1	100
Leica ER2 20 mins	43	67	60	60
Leica ER2 30 mins	16	50	18	50
Leica ER2 40 mins	0	0	3	67
Microwave	4	25	6	33
None	1	100	1	100
Other	1	0	1	100
Pressure Cooker	2	100	4	75
Steamer	2	100	2	100
Ventana CC1 24mins	4	25	7	86
Ventana CC1 32mins	19	63	24	92
Ventana CC1 36mins	8	75	12	83
Ventana CC1 40mins	6	100	4	100
Ventana CC1 48mins	4	100	5	80
Ventana CC1 52mins	4	100	2	100
Ventana CC1 56mins	8	100	6	83
Ventana CC1 64mins	35	91	36	94
Ventana CC1 76mins	1	0	0	0
Ventana CC1 88mins	1	100	1	100
Ventana CC1 8mins	3	67	0	0
Ventana CC1 92mins	0	0	1	100
Ventana CC1 extended	1	100	0	0
Ventana CC1 mild	6	50	12	75
Ventana CC1 standard	30	90	31	84
Ventana CC2 24mins	0	0	1	100
Ventana CC2 36mins	0	0	1	100
Ventana CC2 44mins	1	100	1	100
Ventana CC2 48mins	1	100	0	0
Ventana CC2 64mins	2	100	1	100
Water bath 68 OC	1	100	1	100
Water bath 95-98 OC	1	0	2	50

General Pathology Run: 116

Enzyme Mediated Retrieval	E-Cadherin N	E-Cadherin %	Ki-67 / MIB1 N	Ki-67 / MIB1 %
AS PER KIT	6	83	2	100
Dako Proteinase K (S3020)	0	0	1	100
Menarini protease XXIV (P8038)	1	100	0	0
NOT APPLICABLE	118	71	173	74
Other	1	100	0	0
Ventana Protease	0	0	1	100

General Pathology Run: 116	E-Cadherin		Ki-67 / MIB1	
Detection	**N**	**%**	**N**	**%**
A Menerini Polymer (MP-XCP)	0	0	1	100
AS PER KIT	23	70	17	59
Dako EnVision FLEX (K8000/10)	5	60	10	40
Dako EnVision FLEX+ (K8002/12)	24	58	27	74
Dako Envision HRP/DAB (K5007)	2	100	5	80
Dako Envision+ HRP mouse K4004/5/6/7	4	100	2	50
Dako rb-a-mo Ig (E0354)	0	0	1	100
Dako REAL HRP/DAB (K5001)	0	0	1	0
LabVision UltraVision LP HRP (TL 125 HLJ)	1	100	0	0
LabVision UltraVision ONE Polymer (TL-012/5-HDJ/T	0	0	1	100
Leica Bond Polymer Define (DS9713)	1	100	1	0
Leica Bond Polymer Refine (DS9800)	71	62	80	60
MenaPath X-Cell Plus (MP-XCP)	2	100	1	100
None	0	0	1	100
Novocastra Novolink PDS (RE7-140/150/280/290-K)	1	100	1	100
Other	9	78	9	56
Vector Elite ABC (PK-6102)	1	0	0	0
Ventana iView system (760-091)	2	50	2	100
Ventana OptiView (760-700) + Amp. (7/860-099)	5	60	5	100
Ventana OptiView Kit (760-700)	47	85	54	93
Ventana UltraView Kit (760-500)	67	82	80	84

General Pathology Run: 116	E-Cadherin		Ki-67 / MIB1	
Automation	**N**	**%**	**N**	**%**
Dako Autostainer	1	100	2	50
Dako Autostainer Link 48	32	59	31	81
Dako Autostainer Plus Link	1	100	3	67
Dako Omnis	12	92	14	29
LabVision Autostainer	1	100	2	100
Leica Bond Max	25	64	33	52
Leica Bond-III	55	62	54	61
Menarini - Intellipath FLX	2	100	2	100
None (Manual)	7	43	9	67
Shandon Sequenza	1	100	2	50
Ventana Benchmark GX	4	100	5	100
Ventana Benchmark ULTRA	100	81	101	90
Ventana Benchmark XT	37	81	45	80

General Pathology Run: 116	E-Cadherin		Ki-67 / MIB1	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	39	74	30	73
BioGenex liquid DBA (HK-124-7K)	1	0	1	0
DAKO DAB+	1	100	1	0
Dako DAB+ Liquid (K3468)	3	100	4	100
Dako DAB+ REAL Detection (K5001)	1	100	1	100
Dako EnVision Plus kits	3	0	3	33
Dako FLEX DAB	33	70	37	68
Dako REAL EnVision K5007 DAB	2	100	3	100
Dako REAL K5001 DAB	0	0	1	0
Leica Bond Polymer Refine kit (DS9800)	69	65	81	58
menapath xcell kit DAB (MP-860)	2	100	2	100
Other	15	53	16	94
Sigma DAB (D5637)	1	100	1	100
Sigma DAB (D5905)	0	0	1	100
Ventana DAB	32	84	36	86
Ventana iview	2	50	2	100
Ventana Ultraview DAB	79	82	83	84

BEST METHODS - Gold Standard Antibody

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

Ki-67 / MIB1 - Method 1

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

Primary Antibody: Dako M7240 (clone MIB1) , 30 Mins, ambient ºC Dilution 1: 100

Automation: Dako Autostainer Link 48

Method: Dako FLEX+ kit

Main Buffer: Dako FLEX wash buffer

HMAR: Dako PTLink, Buffer: DAKO target retrieval high pH, PH: 9

EAR:

Chromogen: Dako FLEX DAB, ambie ºC., Time 1: 5 Mins, Time 2: 5 Mins

Detection: Dako EnVision FLEX+ (K8002/12) , 20 Mins, ambient ºC Prediluted

Ki-67 / MIB1 - Method 2

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

Primary Antibody:	Ventana 790-4286 (clone30-9) , 16 Mins, RT ºC Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
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

Ki-67 / MIB1 - Method 3

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

Primary Antibody:	Leica PA0230 (K2) , 30 Mins, rt ºC Prediluted
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), rt ºC., Time 1: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, rt ºC Prediluted

Ki-67 / MIB1 - Method 4

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

Primary Antibody:	Dako M7240 (clone MIB1) , 32 Mins, ROOM ºC Dilution 1: 150
Automation:	Ventana Benchmark ULTRA
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)

BEST METHODS - Secondary Antibody

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

E-Cadherin - Method 1

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

Primary Antibody:	Dako Omnis GA059 (Clone NCH-38) , 25 Mins, 32 ºC Prediluted
Automation:	Dako Omnis
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis, Buffer: Dako TRS High pH, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 32 ºC., Time 1: 5 Mins
Detection:	Dako Envision+ HRP mouse K4004/5/6/7 , 10 Mins, 32 ºC Prediluted

E-Cadherin - Method 2

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

Primary Antibody:	Ventana 790-4497 (Clone 36) , 16 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana apk Wash (250-042)
HMAR:	Ventana CC1 40mins
EAR:	
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700)

E-Cadherin - Method 3

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

Primary Antibody:	Leica/Novocastra E-CAD-L-CE (Clone 36B5) , 15 Mins, 21 ºC Dilution 1: 25
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, 21 ºC

E-Cadherin - Method 4

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

Primary Antibody:	Dako IR059 (Clone NCH-38) , 15 Mins, 20 ºC Prediluted
Automation:	Dako Autostainer Plus Link
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: Dako target retrieval solution high pH
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 20 ºC., Time 1: 10 Mins, Time 2: 10 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 15 Mins, 20 ºC Prediluted

The Breast Hormonal Receptor Module Run 116

Keith Miller 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:	282
Number of Participants This Run	270 (96%)

Table below shows the expected staining characteristics of the UK Neqas distributed tissue for Run 116: This composed of three infiltrating ductal carcinomas (IDCs) with differing levels of ER expression and a normal tonsil section.

Sections	% Positivity	Expected Staining Intensity	Allred / Quick Score
A. IDC	>95%	High	8
B. IDC	11-33%	Mid	6 *
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 occasions be variability in staining from the Allred 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 of in-house material, due to pre-analytics - Poor choice of in-house material	

In-House Tissue Recommendations:

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

The required composite control should consist of the following samples:

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

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

Introduction

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

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

oestrogen based treatments such as Tamoxifen and aromatase-inhibitors (Fisher et al). PR expression is under

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

Choice of Tissue for Assessments

The Run 116 assessment consisted of three invasive breast tumours of varying ER expression level (samples A-C). A tonsil (sample D) was also included and used as a further negative control to help gauge the specificity and sensitivity of the test.

NEQAS tissue samples were tested by staining every 50th serial section from the relevant tissue blocks using the Leica

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

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

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

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

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

- False positive or false 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

270 laboratories submitted their slides for the ER assessment. The acceptable pass rate has risen since the previous ER run (115) with similar results to Run 113. Please see table below:

ER NEQAS Pass Rates :			
	Run 113	**Run 115**	**Run 116**
Acceptable	88%(N=239)	75%(N=197)	84%(N=228)
Borderline	7%(N=18)	18%(N=47)	10%(N=27)
Unacceptable	5%(N=14)	8%(N=20)	6%(N=15)

The borderline and failed marks for this assessment were mostly due to weak ER expression especially in the UK Neqas mid-expressing ER breast tumour sample.

In-House Tissue Assessment Results

99% of participants also submitted their in-house controls for assessment. Overall these showed a similar acceptable pass rate, to the NEQAS section with more participants receiving a borderline pass and fewer laboratories failing on their in-house material. The borderline passes were mostly due to excessive cytoplasmic staining, or when a laboratory had not included a mid-expressing tumour, which is a requirement of the assessment.

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 discordance: For instance, slides may not have been stained soon after receipt, and therefore causing loss of antigenicity. UK NEQAS advises that slides are stained as soon as possible on receipt to the laboratory. If the Neqas samples have been left at room temperature for some time 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, compared to a freshly cut in-house control stained with the same optimal protocol. Another factor to take into consideration is that although the sensitivity of a participant's assay may be acceptable on their own tissue section, the sensitivity of the assay still may not be optimal. A protocol should be in place that will stain effectively on both the NEQAS samples and the labs own samples.

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

Comparing NEQAS Sample Scores and In-house Control Scores.

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

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

References

1. Jennet M. Harvey, Gary M. Clark, C. Kent Osborne, and D. Craig Allred (1999) Estrogen Receptor Status by Immunohistochemistry Is Superior to the Ligand-Binding Assay for Predicting Response to Adjuvant Endocrine Therapy in Breast Cancer J Clin Oncol 17:1474-1481.
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4. Fisher B, Costantino J, Redmond C, Poisson R, Bowman D, Couture J, Dimitrov NV, Wolmark N, Wickerham DL, Fisher ER, et al. (1989) A randomized clinical trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen receptor-positive tumors. N Engl J Med 1989: 479-484.
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7. Ibrahim M, Dodson A, Barnett S, Fish D, Jasani B, Miller K. (2008) Potential for false-positive staining with a rabbit monoclonal antibody to progesterone receptor (SP2): findings of the UK National External Quality Assessment Scheme for Immunocytochemistry and FISH highlight the need for correct validation of antibodies on introduction to the laboratory. Am J Clin Pathol. 129:398-409.
8. Davies C, Godwin J, Gray R et al., (2011) Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. The Lancet 378: 771-784.

Acknowledgments

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

Selected Images showing Optimal and Sub-optimal Immunostaining

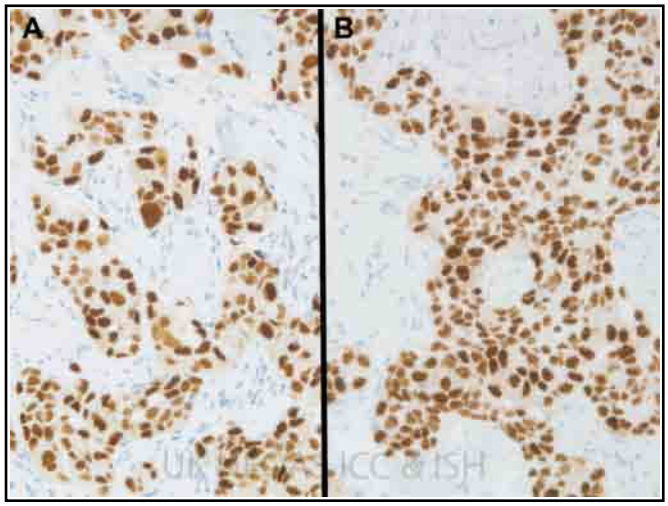


Fig 1. 2 examples showing optimal staining of the UK NEQAS high expressing ER positive tumour. In both images the nuclei are stained strongly in over 95% of the tumour cells. (A) Stained with the Dako EP1 antibody, 1:20, on the Leica Bond III, ER2 retrieval. (B) Stained with the Dako SP1 antibody,1:40, on the Dako autostainer, PT link retrieval.

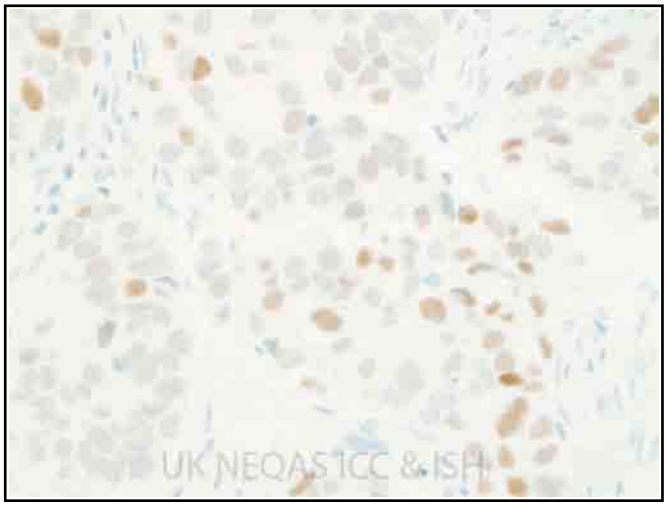


Fig 2. Unacceptable ER staining of the UK NEQAS high expressing tumour (compare to Figs 1A&1B). The staining is very weak with far fewer positive tumour cells demonstrated than expected. The slide also showed no staining of the UK NEQAS mid-high-expressing tumour also present on the slide.

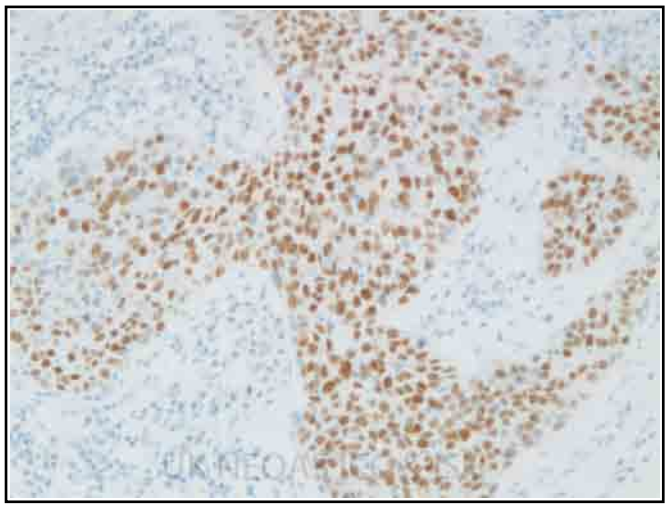


Fig 3. Acceptable level of ER staining in the UK NEQAS distributed breast tumour sample B. The example shows the mid-high expressing tumour. However, some laboratories received sections from a breast tumour sample with an Allred score of 5-6. (Same protocol as Fig 1B).

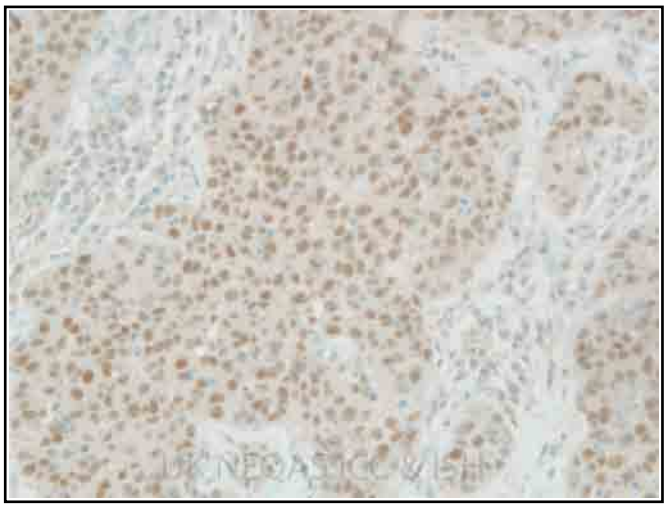


Fig 4. Sub-optimal ER staining of the UK NEQAS distributed breast tumour sample B (compare to Fig 3): The level of staining in the tumour cells is much higher than expected with excessive cytoplasmic staining, and the stromal cells also show inappropriate background staining. Excessive antigen retrieval is most likely to be the cause of the excessive staining.

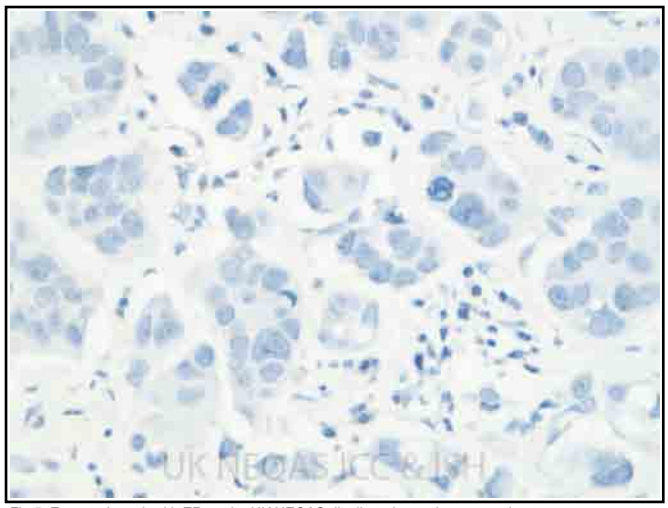


Fig 5. Expected result with ER on the UK NEQAS distributed negative expressing tumour sample C. Although not shown here, the high and mid-expressing tumours also stained as expected. The Ventana SP1 pre-diluted antibody was used on the ULTRA with retrieval for 36 minutes with CC1 buffer.

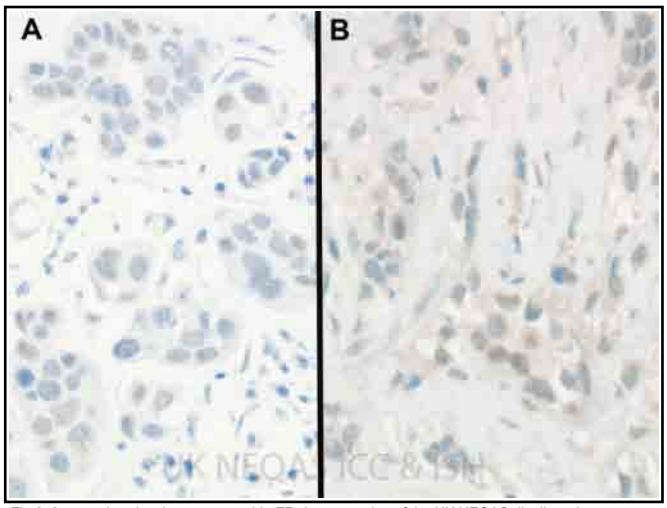
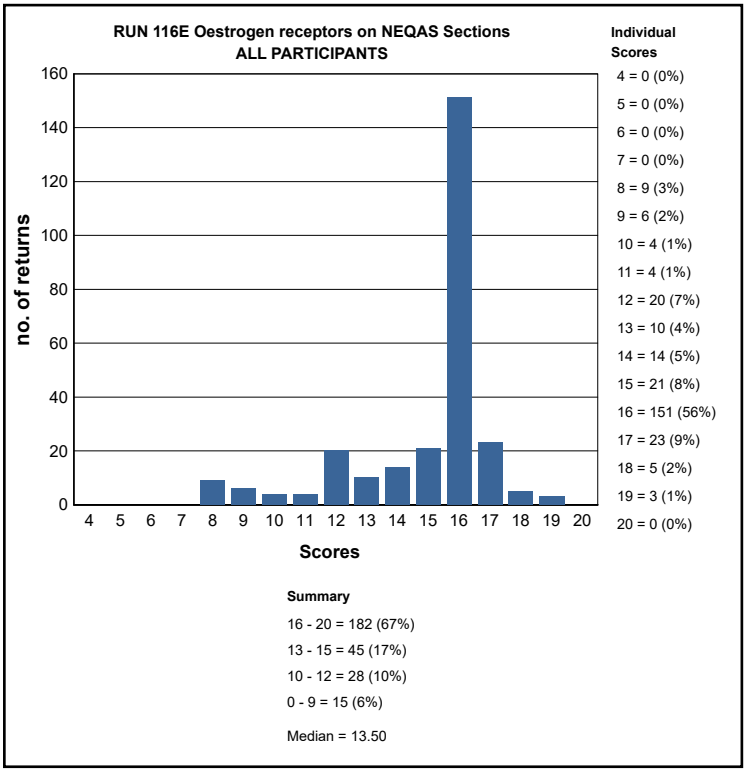
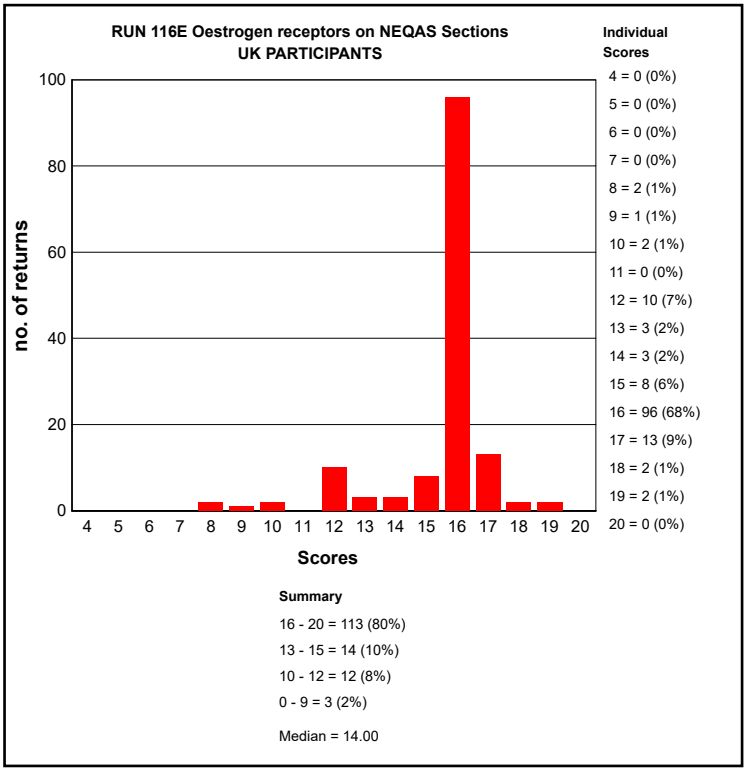
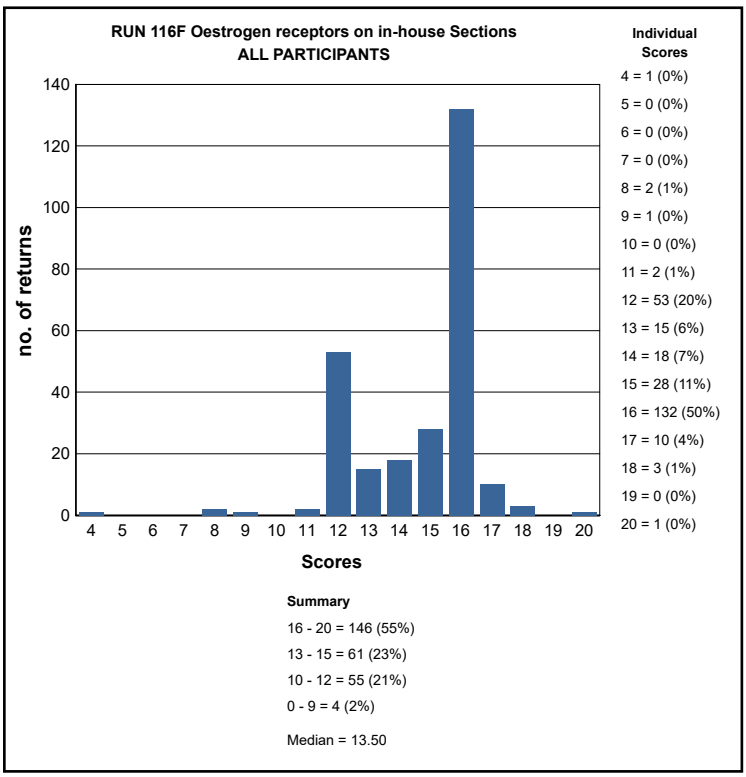


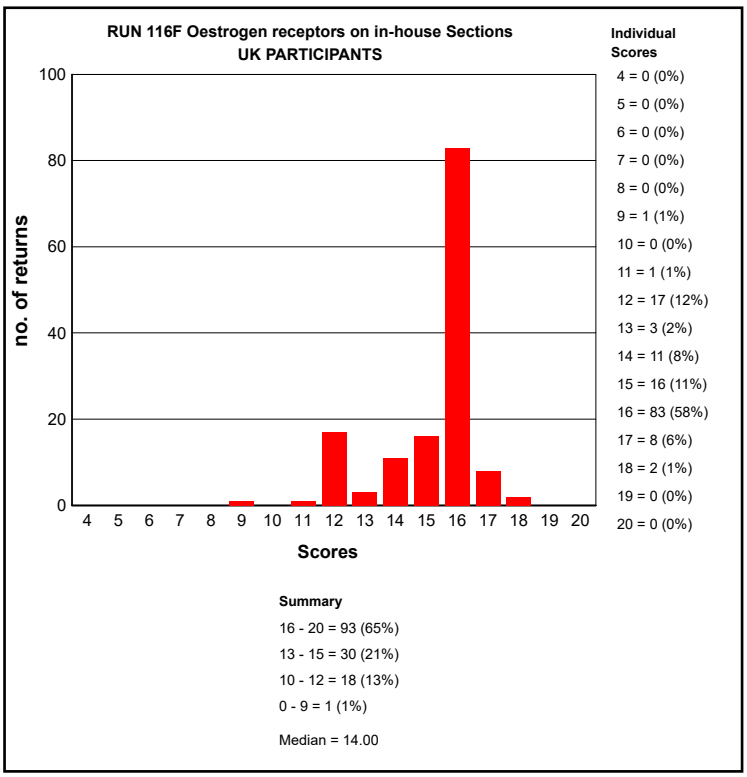
Fig 6. 2 examples showing unacceptable ER demonstration of the UK NEQAS distributed negative expressing tumour sample C (compare both examples to Fig 5). (A) shows false positive staining of the tumour cells known to be negative, and (B) shows excessive non-specific staining and what also appears to be false-staining of the tumour cells.

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: 116

Primary Antibody : Oestrogen receptors

Antibody Details	N	%
Dako M7047 ER (1D5)	5	40
Thermo Fisher/ Neomarkers RM 9101-S (SP1)	14	86
Leica/Novocastra NCL-ER-6F11 (6F11)	12	75
Ventana 250- 2596 ER (6F11)	1	100
Ventana 790-4324 (SP1)	74	92
Leica Bond PA0151 (6F11)	9	67
Dako M3634 (SP1)	5	80
Ventana 790-4325 (SP1)	44	91
Leica/Novocastra NCL-L-ER- 6F11	27	70
Leica/Novocastra RTU-ER-6F11	2	50
Leica/Novocastra NCL-ER-6F11/2	5	80
Dako SK310 pharmDX kit (Link) (1D5 + ER-2-123)	1	100
Dako IR151 Autostainer Link (SP1)	2	100
Dako (EP1) RTU FLEX IR084	22	95
Dako (EP1) M3643	26	88
Dako FLEX (1D5) IR/IS657	1	0
Other	6	50
Dako (EP1) RTU Auto Plus IS084	1	100
Biocare Medical (SP1+6F11) RTU APA 308	1	100
Cell Marque 249-R (SP1)	3	67

Breast Steroid Hormone Receptor Run: 116

Automation	Oestrogen receptors N	%
Dako Autostainer	1	0
Dako Autostainer Link 48	25	80
Dako Autostainer plus	1	100
Dako Autostainer Plus Link	3	100
Dako Omnis	7	86
LabVision Autostainer	3	33
Leica Bond Max	19	79
Leica Bond-III	46	87
Menarini - Intellipath FLX	1	0
None (Manual)	6	50
Shandon Sequenza	1	100
Ventana Benchmark GX	8	100
Ventana Benchmark ULTRA	98	88
Ventana Benchmark XT	48	85

Breast Steroid Hormone Receptor Run: 116

Heat Mediated Retrieval	Oestrogen receptors N	%
Biocare Decloaking Chamber	1	0
Dako Omnis	8	88
Dako PTLink	26	81
Lab vision PT Module	4	50
Leica ER1 20 mins	12	83
Leica ER1 30 mins	15	87
Leica ER1 40 mins	4	75
Leica ER2 10 mins	3	67
Leica ER2 20 mins	25	88
Leica ER2 30 mins	5	80
Leica ER2 40 mins	1	100
Microwave	3	33
Other	2	100
Pressure Cooker	3	100
Ventana CC1 16mins	3	67
Ventana CC1 24mins	3	100
Ventana CC1 32mins	6	50
Ventana CC1 36mins	25	92
Ventana CC1 40mins	4	75
Ventana CC1 48mins	3	67
Ventana CC1 52mins	7	86
Ventana CC1 56mins	4	50
Ventana CC1 64mins	41	88
Ventana CC1 76mins	1	100
Ventana CC1 88mins	1	100
Ventana CC1 92mins	1	0
Ventana CC1 extended	3	100
Ventana CC1 mild	15	100
Ventana CC1 standard	30	93
Ventana CC2 64mins	1	100
Ventana CC2 mild	2	100
Water bath 95-98 OC	4	50

Breast Steroid Hormone Receptor Run: 116

Enzyme Mediated Retrieval	Oestrogen receptors N	%
AS PER KIT	4	75
NOT APPLICABLE	175	81
Ventana Protease 1 (760-2018)	1	100

Breast Steroid Hormone Receptor Run: 116

Detection	Oestrogen receptors	
	N	%
AS PER KIT	9	78
Biocare polymer (M4U534)	1	0
Dako EnVision FLEX (K8000/10)	8	75
Dako EnVision FLEX+ (K8002/12)	18	89
Dako Envision HRP/DAB (K5007)	3	100
Dako REAL HRP/DAB (K5001)	1	0
LabVision UltraVision LP HRP (TL 125 HLJ)	1	0
Leica Bond Polymer Refine (DS9800)	62	85
MenaPath X-Cell Plus (MP-XCP)	1	0
None	1	100
NOT APPLICABLE	2	50
Novocastra Novolink PDS (RE7-140/150/280/290-K)	1	100
Other	7	57
Ventana iView system (760-091)	3	100
Ventana OptiView Kit (760-700)	19	74
Ventana UltraView Kit (760-500)	128	89

Breast Steroid Hormone Receptor Run: 116

Chromogen	Oestrogen receptors	
	N	%
AS PER KIT	17	88
BioGenex liquid DBA (HK-124-7K)	1	100
Dako DAB K3468	1	0
DAKO DAB+	2	100
Dako EnVision Plus kits	3	100
Dako FLEX DAB	23	78
Dako REAL EnVision K5007 DAB	3	67
Dako REAL K5001 DAB	1	0
Leica Bond Polymer Refine kit (DS9800)	61	84
menapath xcell kit DAB (MP-860)	1	0
Other	11	73
Ventana DAB	13	85
Ventana Enhanced Alk. Phos. Red Detection Kit	1	100
Ventana iview	3	100
Ventana Ultraview DAB	126	87

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

Primary Antibody: Dako M3634 (SP1) , 60 Mins, 20 ºC Dilution 1: 40
Automation: Dako Autostainer Link 48
Method: Dako FLEX+ kit
Main Buffer: AS PER KIT
HMAR: Dako PTLink
EAR:
Chromogen: AS PER KIT
Detection: AS PER KIT

Oestrogen receptors - Method 2

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

Primary Antibody: Ventana 790-4325 (SP1) , 32 Mins
Automation: Ventana Benchmark ULTRA
Method: Ventana UltraView DAB
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 64mins
EAR:
Chromogen: Ventana Ultraview DAB
Detection: Ventana UltraView Kit (760-500)

Oestrogen receptors - Method 3

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

Primary Antibody: Dako (EP1) RTU FLEX IR084 , 25 Mins, rt ºC Prediluted
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 20 mins
EAR:
Chromogen: Leica Bond Polymer Refine kit (DS9800), Time 1: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 15 Mins, RT ºC

Oestrogen receptors - Method 4

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

Primary Antibody:	Dako (EP1) RTU FLEX IR084 , 20 Mins, RT ºC Prediluted
Automation:	Dako Omnis
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, Time 1: 5 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , RT ºC

The Breast HER2 ICC Module Run 116

Keith Miller and Suzanne Parry

Antigen Assessed:	HER2
Sections Circulated:	Breast Cancer Cell Line Samples
Number of Registered Participants:	304
Number of Participants this Run	265 (87%)

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 (from left to right on slide)	Assessment of Cell Line Staining Pattern	Assessment of In-House Tissue Sections
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:

The UK NEQAS distributed cell line samples are validated using the Leica Oracle, Ventana Pathway 4B5 and Dako Hercept Test assays and by staining every 50th serial section

UK NEQAS Specific Membrane Scoring Algorithm:

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

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

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

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

Numerical Scoring Criteria

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

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

Introduction

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

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

Recommended HER2 testing guidelines are: The ASCO/CAP guidelines by Wolff et al.(2007), the UK guidelines by Walker et al., (2008), the ASCO/CAP guidelines by Wolff et al., 2013. , and the updated UK guidelines by Rakha et al., (2015). These publications provide invaluable guidelines covering interpretation, tissue fixation and antibody validation. The articles by Walker et. al. and Rhaka et al. also provide guidelines on the minimum number of tests per year that 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 116:	
	NEQAS	**In-House**
Acceptable	72% (N=265)	58% (N=152)
Borderline	13% (N=34)	31% (N=82)
Unacceptable	14% (N=38)	11% (N=29)

As with previous runs the most popular antibody was the Ventana 4B5, used by 64% of participants and showed an overall acceptable pass rate of 86%. 5 laboratories are using the Dako HercepTest, with only one participant achieving an acceptable pass rate. 16 laboratories are using the Leica Oracle assay kit with an acceptable pass rate of 94%. 54 laboratories used laboratory devised tests (LDTs), using a variety of antibodies, pre-treatment methods and staining platforms. These laboratories showed an acceptable pass rate of 28%. All data is based on the methodology information submitted by participants, but unfortunately not all methodology was provided by all labs.

References

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

Acknowledgments

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

Selected Images showing Optimal and Sub-optimal Immunostaining

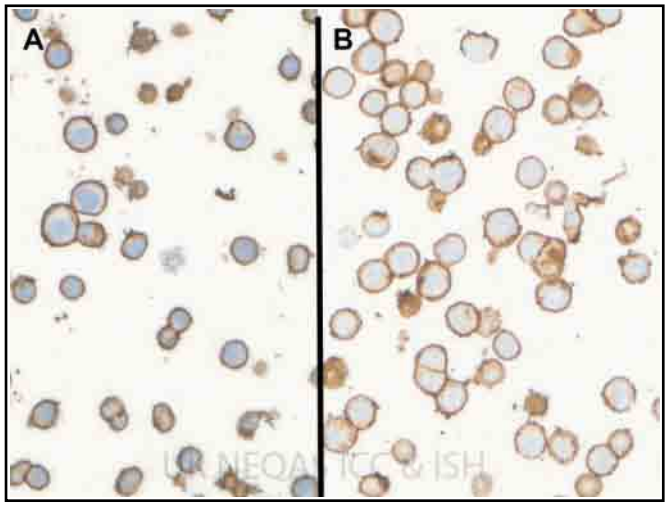


Fig 1. Appropriate staining of the UK NEQAS SK-BR3 (3+) cell line. (A & B) Strong and complete circumferential membrane. Stained using (A) the Dako HercepTest on the autostainer with pre-treatment in the PT link, and (B) Ventana Pathway 4B5 on the ULTRA.

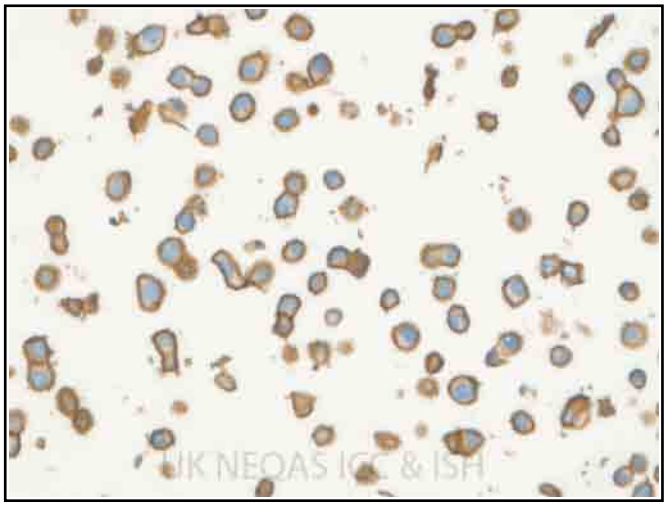


Fig 2. Poor demonstration of HER2 staining of the UK NEQAS 3+ cell line. Although the example shows the expected level of staining, the cells show morphology damage and appear to be over heated.

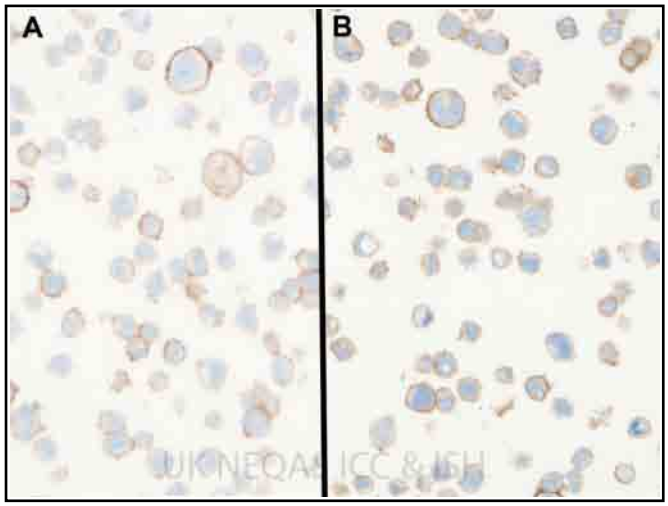


Fig 3. Two examples showing the expected level of staining of the UK NEQAS MDA-MB-453 (2+) cell line. (A & B) The majority of cells show weak to moderate complete membrane staining. (A) stained using the Ventana Pathway 4B5 and (B) the Leica Oracle kit on a BondMax.

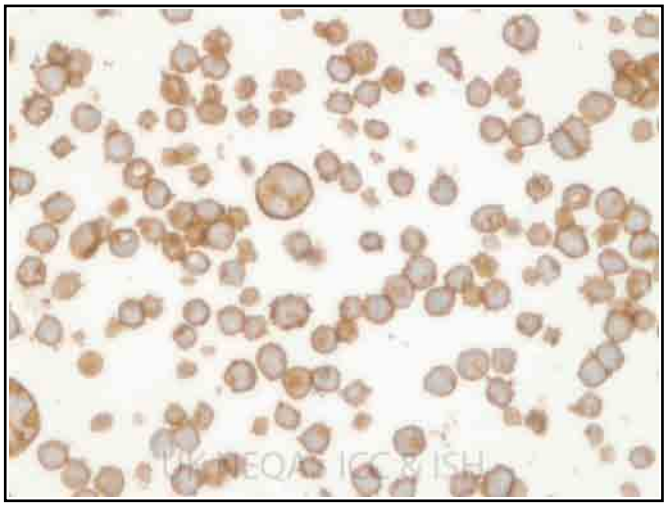


Fig 4. Unacceptable staining of the UK NEQAS 2+ distributed cell line. The example shows excessive membrane staining, more representative of 3+ staining, and also excessive cytoplasmic staining. The staining was carried out using a lab-devised method.

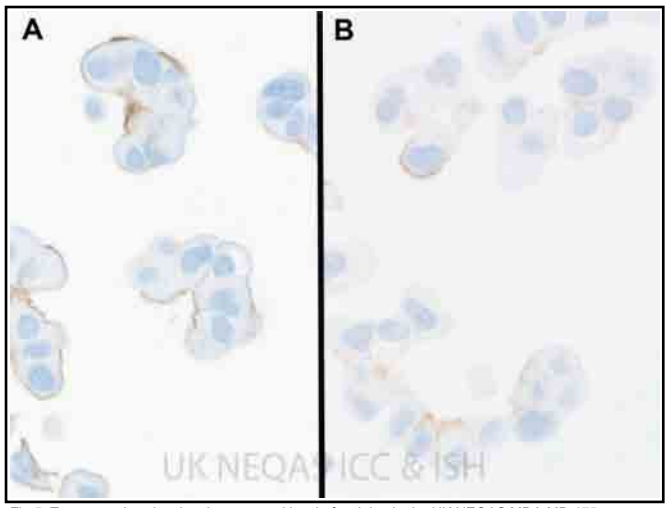


Fig 5. Two examples showing the expected level of staining in the UK NEQAS MDA-MB-175 (1+) cell line, which is partial membranous. Stained using (A) the Dako HercepTest on the autostainer with pre-treatment in the PT link, and (B) Ventana Pathway 4B5 on the ULTRA.

Fig 6. Unacceptable staining of the UK NEQAS MDA-MB-175 (1+) cell line. The staining is too high and more representative of 2+ staining. The laboratory did not provide any methodology details.

Selected Images showing Optimal and Sub-optimal Immunostaining

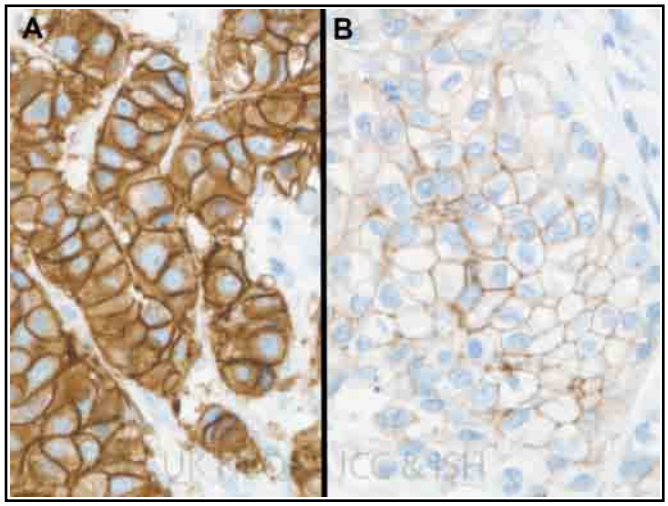


Fig 7. Good in-house breast controls optimally stained with HER2 submitted for assessment: (A) 3+ and (B) 2+ stained using the Dako HercepTest on the autostainer with pre-treatment in the PT link.

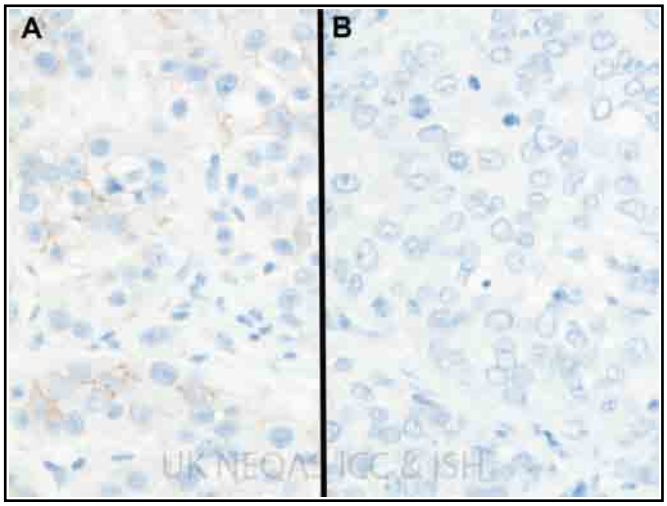


Fig 8. Good in-house breast controls optimally stained with HER2 (A) 1+ and (B) negative (same protocol as Fig 7).

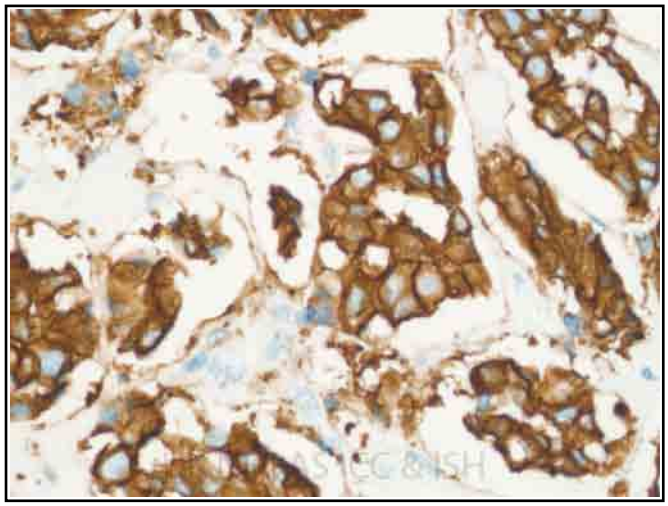


Fig 9. Image showing poor tissue quality of a 3+ in-house section submitted for assessment: Although the membrane staining can be seen as a 3+, due to the poor tissue fixation, the section also shows excessive cytoplasmic staining and retraction artefact.

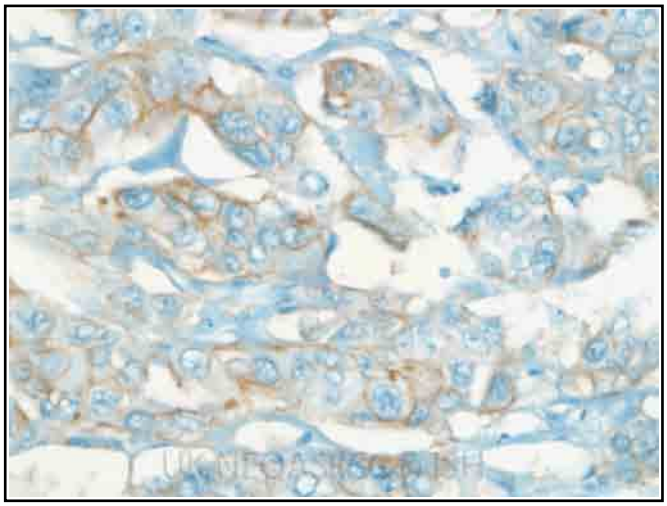
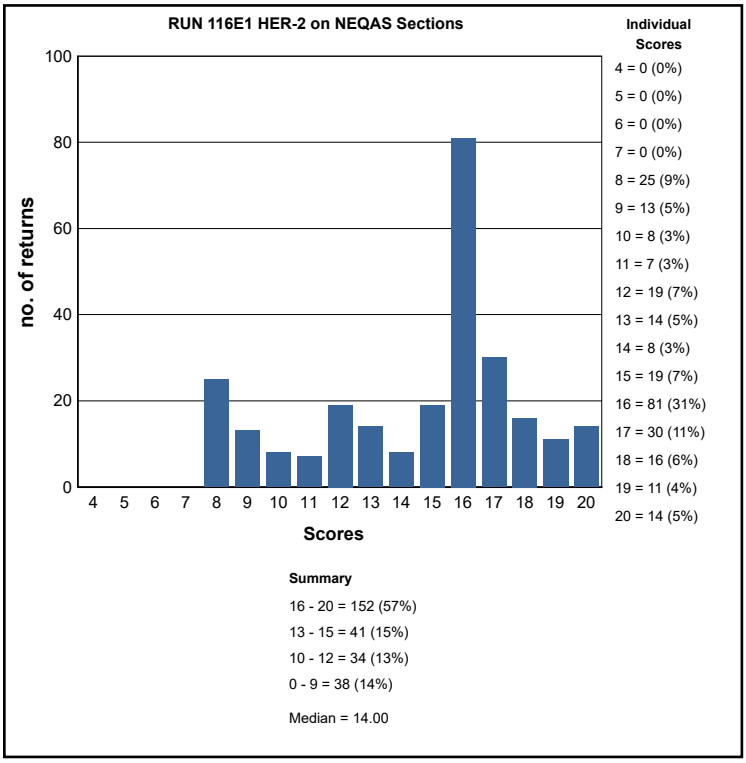
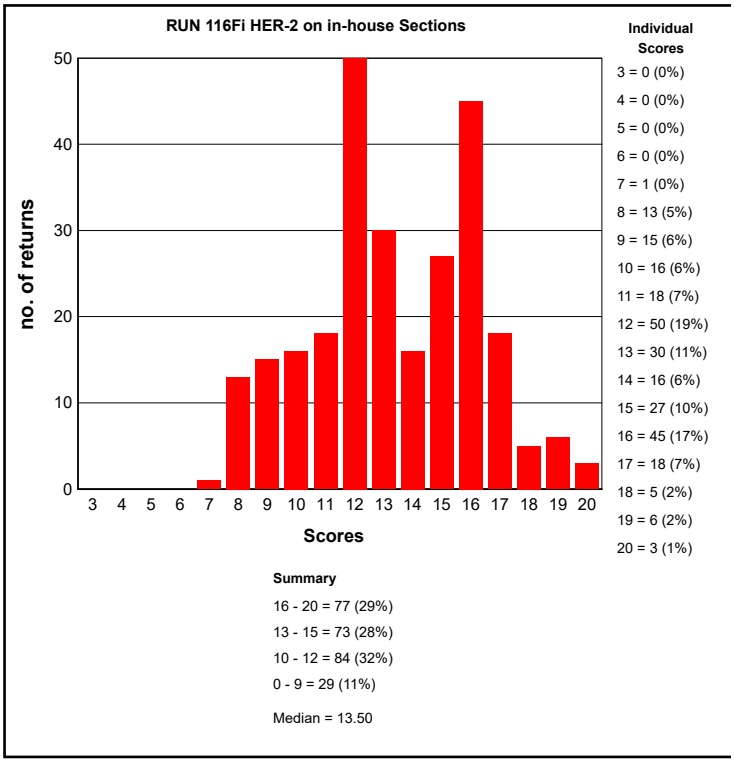


Fig 10. Image showing poor tissue quality and excessive counterstain on a 2+ in-house section submitted for assessment.

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: 116

Primary Antibody	N	%
Dako HercepTest K5204 (poly)	3	0
Dako HercepTest K5205 (poly)	1	0
Dako HercepTest K5207 (poly)	1	100
Dako A0485 C-erB-2 (poly)	24	25
Cell Marque CMA 601 (CB11)	1	0
Ventana Pathway 790-100 (4B5)	12	75
Labvision / Neomarkers RM-9103 (SP3)	3	0
Leica Oracle HER2 Bond IHC (CB11)	16	94
Dako Link HercepTest SK001 (poly)	8	75
BioGenex (EP1045Y) rb mono	2	50
Ventana Confirm 790-4493 (4B5)	40	83
Ventana Pathway 790-2991 (4B5)	130	89
Novocastra NCL-L-CB11 (CB11)	4	0
Biocare CME 342 A,B (EP1045Y)	4	25
Other	8	13

Breast HER2 ICC Run: 116

Automation	N	%
Dako Autostainer	3	33
Dako Autostainer Link 48	14	36
Dako Autostainer plus	2	0
Dako Autostainer Plus Link	3	67
Dako Omnis	3	100
LabVision Autostainer	2	0
Leica Bond Max	11	36
Leica Bond-III	16	81
None (Manual)	13	0
Other	2	50
Ventana Benchmark GX	14	79
Ventana Benchmark ULTRA	99	94
Ventana Benchmark XT	75	75

Breast HER2 ICC Run: 116

Heat Mediated Retrieval	N	%
Biocare Decloaking Chamber	3	33
Dako Omnis	3	100
Dako Pascal	1	0
Dako PTLink	18	44
Lab vision PT Module	4	0
Leica ER1 10 mins	1	100
Leica ER1 20 mins	7	29
Leica ER1 25 mins	13	100
Leica ER1 30 mins	1	0
Leica ER1 40 mins	1	0
Leica ER2 30 mins	1	0
Microwave	5	0
None	4	50
Other	5	60
Pressure Cooker	1	0
Ventana CC1 16mins	2	100
Ventana CC1 20mins	1	0
Ventana CC1 24mins	2	100
Ventana CC1 32mins	18	72
Ventana CC1 36mins	47	94
Ventana CC1 40mins	2	100
Ventana CC1 48mins	1	0
Ventana CC1 52mins	3	100
Ventana CC1 56mins	7	100
Ventana CC1 64mins	7	100
Ventana CC1 76mins	1	100
Ventana CC1 8mins	5	60
Ventana CC1 92mins	1	100
Ventana CC1 mild	66	82
Ventana CC1 standard	14	86
Ventana CC2 36mins	1	100
Water bath 95-98 OC	6	0

Breast HER2 ICC Run: 116

Detection	N	%
AS PER KIT	19	79
Biocare SLAB (STU HRP 700H,L10)	2	0
BioGenex SS Polymer (QD 430-XAKE)	1	0
Dako HerCep Test (K5204)	1	0
Dako EnVision FLEX (K8000/10)	5	20
Dako EnVision FLEX+ (K8002/12)	9	33
Dako Envision HRP/DAB (K5007)	3	0
Dako HerCep Test Autor (SK001)	7	71
LabVision UltraVision ONE Polymer (TL-012/5-HDJ/T	1	0
Leica Bond Polymer Refine (DS9800)	16	44
Other	8	25
Ventana iView system (760-091)	5	80
Ventana OptiView Kit (760-700)	10	90
Ventana UltraView Kit (760-500)	163	85

Breast HER2 ICC Run: 116

Enzyme Retrieval	N	%
AS PER KIT	18	83
NOT APPLICABLE	138	71
Ventana Protease	1	0
Ventana Protease 1 (760-2018)	3	67

Breast HER2 ICC Run: 116

Chromogen	N	%
AS PER KIT	40	85
BioGenex Liquid DAB (HK153-5K)	1	0
BioGenex liquid DBA (HK-124-7K)	1	0
DAKO DAB+	1	100
Dako DAB+ Liquid (K3468)	1	0
Dako EnVision Plus kits	1	0
Dako FLEX DAB	14	29
Dako REAL EnVision K5007 DAB	5	0
Leica Bond Polymer Refine kit (DS9800)	17	47
Other	10	40
Ventana DAB	7	86
Ventana iview	3	67
Ventana Ultraview DAB	156	83

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

Primary Antibody:	Ventana Pathway 790-2991 (4B5) , 16 Mins Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 standard
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

HER-2 - Method 2

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

Primary Antibody:	Leica Oracle HER2 Bond IHC (CB11) , 15 Mins, r/t ºC Prediluted
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER1 25 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	AS PER KIT

HER-2 - Method 3

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

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

HER-2 - Method 4

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 UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 mild
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

The Gastric HER2 ICC Module Run 116

Suzanne Parry and Dawn Wilkinson

Antigen Assessed:	HER2
Sections Circulated:	Composite slide consisting of 4 surgical intestinal gastric carcinomas with different levels of HER2 expression
Number of Registered Participants:	78
Number of Participants this Run	62 (79%)

Expected staining characteristics of the UK Neqas distributed tissue for Run 116:

Sample Position (from left to right on slide)	Expected HER2 IHC Expression Level
A **B** **C** **D**	**0** **0 or 1+ depending on the serial section received by the laboratory*** **2+** **3+**

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

Table1: Gastric HER2 ICC Membrane Scoring Guidelines: Hoffman et al., (2008) and Rüschoff et al., (2010)

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

Validation of Distributed Samples

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

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

Table 2 showing the HER2 IHC staining and & gene expression with ISH

Assessment Procedure

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

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

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

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

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

Introduction

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

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

Assessment Results

Features Of Acceptable Staining: (Figs 1, 2, 4 & 6)

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

Features Of Suboptimal or Unacceptable Staining: (Figs 3 & 5)

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

Additional Comment:

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

Pass Rates

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

NEQAS Pass Rates Run 116:	
Acceptable	86% (N=53)
Borderline	11% (N=7)
Unacceptable	3% (N=2)

There has been a remarkable significant improvement in pass rates over the past several runs. Improvement has increased from 65% in Run 112 to 86% in Run 116. Only 2 laboratories (3%) obtained an unacceptable result due to weak staining. In the clinical setting, false negative results may 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 ISH reflex testing.

Most labs are using the recommended standardised protocols for their particular automated systems, such as CC1 Mild antigen retrieval on the Ventana XT with the 4B5 pre-diluted antibody clone. However, a few labs are not using the recommended protocols, and this may have the potential to induce weaker or stronger membrane staining than expected. 9 laboratories did not submit in-house control material for assessment. Participant results for those that did submit in-house results are summarised in the following table:

In-House Pass Rates Run 116:	
Acceptable	72% (N=44)
Borderline	28% (N=17)
Unacceptable	0% (N=0)

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

Methodologies

The majority of labs are using the Ventana 4B5 standardised kit for their gastric HER2 testing: In this particular assessment run the Ventana assay was used by 89% of participants with an average acceptable pass rate of 87%. 3 laboratories used the Dako Hercept test with 100% acceptable pass rate.

Control Tissue and Recommendations

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

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

References:

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

Acknowledgments

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

Selected Images showing Optimal and Sub-optimal Immunostaining

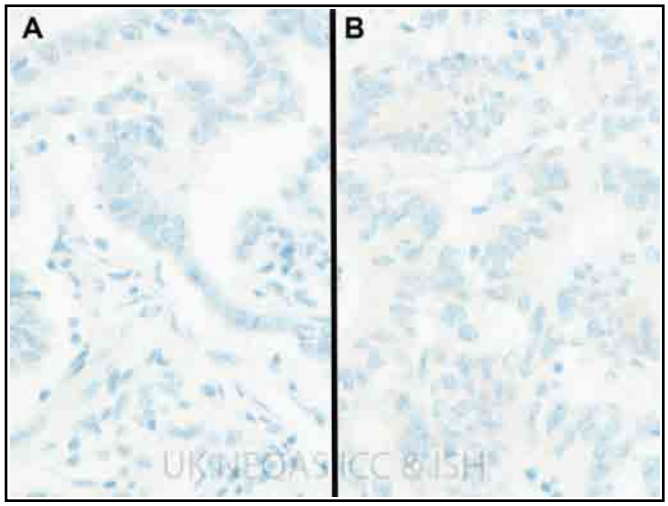


Fig 1. Expected HER2 negative UK NEQAS gastric tumour samples A and B. Sample B showed features of 1+ membrane staining on some of the distributed serial sections. Both sections stained with the Ventana 4B5 assay on the ULTRA Benchmark with CC1 retrieval for 31 minutes.

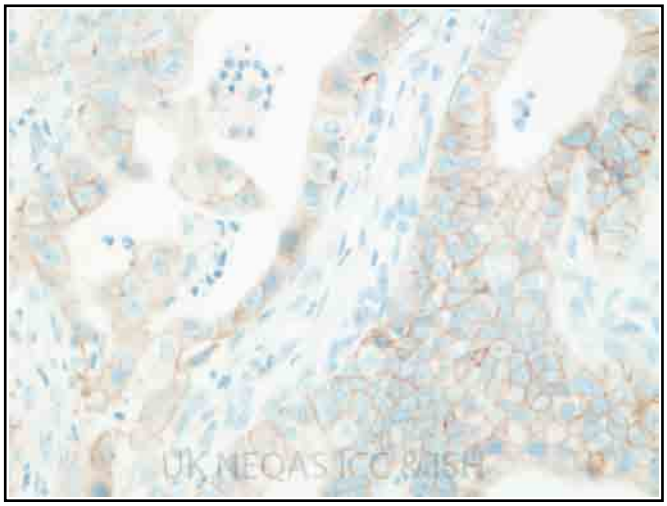


Fig 2. Optimal demonstration of HER2 IHC on the UK NEQAS distributed 2+ gastric tumour (sample C). As expected the staining is complete and membranous (stained with the same method as in Fig 1A).

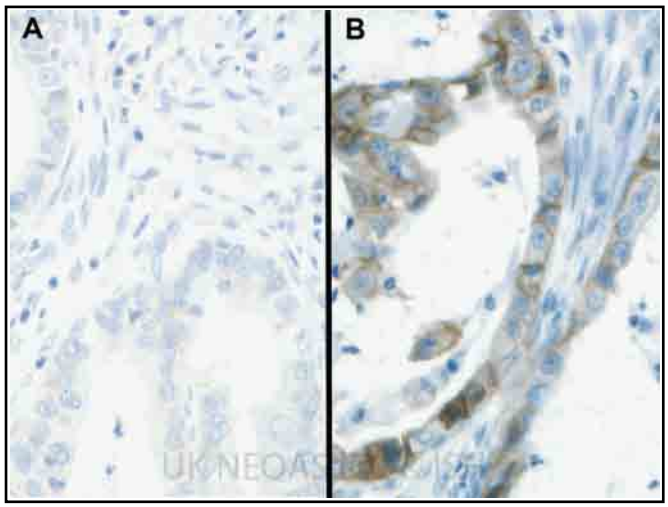


Fig 3. Two examples showing unacceptable demonstration of HER2 IHC in the UK NEQAS distributed gastric tumour sample C (compare to Fig 2). This sample should be a 2+, but the staining in example A is too weak and therefore negative, while the staining in example B is stronger than the expected level, and therefore more representative of 3+ staining.

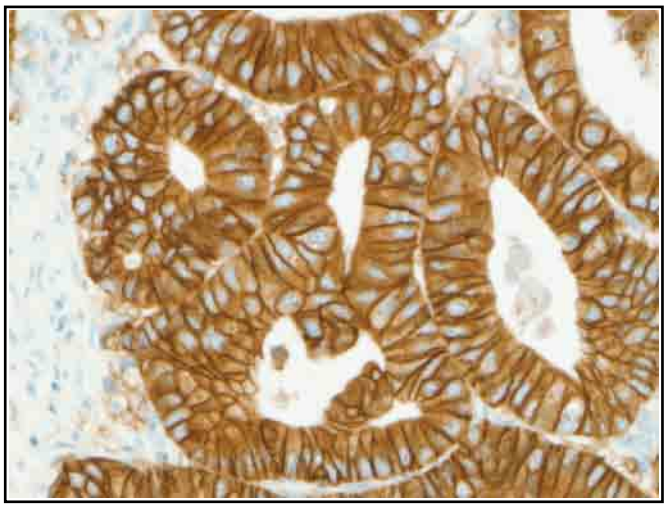


Fig 4. Optimal demonstration of HER2 IHC staining in the UK NEQAS distributed gastric tumour sample D. As expected the example shows 3+ HER2 IHC staining expression, which is strong and complete membranous staining (same protocol as Figs 1A&2).

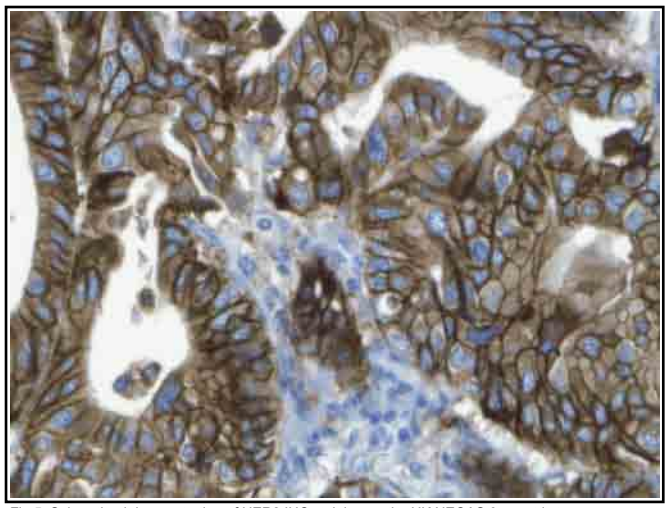


Fig 5. Sub-optimal demonstration of HER2 IHC staining on the UK NEQAS 3+ gastric tumour sample D. Although the membranous staining is very strong and in keeping with a 3+ expression level, the counterstain is excessive. Interpretation was made difficult on the rest of the NEQAS samples due to the excessive counterstain masking the membranes.

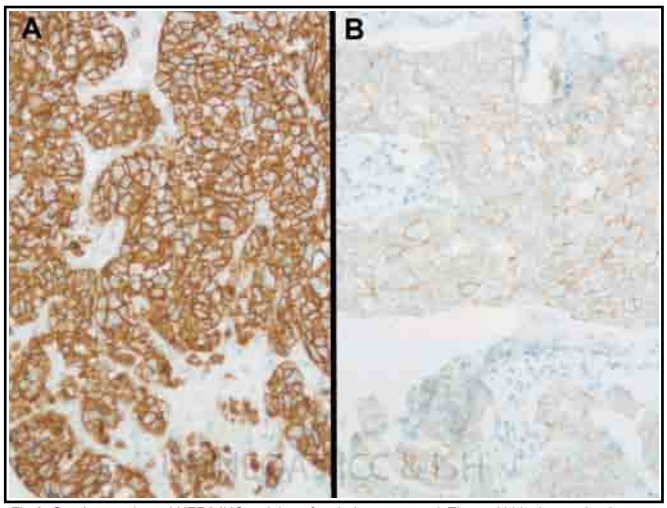
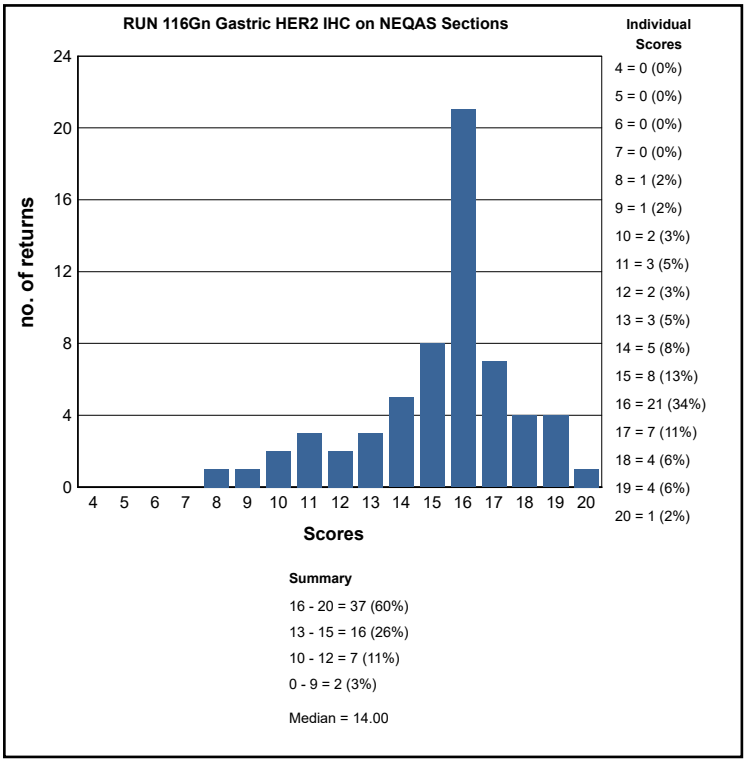
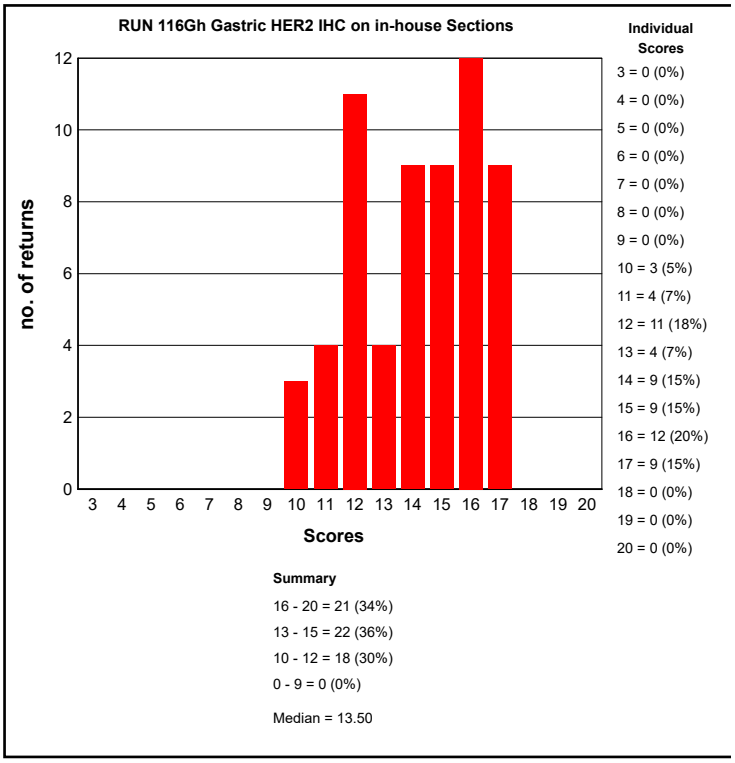


Fig 6. Good example and HER2 IHC staining of an in-house control. The multi-block contained (A) 3+, (B) 2+, and negative expressing gastric tumours (negative tumour not shown). Stained with the Ventana 4B5 assay on the Benchmark ULTRA using the recommended protocol.

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: 116

Primary Antibody	N	%
Labvision / Neomarkers RM-9103 (SP3)	1	0
Dako Link HercepTest SK001 (poly)	3	100
Ventana Pathway 790-100 (4B5)	3	100
Ventana Pathway 790-2991 (4B5)	34	82
Other	1	100
Ventana Confirm 790-4493 (4B5)	18	94

Gastric HER2 ICC Run: 116

Automation	N	%
Dako Autostainer Link 48	1	100
Dako Autostainer plus	1	0
Dako Autostainer Plus Link	2	100
Dako Omnis	1	0
Leica Bond Max	1	0
None (Manual)	1	100
Ventana Benchmark GX	2	100
Ventana Benchmark ULTRA	31	87
Ventana Benchmark XT	21	90

Gastric HER2 ICC Run: 116

Heat Mediated Retrieval	N	%
Dako Omnis	1	0
Dako PTLink	3	100
Lab vision PT Module	2	50
Leica ER2 30 mins	1	0
Ventana CC1 16mins	1	0
Ventana CC1 24mins	1	100
Ventana CC1 32mins	6	83
Ventana CC1 36mins	14	79
Ventana CC1 56mins	2	100
Ventana CC1 64mins	6	100
Ventana CC1 mild	19	95
Ventana CC1 standard	5	100

Gastric HER2 ICC Run: 116

Detection	N	%
AS PER KIT	3	33
Dako EnVision FLEX+ (K8002/12)	1	0
Dako HerCep Test Autor (SK001)	2	100
Leica Bond Polymer Refine (DS9800)	1	0
Other	2	50
Ventana iView system (760-091)	2	100
Ventana OptiView Kit (760-700)	3	100
Ventana UltraView Kit (760-500)	45	91

Gastric HER2 ICC Run: 116

Enzyme Retrieval	N	%
AS PER KIT	5	100
NOT APPLICABLE	29	79
Ventana Protease 1 (760-2018)	1	100

Gastric HER2 ICC Run: 116

Chromogen	N	%
AS PER KIT	9	89
DAKO DAB+	1	0
Dako FLEX DAB	2	100
Leica Bond Polymer Refine kit (DS9800)	1	0
Other	2	50
Ventana DAB	2	100
Ventana iview	1	100
Ventana Ultraview DAB	43	88

BEST METHODS

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

Gastric HER2 IHC - Method 1

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

Primary Antibody:	Ventana Confirm 790-4493 (4B5) , 12 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 36mins
EAR:	
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

Gastric HER2 IHC - Method 2

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

Primary Antibody:	Ventana Pathway 790-2991 (4B5)
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 32mins
EAR:	
Chromogen:	AS PER KIT
Detection:	

Gastric HER2 IHC - Method 3

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

Primary Antibody:	Ventana Confirm 790-4493 (4B5)
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 36mins
EAR:	AS PER KIT
Chromogen:	AS PER KIT
Detection:	Ventana UltraView Kit (760-500)

The Lymphoma Module Run 116

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	Gold Standard	Second Antibody
Antigens Assessed:	BCL-2	CD10
Tissue Sections circulated:	Follicular Lymphoma & Reactive Tonsil	Follicular Lymphoma & Reactive Tonsil
Number of Registered Participants:	215	
Number of Participants this Run	206 (96%)	

Introduction

Gold Standard: BCL-2

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

Features of Optimal Immunostaining (Figs 1, 3, 5 & 6):

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

Features of Sub-optimal Immunostaining (Figs 2 & 4):

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

References:

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

Second Antigen: CD10

CD10 is a transmembrane glycoprotein with metalloproteinase activity, which can modulate the effect of bioactive peptides including hormones and growth factors. It is transitionally expressed on immature lymphocyte precursors of both B– and T– cell type and then re-expressed in proliferation B-cells found mainly in the lymphoid germinal centres. It is also detected in mature neutrophil granulocytes and in several epithelial cell types including liver, kidney, prostate, intestine and breast myoepithelium, as well as some stromal cells and their tumours. CD10, also known as the acute lymphoblastic leukaemia antigen (CALLA): It is expressed in acute lympyhoblastic leukaemia (ALL) arising from B cells, but is also found in a proportion of T-cell ALL's[1, 2]. It is a useful marker for identifying precursor B-cell lymphoblastic lymphoma/leukaemias , follicular lymphomas and Burkitt's lymphoma[3].

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

- Moderate-strong membrane staining of all germinal centre B -lymphocytes and some dispersed in the peri-follicular region of the reactive tonsil. Intense membrane staining in most of the neoplastic B-cells of the follicular lymphoma.
- Clean background

Features of Sub-optimal Immunostaining (Figs 9 & 11):

- Weak, uneven, partially missing staining of relevant cells.
- Poor / diffuse localisation of staining.
- 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. Chui P, Arber DA. Paraffin-section detection of CD10 in 505 non-hematopoietic neoplasms. Frequent expression in renal cell carcinoma and endometrial stromal sarcoma. Am J Clin Pathol 2000; 113: 374-382.
2. Kaufamann O, et al. Immunohistochemical detection of CD10 with monoclonal anditoby 56C6 on paraffin sections. Am J Clin Pathol 1999; 11: 117-22.
3. Jaffe ES, Harris NL, Stein H, Vardiman JW (Eds.) WHO Classification of Tumours. Pathology and Genetics of tumours of Haematopoietic and Lymphoid Tissues. Lyon, IARC Press 2001

Assessment Summary:

BCL-2

204 laboratories submitted their slides for the BCL-2 assessment. Similarly to previous results, weak staining or poor localisation with diffuse uneven staining were the main reasons for receiving a borderline pass or in some cases, where there was very little staining at all, the labs received a failed score. This was not particularly attributable to a particular antibody clone, but mostly due to an inappropriate dilution or antigen retrieval protocol.

All laboratories submitted an in-house control with a higher acceptable pass rate of 89% compared to the NEQAS scores for this run, no laboratories failed on their in-house material.

Pass rates on the Neqas material are shown in the table below:

NEQAS Pass Rates Run 116: BCL-2	
Acceptable	83% (N=170)
Borderline	15% (N=30)
Unacceptable	2% (N=5)

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

CD10

205 laboratories submitted both the NEQAS and in house sections for assessment, and the pass rates were very similar for both: 80% of participants received an acceptable pass on their Neqas sections and 83% for the in-house sections. The borderline and unacceptable pass rates on the Neqas material and in-house sections was also similar, although slightly

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lower on the Neqas material compared to the NEQAS sections.

Results from the CD10 assessment are summarised in the table below:

NEQAS and In-house Pass Rates Run 116: CD10		
	NEQAS	In-House
Acceptable	80% (N=163)	83% (N=170)
Borderline	18% (N=38)	16% (N=33)
Unacceptable	2% (N=5)	1% (N=2)

It was noted that borderline scores were mostly marked down due to weak demonstration of the antibody. However, only a very small number of laboratories failed. This was due to a combination of very weak, uneven staining, or a lower percentage of cells staining than expected. The most popular primary antibody for this run was the Novocastra/Leica (Clone 56C6) used as either the concentrate form or the ready -to-use form. This was used by 110 participants and showed an acceptable pass rate of 84%. The second most popular antibody was the Ventana 790-4506 (SP67); with 42 participants using this clone, and showed an average 67% acceptable pass rate.

Selected Images showing Optimal and Sub-optimal Immunostaining

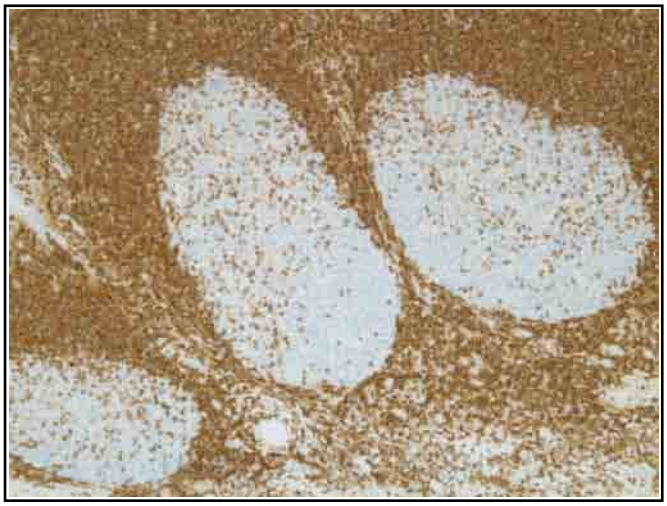


Fig 1. Optimal demonstration of BCL2 in the reactive tonsil section. The example shows strong cytoplasmic staining of the peripheral B-cells and intra-follicular T-cells. Stained with the Dako 124 antibody, 1:20, on the Dako autostainer with pre-treatment in the PT Link with high pH buffer.

Fig 2. Sub-optimal staining with BCL2 on the UK NEQAS reactive tonsil: The staining is weak, and several of the lymphocytes expected to stain are not demonstrated (compare to Fig 1). Section stained with the Novocastra BCl2/100/D5 clone, 1:40, on Leica BondMax, ER1 retrieval for 30 minutes.

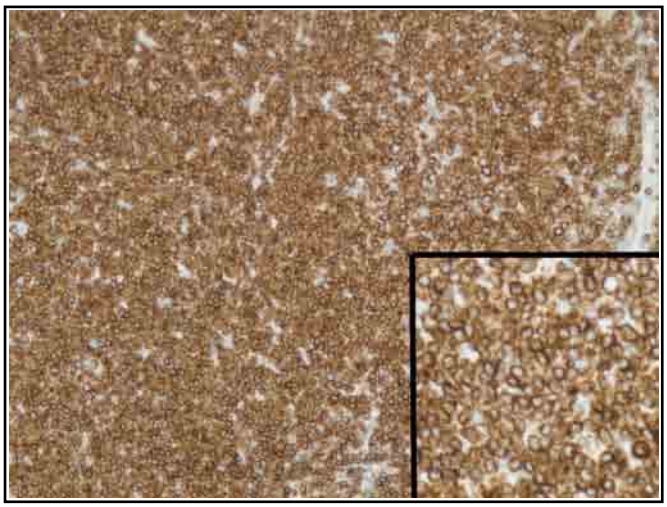


Fig 3. Optimal staining for BCL2 on the UK NEQAS distributed follicular lymphoma section: Almost all the neoplastic cells show strong and well localised cytoplasmic staining (shown to better advantage in the high power insert). Stained with Dako 124 antibody, 1:50, on the Leica BondMax with ER2 retrieval for 20 minutes.

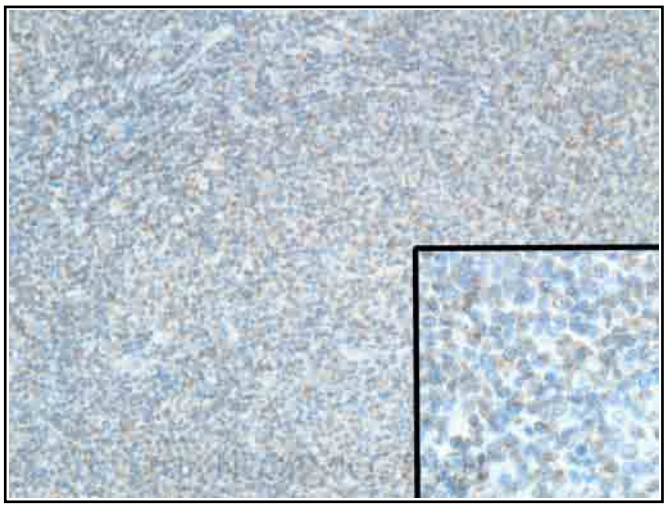


Fig 4. Unacceptable staining for BCL2 on the UK NEQAS distributed follicular lymphoma section (compare to Fig 3). Not only is the staining weak, but many of the tumour cells expected to stain are not demonstrated. Same antibody and pre-treatment as Fig 2, although the antibody dilution used was at 1:80.

Fig 5. Optimal staining of a participants' in-house tonsil control section. The example shows strong staining of the mantle zone lymphocytes, and as expected the germinal centre cells remain negative apart from some isolated T-cells staining. (Same protocol as Fig 2).

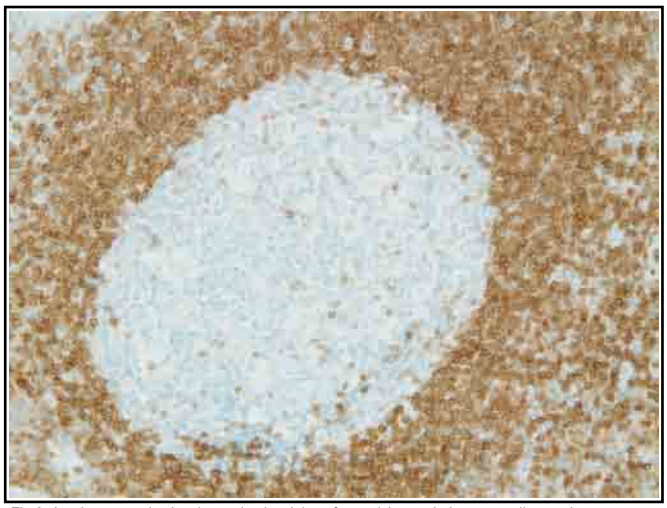


Fig 6. Another example showing optimal staining of a participants' in-house tonsil control section. Using the Ventana pre-diluted 124 clone on the ULTRA with CC1 pre-treatment for 24 minutes and Optiview detection.

Selected Images showing Optimal and Sub-optimal Immunostaining

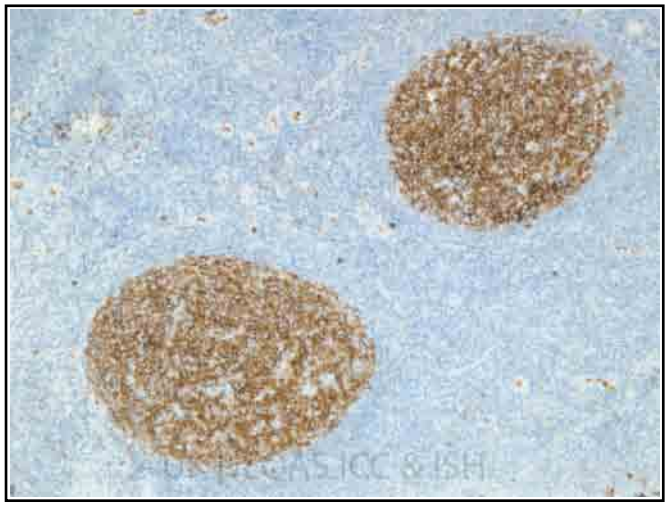


Fig 7. Optimal demonstration of CD10 on the UK NEQAS tonsil section. Virtually all the germinal centre B-cells show a strong membranous staining while the background remains clean. Stained with the Novocastra 56C6 antibody, 1:20, on the Dako autostainer and pre-treatment in the PT link using high pH buffer solution.

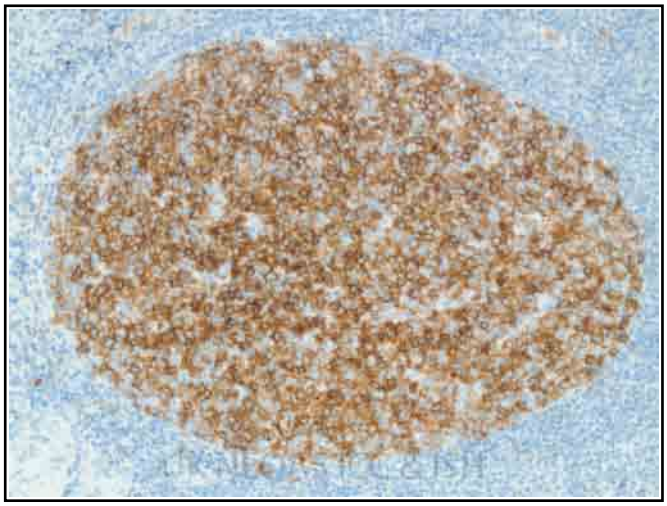


Fig 8. Higher power view of the CD10 staining on the UK NEQAS tonsil section. The image highlights the strong membranous staining of the B-cells, while the mature lymphocytes remain unstained. Stained using Dako Omnis RTU 56C6 antibody on the Dako Omnis stainer and high pH retrieval solution.

Fig 9. Poor staining for CD10 on the UK NEQAS tonsil section: Although most of the cells expected to stain are demonstrated, the staining is very weak (compare to Figs 7&8). Stained with the Ventana pre-diluted SP67 antibody on the ULTRA with CC1 retrieval for 72 minutes, ultraview detection.

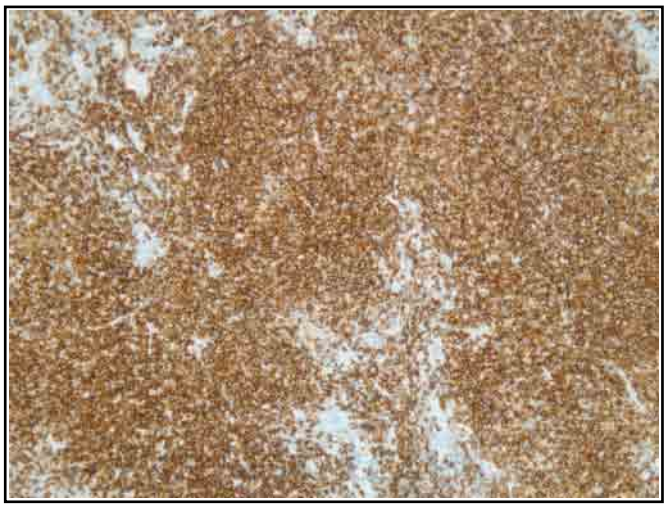


Fig 10. Optimal CD10 staining of the UK NEQAS follicular lymphoma section: The majority of neoplastic cells show strong distinct membranous staining. (Same protocol as Fig 8)

Fig 11. Sub-optimal demonstration of CD10 on the UK NEQAS follicular lymphoma. The neoplastic cells only show weak and diffuse staining (compare to Fig 10). Stained with the Novocastra 56C6 antibody, 1:40, stained with the Menapath X Cell method with pre-treatment in the Decloaking chamber.

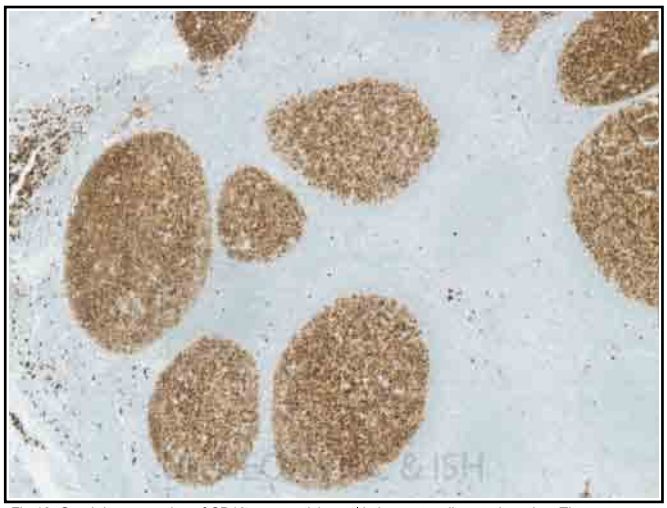
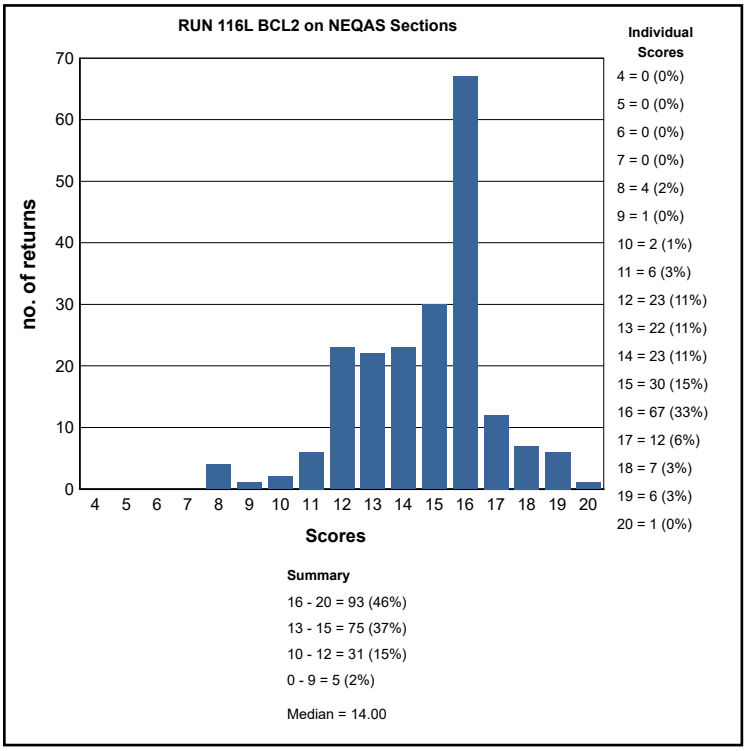
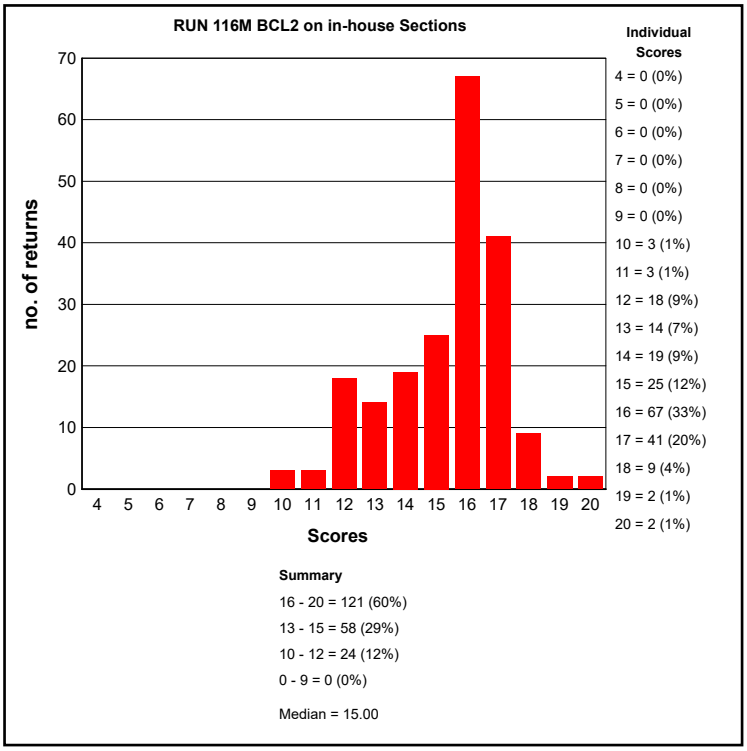
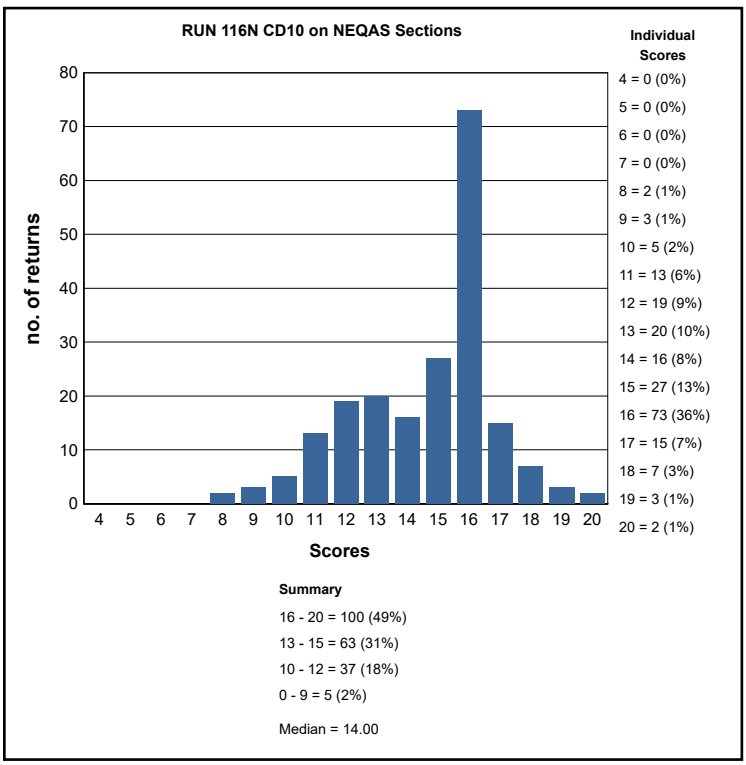


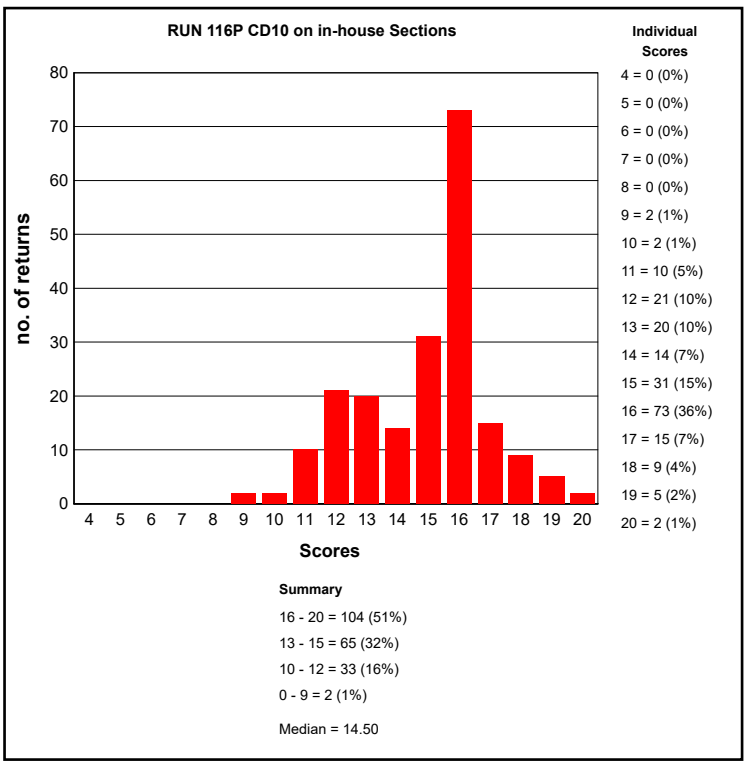
Fig 12. Good demonstration of CD10 on a participants' in house tonsil control section. The example shows strong distinct staining of the B-cells, while the background remains clean. Stained with the Ventana pre-diluted SP67 antibody on the Benchmark XT with CC1 retrieval for 64 minutes, Optiview detection.

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: 116

Primary Antibody : BCL2

Antibody Details	N	%
Dako M0887 (124)	82	82
Labvision/Neomarkers MS-123-P (100/D5)	1	100
Leica/Novocastra NCL-BCl-2 (BCl2/100/D5)	15	73
Ventana (SP66) 790-4604	25	92
Ventana 760-4240 (124)	1	100
Other	4	75
Cell marque CMC329 (124)	2	50
Dako FLEX IR614 (124)	16	88
Leica/Novocastra NCL-BCl-2-486 (3.1)	5	80
Leica RTU (BCl2/100/D5) PA0117	21	81
Ventana (124) 790-4464	24	96
Cell Marque 226R-26 (SP66)	5	40
Abcam ab32124 (E17)	1	100

Lymphoma Run: 116

Primary Antibody : CD10

Antibody Details	N	%
BioGenex MU/AM451 (56C6)	1	100
Dako M0727 (SS2/36)	2	50
Novocastra NCL-CD10-270 (56C6)	35	71
Novocastra NCL-L-CD10-270 (56C6)	51	88
Novocastra RTU-CD10-270-R (56C6)	8	88
Vector VP-C328 (56C6)	2	100
Ventana 760-2705 (56C6)	2	50
Other	6	83
Leica RTU PA0270 (56C6)	24	92
Dako M7308 (56C6)	11	91
Dako RTU FLEX Link IR648 (56C6)	7	71
Dako RTU FLEX Auto Plus IS648 (56C6)	2	100
Ventana 790-4506 (SP67)	42	67
Cell Marque 110M-16/18 (56C6)	2	100
DAKO RTU Flex Omnis GA648 (56C6)	5	100
Gennova AP10076C	1	100

Lymphoma Run: 116

Heat Mediated Retrieval	BCL2 N	BCL2 %	CD10 N	CD10 %
Biocare Decloaking Chamber	1	100	1	0
Dako Omnis	7	57	5	80
Dako PTLink	22	91	21	76
Lab vision PT Module	1	100	1	100
Leica ER1 10 mins	1	100	1	100
Leica ER1 20 mins	3	67	2	100
Leica ER1 30 mins	5	40	0	0
Leica ER1 40 mins	0	0	1	0
Leica ER2 20 mins	46	85	44	86
Leica ER2 30 mins	8	100	15	73
Leica ER2 40 mins	1	100	2	100
Microwave	3	67	2	50
Other	1	100	0	0
Pressure Cooker	2	100	2	100
Pressure Cooker in Microwave Oven	1	100	0	0
Ventana CC1 24mins	3	33	0	0
Ventana CC1 32mins	9	89	5	60
Ventana CC1 36mins	2	50	0	0
Ventana CC1 40mins	3	100	8	75
Ventana CC1 48mins	10	90	6	100
Ventana CC1 52mins	1	100	0	0
Ventana CC1 56mins	5	80	5	80
Ventana CC1 64mins	34	94	32	91
Ventana CC1 72mins	3	67	4	100
Ventana CC1 76mins	4	75	1	100
Ventana CC1 80mins	0	0	2	100
Ventana CC1 88mins	1	100	0	0
Ventana CC1 92mins	2	50	8	50
Ventana CC1 extended	4	75	5	60
Ventana CC1 mild	2	0	0	0
Ventana CC1 standard	13	85	18	78
Ventana CC2 56mins	1	100	1	100
Ventana CC2 64mins	0	0	1	100
Ventana CC2 72mins	1	100	0	0
Water bath 95-98 OC	2	50	2	50

Lymphoma Run: 116

Enzyme Mediated Retrieval	BCL2 N	BCL2 %	CD10 N	CD10 %
AS PER KIT	3	100	2	100
NOT APPLICABLE	120	82	101	79

Lymphoma Run: 116	BCL2		CD10	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	10	90	11	82
BioGenex SS Polymer (QD 420-YIKE)	1	100	1	100
Dako EnVision FLEX (K8000/10)	4	50	2	50
Dako EnVision FLEX+ (K8002/12)	18	89	16	81
Dako Envision HRP/DAB (K5007)	3	100	3	67
Dako Envision+ HRP mouse K4004/5/6/7	2	100	2	100
Dako REAL HRP/DAB (K5001)	1	0	0	0
Leica Bond Polymer Define (DS9713)	2	100	0	0
Leica Bond Polymer Refine (DS9800)	59	83	61	85
MenaPath X-Cell Plus (MP-XCP)	1	100	1	0
Other	5	60	7	57
Ventana iView system (760-091)	1	0	3	33
Ventana OptiView (760-700) + Amp. (7/860-099)	6	100	12	83
Ventana OptiView Kit (760-700)	47	85	36	92
Ventana UltraView Kit (760-500)	41	83	41	76

Lymphoma Run: 116	BCL2		CD10	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	25	92	26	81
BioGenex Liquid DAB (HK153-5K)	1	100	2	100
BioGenex liquid DBA (HK-124-7K)	1	0	1	100
DAKO DAB+	1	100	0	0
Dako DAB+ Liquid (K3468)	0	0	1	0
Dako EnVision Plus kits	2	50	1	0
Dako FLEX DAB	23	83	19	84
Dako REAL EnVision K5007 DAB	2	100	2	50
Dako REAL K5001 DAB	1	0	0	0
Leica Bond Polymer Refine kit (DS9800)	57	82	59	85
menapath xcell kit DAB (MP-860)	1	100	1	0
Other	16	81	15	93
Sigma DAB (D5637)	0	0	1	0
Ventana DAB	25	100	24	71
Ventana iview	1	0	0	0
Ventana Ultraview DAB	45	76	48	81
Vision BioSystems Bond X DAB	1	100	1	100

Lymphoma Run: 116	BCL2		CD10	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	1	100	1	100
Dako Autostainer	0	0	1	100
Dako Autostainer Link 48	21	90	17	82
Dako Autostainer plus	0	0	1	0
Dako Autostainer Plus Link	3	100	3	67
Dako Omnis	6	50	6	83
LabVision Autostainer	2	100	1	100
Leica Bond Max	26	81	32	84
Leica Bond X	0	0	1	100
Leica Bond-III	39	85	35	83
Menarini - Intellipath FLX	2	50	2	0
None (Manual)	3	67	3	67
Ventana Benchmark GX	1	100	1	100
Ventana Benchmark ULTRA	70	86	65	83
Ventana Benchmark XT	27	78	31	74

BEST METHODS - Gold Standard Antibody

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

BCL2 - Method 1

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

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

BCL2 - Method 2

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

Primary Antibody: Dako M0887 (124) , 30 Mins, 23 ºC Dilution 1: 1:20
Automation: Dako Autostainer Link 48
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer, PH: 7.6
HMAR: Dako PTLink, Buffer: ENVISION TR SOLN 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

BCL2 - Method 3

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

Primary Antibody:	Leica RTU (BCl2/100/D5) PA0117 , 20 Mins, RT ºC Prediluted
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

BCL2 - Method 4

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

Primary Antibody:	Ventana (SP66) 790-4604 , 60 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.5
HMAR:	Ventana CC2 72mins
EAR:	
Chromogen:	Ventana DAB, 37 ºC., Time 1: 8 Mins
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099) , 8 Mins, 37 ºC Prediluted

BEST METHODS - Secondary Antibody

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

CD10 - Method 1

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

Primary Antibody:	Novocastra NCL-L-CD10-270 (56C6) Dilution 1: 50
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

CD10 - Method 2

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

Primary Antibody:	DAKO RTU Flex Omnis GA648 (56C6) , 20 Mins Prediluted
Automation:	Dako Omnis
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis, Buffer: dako high ph
EAR:	
Chromogen:	Dako FLEX DAB, Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Other , 10 Mins Prediluted

CD10 - Method 3

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

Primary Antibody:	Novocastra NCL-L-CD10-270 (56C6) , 30 Mins, 20 ºC Dilution 1: 20
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer, PH: 7.6
HMAR:	Dako PTLink, Buffer: High PH TRS, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 20 ºC., Time 1: 10 Mins, Time 2: 10 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 15 Mins, 20 ºC Prediluted

CD10 - Method 4

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

Primary Antibody:	Ventana 790-4506 (SP67) , 36 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 72mins, Buffer: CC1
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099)

Neil Bilbe

	Gold Standard	Second Antibody
Antigens Assessed:	GFAP	Tau protein
Tissue Sections circulated:	Low grade glioma.	Alzheimer's brain.
Number of Registered Participants:	62	
Number of Participants this Run	62 (100%)	

Introduction

Gold Standard: GFAP

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

Features of Optimal Immunostaining:

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

Features of Sub-optimal Immunostaining:

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

References:

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

Second Antigen: Tau Protein

Tau protein is a microtubule (MT)-associated protein present in brain and other neuronal tissues. It is expressed predominantly in axons of both the central and the peripheral nervous system, but is barely detectable in astrocytes and oligodendrocytes of the central nervous system. In the adult brain, alternative splicing of 3 exons generates 6 tau isoforms ranging from 352 to 441 amino acids in length, however, only the shortest tau isoform is expressed in the fetal brain. Intracellular accumulations of abnormal tau filaments are characteristic in a heterogeneous group of diseases known as neurodegenerative tauopathies; including Alzheimer´s disease. Binding of tau to MTs is related to the degree of phosphorylation, and hyperphosphorylation, of tau may lead to disengagement of tau from MTs, thereby increasing the pool of unbound tau which then may aggregate into insoluble filamentous inclusions. The precise mechanisms whereby tau assembles into filaments and causes neurodegeneration in the human brain remain to be elucidated.

Features of Optimal Immunostaining:

- Staining of NFTs in axons and neurophil threads in plaques
- Clean background with minimal non-specific staining.

Features of Sub-optimal Immunostaining:

- Weak or uneven staining of Tau protein
- Diffuse, poorly-localised staining
- Excessive background or non-specific specific staining of glia

References:

1. **Butner KA, Kirschner MW.** Tau protein binds to microtubules through a flexible array of distributed weak sites. J Cell Biol 1991;115:717-30.
2. **Trojanowski JQ, Lee VM-Y.** The role of tau in Alzheimer's disease. Med Clin N Am 2002;86:615-27.
3. **Biernat J, Wu Y-Z, Timm T, Zheng-Fischhöfer Q, Mandelkow E, Meijer L**, et al. Protein kinase MARK/PAR-1 is required for neurite outgrowth and establishment of neuronal polarity. Mol Biol Cell 2002;13:4013-28.
4. **Liu, F., et al.** (2002) Role of glycosylation in hyperphosphorylation of tau in Alzheimer's disease. FEBS Lett. 512:101-106 (cites the use of cat. # 44734G, 44742G, 44750G, 44752G and 44754G).
5. **Alonso, A.D., et al.** (2001) Interaction of tau isoforms with Alzheimer's disease abnormally hyperphosphorylated tau and *in vitro* phosphorylation into the disease-like protein. J. Biol. Chem. 276(41

Assessment Summary:

All 62 participants submitted GFAP (**G & H**) slides. 36 (58%) submitted Tau slides; 18 (29%) labs requested an alternative antibody for Tau (**J & K**), (Synaptophysin) but 8 (13%) labs did not submit any slides for Tau or contact NEQAS for an alternative, including one which stained for Neurofilament, which was not the permitted alternative marker, and therefore not scored. This left 232 slides to assess: **G=62 H=62 J=54 K=54**.

The overall pass rate was 94% (218 slides), borderline 5% (10), and the failed slides rate was 2% (4). The average score for all slides was 16/20.

(1) Summary Table - All Slides:

Slide	Antigen	Pass	Borderline	Fail
G (NEQAS)	GFAP (62)	90%	8%	2%
H (In-House)	GFAP (62)	95%	2%	3%
J (NEQAS)	Tau (54)	93%	6%	2%
K (In-House)	Tau (54)	98%	2%	0%
Total (Average)	**232**	**94%**	**5%**	**2%**

GFAP (**G & H**)

The NEQAS (**G**) GFAP pass rate has dropped from 95% (Run 115) to 90% which is the same level as for Run 114. The primary factor is an increased number of borderline scores (↑3 slides) and a single failed section (none previously). All borderline sections showed one or more of: weak/incomplete/uneven staining (see Image: Fig 4). The single failed slide was so heavily counterstained as to make the slide unreadable (see Image: Fig 5). The average scores for the NEQAS GFAP (**G**) dropped from 16/20 (115, 114) to 15/20.

Tau Protein (**J & K**)

Tau was last requested in 2012 (Run 98). The Run 116 results were better than for Run 98, for the NEQAS (**J**) material, with only one slide failing (three previously), and with a pass rate of 89% slightly better than 85% (Run 98).

(2) Summary Table - Tau Protein slides only (N = 36):

Slide	Antigen	Pass	Borderline	Fail
J (NEQAS)	Tau (36)	89% (32)	8% (3)	3% (1)
K (In-House)	Tau (36)	98% (35)	2% (1)	0% (0)
Total (Average)	**72**	**93.5%**	**5.0%**	**1.5%**

The single 116 failed slide had no Tau staining (see Images: Fig 11) and used the Tau-2 clone. There were three borderline slides. One each: Novocastra Tau-2, Autogen-Bioclear AT8 clone, and the Dako polyclonal. The average score for the NEQAS (**J**) Tau slides was 15/20. A pass rate of 89% was the worse, but only just lower than GFAP 90% (**G**).

N.B. Those submitting Synaptophysin (N = 18) had a 100% pass rate for their NEQAS and In-house slides **(J&K)** averaging 17/20, which obviously improves the overall rates for the 2nd antigen.

Selected Images showing Optimal and Sub-optimal Immunostaining

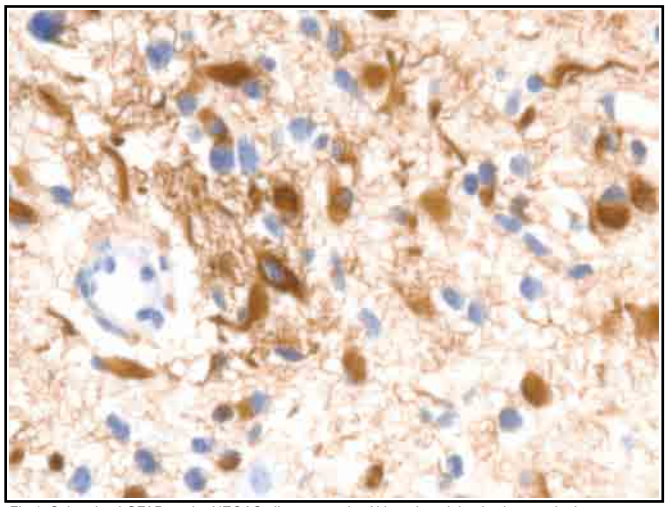


Fig 1. Suboptimal GFAP on the NEQAS glioma sample. Although staining is clean and crisp, some of the tumour is not demonstrated. Therefore the assessors deducted a mark for this. Dako polyclonal, without heat retrieval, but with VBS Bond Enzyme pretreatment, Bond Polymer Refine kit on the Leica Bond Max.

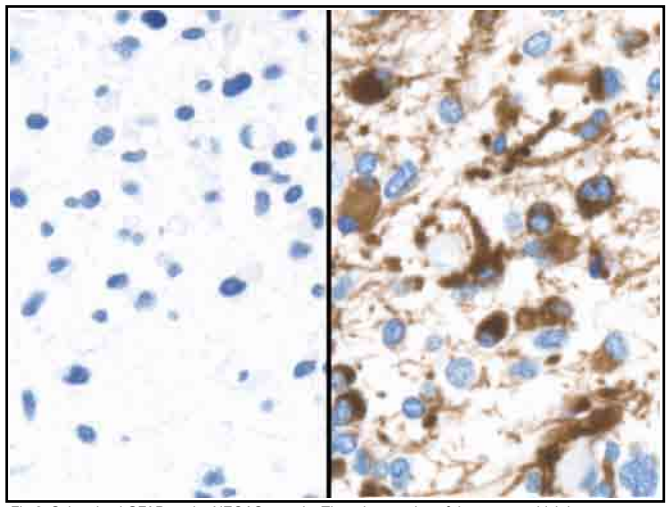


Fig 2. Suboptimal GFAP on the NEQAS sample. There is a portion of the tumour which has failed to stain (L) compared to other areas (R). Slide was assessed as borderline. Dako monoclonal (6F2), 1:100, Leica ER1 30 mins, RTU Bond Polymer Refine 8 mins, on the Leica Bond-III.

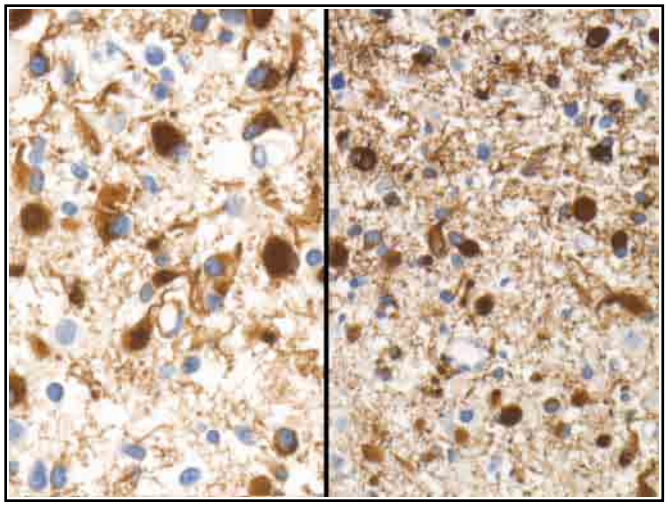


Fig 3. Optimal demonstration of GFAP on NEQAS tissue; high (L) and low power (R) views. Staining is very selective, and clean, with little or no discernible background, and optimal counterstain intensity. Dako poly, 1:1500, Bond Enzyme 1 retrieval, Bond Polymer Refine, on the Leica Bond-III.

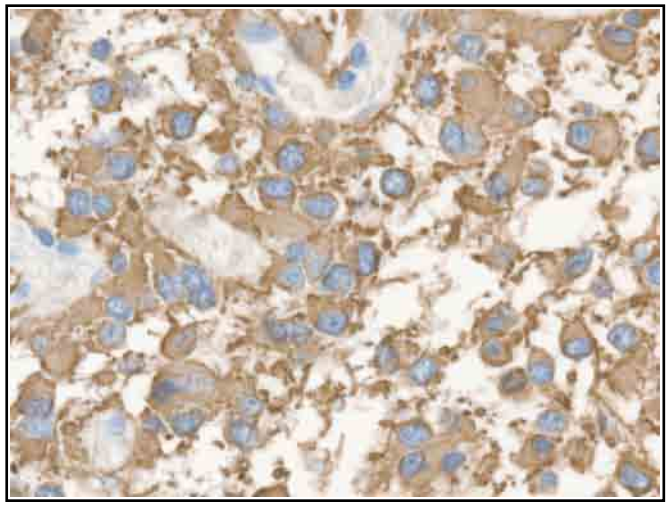


Fig 4. Suboptimal GFAP on the NEQAS glioma sample. Staining is weak and muddy (comp Fig 3), possibly due to over retrieval, weak primary* (1:400 recommended by supplier). Novocastra monoclonal (GA5), *1:1000, Leica ER1 20 mins, RTU Bond Polymer Refine, on the Leica Bond-III.

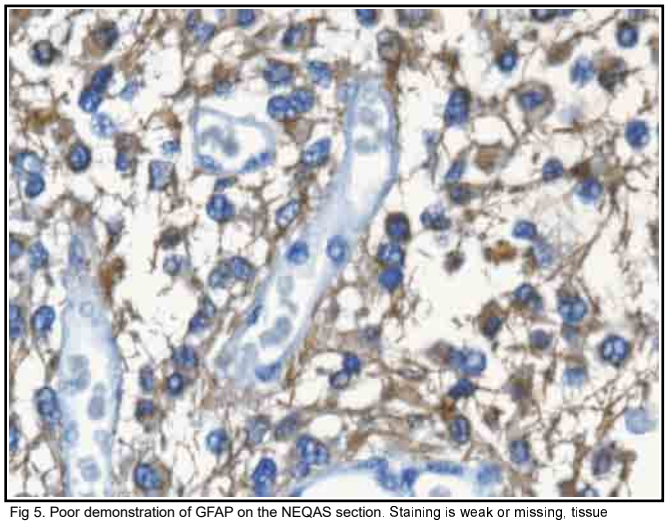


Fig 5. Poor demonstration of GFAP on the NEQAS section. Staining is weak or missing, tissue morphology is compromised, and the counterstain is too heavy. Slide failed the assessment. RTU Dako IR524 poly, pH 9 Dako PTLink, RTU Dako EnVision FLEX+ kit, on the Dako Autostainer Link 48.

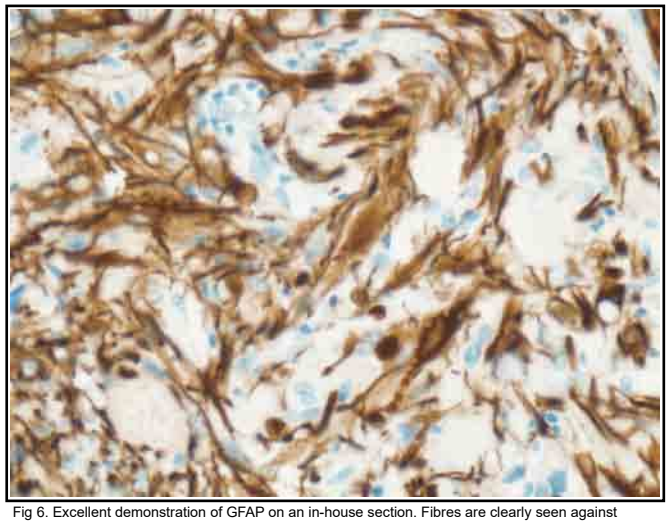


Fig 6. Excellent demonstration of GFAP on an in-house section. Fibres are clearly seen against a clean background, with an optimal counterstain level. Dako Z0334 poly, 1:1000, Ventana Protease 1 retrieval, the Ventana OptiView Kit, and on the Ventana Benchmark ULTRA.

Selected Images showing Optimal and Sub-optimal Immunostaining

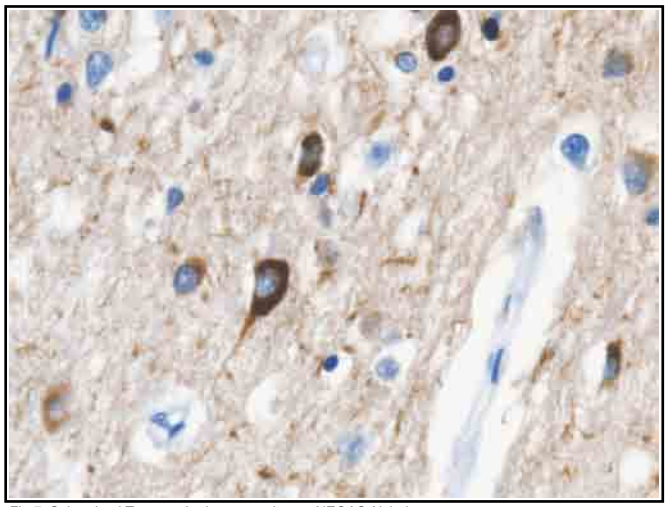


Fig 7. Suboptimal Tau protein demonstration on NEQAS Alzheimer's section. Not all tangles are seen and there is some background staining, although still adequate for clinical use. Dako A0024 polyclonal, 1:6000, Leica ER1 10 mins, with Leica Bond Polymer Refine kit, on the Leica Bond-III.

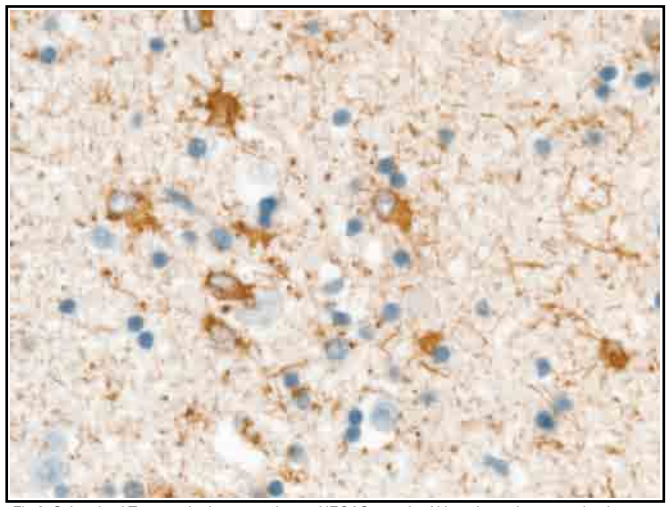


Fig 8. Suboptimal Tau protein demonstration on NEQAS sample. Although tangles are stained, there is also some non-specific staining of other processes. Sigma T6402 (AT8) polyclonal, 1:500, with microwave pretreatment, Vector Elite Universal ABC (PK-6200) kit, employing a manual method.

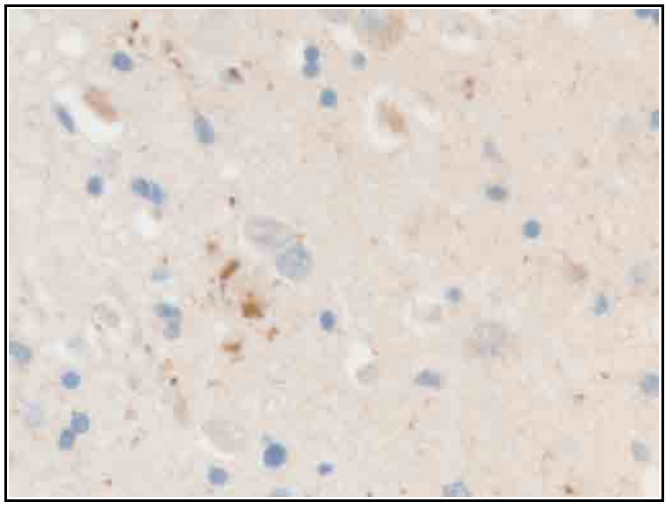


Fig 9. Suboptimal Tau protein demonstration on NEQAS tissue. Staining is too weak, or barely visible in some areas. Slide received a low borderline score, and needs improving. Novocastra (Tau-2) monoclonal, 1:50, with Leica ER2 20 mins, the Leica Bond Polymer Refine kit, on the Leica Bond-III.

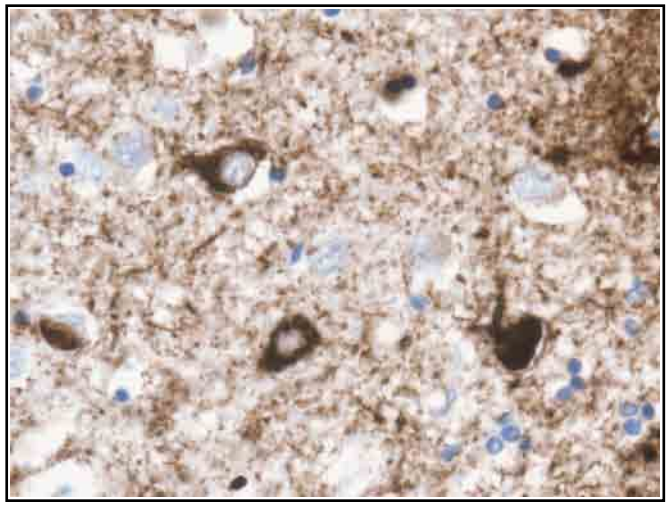


Fig 10. Suboptimal Tau protein demonstration on NEQAS Alzheimer's. Tangles and threads are seen, but the background levels are a little high. Slide was adequate for diagnosis and scored as a pass. Thermo (AT8) monoclonal, 1:300, Leica ER2 20 mins, Bond Polymer Refine and the Bond Max.

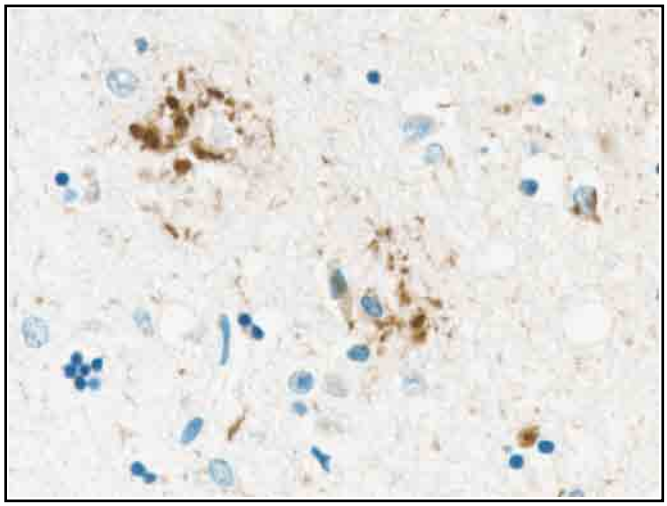


Fig 11. Poor Tau protein demonstration on NEQAS sample. There is little or no NFT staining in the majority of the section, but bits of plaque were picked up. Slide failed the assessment. Novocastra (Tau-2) mono, 1:75, Leica ER2 20 mins, RTU Leica Bond Polymer Refine, on the Leica Bond Max.

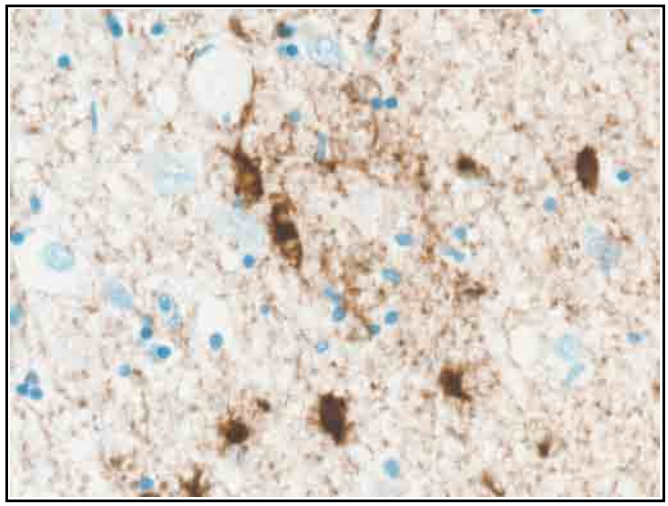
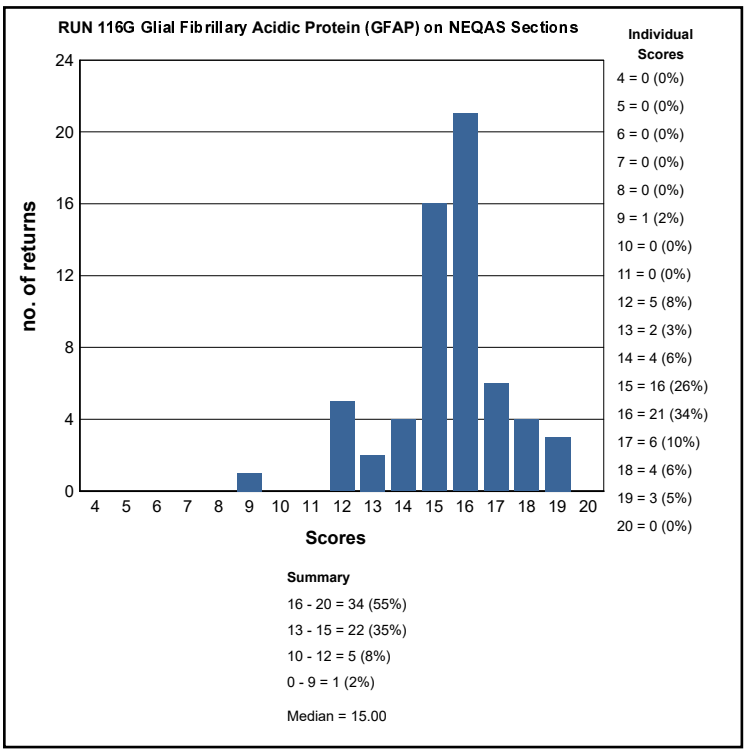
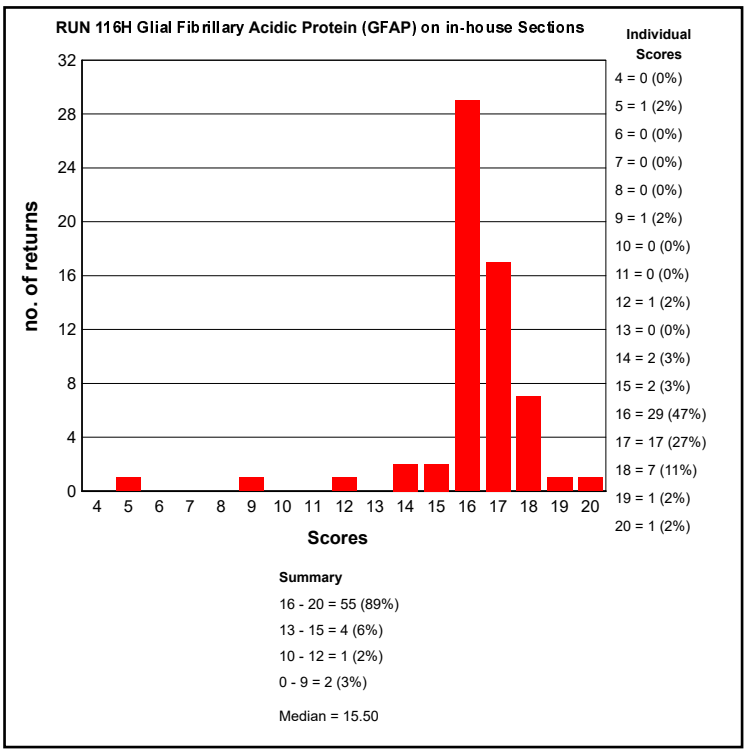
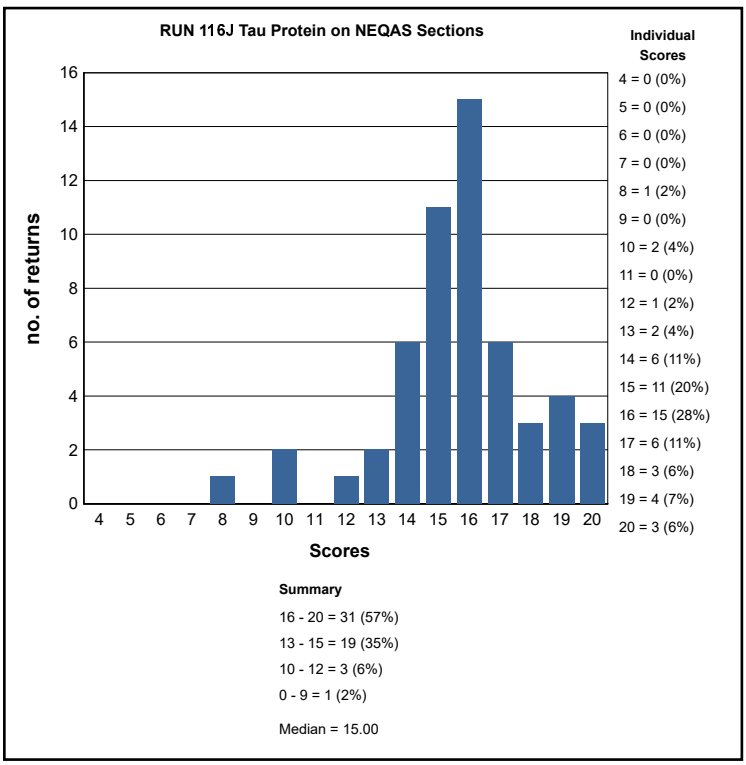


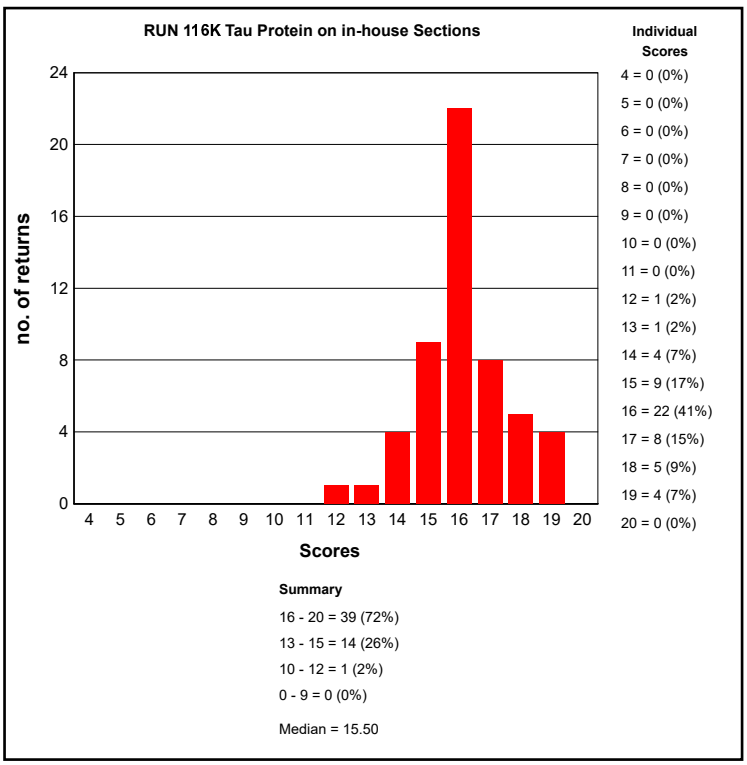
Fig 12. Optimal Tau protein demonstration on NEQAS sample. Tangles and threads are nicely stained and there is little or no non-specific staining of glial processes. Thermo (AT8) monoclonal, 1:20, Ventana CC1 standard, with Ventana iView system, and the Ventana Benchmark XT.

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: 116

Primary Antibody : Glial Fibrillary Acidic Protein (GFAP)

Antibody Details	N	%
Dako M0761 (6F2)	12	83
Dako Z0334 (R Poly)	26	96
Immunon 490740RB	1	100
Novocastra NCL-GFAP-GA5 (GA5)	2	50
Sigma G3895 (GA5)	2	50
Zymed/Invitrogen 08-1021 (ZCG29)	1	100
Dako IR524 (R Poly)	2	50
Novocastra PA0026 RTU (GA5)	4	100
Ventana 760-4345 (EP672Y)	6	100
Cell Marque (EP672Y) 258R	2	100
Dako Omnis GA524 (R Poly)	3	100

Neuropathology Run: 116

Primary Antibody : Tau Protein

Antibody Details	N	%
Dako A0024 (rb poly)	13	92
In House produced	1	100
Innogen.Autogen Bioclear BR-003 (AT8)	4	75
Novocastra NCL-TAU 2 (Tau-2)	3	33
Sigma T5530 (Tau-2)	3	100
Other	17	100
Sigma T6402 (AT8)	1	100
Thermo Scien. MN1020 PHF-tau (AT8)	8	100

Neuropathology Run: 116

Heat Mediated Retrieval	Glial Fibrillary Acidic Protein (GFAP)		Tau Protein	
	N	%	N	%
_Leica Bond III ER2	0	0	1	100
Dako Omnis	5	100	1	100
Dako PTLink	5	60	3	67
Leica ER1 10 mins	1	100	1	100
Leica ER1 20 mins	4	75	1	100
Leica ER1 30 mins	2	50	3	67
Leica ER1 40 mins	0	0	1	100
Leica ER2 10 mins	1	100	0	0
Leica ER2 20 mins	4	100	4	50
Leica ER2 30 mins	0	0	1	100
Microwave	3	100	2	100
None	11	91	13	92
Other	1	100	1	100
Ventana CC1 20mins	1	100	0	0
Ventana CC1 32mins	2	100	4	100
Ventana CC1 36mins	2	100	0	0
Ventana CC1 40mins	1	100	1	100
Ventana CC1 52mins	0	0	1	100
Ventana CC1 64mins	4	100	3	100
Ventana CC1 76mins	1	100	0	0
Ventana CC1 8mins	4	75	0	0
Ventana CC1 92mins	0	0	1	100
Ventana CC1 mild	4	100	2	100
Ventana CC1 standard	2	100	3	100
Ventana CC2 mild	0	0	1	0
Water bath 95-98 OC	1	100	0	0

Neuropathology Run: 116

Enzyme Mediated Retrieval	Glial Fibrillary Acidic Protein (GFAP)		Tau Protein	
	N	%	N	%
AS PER KIT	0	0	1	100
NOT APPLICABLE	30	87	25	88
Other	1	100	1	100
VBS Bond Enzyme 1	5	80	0	0
Ventana Protease 1 (760-2018)	5	100	1	100

Neuropathology Run: 116 Detection	Glial Fibrillary Acidic Protein (GFAP) N	%	Tau Protein N	%
AS PER KIT	5	100	5	80
Dako EnVision FLEX (K8000/10)	4	75	3	67
Dako EnVision FLEX+ (K8002/12)	4	50	3	100
Dako Envision HRP/DAB (K5007)	2	100	4	100
Leica Bond Polymer Refine (DS9800)	18	89	15	80
MenaPath X-Cell Plus (MP-XCP)	0	0	1	100
Other	2	100	0	0
Vector Elite ABC Kit (PK-7200)	1	100	0	0
Vector Elite Universal ABC (PK-6200)	0	0	1	100
Ventana iView system (760-091)	0	0	1	100
Ventana OptiView (760-700) + Amp. (7/860-099)	0	0	1	100
Ventana OptiView Kit (760-700)	8	100	6	100
Ventana UltraView Kit (760-500)	17	94	11	91

Neuropathology Run: 116 Automation	Glial Fibrillary Acidic Protein (GFAP) N	%	Tau Protein N	%
Dako Autostainer Link 48	6	67	7	86
Dako Autostainer Plus Link	1	0	0	0
Dako Omnis	5	100	1	100
Leica Bond Max	6	100	4	50
Leica Bond-III	13	85	11	91
None (Manual)	3	100	7	100
Ventana Benchmark ULTRA	20	100	13	92
Ventana Benchmark XT	7	86	8	88

Neuropathology Run: 116 Chromogen	Glial Fibrillary Acidic Protein (GFAP) N	%	Tau Protein N	%
AS PER KIT	8	100	11	82
DAKO DAB+	1	100	0	0
Dako DAB+ REAL Detection (K5001)	1	100	2	100
Dako EnVision Plus kits	2	100	0	0
Dako FLEX DAB	7	57	3	100
Dako REAL EnVision K5007 DAB	1	100	3	100
Leica Bond Polymer Refine kit (DS9800)	18	89	13	77
menapath xcell kit DAB (MP-860)	0	0	1	100
Other	3	100	2	100
Sigma DAB (D4168)	0	0	1	100
Sigma DAB (D5637)	0	0	1	100
Ventana DAB	2	100	4	100
Ventana iview	0	0	1	100
Ventana Ultraview DAB	18	94	10	90

BEST METHODS - Gold Standard Antibody

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

Glial Fibrillary Acidic Protein (GFAP) - Method 1

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

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

Glial Fibrillary Acidic Protein (GFAP) - Method 2

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

Primary Antibody: Dako Z0334 (R Poly) , 15 Mins, r/t ºC Dilution 1: 1500
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: None
EAR: Other Digestion Time NEQAS: 10 Mins. In-House: 10 Mins
Chromogen: Leica Bond Polymer Refine kit (DS9800)
Detection: Leica Bond Polymer Refine (DS9800)

Glial Fibrillary Acidic Protein (GFAP) - Method 3

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

Primary Antibody:	Dako Z0334 (R Poly) Dilution 1: 1000
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	AS PER KIT
HMAR:	
EAR:	Ventana Protease 1 (760-2018)
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

Glial Fibrillary Acidic Protein (GFAP) - Method 4

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

Primary Antibody:	Dako M0761 (6F2) , 28 Mins, 37 ºC Dilution 1: 200
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
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC Prediluted

BEST METHODS - Secondary Antibody

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

Tau Protein - Method 1

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

Primary Antibody:	Thermo Scien. MN1020 PHF-tau (AT8) , 40 Mins Dilution 1: 800
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	None
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 30 Mins Prediluted

Tau Protein - Method 2

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

Primary Antibody:	Thermo Scien. MN1020 PHF-tau (AT8) , 60 Mins, 23 ºC Dilution 1: 100
Automation:	None (Manual)
Method:	Envision
Main Buffer:	Phosphate Buffered Saline (PBS), PH: 7.6
HMAR:	Microwave, Buffer: Citrate, PH: 6
EAR:	
Chromogen:	Dako DAB+ REAL Detection (K5001), 23 ºC., Time 1: 2 Mins
Detection:	Dako Envision HRP/DAB (K5007) , 30 Mins, 23 ºC Prediluted

Tau Protein - Method 3

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

Primary Antibody:	Innogen.Autogen Bioclear BR-003 (AT8)
Automation:	None (Manual)
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	None
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

Tau Protein - Method 4

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

Primary Antibody:	Thermo Scien. MN1020 PHF-tau (AT8) Dilution 1: 20
Automation:	Ventana Benchmark XT
Method:	Ventana iView Kit
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 standard
EAR:	
Chromogen:	Ventana iview
Detection:	Ventana iView system (760-091)

Neil Bilbe and Irena Srebotnik Kirbis

	Gold Standard	Second Antibody
Antigens Assessed:	CK	CD45
Sample circulated; cytospins and cell block sections:	Four effusion samples with carcinoma, mesothelial cells, lymphocytes and RBCs.	Four effusion samples with carcinoma, mesothelial cells, lymphocytes and RBCs.
Number of Registered Participants:	84- Cell block 62 (74%), Cytospin 22 (26%)	
Number of Participants this Run	84 (100%)	

Introduction

Gold Standard: Cytokeratin

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

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

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

References

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

Second Antigen: CD45

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

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

Features of Suboptimal Immunostaining:
- Weak, diffuse or partial membrane staining of lymphocytes
- Uneven staining
- Excessive background staining
- Non-specific staining of cell types or components not expected to stain
- Inadequate nuclear counterstain

References

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

References (cell blocks in cytology)

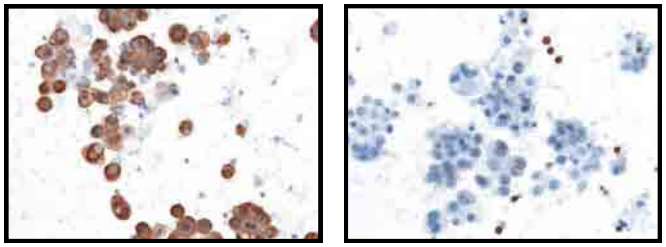
1. Xin Jing, et al,. Morphologic and Immunocytochemical Performances of Effusion Cell Blocks Prepared Using 3 Different Methods. (2013) American Journal of Clinical Pathology, *139,* 177-182
2. Nithyananda A. Nathan, et al, Cell Block Cytology. Improved Preparation and Its Efficacy in Diagnostic Cytology.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

Assessment Summary:

There are currently 84 labs registered on the Cytopathology module. For Run 116 all participants submitted at least two slides. One participant did not submit any in-house **(S & U)** samples. Interestingly, several labs placed their in-house sections beneath the NEQAS section (CB). A total of 334 slides were scored: **R = 84, S = 83, T = 84, U = 83.**

The overall pass rate was 89% (297 slides), 8% borderline (26), and 3% failure rate (11). The average score for all slides was 16/20. See Table (3) for cell block v cytospin averages.

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



Figs 1 & 2: Run 116 samples: CK MNF116 (L) and CD45 (R)

There are approximately 80 % of cells in this mixture expressing CK, and 10 % of lymphocytes expressing CD45.

Assessment Outcomes:

The overall assessment outcomes were slightly worse than for the previous two runs (114, 115). For the first time this EQA year (2016/2017) pass levels have fallen below 90% for both the overall rates, and the NEQAS Gold Cytokeratin (**R**).
A full review will be carried out after the last run of the year (117) in the Summer.

(1) Summary Table - All Slides:

Slide	Antigen	Pass	Borderline	Fail
R (NEQAS)	CK (84)	88%	6%	6%
S (In-House)	CK (83)	93%	6%	1%
T (NEQAS)	CD45 (84)	86%	11%	4%
U (In-House)	CD45 (83)	89%	8%	2%
Total (Average)	334	89%	8%	3%

Following two relatively consistent runs, where pass rates, and failure levels, were comparable for the Cytokeratin and 2nd antigens (ER and Ki 67), it is difficult to know why there has been an increase in failed slides, and therefore a corresponding drop in overall pass rates - overall borderline rates have remained consistent: 7%, 5%, 8%.
Given this increased number of *failed* NEQAS slides (**R & T**) particularly for the Gold CK slides (**R)**, which increased to five for this run, but was zero (115), and one (114), previously, the protocols have been summarised below in Table 3.

CK (R & S)

Six slides failed the assessment for this Run (zero previously), five NEQAS slides (**R)**, and a single in-house (**S**); the pass rate for the NEQAS slides (**R**) decreased from 94% (115) to 88%. All the failed NEQAS slides were on cell block sections, the primary reason was very weak CK demonstration (see Fig 3), although one or two also showed patchy and/or non-specific reactions, allied to the use of excessive pre-treatments. The single failed in-house control slide (**S**) was a cytospin, which had simply failed to stain, though the same lab did score 15 for their NEQAS cytospin (**R**) sample.

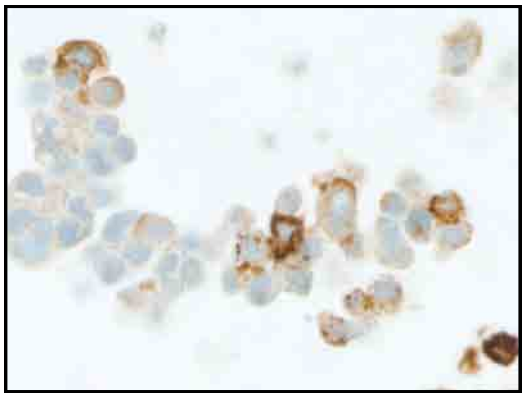


Fig 3: Example of a failed CB section stained with CK. Only a very low percentage of epithelial cells are suitably demonstrated.

A similar number of slides scored 20/20 for the CK as for Run 115, nine, (eight previously), although only two of these were NEQAS (**R**) samples, but both were CB sections; the seven in -house slides (**S**) were from a variety of sample types: FFPE (1), cell blocks (3), cytospin (2), smear (1).
Both the NEQAS slides (**R**) used a Dako marker: MNF116 on a Ventana platform; and the Omnis AE1/AE3 on the Omnis system. Similarly for the in-house (**S)**, five used the Dako MNF116 (variety of platforms), and one each employed a Ventana AE1/AE3 and a Novocastra AE1/AE3.

CD45 (T & U)

There were five failed slides for the CD45 submissions. Three NEQAS (**T**) slides, two cell blocks and a cytospin, and two in-house samples (**U**), one smear and one cytospin.
The assessors found that two of the NEQAS slides (**T**) were both weakly stained and had non-specific staining; the third slide was so heavily counterstained that all primary staining was completely masked and therefore unreadable. The in-house (**U**) samples were too weakly stained for diagnostic purposes, and both had some inappropriate staining.
The number of slides scoring 20/20 totalled six, of which two were on NEQAS (**T**) cell block slides. The four in-house (**U**) samples were on cytospin controls (3) and a single FFPE section.
The two NEQAS slides (**T**) used contrasting protocols; one employed a Dako RTU monoclonal (2B11+PD7/26), on a Ventana platform: CC1 mild 30 mins, with the UltraView Kit on the benchmark XT.
The other employed the Novocastra NCL-L-LCA (X16/99), at 1:80, with ER1 30 mins, a Refine kit, and the Bond Max.
A common theme with the CD45 slides was the presence of some nuclear staining in the cytospins. This was seen in the evaluation slides sent by the supplier (see Fig 2). Assessors were made aware and attuned to this, and therefore scored the CD45 slides accordingly; only poor or excessive nuclear staining was marked down, but still accounted for the relatively high number of borderline slides for the NEQAS CD45 **(T)** samples:

(2) Summary Table - Borderline Scores NEQAS CD45 (**T**):

Sample	Type	No Slides	Borderline	% By Type	% Overall (84)
Cell Block (CD45)	NEQAS	62	2	3%	2%
Cytospin (CD45)	NEQAS	22	7	32%	8%
Total		84	9		

Out of the 22 CD45 cytospin slides assessed, seven were given a borderline score of between 10-12; which equates to 32% of NEQAS cytospins, and 8% of *all* CD45 slides (N=84).

When looking at the comments given by the assessors on the cytospins, most had inappropriate/nuclear staining (Fig 9), some excessive background, one poor counterstain due to insufficient blueing, giving an unpleasant purple/grey hue to the cells, and resulting in poor contrast.
For the two cell block sections, one had non-specific/diffuse staining (Fig 7), the other had excessive haematoxylin staining which masked the positive reactions (Fig 11).

Summary

(3) Summary Table - NEQAS Slides Failing ≤ 9:

Letter	Antigen	Type	Sample	Primary	Dilution	Retrieval	Automation	Detection
R	CK	NEQAS	Cell block	Dako M3515 (AE1/AE3)	Not Given	Ventana CC1 20mins	Ventana Benchmark ULTRA	Ventana UltraView Kit
R	CK	NEQAS	Cell block	Ventana 760-2135 (AE1/AE3/PCK26)	RTU	None	Ventana Benchmark ULTRA	Ventana OptiView Kit
R	CK	NEQAS	Cell block	Ventana 760-2595 AE1/AE3/PCK26	Not Given	None	Ventana Benchmark XT	Ventana OptiView Kit
R	CK	NEQAS	Cell block	Leica CK14 (LL002)	RTU	Leica ER1 20 mins	Leica Bond-III	Leica Bond Polymer Refine
R	CK	NEQAS	Cell block	Dako CK5/6 (D5/10 B4)	50	Ventana CC1 64mins	Ventana Benchmark XT	Ventana UltraView Kit
T	CD45	NEQAS	Cytospin	Dako M0701 (2B11+PD7/26)	300	None	Leica Bond-III	Leica Bond Polymer Refine
T	CD45	NEQAS	Cytospin	Dako M0701 (2B11+PD7/26)	1200	None	Leica Bond Max	Leica Bond Polymer Refine
T	CD45	NEQAS	Cell block	Dako RTU FLEX LINK (PD7/26 + 2B11)	RTU	Dako PTLink	Dako Autostainer Link 48	Dako EnVision FLEX+

(4) Summary Table - Average scores for CK and CD45 on NEQAS Cytospins and Cell Block sections:

Letter	Antigen	Type	Sample	Average All	Average (UK)
R	CK	NEQAS	Cell Block	16	16
R	CK	NEQAS	Cytospin	16	15
T	CD45	NEQAS	Cell Block	17	17
T	CD45	NEQAS	Cytospin	14	13

Table (4) reflects the overall impression formed at the assessments; the quality of the cytokeratin slides was generally better than those stained for CD45.

(5) Summary Table - Current levels of samples by region:

Sample	Totals	UK (55%)	EIRE (8%)	EUR (32%)	ROW (5%)
CB	62 (74%)	41 (67%)	7 (11%)	9 (15%)	4 (7%)
CS	22 (26%)	5 (22%)	0	18 (78%)	0
	84	46	7	27	4

Selected Images showing Optimal and Sub-optimal Immunostaining

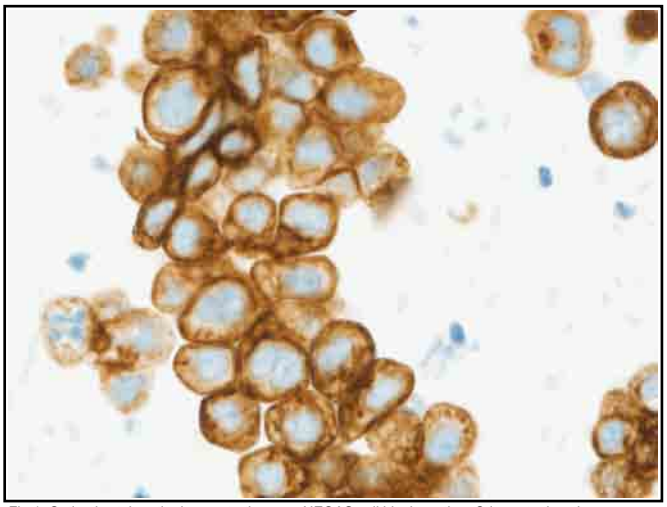


Fig 1. Optimal cytokeratin demonstration on a NEQAS cell block section. Crisp cytoplasmic staining is seen with no background, and an ideal counterstain intensity. Dako M0821 (MNF116), 1:200, Dako PTLink pH 9 20 mins, RTU Dako EnVision FLEX+ kit, on the Dako Autostainer Link 48.

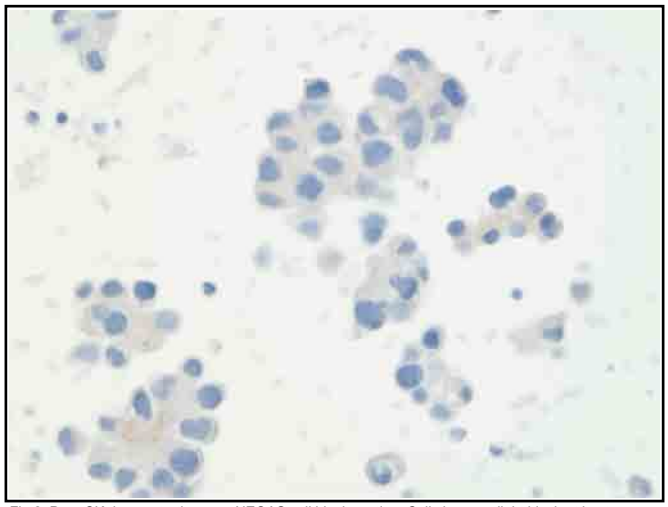


Fig 2. Poor CK demonstration on a NEQAS cell block section. Cells have a slight blush at best (comp Fig 1). Participant used a CK14 targeted marker, as against a pan CK. Prediluted Leica CK14 (LL002) monoclonal, Leica ER1 20 mins, Leica Bond Polymer Refine kit, on the Leica Bond-III.

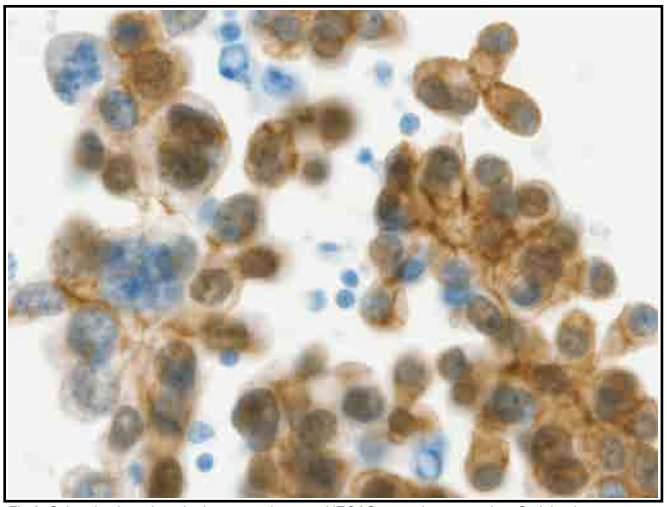


Fig 3. Suboptimal cytokeratin demonstration on a NEQAS cytospin preparation. Staining is patchy and not all epithelia cells are shown, but overall is adequate for diagnosis. Slide passed the assessment. Dako monoclonal CK5/6, 1:500, no RT, Bond Polymer Refine kit, on the Leica Bond Max.

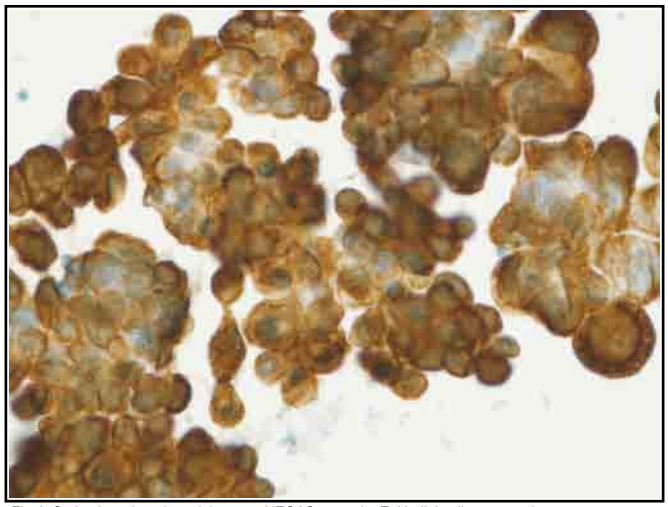


Fig 4. Optimal cytokeratin staining on a NEQAS cytospin. Epithelial cells are evenly demonstrated, non-staining cell types are negative, and the counterstain is ideal. Dako M0821(MNF116), 1:50, without retrieval, with Ventana UltraView Kit, on the Ventana Benchmark ULTRA.

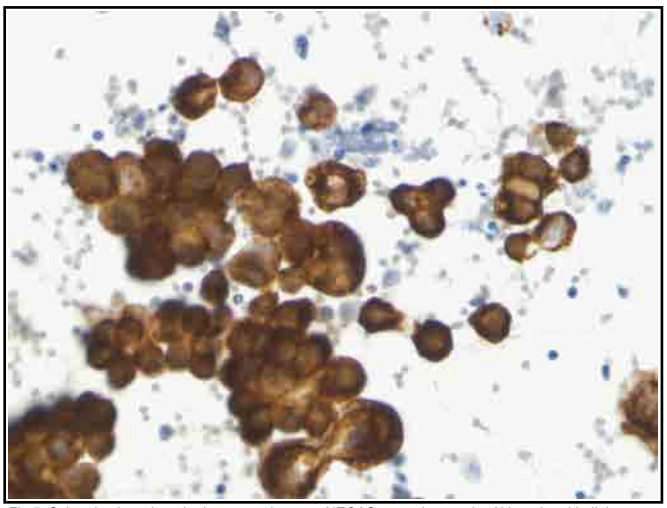


Fig 5. Suboptimal cytokeratin demonstration on a NEQAS cytospin sample. Although epithelial cells are stained there is evidence of non-specific staining, counterstain is heavy with RBCs being picked up. Dako M3515 (AE1/AE3), 1:100, MW 10 mins, RTU Envision+ HRP mouse kit, manual method.

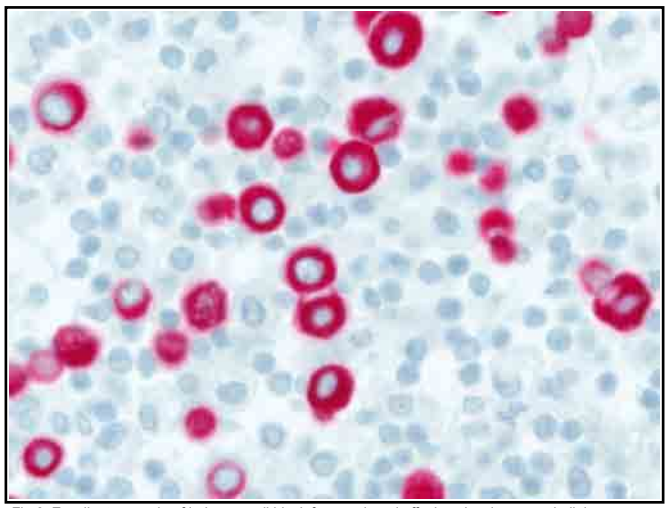


Fig 6. Excellent example of in-house cell block from a pleural effusion showing mesothelial cells. Cells are clearly seen, there is little or no bleeding of chromogen and the counterstain is ideal. Diagnostic Biosystems MNF116, 1:200, CC1 standard, RTU UV AP Red Detection Kit, on Ventana Benchmark XT.

Selected Images showing Optimal and Sub-optimal Immunostaining

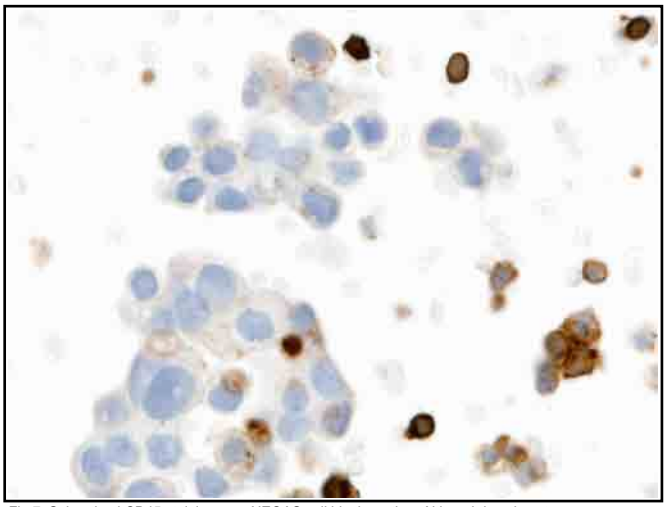


Fig 7. Suboptimal CD45 staining on a NEQAS cell block section. Although lymphocytes are demonstrated (10% overall), there is non-specific and some background staining (comp Fig 8). Dako M0701 (2B11+PD7/26), 1:100, Leica ER1 30 mins, Leica Bond Polymer Refine, on the Leica Bond-III.

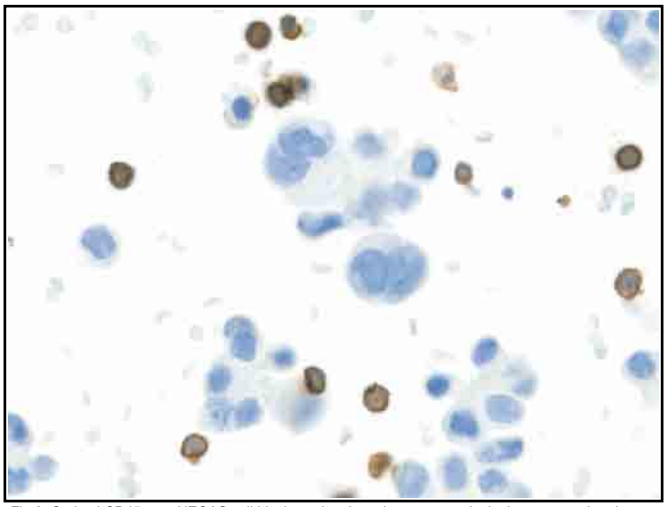


Fig 8. Optimal CD45 on a NEQAS cell block section. Lymphocytes are nicely demonstrated and the slide is generally clean, and the counterstain intensity is ideal. Dako M0701 (2B11+PD7/26), 1:500, Leica ER1 20 mins, Leica Bond Polymer Refine, on Leica Bond-III.

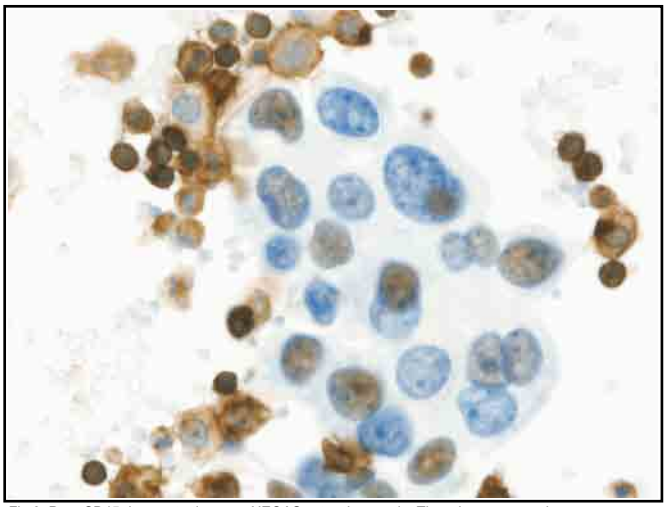


Fig 9. Poor CD45 demonstration on a NEQAS cytospin sample. There is pronounced non-specific staining beyond the expected level (comp Fig 10). The slide failed the assessment. Same Dako monoclonal marker (2B11+PD7/26), 1:500, no RT, Leica Bond Polymer Refine, on a Leica Bond-III.

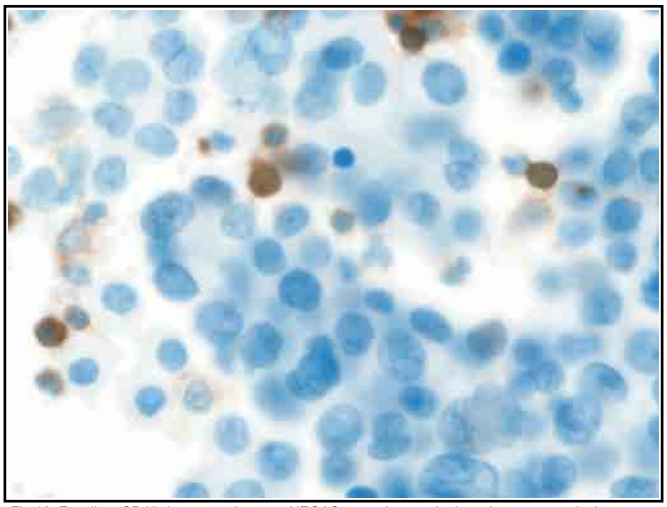


Fig 10. Excellent CD45 demonstration on a NEQAS cytospin sample. Lymphocytes are nicely seen and there is virtually no staining in the epithelial cells. The counterstain provides an good contrast. Dako M0701 (2B11+PD7/26), 1:400, no RT, RTU Leica Bond Polymer Refine kit, on the Leica Bond-III.

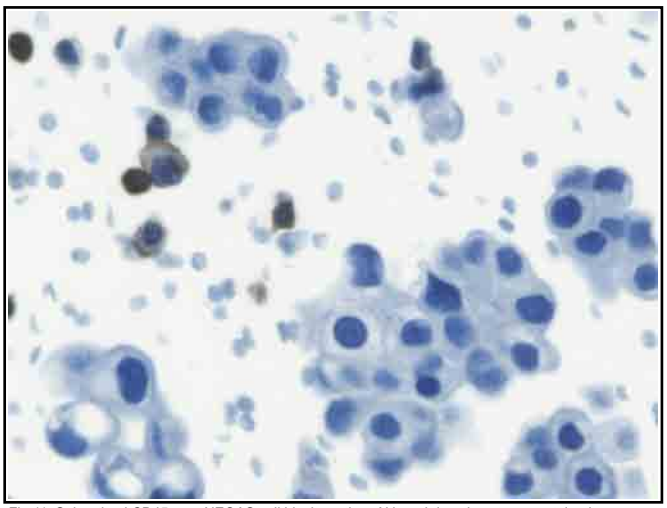


Fig 11. Suboptimal CD45 on a NEQAS cell block section. Although lymphocytes are stained, the section is too heavily counterstained masking some reactions, therefore slide assessed as borderline. Dako monoclonal, 1:400, Leica ER2 20 mins, RTU Leica Bond Polymer Refine, on the Leica Bond Max.

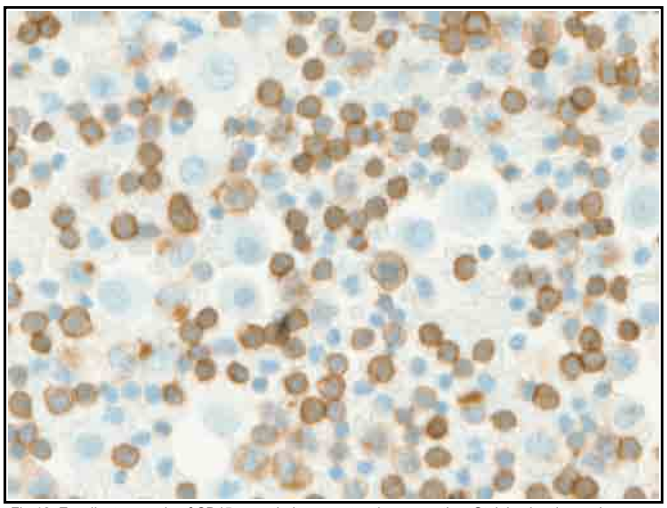
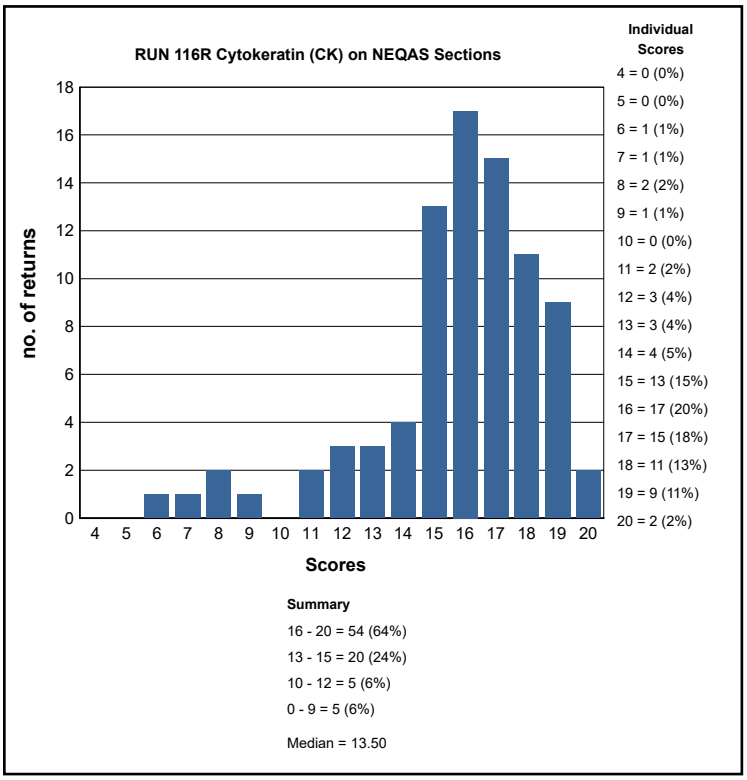
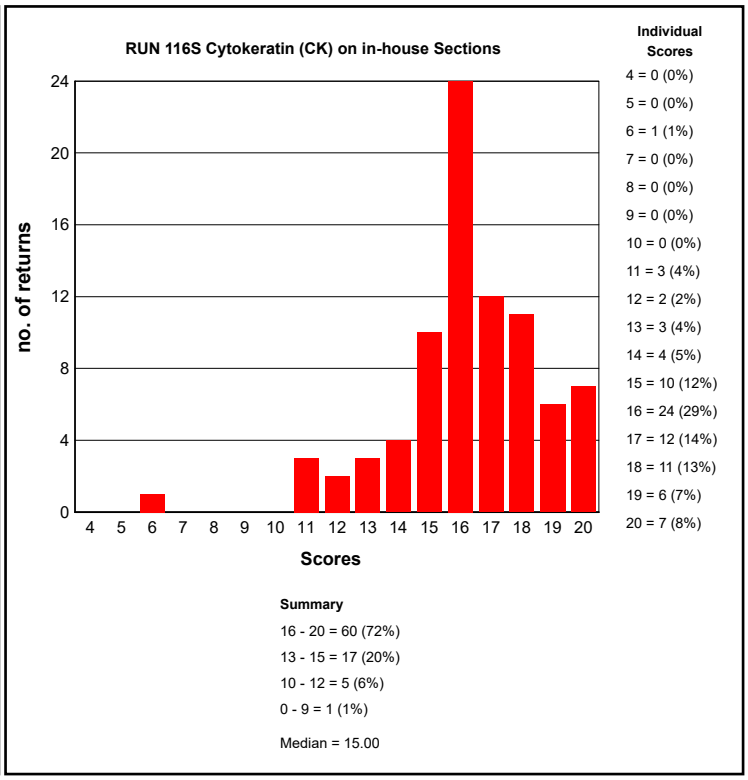
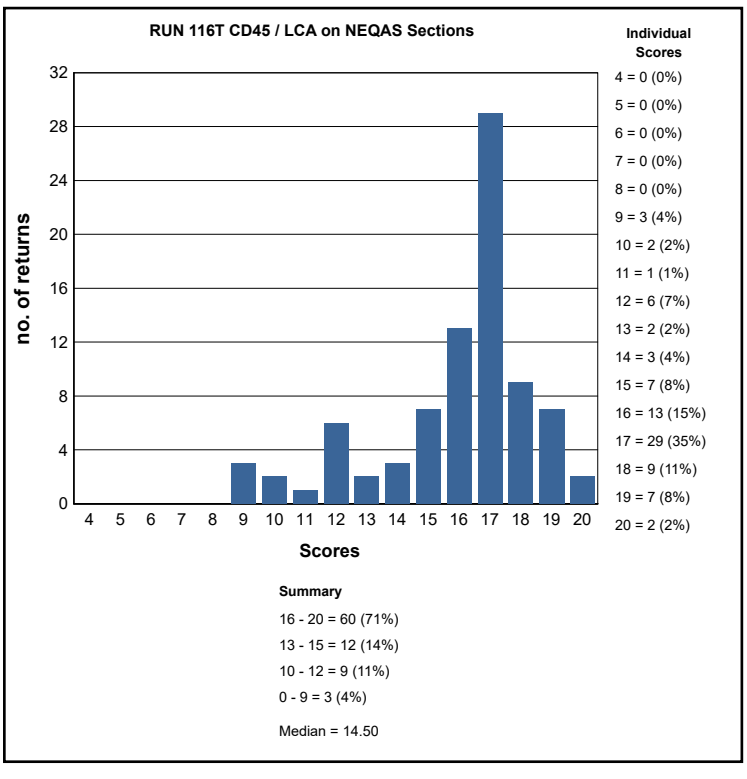


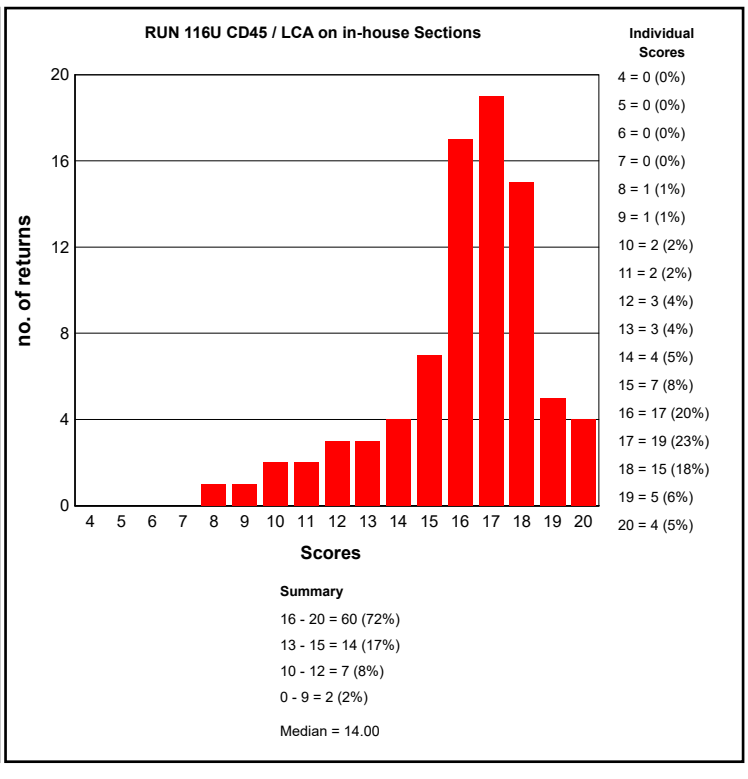
Fig 12. Excellent example of CD45 on an in-house cytospin preparation. Staining is crisp and selective with no background and good counterstain. Dako monoclonal, 1:1000, 32 mins, no RT, Ventana UltraView kit, on a Ventana Benchmark XT.

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: 116

Primary Antibody : Cytokeratin (CK)

Antibody Details	N	%
Becton Dickinson 349205 (CAM5.2)	2	100
BioGenex MU071-UC (clones AE1/AE3)	1	100
Dako M3515 (AE1/AE3)	17	94
Dako M0821(MNF116)	23	100
Leica/Novocastra RTU PA0909 (AE1/AE3)	1	100
Leica/Novocastra NCL-L-AE1/AE3	4	100
Ventana 760 2135 (AE1/AE3/PCK26)	3	33
Ventana 760 2595 AE1/AE3/PCK26	8	75
Other	5	40
Cell Marque 313M- (AE1/AE3)	1	100
Leica/Novocastra NCL-AE1/AE3	1	100
Biomedicals BMA-T-1302	1	100
Ventana CONFIRM 790-4373 (34BE12)	1	100
Dako FLEX RTU IR053 (AE1/AE3)	4	75
ImmunoBS MM-1012 (CK cocktail)	1	100
Leica/Novacastra NCL- CK5/6/8/18 (Multi 5D3/LP34)	1	100
Ventana 790-4555 (CAM 5.2)	3	100
Leica/Novacastra NCL- L-CK5/6/8/18 (Multi 5D3/LP34	3	67
Dako Omnis FLEX GA053 (AE1/AE3)	3	100

Cytology Run: 116

Primary Antibody : CD45 / LCA

Antibody Details	N	%
Dako M0701 (2B11+PD7/26)	48	83
Dako M0754 (4KB5) CD45RA	1	0
Dako M0833 (PD7/26) CD45RB	1	100
Leica/Novocastra NCL-L-LCA (X16/99)	5	80
Ventana CONFIRM 760-2505 (RP2/18)	13	100
Other	2	50
Ventana 760-4279 (2B11 & PD7/26)	2	100
Cell Marque 145M-97	1	100
Leica/Novocastra Bond RTU PA0042 (X16/99)	2	100
Dako RTU FLEX LINK IR751 (2B11 + PD7/26)	4	75
Dako Omnis RTU GA751 (2B11+PD7/26)	4	100

Cytology Run: 116

Primary Antibody : Cytokeratin (CK)

Antigen Retrieval	N	%
YES	22	26
NO	62	74
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	22	
Not Specified	0	

Cytology Run: 116

Primary Antibody : CD45 / LCA

Antigen Retrieval	N	%
YES	26	31
NO	58	69
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	26	
Not Specified	0	

Cytology Run: 116

Heat Mediated Retrieval

Cytology Run: 116

Heat Mediated Retrieval

Cytology Run: 116

Enzyme Mediated Retrieval

Cytology Run: 116

Enzyme Mediated Retrieval

Cytology Run: 116

Detection	CD45 / LCA		Cytokeratin (CK)	
	N	%	N	%
AS PER KIT	7	100	5	100
BioGenex HRP (HK 519-06K)	0	0	1	100
BioGenex SS Polymer (QD 430-XAKE)	1	100	0	0
Dako EnVision FLEX (K8000/10)	1	100	2	100
Dako EnVision FLEX+ (K8002/12)	8	88	7	86
Dako Envision HRP/DAB (K5007)	1	0	1	100
Dako Envision+ HRP mouse K4004/5/6/7	2	0	2	100
Leica Bond Polymer AP Red Detection (DS9305)	0	0	1	100
Leica Bond Polymer Refine (DS9800)	23	70	21	90
Other	2	100	2	50
Ventana iView system (760-091)	2	100	2	100
Ventana OptiView (760-700) + Amp. (7/860-099)	0	0	1	0
Ventana OptiView Kit (760-700)	14	100	15	87
Ventana UltraView Kit (760-500)	20	100	21	86

Cytology Run: 116

Automation	CD45 / LCA		Cytokeratin (CK)	
	N	%	N	%
BioGenex GenoMX 6000i	1	100	1	100
Dako Autostainer Link 48	8	63	10	90
Dako Autostainer Plus Link	2	100	1	100
Dako Omnis	4	100	3	100
Leica Bond Max	9	67	7	100
Leica Bond-III	16	69	16	88
None (Manual)	1	0	1	100
Ventana Benchmark GX	3	100	3	100
Ventana Benchmark ULTRA	27	100	29	86
Ventana Benchmark XT	12	100	12	75

Cytology Run: 116

Chromogen	CD45 / LCA		Cytokeratin (CK)	
	N	%	N	%
AS PER KIT	7	86	9	100
BioGenex Liquid DAB (HK153-5K)	1	100	1	100
DAKO DAB+	2	0	2	100
Dako EnVision Plus kits	1	100	0	0
Dako FLEX DAB	9	89	10	90
Dako REAL EnVision K5007 DAB	1	0	1	100
Leica Bond Polymer Refine kit (DS9800)	23	70	22	91
Other	6	100	5	60
Ventana DAB	9	100	10	80
Ventana iview	3	100	2	100
Ventana Ultraview DAB	21	100	21	86

BEST METHODS - Gold Standard Antibody

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

Cytokeratin (CK) - Method 1

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

Primary Antibody:	Leica/Novocastra NCL-L-AE1/AE3 , 15 Mins, RT ºC Dilution 1: 250
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER1 30 mins, Buffer: 0, 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

Cytokeratin (CK) - Method 2

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

Primary Antibody:	Ventana 790-4555 (CAM 5.2) , 16 Mins
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	
EAR:	Ventana Protease 1 (760-2018) Digestion Time NEQAS: 8 Mins. In-House: 8 Mins
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

Cytokeratin (CK) - Method 3

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

Primary Antibody:	Dako M0821(MNF116) , 30 Mins, 23 ºC Dilution 1: 1:200
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer, PH: 7.6
HMAR:	Dako PTLink, Buffer: ENVISION TR SOL 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

Cytokeratin (CK) - Method 4

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

Primary Antibody:	Dako M0821(MNF116) , 48 Mins, 32 ºC Dilution 1: 75
Automation:	Ventana Benchmark ULTRA
Method:	Ventana iView Kit
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	None
EAR:	Ventana Protease 1 (760-2018)
Chromogen:	Ventana iview
Detection:	Ventana iView system (760-091) Prediluted

BEST METHODS - Secondary Antibody

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

CD45 / LCA - Method 1

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

Primary Antibody:	Dako M0701 (2B11+PD7/26) , 32 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 mild
EAR:	
Chromogen:	Ventana Ultraview DAB, 37 ºC., Time 1: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC Prediluted

CD45 / LCA - Method 2

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

Primary Antibody:	Ventana CONFIRM 760-2505 (RP2/18) , 24 Mins Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 mild
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

CD45 / LCA - Method 3

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

Primary Antibody:	Leica/Novocastra Bond RTU PA0042 (X16/99)
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER1 20 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

CD45 / LCA - Method 4

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

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

The Alimentary Tract Module: GIST Run 116

Suzanne Parry

	First Antibody	Second Antibody
Antigens Assessed:	CD117	DOG-1
Tissue Sections circulated:	Normal appendix, GIST and Desmoid Tumour	
Number of Registered Participants:	117	
Number of Participants this Run	112 (96%)	

Introduction

Gold Standard: CD117

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

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

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

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

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

Second Antibody: DOG-1

"Discovered on GIST 1" (DOG-1) antibody was initially described in 2004[4] and has now started to be recognized as a more specific marker of GISTs than CD117 [4,5,6]. A recent study has shown DOG-1 sensitivity of 99% and found to be strong and easier to interpret than CD117[6]. The groups also showed that seven CD117 negative GIST cases were found to be DOG-1 positive, and six of the tumours had PDGFRA mutations. Furthermore, DOG-1 is not expressed in Kaposi sarcomas of the GI tract which have been shown to stain for CD117[7].

In more cases, combined CD117 and DOG-1 immunohistochemistry has been suggested as a sufficient panel to confirm diagnosis, with CD117 and DOG-1 negative cases tested further with a panel of antibodies including SMA, desmin, S100 and molecular analysis, should be considered[6].

Features of Optimal Immunostaining (see Figs 7-8)

- Good localisation of DOG-1 to cells of the GIST
- Good localisation of DOG-1- to interstitial cells of Cajal
- No staining of desmoid tumour

Features of Sub-optimal Immunostaining (see Figs 9-10)

- Weak and/or patchy staining of the tumour cells of the GIST
- Excessive background or non specific staining
- Staining of the desmoid tumour
- Staining of the mast cells (Note: Mast cells are not expected to stain with DOG-1)

Tissue Distribution and Assessment Procedure

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

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

Assessment Summary:

Results from the **CD117** assessment showed an overall acceptable pass rate of 83% similar to the previous run on the Neqas distributed material. See results table summary below:

NEQAS Pass Rates Run 115 v 116 CD117		
Run	115	116
Acceptable	84% (N=83	83% (N=93)
Borderline	14% (N=15)	13% (N=15)
Unacceptable	3% (N=3)	4% (N=4)

Sub-optimal marks were given to slides showing very weak staining of the GIST or non-specific and background staining, predominantly in the desmoid tumour. Inappropriate antibody dilution factor and antigen retrieval were the main causes of these issues. The most popular CD117 antibody choice still remains the Dako polyclonal, used by 89 participants and showed an acceptable pass rate of 89% in this assessment run.

The **DOG-1** antibody was chosen as the secondary antibody for this current run 116 assessment. The acceptable pass rate was slightly higher to that of the CD117 assessment, and only 3 laboratories failed the assessment. It was encouraging to see that most labs are using a composite control with a positive GIST tumour along with normal tissue.

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. West RB, Corless CL, Chen X et al. The novel marker, DOG1, is expressed ubiquitously in gastrointestinal stromal tumors irrespective of KIT or PDGFRA mutation status. Am. J. Pathol. 2004; 65; 107–113.
5. Espinosa I, Lee CH, Kim MK et al. A novel monoclonal antibody against DOG1 is a sensitive and specific marker for gastrointestinal stromal tumors. Am. J. Surg. Pathol. 2008; 32; 210– 218.
6. 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.
7. Parfitt JR, Rodriguez-Justo M, Feakins R, Novelli MR. Gastrointestinal Kaposi's sarcoma: CD117 expression and the potential for misdiagnosis as gastrointestinal stromal tumour. Histopathology 2008; 52; 816–823.

Selected Images showing Optimal and Sub-optimal Immunostaining

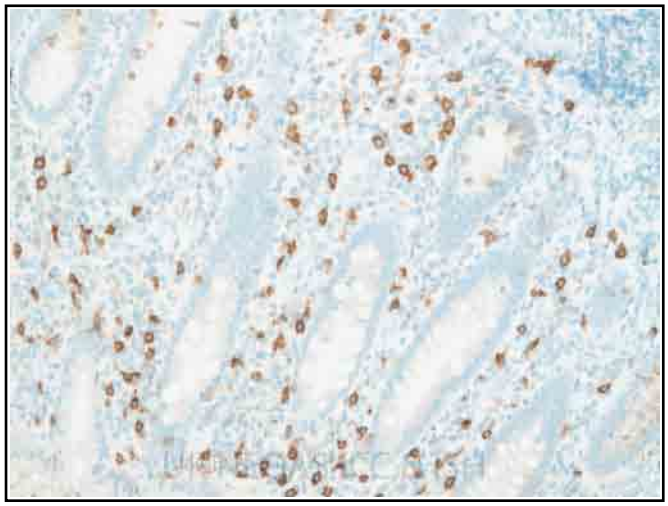


Fig 1. Optimal demonstration of CD117 in the UK NEQAS distributed appendix section. As expected, the mast cells show strong positive membranous staining for CD117. Alongside a positive GIST tumour, appendix is recommended as an appropriate in-house control. This example was stained with the Dako polyclonal antibody, 1:300, on the Ventana Benchmark,

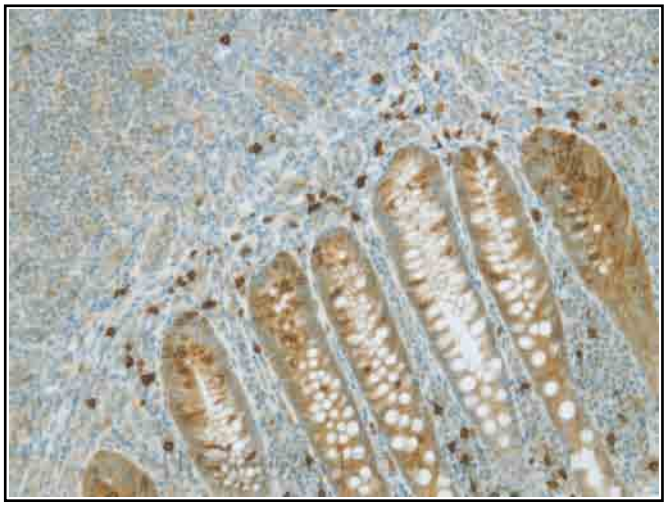


Fig 2. Sub-optimal staining of CD117 in the UK NEQAS distributed appendix (compare to Fig 1). Although the mast cells are staining as expected, the section also shows inappropriate background staining. This is most likely the result of excessive antigen retrieval. Stained with the Leica/Novocastra RTU antibody, antigen retrieval in a pressure cooker.

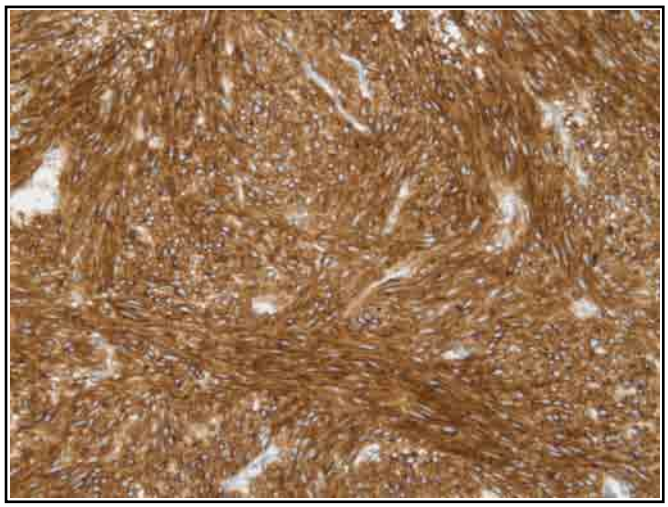


Fig 3. Optimal staining of the UK NEQAS distributed GIST tumour: All neoplastic cells show strong, crisp cytoplasmic and membranous staining, while the background remains clean. Section stained with the Dako polyclonal antibody, 1:200, on the Leica Bond with ER2 retrieval for 20 minutes.

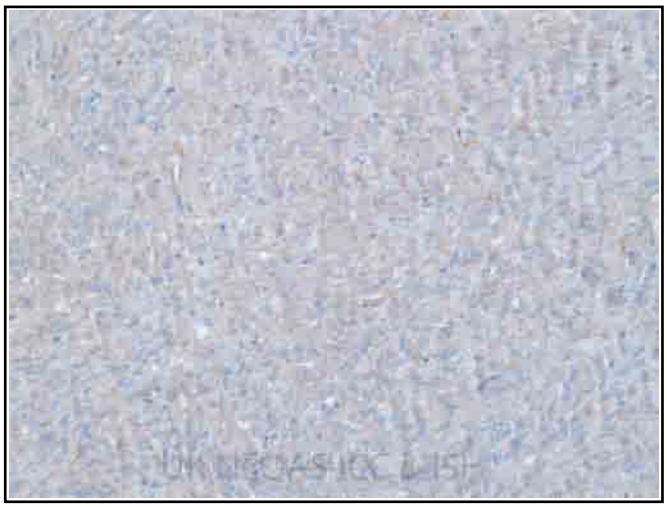


Fig 4. Unacceptable demonstration of CD117 in the UK NEQAS distributed GIST section (compare to Fig 3). The staining is very weak, and therefore considered a false negative result. No antigen retrieval was carried out using the Dako CD117 clone. Recommendations from the supplier of this antibody are to use antigen retrieval.

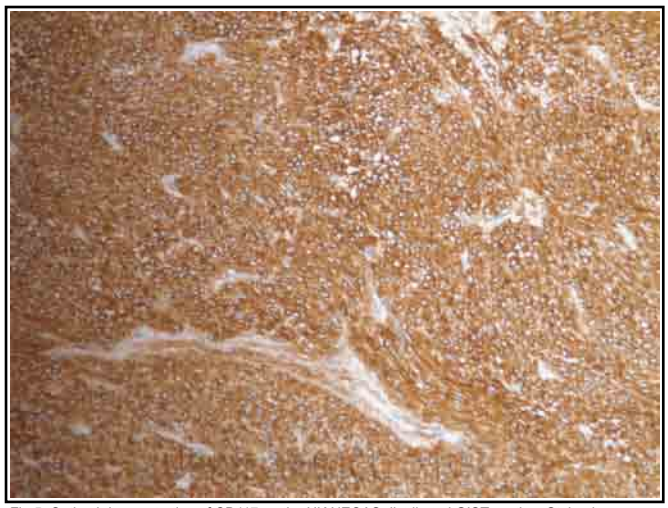


Fig 5. Optimal demonstration of CD117 on the UK NEQAS distributed GIST section. Stained with the Leica RTU antibody on the Bond with the Leica RTU antibody. Similarly to Fig 3, the example shows very strong staining of the tumour cells.

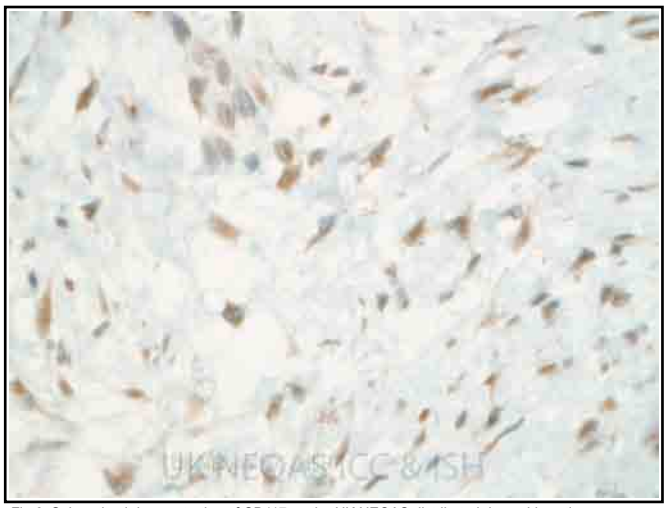


Fig 6. Sub-optimal demonstration of CD117 on the UK NEQAS distributed desmoid section. This is a known negative tumour for CD117, but as seen in the example image, the section shows inappropriate non-specific staining of the stromal and tumour cells. This was most likely caused by excessive antigen retrieval (same protocol as Fig 2).

Selected Images showing Optimal and Sub-optimal Immunostaining

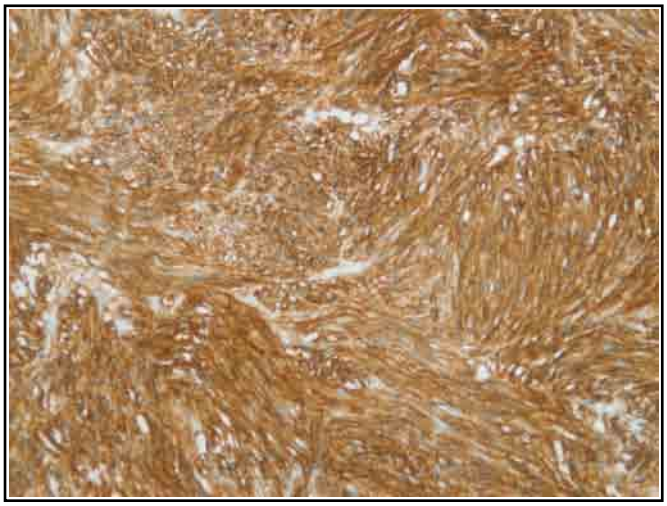


Fig 7. Optimal demonstration of DOG-1 in the UK NEQAS distributed GIST section. Virtually all the neoplastic cells show strong membranous and cytoplasmic staining. Section stained with the Leica K9 antibody, 1:50, on the Dako Omnis with high pH antigen retrieval.

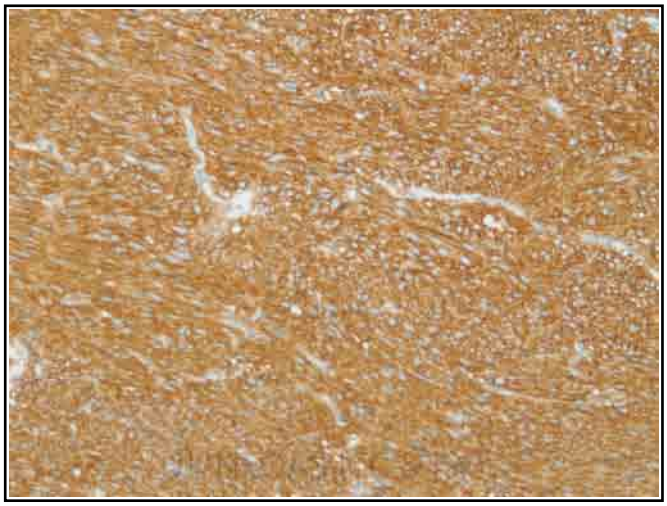


Fig 8. Another example of optimal staining of the UK NEQAS GIST section, stained with the Leica K9 antibody at a dilution of 1:100 on the Ventana ULTRA, CC1 64 minutes. All the tumour cells are staining strongly while the background remains clean.

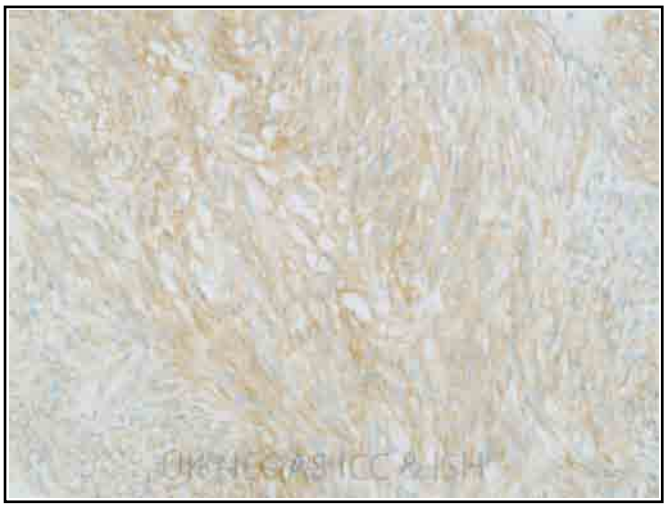


Fig 9. Sub-optimal demonstration of DOG-1 in the UK NEQAS GIST section. The example shows only weak and patchy equivocal staining (compare to Figs 7&8). The section was stained with the Leica K9 antibody, 1:100, on the Dako autostainer with antigen retrival in the PT Link.

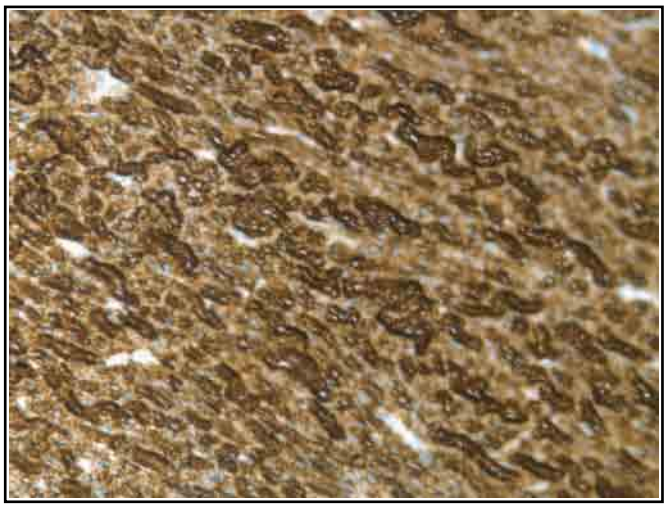


Fig 10. Sub-optimal demonstration of DOG-1 in the UK NEQAS GIST section. Although the tumour cells are staining strongly as expected, the section also shows heat artefact, caused by excessive antigen retrieval. Stained with the Leica K9 antibody on the Dako autostainer with antigen retrieval in the PT Link. Specific details of the antigen retrieval were not provided.

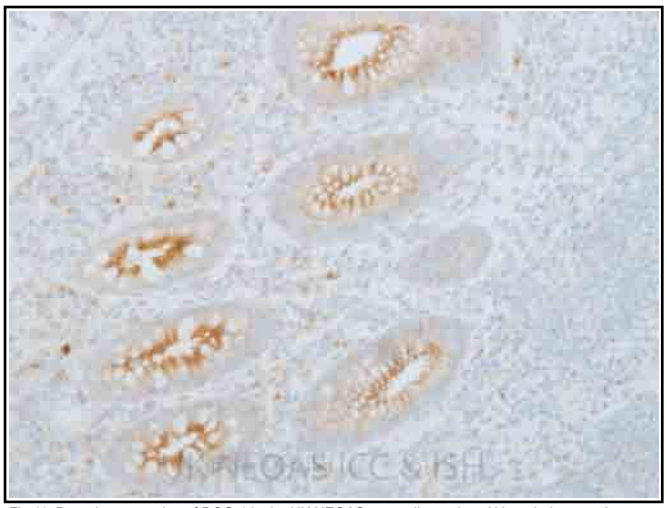


Fig 11. Poor demonstration of DOG-1 in the UK NEQAS appendix section. Although the sample is negative, the example also shows inappropriate non-specific and background staining. Stained using the Abcam polyclonal antibody on the Dako autostainer with pretreatment in the PT link. Unfortunatley, not all details of the method were provided.

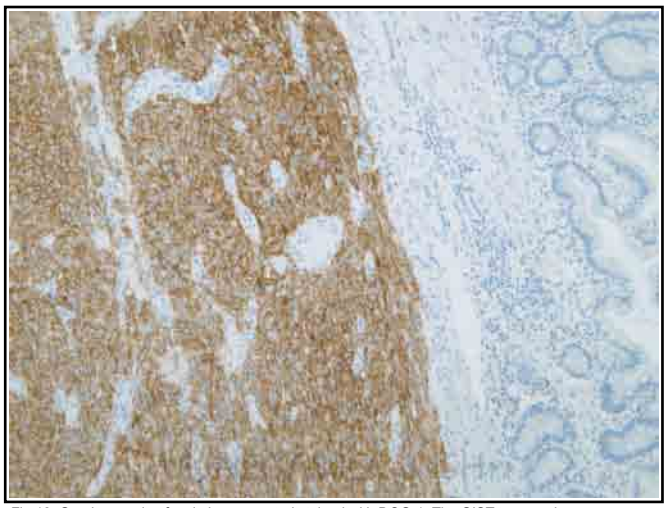
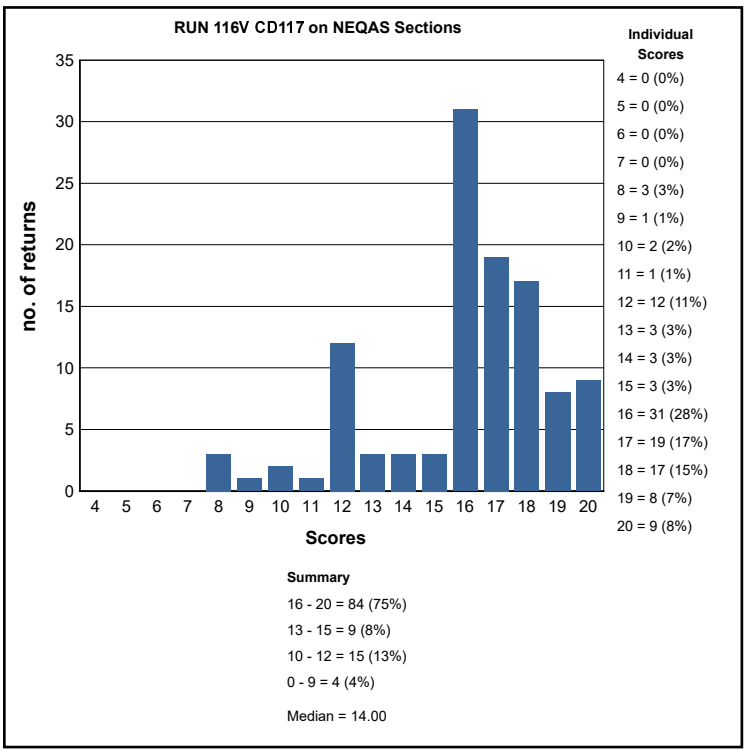
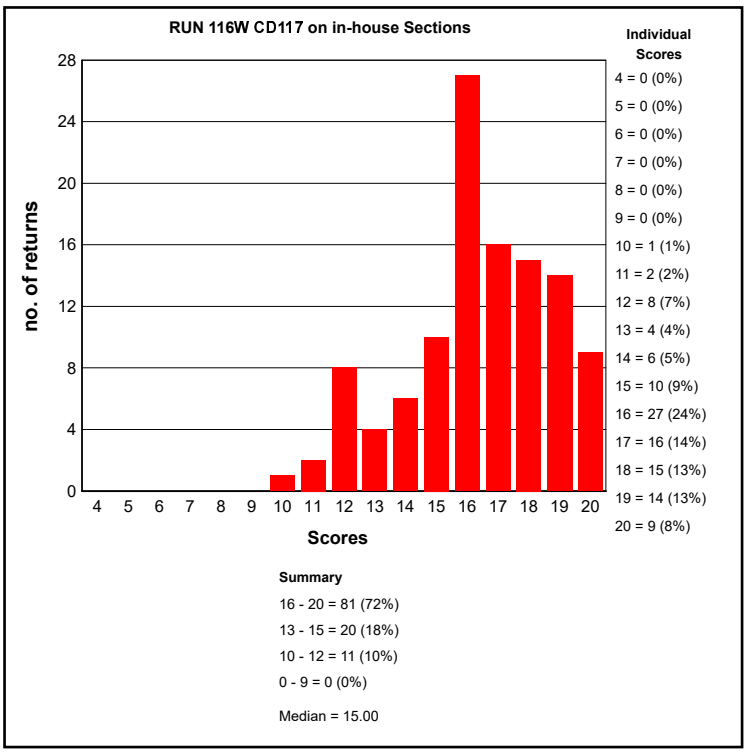
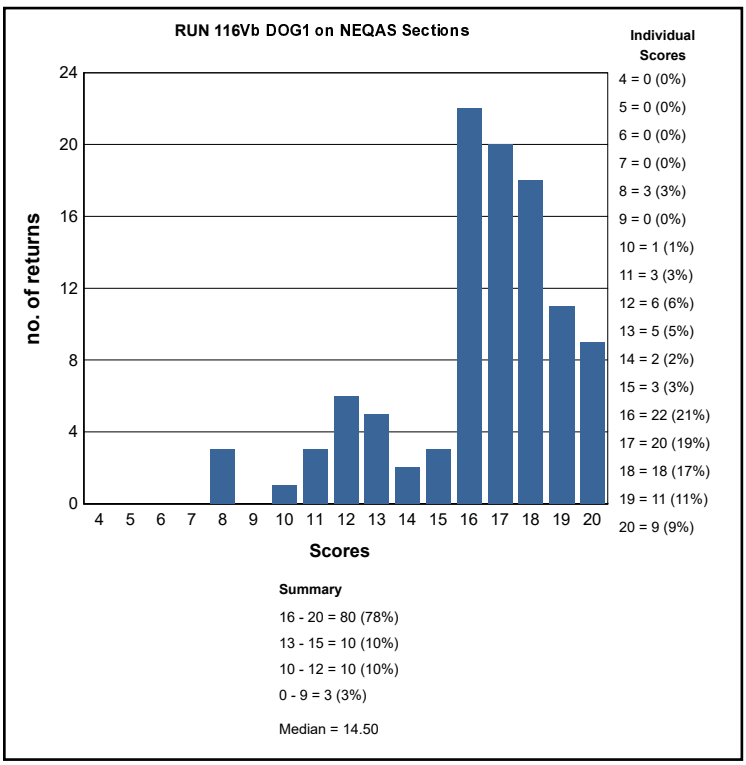


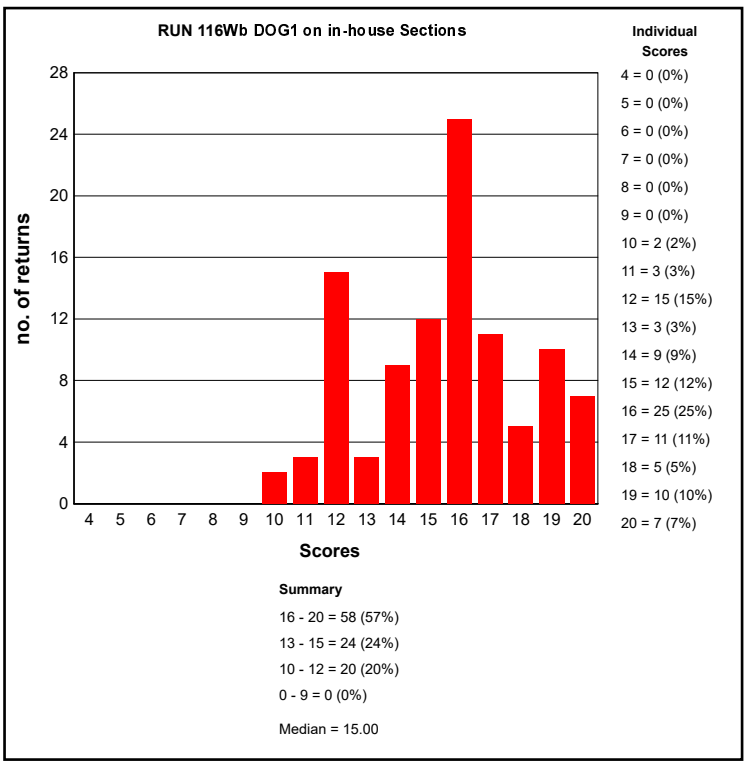
Fig 12. Good example of an in house control stained with DOG-1. The GIST tumour shows strong staining of the neoplastic cells, while the normal epithelium of the gastric sample remains unstained. Section stained with the Leica K9 antibody on the BondMax with ER2 retreival for 20 minutes.

GRAPHICAL REPRESENTATION OF PASS RATES









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

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

Alimentary Tract Pathology Run: 116

Primary Antibody : CD117

Antibody Details	N	%
Dako A4502 (rb poly)	89	89
Leica/Novocastra NCL-CD117 (T595)	1	0
Ventana 790-2939 (rb poly)	1	0
Cell Marque 117R/S-xx (YR145)	6	83
Leica/Novocastra RTU-CD117 (T595)	1	100
Ventana 790-2951 (9.7)	7	71
Epitomics AC-0029 (EP10)	1	100
Leica RTU (EP10) PA0007	5	40

Alimentary Tract Pathology Run: 116

Primary Antibody : DOG1

Antibody Details	N	%
Biocare CM 385 (1.1)	1	0
Cell Marque 244R-14/15/16 (SP31)	1	100
Cell Marque 244R-17/18 (SP31)	1	100
Leica NCL-L-DOG-1 (K9)	50	86
Leica PA0219 (K9)	20	90
Thermo RM-9132-R7 (SP31)	1	100
Other	4	75
Spring Biosciences M3311 (SP31)	1	100
Abcam TMEM16A (ab53212)	1	0
Ventana (SP31) 760-4590	20	95
Menarini MP-385-CM01/1	1	100

Alimentary Tract Pathology Run: 116

Heat Mediated Retrieval	CD117 N	CD117 %	DOG1 N	DOG1 %
Dako Omnis	2	50	2	100
Dako PTLink	14	100	15	80
Leica ER1 10 mins	1	100	0	0
Leica ER1 20 mins	4	75	5	40
Leica ER1 30 mins	4	100	0	0
Leica ER2 10 mins	2	100	0	0
Leica ER2 20 mins	22	77	24	88
Leica ER2 30 mins	6	100	6	100
None	3	33	2	50
Pressure Cooker	1	0	1	100
Ventana CC1 16mins	1	100	0	0
Ventana CC1 24mins	0	0	2	100
Ventana CC1 32mins	8	88	4	100
Ventana CC1 36mins	5	80	3	67
Ventana CC1 40mins	2	100	0	0
Ventana CC1 48mins	3	67	3	100
Ventana CC1 52mins	1	0	1	0
Ventana CC1 56mins	4	100	1	100
Ventana CC1 64mins	13	85	16	94
Ventana CC1 72mins	1	100	0	0
Ventana CC1 76mins	0	0	1	100
Ventana CC1 88mins	1	100	1	100
Ventana CC1 8mins	0	0	2	100
Ventana CC1 92mins	1	100	0	0
Ventana CC1 mild	4	75	3	100
Ventana CC1 standard	8	88	8	100
Ventana CC2 32mins	0	0	1	100
Ventana CC2 mild	0	0	1	0

Alimentary Tract Pathology Run: 116

Enzyme Mediated Retrieval	CD117 N	CD117 %	DOG1 N	DOG1 %
NOT APPLICABLE	82	87	69	87

Alimentary Tract Pathology Run: 116	CD117		DOG1	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	8	100	8	100
BioGenex SS Polymer (QD 420-YIKE)	0	0	1	100
BioGenex SS Polymer (QD 430-XAKE)	1	0	0	0
Dako EnVision FLEX (K8000/10)	2	50	2	50
Dako EnVision FLEX+ (K8002/12)	8	100	7	71
Dako Envision+ HRP mouse K4004/5/6/7	0	0	1	100
Dako Envision+ HRP rabbit K4008/9/10/11	1	100	0	0
Leica Bond Intense R Detection (DS9263)	0	0	1	100
Leica Bond Polymer Define (DS9713)	0	0	1	100
Leica Bond Polymer Refine (DS9800)	39	82	32	84
Other	1	100	3	67
Ventana iView system (760-091)	1	100	1	100
Ventana OptiView (760-700) + Amp. (7/860-099)	1	100	0	0
Ventana OptiView Kit (760-700)	23	87	17	100
Ventana UltraView Kit (760-500)	25	76	27	85

Alimentary Tract Pathology Run: 116	CD117		DOG1	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	1	0	1	100
Dako Autostainer Link 48	12	100	11	91
Dako Autostainer Plus Link	3	100	3	67
Dako Omnis	2	50	2	100
LabVision Autostainer	0	0	1	0
Leica Bond Max	13	69	11	73
Leica Bond-III	27	89	25	88
Ventana Benchmark GX	1	0	1	100
Ventana Benchmark ULTRA	36	89	34	91
Ventana Benchmark XT	16	75	12	83

Alimentary Tract Pathology Run: 116	CD117		DOG1	
Chromogen	**N**	**%**	**N**	**%**
A. Menarini Liquid Stable DAB kit	1	0	0	0
AS PER KIT	15	87	12	92
BioGenex liquid DBA (HK-124-7K)	0	0	1	100
DAKO DAB+	1	100	0	0
Dako EnVision Plus kits	1	100	2	50
Dako FLEX DAB	10	90	10	80
Leica Bond Polymer Refine kit (DS9800)	38	84	34	85
NOT APPLICABLE	1	0	0	0
Other	5	80	4	100
Ventana DAB	11	100	11	100
Ventana iview	1	100	1	100
Ventana Ultraview DAB	27	78	27	81

BEST METHODS - Gold Standard Antibody

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

CD117 - Method 1

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

Primary Antibody: Dako A4502 (rb poly) , 30 Mins, RT ºC Dilution 1: 200
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), RT ºC., Time 1: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins, RT ºC Prediluted

CD117 - Method 2

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

Primary Antibody: Cell Marque 117R/S-xx (YR145) , 60 Mins, amb ºC Dilution 1: 50
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 20/20 (UK NEQAS Slide) and 20/20 (In House slide) using this method.

Primary Antibody:	Dako A4502 (rb poly)
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

CD117 - Method 4

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

Primary Antibody:	Leica RTU (EP10) PA0007
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

BEST METHODS - Secondary Antibody

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

DOG1 - Method 1

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

Primary Antibody:	Leica NCL-L-DOG-1 (K9) , 20 Mins Dilution 1: 50
Automation:	Dako Omnis
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako Omnis, Buffer: DAKO HIGH PH TRS
EAR:	
Chromogen:	Dako FLEX DAB, Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Other , 10 Mins Prediluted

DOG1 - Method 2

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

Primary Antibody:	Leica PA0219 (K9) , 60 Mins, amb ºC Prediluted
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), amb ºC., Time 2: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 15 Mins, amb ºC Prediluted

DOG1 - Method 3

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

Primary Antibody:	Leica NCL-L-DOG-1 (K9) , 32 Mins, 36 ºC Dilution 1: 50
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 48mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700)

DOG1 - Method 4

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

Primary Antibody:	Leica NCL-L-DOG-1 (K9) , 56 Mins, 36 ºC Dilution 1: 30
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500) Prediluted

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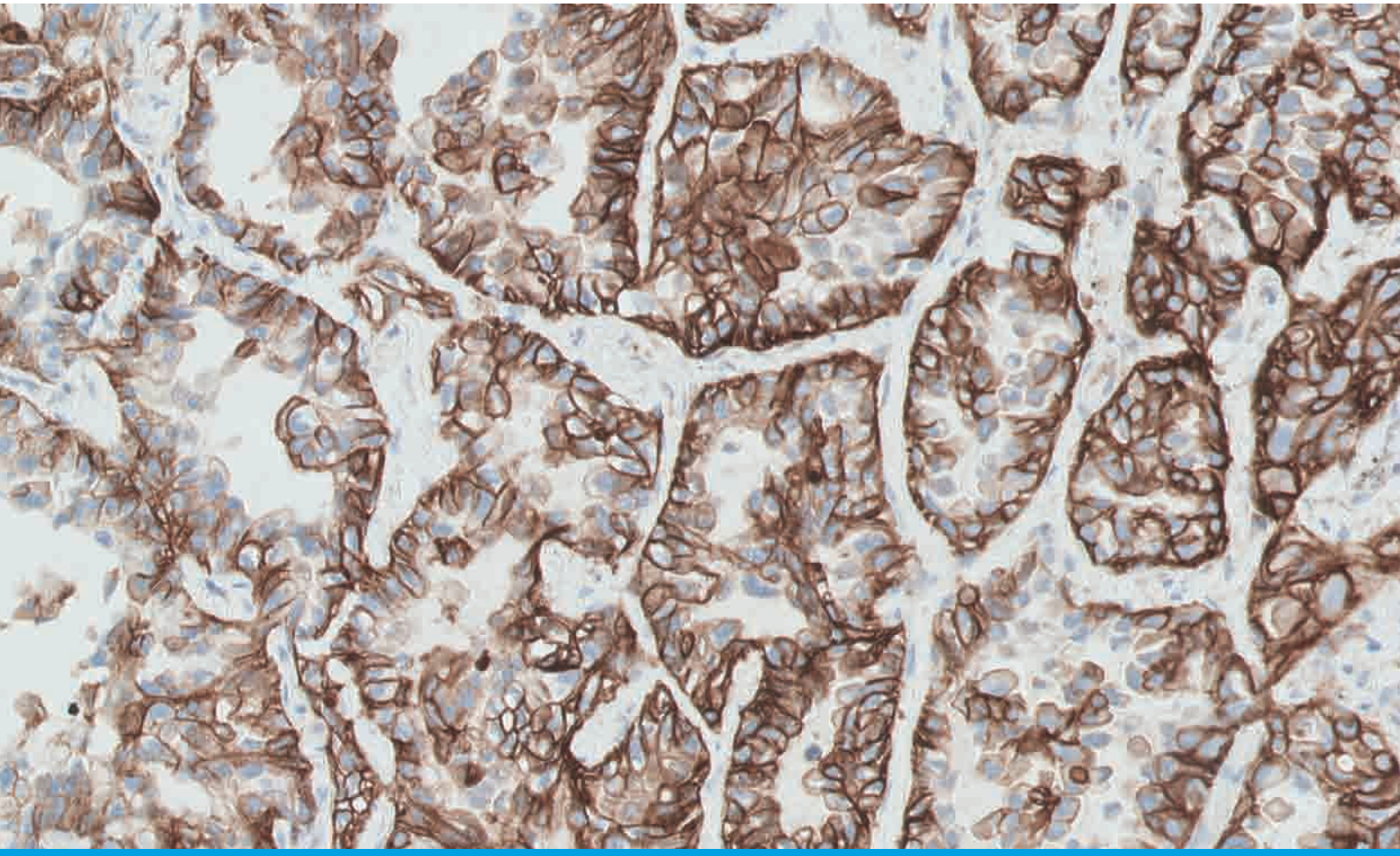

Agilent Technologies

50249 2016JUN09

Lynch Syndrome/HNPCC Run 116

Keith Miller and Suzanne Parry

	Gold Standard	Second Antibody
Antigens Assessed:	MLH1	PMS2
Tissue Sections circulated:	Positive and negative colonic tumours & normal appendix	
Number of Registered Participants:	91	
Number of Participants This Run:	84 (92%)	

General Introduction

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

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

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

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

Mismatch Repair Markers

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

Clinical Interpretation & Reporting Guidelines

We recommend reporting MMR IHC findings in tumours as either:

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

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

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

Clinical Reporting

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

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

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

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

Further Discussion on MMR proteins

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

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

Assessment Procedure:

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

Features of Optimal Immunostaining: (Figs 1, 3, 5, 7,8, &11)
Appendix:
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:

. Strong nuclear staining in the tumour cells.

- Strong nuclear staining in the lymphocytes and stromal cells.

Tumour with loss of MMR protein:

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

Features of Suboptimal Immunostaining: (Figs 2, 4, 6, 9, 10 & 12)
Appendix:

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

Tumour without loss of MMR protein:

- 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:

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

Assessment Summary:

The pass rates for the **MLH1** assessment were higher than the previous time (Run 114) this antibody was assessed, with 77% (n=65) of laboratories achieving an acceptable pass, and a further 17% of participants receiving a borderline score (10-12/20), and therefore an overall pass of 94%. The Ventana 790 -4535 (M1) clone was the most popular choice of antibody, and showed a pass rate of 72%. The Dako (ES05) in both concentrate and ready-to-use (RTU) form was used by 28 laboratories with a pass rate of 82%.

The **PMS2** assessment showed an acceptable pass rate of 80%, and a further 12% received a borderline pass. Similarly to the **MLH1** assessment, the fail rate was quite low with 7 laboratories (8%) receiving a score of under 10. Again, weak staining was the main reason for failure or borderline scores (depending on the severity). The Ventana (EPR3947) clone was the most popular choice of PMS2 antibody used in this assessment by 32 laboratories, and showed a pass rate of 84%. The DakoEP51 clone in both concentrate and RTU was also commonly used, and showed an overall pass rate of 84%.

In-House Control Tissue Recommendations

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

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6. Vasen F, et al. Revised guidelines for the clinical management of Lynch syndrome (HNPCC): recommendations by a group of European experts. Gut 2013;62: 812-823.

Selected Images showing Optimal and Sub-optimal Immunostaining

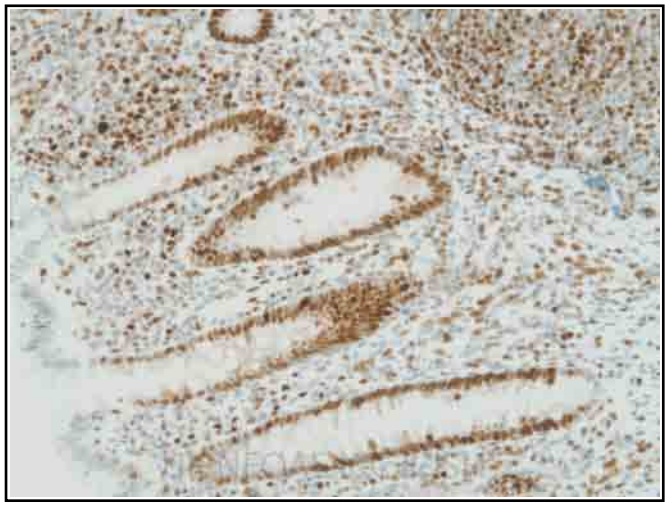


Fig 1. Optimal demonstration of MLH1 in the UK NEQAS distributed appendix, showing strong staining of the basal and lower half of the epithelial crypts, fading towards the luminal surface. The Ventana pre-diluted M1 antibody was used on the ULTRA, pre-treatment CC1 for 64 mins with Optiview detection.

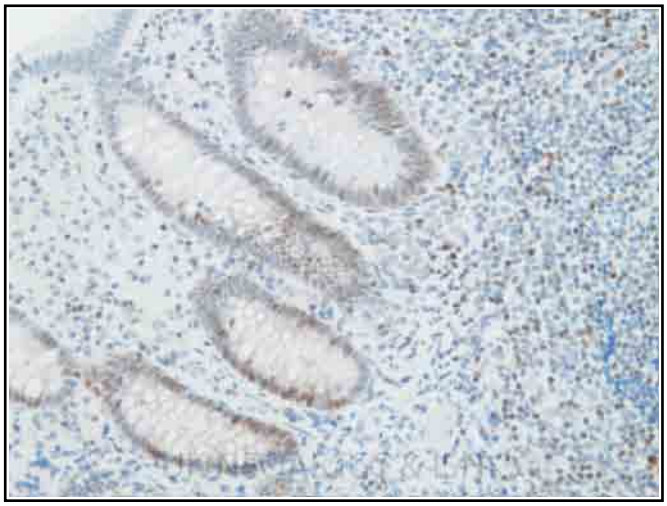


Fig 2. Sub-optimal demonstration of MLH1 in the UK NEQAS distributed appendix (compare to Fig 1). Not only is the staining weak, but far fewer lymphocytes than expected are demonstrated. Section stained with the Ventana pre-diluted M1 antibody with CC1 retrieval for 32 minutes, and Optiview detection on the Benchmark XT.

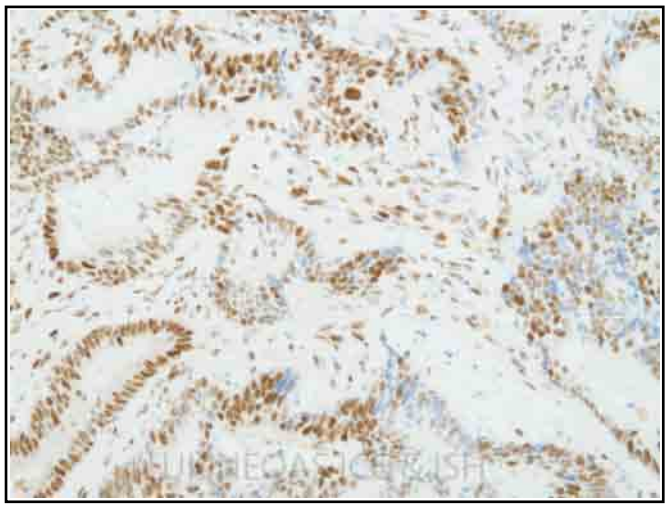


Fig 3. Optimal demonstration of MLH1 in the UK NEQAS distributed positive tumour. The example shows strong nuclear staining in the tumour cells as well as the intra-tumoral lymphoid and stromal cells. Staining carried out using the Dako ES05 clone, 1:25, on the Leica BondMax with retrieval for 60 minutes with ER2 buffer.

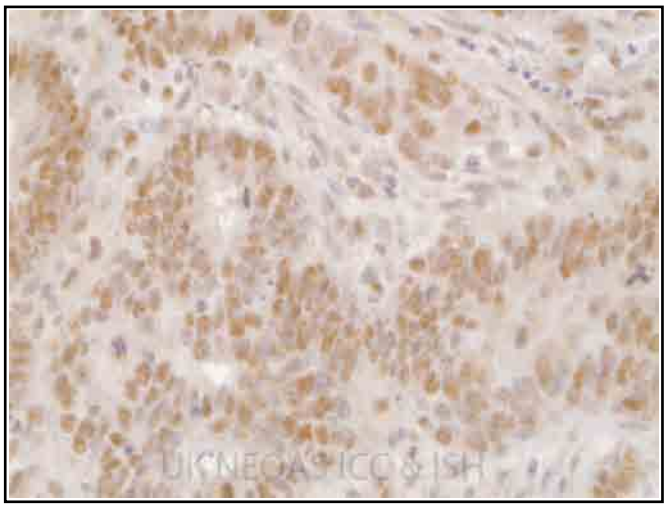


Fig 4. Unacceptable staining with MLH1 in the UK NEQAS distributed positive tumour. Although the tumour nuclei and stromal cells are staining as expected, the section also shows inappropriate and non-specific background staining. Section stained on the Ventana ULTRA using the Novocastra antibody, 1:25, and CC1 retrieval for 64 minutes.

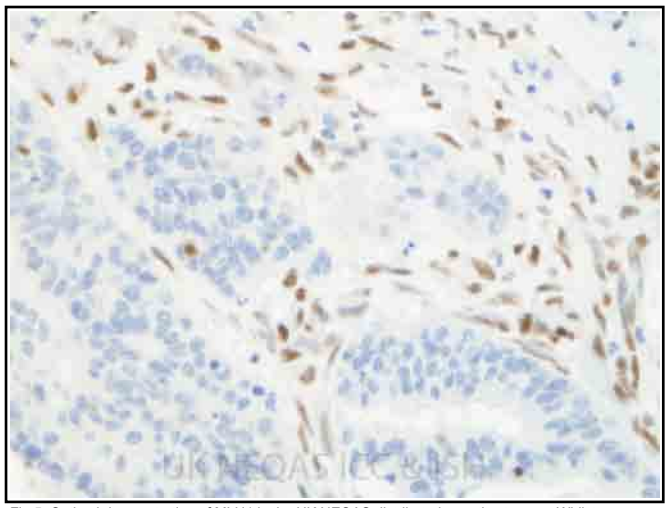


Fig 5. Optimal demonstration of MLH1 in the UK NEQAS distributed negative tumour. Whilst the tumour is negative, the intra-tumoural lymphoid and stromal cells are staining strongly positive, acting as the internal tissue control. Stained with Dako ES05 antibody, 1:50, on the Dako autostainer with pre-treatment in the PT Link with high pH buffer.

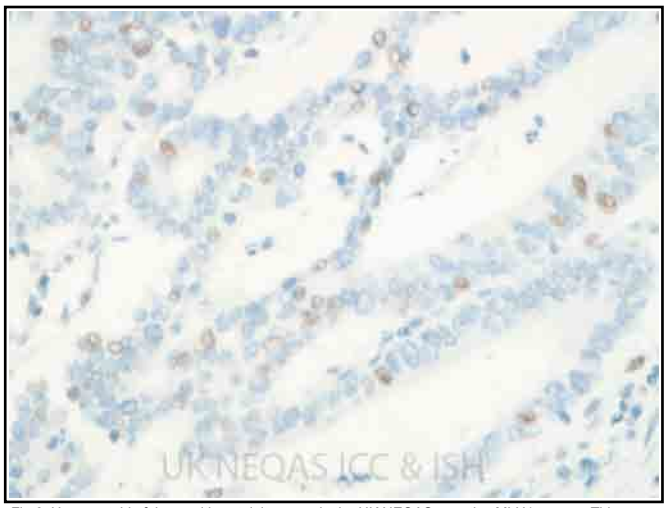


Fig 6. Unacceptable false positive staining seen in the UK NEQAS negative MLH1 tumour. This inappropriate staining may be due to unsuitable antigen retrieval, which was carried out in the microwave with ETDA buffer. A manual assay was used with the Dako ES05 antibody.

Selected Images showing Optimal and Sub-optimal Immunostaining

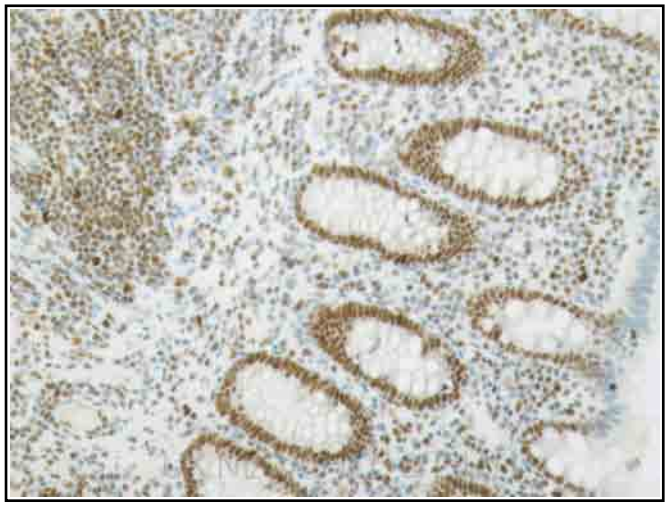


Fig 7. Optimal demonstration of PMS2 in the UK NEQAS distributed appendix, showing strong staining of the basal and lower half of the epithelial crypts and fading towards the luminal surface. The method used was with the Dako EP51 antibody on the autostaininer, and pre-treatment in the PT link.

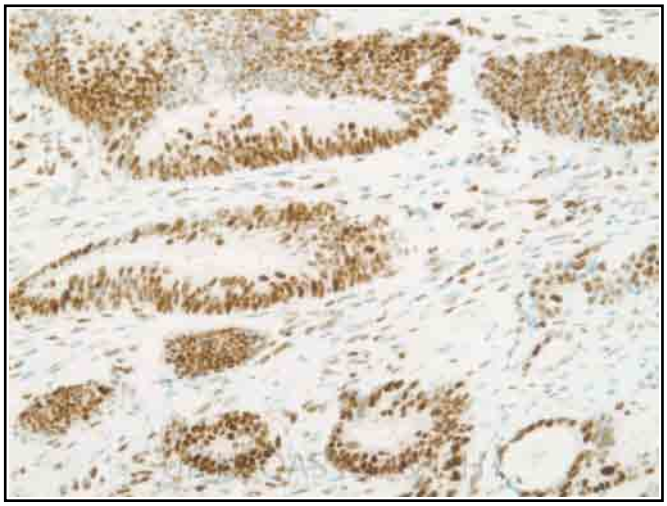


Fig 8. Optimal demonstration of PMS2 in the positive tumour. Not only is the nuclear staining strong in the tumour cells, but the intra-tumoural lymphocytes and stromal cells are also showing strong expression. Stained with the Dako EP51 antibody on the Ventana ULTRA with Optiview detection.

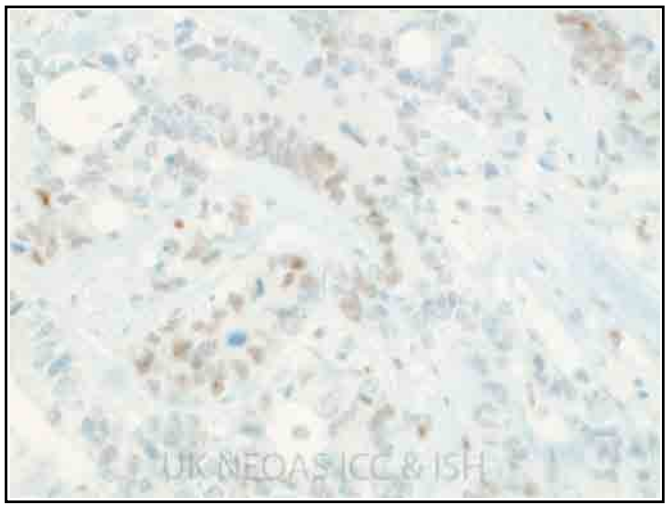


Fig 9. Unacceptable weak staining of the UK NEQAS positive tumour stained with PMS2 (compare to Fig 8). Stained using the Ventana pre-diluted EPR3947 antibody on the Benchmark GX, CC1 for 64 minutes and Optiview detection.

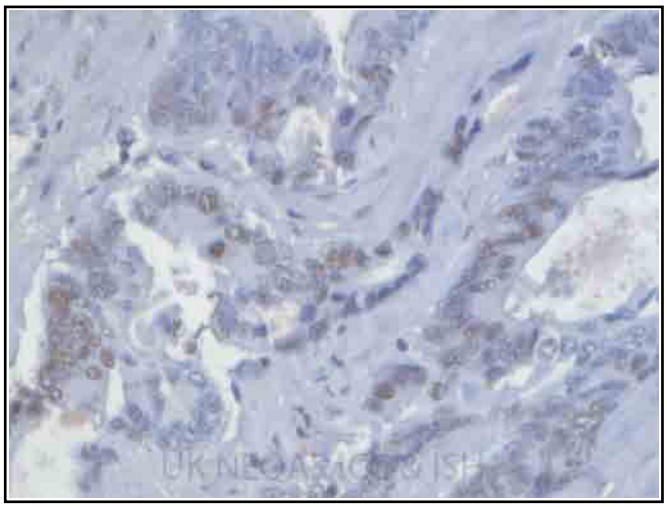


Fig 10. Unacceptable demonstration of PMS2 in the positive tumour. Not only is the staining weak, but the excessive counterstain is masking the staining reaction. Stained with the Dako EP51 clone on the Leica BondMax, ER2 40 minutes.

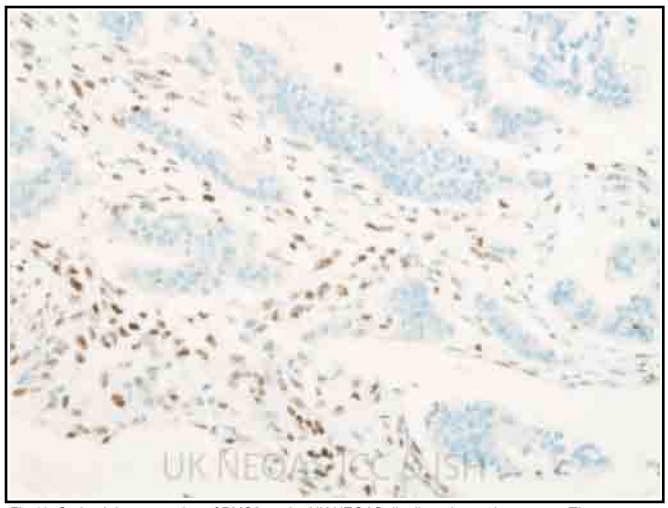


Fig 11. Optimal demonstration of PMS2 on the UK NEQAS distributed negative tumour. The tumour nuclei remain negative, but the stromal cells and lymphocytes are staining strongly and act as an internal tissue control. Stained with the Ventana EPR3947 antibody on the ULTRA, CC1 for 92 minutes and Optiview detection.

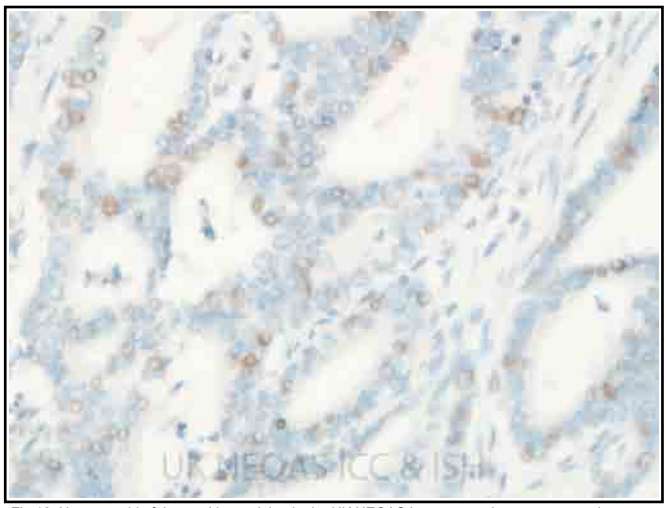
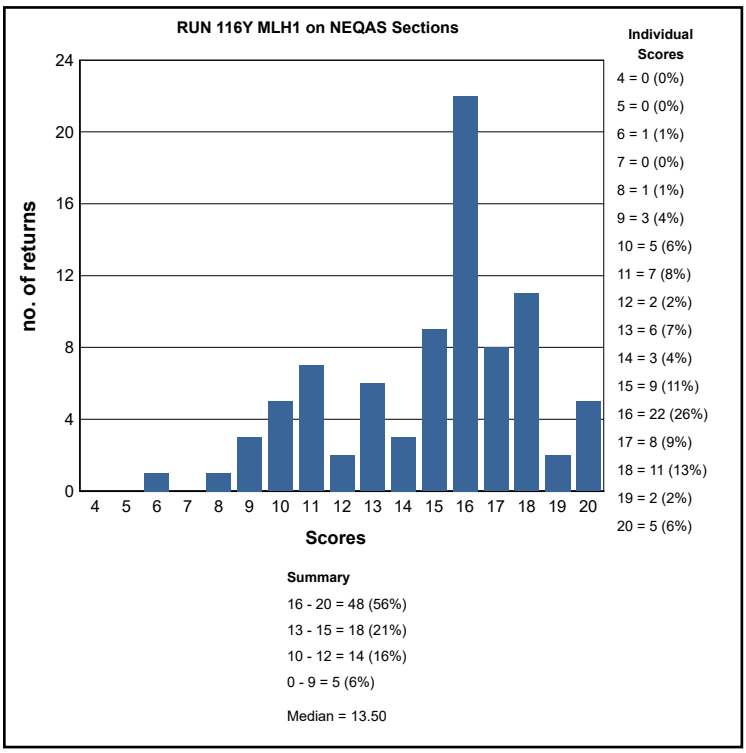
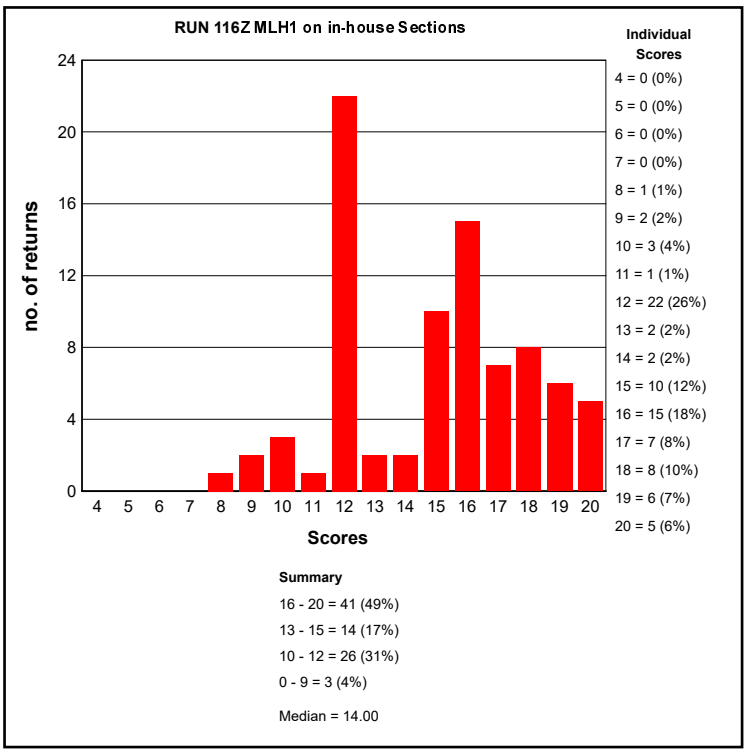
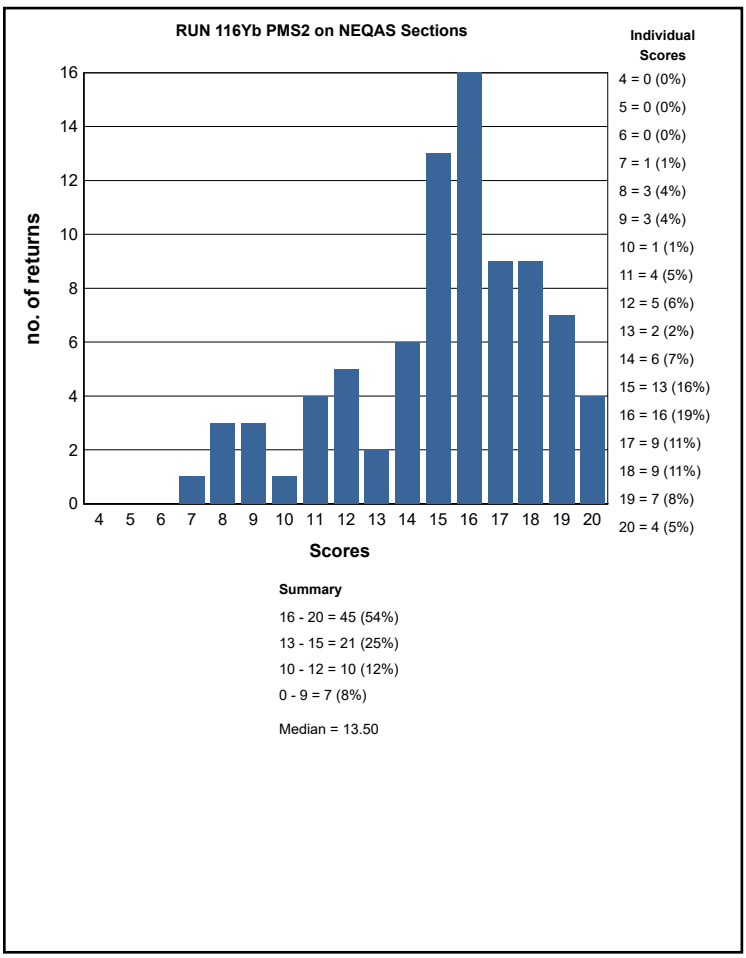


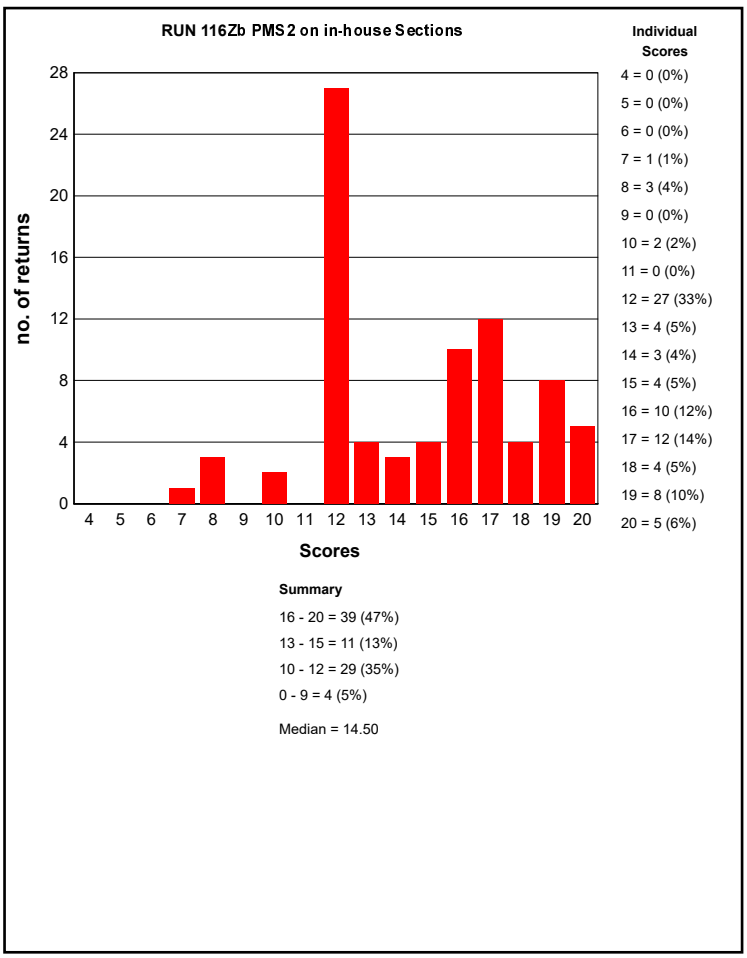
Fig 12. Unacceptable false positive staining in the UK NEQAS known negative tumour sample. This inappropriate staining may be due to unsuitable antigen retrieval carried out in the microwave with ETDA buffer. A manual assay was used with the Dako EP51 antibody.

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: 116

Primary Antibody : MLH1

Antibody Details	N	%
Other	1	0
BD Pharmingen (G168-15)	3	100
Biocare medical CM/PM 220 (G168-15)	1	100
Novocastra NCL-L-MLH1 (ES05)	12	75
Dako M3640 (ES05)	15	80
Leica Bond RTU PA0610 (ES05)	3	100
Dako Flex RTU IR079/IS079 (ES05)	13	85
Ventana 790-4535 (M1)	36	72

Primary Antibody : PMS2

Antibody Details	N	%
BD Bio/Pharmingen 556415 (A16-4)	11	82
Cell Marque 288R -17/18 (EPR3947)	4	75
Leica/Novoca NCL-L-PMS2 (MOR4G)	2	0
Other	2	50
Ventana 760-4531 (EPR3947)	32	84
Cell Marque 288M -16 (MRQ28)	1	0
Dako M3647 (EP51)	19	89
Dako RTU FLEX IR087 (EP51)	11	73
Epitomics AC-0049 (EP51)	1	100

HNPCC Run: 116

Heat Mediated Retrieval	MLH1 N	MLH1 %	PMS2 N	PMS2 %
Dako Omnis	2	100	3	33
Dako PTLink	9	100	9	78
Leica ER1 30 mins	1	100	0	0
Leica ER2 20 mins	7	86	7	57
Leica ER2 30 mins	11	73	8	100
Leica ER2 40 mins	6	83	9	89
Microwave	1	0	1	0
Other	1	100	1	100
Pressure Cooker	1	100	1	0
Ventana CC1 24mins	1	0	0	0
Ventana CC1 32mins	4	25	0	0
Ventana CC1 40mins	5	100	1	100
Ventana CC1 48mins	3	33	4	75
Ventana CC1 56mins	3	67	1	0
Ventana CC1 64mins	19	74	14	79
Ventana CC1 72mins	0	0	3	100
Ventana CC1 80mins	2	50	3	100
Ventana CC1 88mins	1	100	3	100
Ventana CC1 92mins	1	100	11	82
Ventana CC1 extended	0	0	1	100
Ventana CC1 standard	6	100	3	67
Ventana CC2 64mins	0	0	1	0
Ventana CC2 92mins	0	0	1	100

HNPCC Run: 116

Enzyme Mediated Retrieval	MLH1 N	MLH1 %	PMS2 N	PMS2 %
AS PER KIT	3	100	2	50
NOT APPLICABLE	45	84	47	83

HNPCC Run: 116	MLH1		PMS2	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	3	100	2	100
Dako EnVision FLEX (K8000/10)	1	100	1	0
Dako EnVision FLEX+ (K8002/12)	7	100	7	86
Dako Envision HRP/DAB (K5007)	1	100	1	0
Dako Envision+ HRP mouse K4004/5/6/7	1	100	1	0
Leica Bond Polymer Define (DS9713)	0	0	1	100
Leica Bond Polymer Refine (DS9800)	24	79	23	83
None	0	0	1	0
Other	2	100	2	100
Ventana OptiView (760-700) + Amp. (7/860-099)	9	78	10	80
Ventana OptiView Kit (760-700)	29	72	27	89
Ventana UltraView Kit (760-500)	6	50	5	20

HNPCC Run: 116	MLH1		PMS2	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	0	0	1	100
Dako Autostainer Link 48	7	100	7	86
Dako Autostainer plus	1	100	1	0
Dako Autostainer Plus Link	1	100	1	100
Dako Omnis	2	100	3	33
Leica Bond Max	8	75	7	86
Leica Bond-III	18	83	18	83
None (Manual)	2	50	2	0
Ventana Benchmark GX	2	50	2	50
Ventana Benchmark ULTRA	34	74	35	83
Ventana Benchmark XT	9	67	8	75

HNPCC Run: 116	MLH1		PMS2	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	15	73	16	88
Dako EnVision Plus kits	2	100	2	100
Dako FLEX DAB	6	100	7	57
Dako REAL EnVision K5007 DAB	2	50	2	0
Leica Bond Polymer Refine kit (DS9800)	25	80	24	83
Other	5	60	4	100
Ventana DAB	20	75	21	81
Ventana Ultraview DAB	9	78	9	56

BEST METHODS - Gold Standard Antibody

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

MLH1 - Method 1

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

Primary Antibody: Novocastra NCL-L-MLH1 (ES05) , 32 Mins, 37 ºC Dilution 1: 1/50
Automation: Leica Bond Max
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 30 mins
EAR: NOT APPLICABLE
Chromogen: Leica Bond Polymer Refine kit (DS9800)
Detection: AS PER KIT

MLH1 - Method 2

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

Primary Antibody: Dako Flex RTU IR079/IS079 (ES05) Prediluted
Automation: Dako Autostainer Link 48
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako PTLink
EAR: NOT APPLICABLE
Chromogen: Dako EnVision Plus kits
Detection: Dako EnVision FLEX+ (K8002/12)

MLH1 - Method 3

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

Primary Antibody:	Dako M3640 (ES05) , 30 Mins, r/t ºC Dilution 1: 25
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Other
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

MLH1 - Method 4

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

Primary Antibody:	Ventana 790-4535 (M1) , 12 Mins
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	
Chromogen:	AS PER KIT
Detection:	Ventana OptiView Kit (760-700)

BEST METHODS - Secondary Antibody

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

PMS2 - Method 1

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

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

PMS2 - Method 2

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

Primary Antibody:	Dako M3647 (EP51)
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	AS PER KIT
HMAR:	Dako PTLink
EAR:	
Chromogen:	AS PER KIT
Detection:	AS PER KIT

PMS2 - Method 3

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

Primary Antibody:	Dako RTU FLEX IR087 (EP51) , 24 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins, Buffer: CC1 ULTRA
EAR:	NOT APPLICABLE
Chromogen:	Other, 37 ºC., Time 1: 8 Mins, Time 2: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 37 ºC Prediluted

PMS2 - Method 4

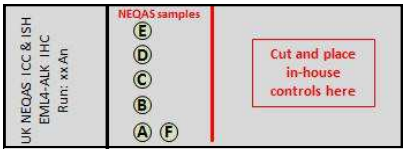
Participant scored 20/20 (UK NEQAS Slide) and 20/20 (In House slide) using this method.

Primary Antibody:	Ventana 760-4531 (EPR3947) , 60 Mins, 36 ºC
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 92mins
EAR:	
Chromogen:	Ventana DAB
Detection:	Ventana OptiView (760-700) + Amp. (7/860-099)

Suzanne Parry

Antigen Assessed:	ALK
Sections Circulated:	Composite slide consisting of cell lines and Non-small cell lung carcinoma (NSCLC) tissue samples with different levels of ALK expression . An appendix tissue was also included.
Number of Registered Participants:	66
Number of Participants this Run	56 (85%)

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	**Cell line: Adenocarcinoma**	-ve
C	**Cell line: Adenocarcinoma**	+ve
D	**NSCLC: Adenocarcinoma**	-ve
E	**NSCLC: Adenocarcinoma**	+ve
F	**NSCLC: Adenocarcinoma**	+ve

Introduction

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

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

Assessment Criteria

Same slide NEQAS & In-house controls

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

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

Interpretation criteria incorporating staining intensity

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

Assessment scoring guidelines

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

Table 2: Assessment interpretation

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

Results & Discussion

Distributed NEQAS Sample Results

There was a continued increase in number of participants from the previous run, with an 89% (N=48) acceptable pass rate for the NEQAS section. Two laboratories (4%) received a borderline pass and 4 laboratories (7%) failed the assessment. This was mainly due to weak or very weak staining compared to the expected level of staining this was mainly due to laboratories not following the recommended staining protocols or inadequate antigen retrieval. The most popular antibody of choice was the Ventana/Roche (D5F3) with 41 participants using this antibody with a 100% pass rate. The Cell Signalling Technology (D5F3) was also used by 3 participants achieving a 100% pass rate.

Features of Optimal Staining. (Figs 1, 3, 5, 7, 9, 11 &12):

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

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Features of Sub-optimal Staining (Figs 2, 4, 6, 8 &10):

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

In-house Control Results

Of the 54 participants taking part in this assessment, 37(69%) achieved an acceptable result. 15 (28%) participants obtained a borderline pass result and 2 participants failed with the in-house sections. This was mainly due to the use of an inappropriate control for the lung cancer setting.

Lymphoma control

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

ALK IHC Control Recommendation

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

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

Appendix is also recommended to 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.

References:

1. Jemal A, Bray F, Center MM, et al. Global cancer statistics. CA Cancer J Clin 2011;61:69–90
2. American Cancer Society. Cancer Facts & Figures 2011. Atlanta: American Cancer Society; 2011
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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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.

Selected Images showing Optimal and Sub-optimal Immunostaining

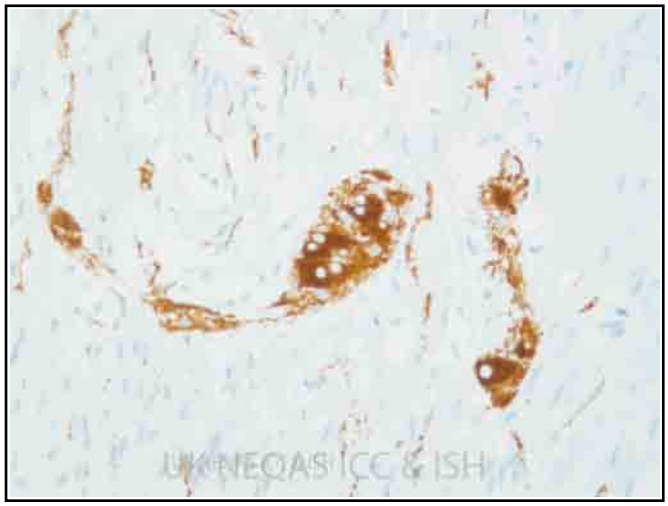


Fig 1. Good example of ALK staining in the UK NEQAS distributed appendix sample A. As expected there is strong staining of the ganglion cells and axons. Stained with the Ventana D5F3 assay and recommended protocol on the Ventana ULTRA with Optiview detection and amplification.

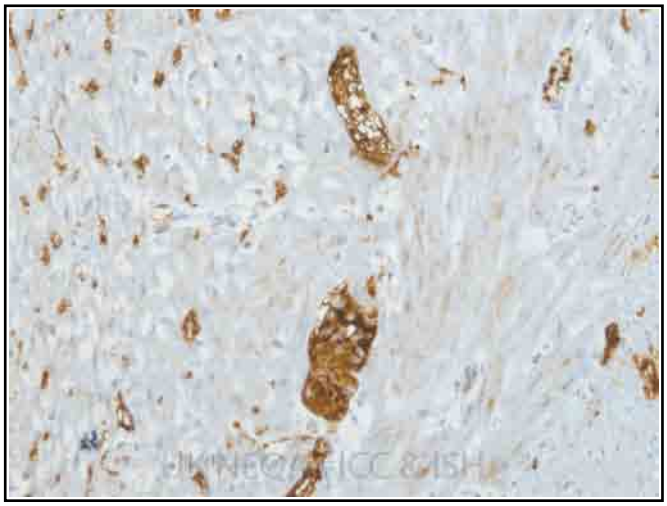


Fig 2. Sub-optimal staining of the UK NEQAS distributed appendix sample A. Although the ganglion cells and axons are staining as expected, the section also shows non-specific background staining. Unfortunately, the laboratory did not provide any methodology details.

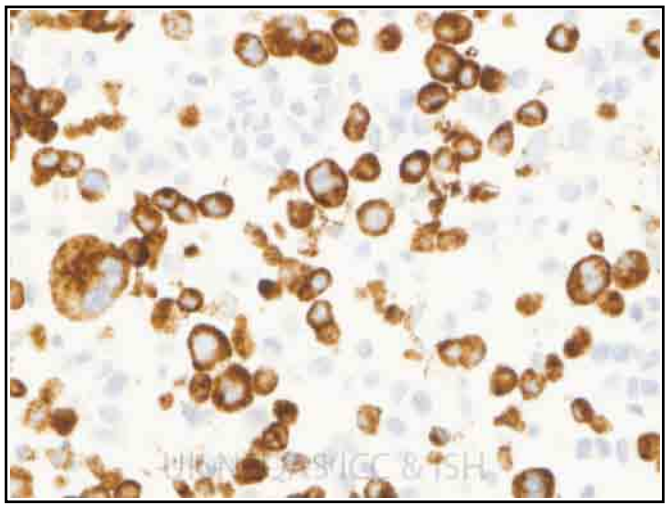


Fig 3. Optimal demonstration of ALK in the UK NEQAS distributed positive cell line sample B. As expected the example shows strong membranous and cytoplasmic staining of the neoplastic cells. Stained with the Ventana D5F3 assay on the Ventana Benchmark GX with CC1 retrieval for 92 minutes and Optiview detection.

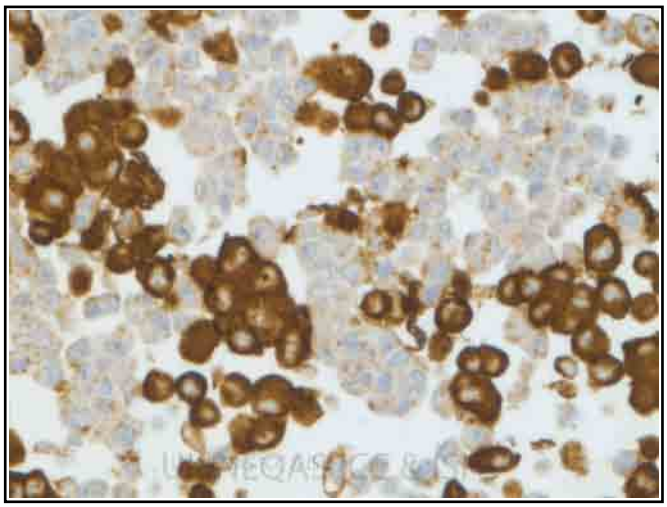


Fig 4. Sub-optimal demonstration of ALK in the UK NEQAS distributed positive cell line sample B. Although the neoplastic cells are staining strongly as expected, the sample also shows excessive non-specific teramide deposit seen in the negative cells. No methodology was provided.

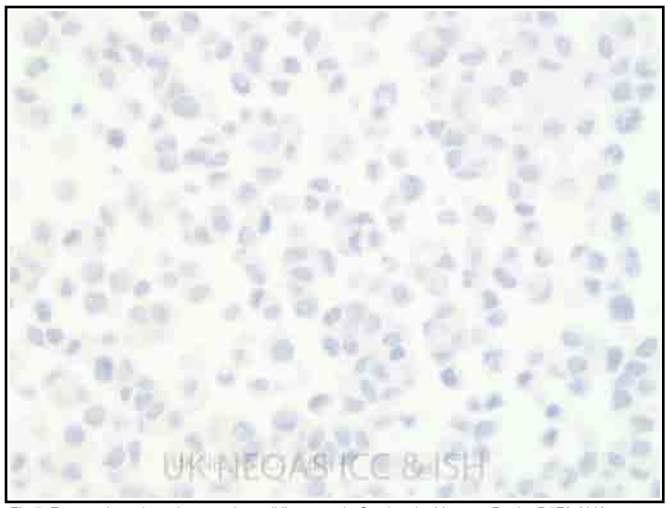


Fig 5. Expected result on the negative cell line sample C using the Ventana Roche D5F3 ALK assay. All cells are negative and the sample is clean. (Same protocol as Fig 3).

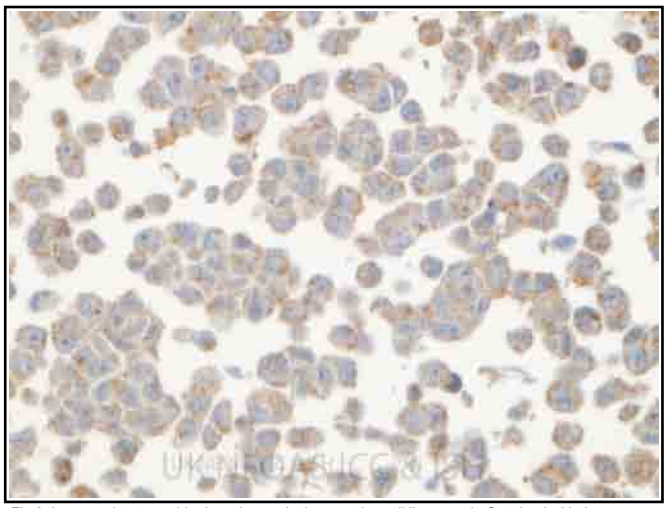


Fig 6. Inappropriate teramide deposit seen in the negative cell line sample C stained with the Ventana D5F3 ALK assay (compare to Fig 5). The laboratory was obviously aware of the non-specific staining as they provided the correct interpretation result negative. However, improvements are required.

Selected Images showing Optimal and Sub-optimal Immunostaining

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

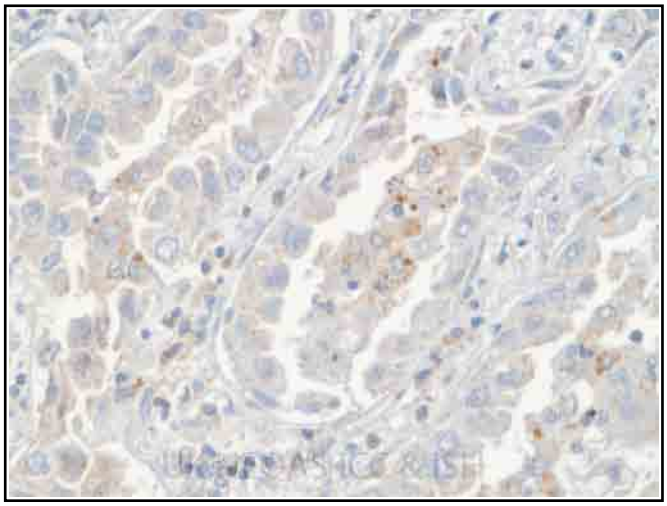


Fig 8. Unacceptable ALK IHC result in the UK NEQAS distributed negative tumour sample D. The example shows inappropriate background staining, which could potentially be misinterpreted as a low positive result. (Same laboratory as Fig 6).

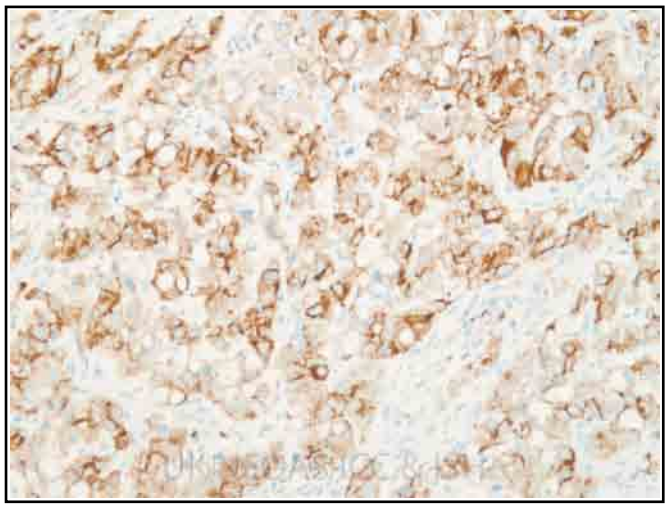


Fig 9. Expected level of staining in the UK NEQAS distributed positive tumour sample E. The example shows moderate and strong membranous and cytoplasmic staining of the neoplastic cells. (Same protocol as Fig 1).

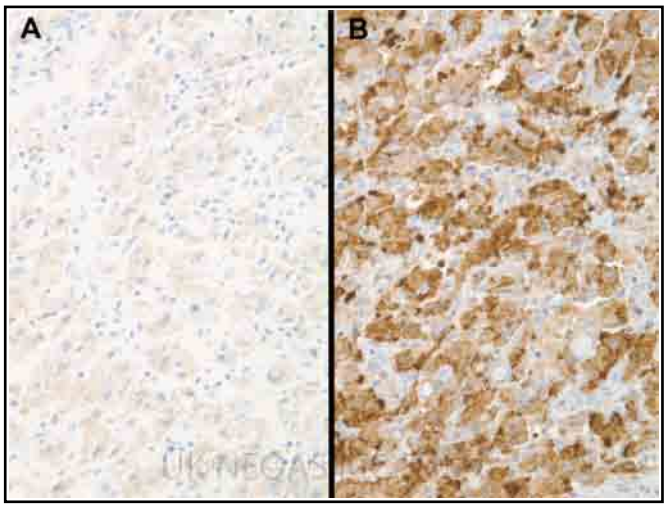


Fig 10. Two examples of sub-optimal staining with ALK on the UK NEQAS distributed positive NSCLC tumour sample E (compare both images to Fig 9). Although both examples are positive, (A) shows weak demonstration, while the staining in example (B) is much stronger than expected.

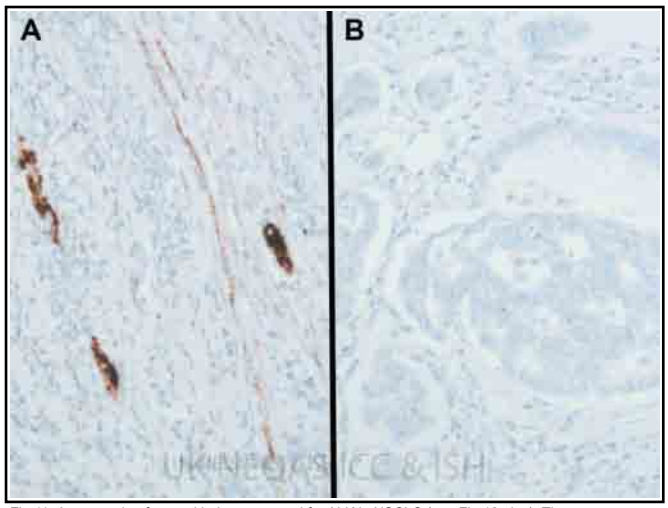


Fig 11. An example of a good in-house control for ALK in NSCLC (see Fig 12 also). The multi-tissue control included appendix (image A), and both negative (image B) and positive (Fig 12) NSLC tumour samples. All sections were stained appropriately.

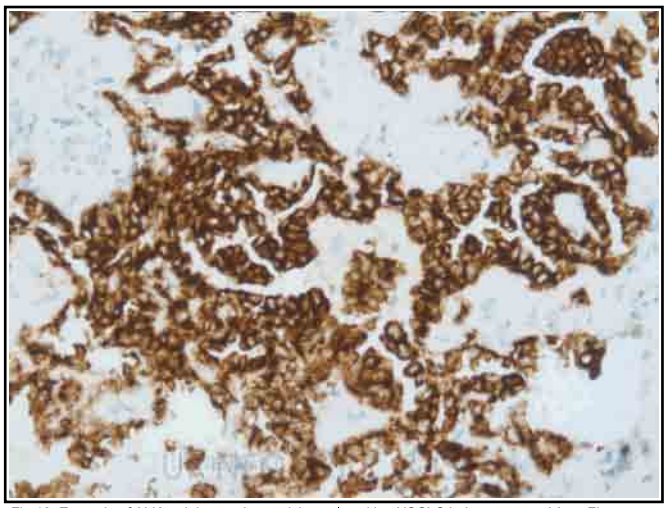
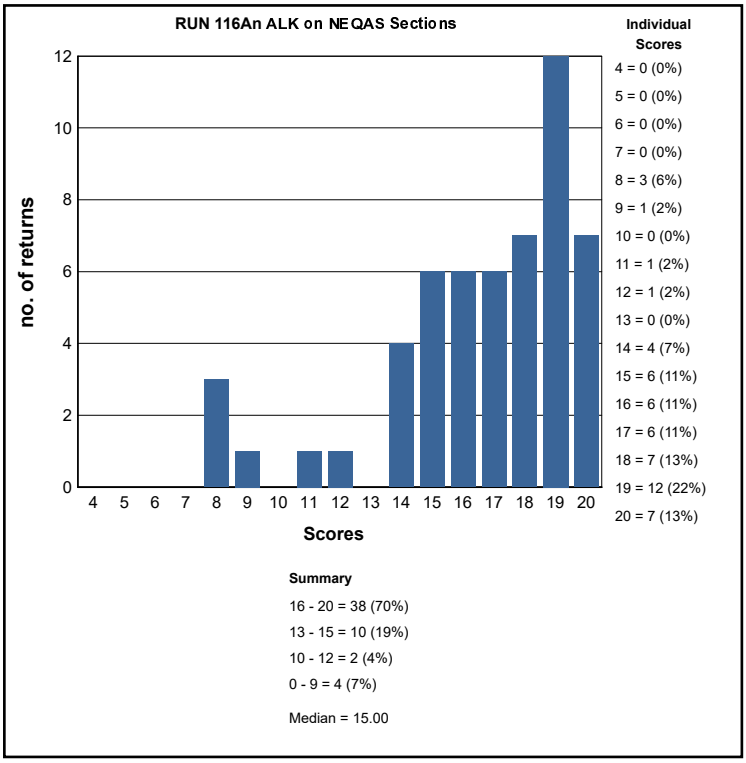
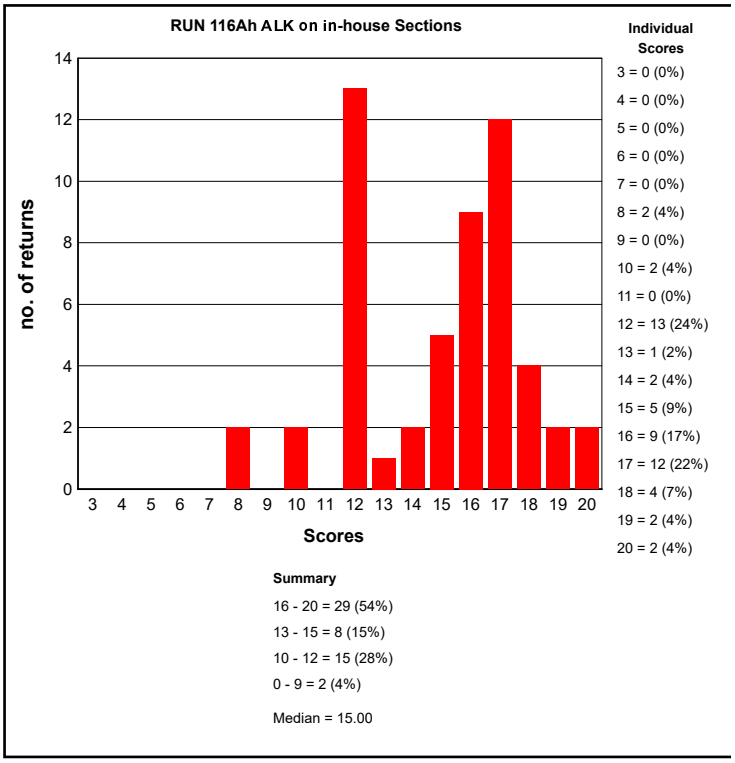


Fig 12. Example of ALK staining on the participants' positive NSCLC in-house control (see Fig 11 also).

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: 116

Primary Antibody	N	%
Abcam (5A4)	1	0
Cell Sign. Tech. (D5F3)	3	100
Dako M7195 (ALK1)	1	0
Novocastra NCL-ALK (5A4)	6	50
Ventana/Roche (D5F3)	41	100

ALK NSCLC Run: 116

Automation	N	%
Dako Autostainer Link 48	3	33
Leica Bond Max	1	0
Ventana Benchmark GX	4	100
Ventana Benchmark ULTRA	14	86
Ventana Benchmark XT	30	100

ALK NSCLC Run: 116

Heat Mediated Retrieval	N	%
Dako PTLink	2	50
Leica ER2 20 mins	1	0
None	1	0
Ventana CC1 64mins	1	100
Ventana CC1 88mins	1	100
Ventana CC1 92mins	36	97
Ventana CC1 extended	5	80
Ventana CC1 standard	4	100
Ventana CC2 92mins	1	100

ALK NSCLC Run: 116

Detection	N	%
AS PER KIT	1	0
Dako EnVision FLEX (K8000/10)	1	100
Leica Bond Polymer Refine (DS9800)	1	0
Other	1	0
Ventana OptiView (760-700) + Amp. (7/860-099)	29	97
Ventana OptiView Kit (760-700)	17	100
Ventana UltraView Kit (760-500)	1	0

ALK NSCLC Run: 116

Enzyme Retrieval	N	%
AS PER KIT	2	50
NOT APPLICABLE	27	93
Ventana Protease	2	100

ALK NSCLC Run: 116

Chromogen	N	%
AS PER KIT	17	94
Dako EnVision Plus kits	1	100
Dako FLEX DAB	1	0
Leica Bond Polymer Refine kit (DS9800)	1	0
Ventana DAB	29	97
Ventana Ultraview DAB	3	67

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

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

ALK - Method 2

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

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

ALK - Method 3

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

Primary Antibody:	Cell Sign. Tech. (D5F3) , 16 Mins Dilution 1: 100
Automation:	Ventana Benchmark XT
Method:	
Main Buffer:	
HMAR:	Ventana CC1 extended
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	Ventana OptiView Kit (760-700)

ALK - Method 4

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

Primary Antibody:	Ventana/Roche (D5F3) , 16 Mins Prediluted
Automation:	Ventana Benchmark GX
Method:	
Main Buffer:	
HMAR:	Ventana CC1 92mins
EAR:	
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700) , 12 Mins

The HER2 ISH Module - Interpretive Run 45

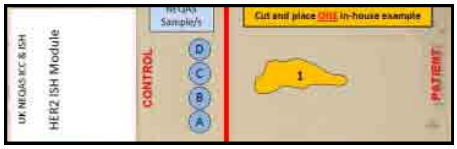
Suzanne Parry and Dawn Wilkinson

Gene Assessed:	HER2
Sections Circulated:	Composite slide consisting of 4 breast carcinoma sections (see table below)
Number of Registered Participants:	174
Number of Participants This Run	160 (92%)

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

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

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



Introduction

Breast cancer HER2 status should be determined in all newly diagnosed and recurrent and metastatic breast cancers[1] . In-situ hybridization (ISH), using either fluorescent (FISH)[4] or brightfield chromogenic methods (CISH)[5] are now recognised techniques to determine the amplification status of breast cancers in either 2+ IHC equivocal cases or as an upfront method.

Although single HER2 probe methods are available, such as Ventana Silver ISH (SISH)[3], the dual probe (ratio) based techniques are the method of choice, with chromogenic assays now used as abundantly as **FISH**

For UK laboratories who are accredited to carry out diagnostic HER2 ISH testing, participation in an external quality assessment (such as the UK NEQAS ICC & ISH) is a requirement, and therefore poor performance is monitored on a regular basis to enhance confidence in HER2 ISH testing. It should also be emphasised that the scheme remains very much open to non-UK laboratories as an aid to monitoring and improving performance.

Recommendations

There are several recent recommendations regarding HER2 ISH testing including those by ASCO/CAP (USA) and UK Recommendations and Guidelines[6,7]. It is advisable that these guidelines are followed and the processes of introducing and maintaining a clinically validated HER2 ISH assay or laboratory developed test (LDT) are properly validated within the prior to their introduction into the laboratory as a diagnostic test.

Updated Assessment Procedure

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

Assessment Results

All laboratories employed a dual probe and a ratio scoring algorithm. 70% of participants achieved excellent or acceptable results. 27% received a borderline pass and only four laboratories (3%) had an unacceptable interpretation result. There were no unacceptable results from any UK laboratory. The most common brightfield method was the Ventana DDISH with 52 (33%) laboratories using this technique. The most popular FISH method was the Pathvysion Vysis Kit with 45 (28%) laboratories using this technique. Both of these methods had very similar pass rates of 67% and 69% respectively.

The most notable difference when comparing the interpretative results of Run 45 to the previous Run 44 is the drop in unacceptable results which has fallen from 13 laboratories (9%) to 4 (3%) respectively.

Frequency Histograms

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

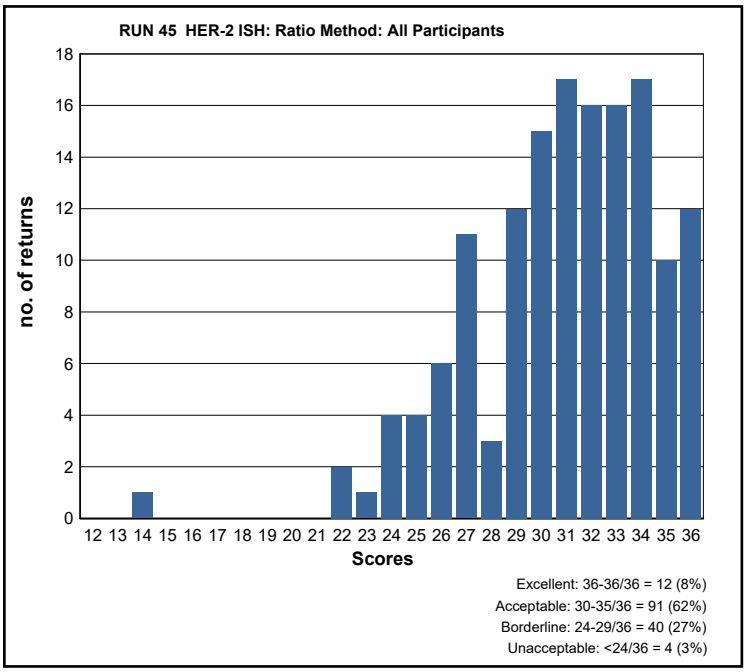
HER2 ISH Method and Probe Enumeration

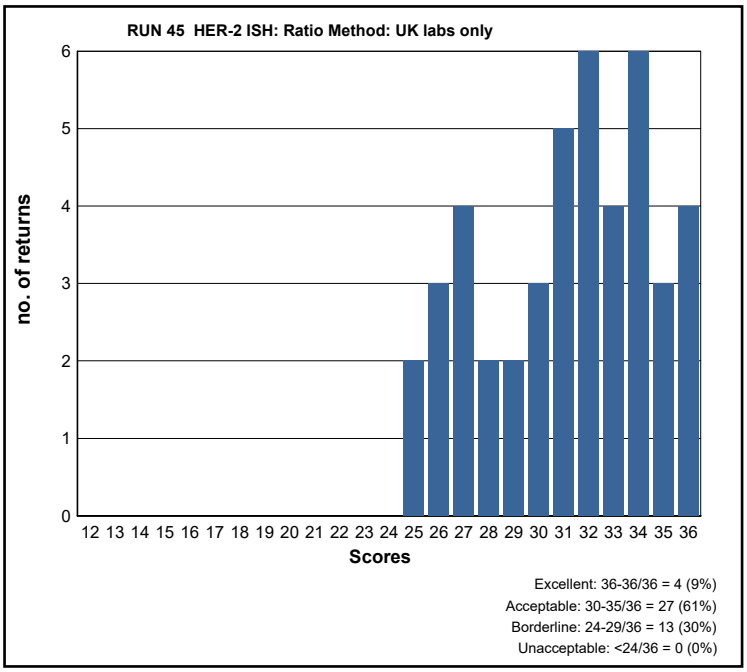
Table 1 shows the various ISH methods employed along with mean HER2, Cen17 counts and ratio calculations.

References

1. Walker RA, Bartlett JM, Dowsett M, Ellis IO, Hanby AM, Jasani B, Miller K. Pinder SE. (2015) HER2 testing in the UK: further update recommendations. J Clin Pathol;61818-824.
2. Bartlett JM, Starczynski J, Atkey N, Kay E, O'Grady A, Gandy M, Ibrahim M, Jasani B, Ellis IO, Pinder SE, Walker RA. (2011) HER2 testing in the UK: recommendations for breast and gastric in-situ hybridisation methods. J Clin Pathol; 64: 649-653.
3. Moore, D. S. and McCabe, G. P. Introduction to the Practice of Statistics, 4th ed. New York: W. H. Freeman, 2002.

GRAPHICAL REPRESENTATION OF PASS RATES (ratio methods only)





METHODS USED and PASS RATES

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

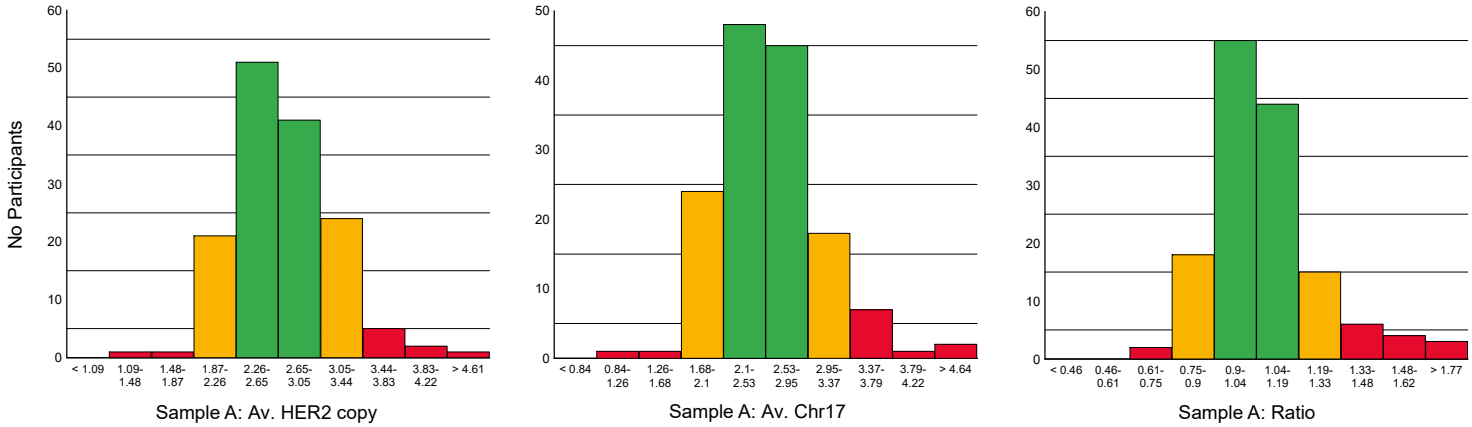
Ratio Method	N	% Pass (score ≥ 30/36)
Ratio: Dako DuoCISH	1	0%
Ratio: Dako IQFISH pharmDX	11	73%
Ratio: Dako Pharm Dx	1	100%
Ratio: In house FISH	3	67%
Ratio: Kreatech Probes	6	100%
Ratio: Leica HER2 FISH TA9217	7	100%
Ratio: Other - CISH	1	100%
Ratio: Other - FISH	5	80%
Ratio: Pathvysion Vysis Kit	45	69%
Ratio: Ventana BDISH 800-098/505	5	80%
Ratio: Ventana DDISH (780/800-4422)	52	67%
Ratio: Ventana Inform Silver ISH	3	67%
Ratio: Zytovision ZytoDot 2C	1	100%
Ratio: Zytovision ZytoLight	6	17%

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

FREQUENCY HISTOGRAMS

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

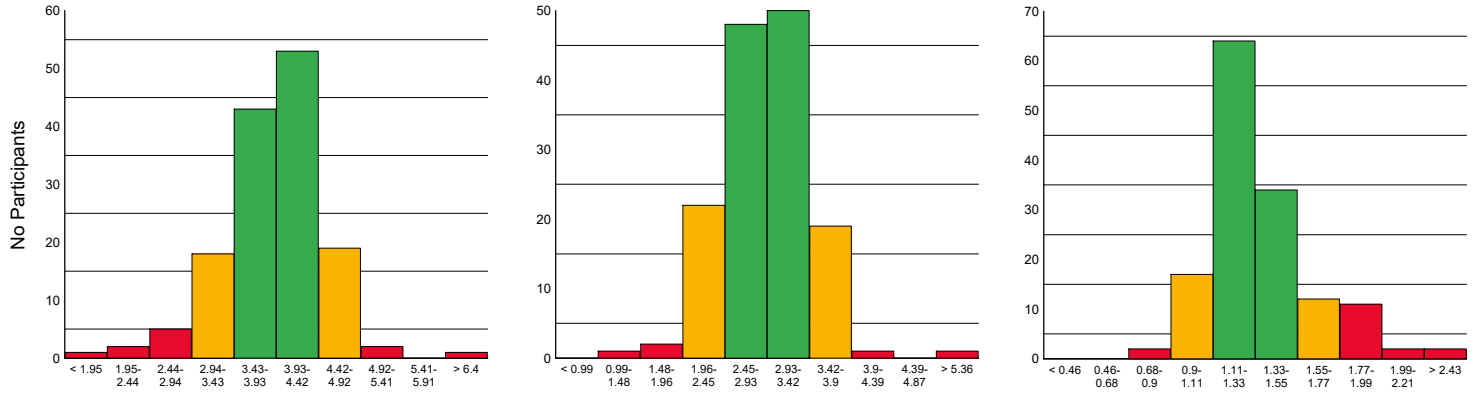
Green = mean +/- 1 Stdev Amber = mean +/- 2 Stdev Red = mean ≥ +/- 3 stdev



Sample A: Av. HER2 copy

Sample A: Av. Chr17

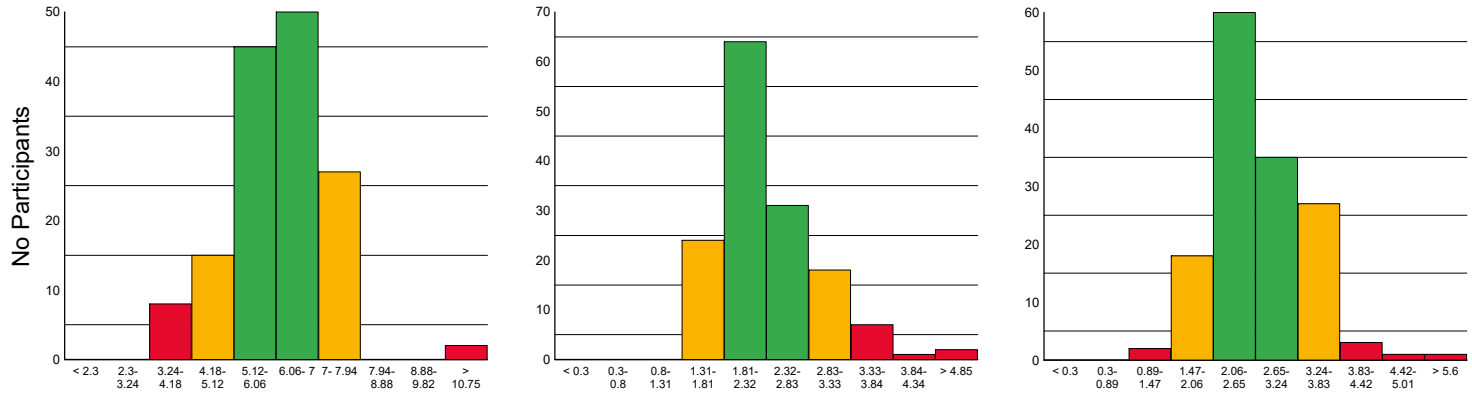
Sample A: Ratio



Sample B: Av. HER2 copy

Sample B: Av. Chr17

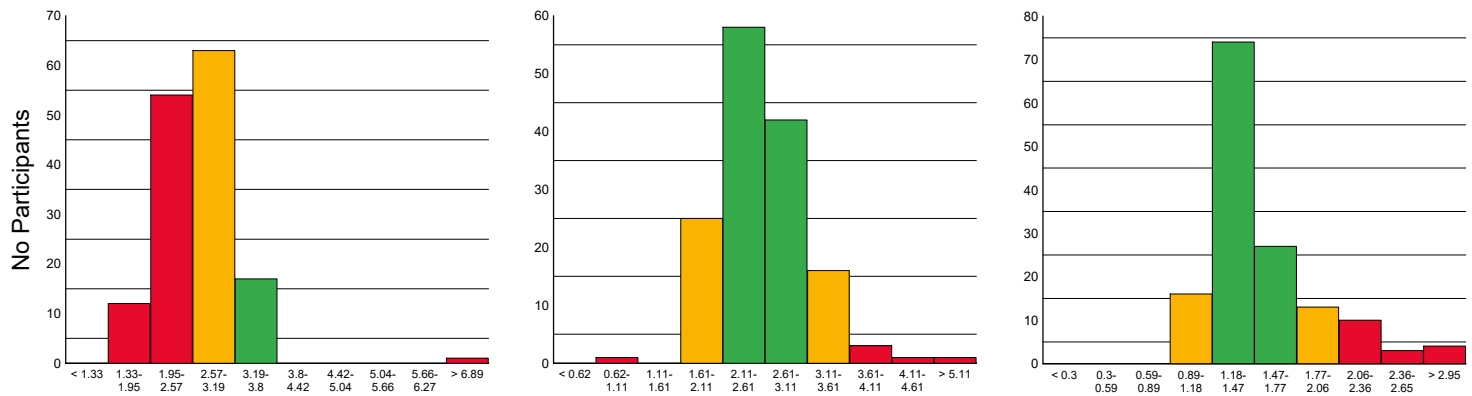
Sample B: Ratio



Sample C: Av. HER2 copy

Sample C: Av. Chr17

Sample C: Ratio



Sample D: Av. HER2 copy

Sample D: Av. Chr17

Sample D: Ratio

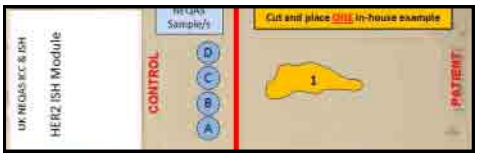
The HER2 ISH Module - Technical Run 45

Suzanne Parry and Dawn Wilkinson

Gene Assessed:	HER2
Sections circulated:	Composite slide consisting of 4 breast carcinoma sections (see table below)
Number of Registered Participants:	174
Number of Participants Taking Part this Run	160 (92%) (82 Fluorescent and 68 Chromogenic)

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

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



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

Assessment Procedure

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

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

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

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

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

Results Summary

CISH Results

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

The overall results showed an improvement in pass rates compared to the previous run (Run 44). 33 (50%) of laboratories achieved an acceptable pass rate on this Run (45) compared to 36% on previous run on the UK NEQAS distributed material. Again, the failures were predominantly due to weak or no Cen17 signals, as seen in previous assessment runs over the last 12-18 months. The borderline passes (30%, n=20) were given for weak, but diagnostically readable signals; again, this was mostly due to weak Cen17 signals. Some slides showed dust-like silver chromogen signals, but unless obscuring the specific HER2 signals and the slide was deemed still readable, the section did not fail, but was given a borderline pass. These observational results refer to the Ventana BDISH, Inform or DDISH methods, which 88% of laboratories have adopted who submitted brightfield ISH slides for this technical assessment.

The Dako DuoCISH method was used by a small number of laboratories, but official data for this assay has only been collected from two laboratories as other participants using this assay did not correctly submit their methodology details. However, these slides were recognised as being stained with the Dako DuoCISH kit by the assessment team as the staining is very distinct: The HER2 signals are red and the Cen17 signals are blue. The cytoplasm also has a very red blush which is inherent of this method. Both of the laboratories who entered correct methodology data achieved an acceptable score. The Zytovision ZytoDot 2C method was used by one laboratory, and this achieved a borderline pass due to weak signals in some of the cores. This CISH methodology is easily recognised by the assessment team, as the HER2 signals are green and the Cen17 signals are red.

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

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

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

FISH Results

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

The previous Run (44) noted an unusually high level of FISH results with weak or no HER2 and/or Cen17 signals, which contributed to a higher number of laboratories receiving an unacceptable mark (31%). This problem was not noted in the current Run and could most likely be attributed to technical, storage or transport errors, as it does not appear to be assay specific.
This is reflected in the overall pass rate with an increase of acceptable pass rates from 60% (Run 44) to 73% (Run 45).
The Pathvysion Vysis Kit is most commonly used by laboratories. The Dako IQFISH and Leica FISH assays were used by 13% and 9% of participants respectively. They both achieved a 100% acceptable pass rate. Other FISH assays used include the Dako Pharm Dx, Kreatech Probes and the Zytovision ZytoLight, all of which performed well, although the numbers of laboratories adopting these methods are low.

Validating ISH

It is crucial to emphasise the importance of undertaking a robust validation and verification protocol when introducing ISH into a diagnostic laboratory for the first time or a new assay/method is being introduced. For example, changing from a FISH assay to a brightfield ISH asssay, the new technique must be fully validated and staff must undergo extra training to gain expertise with the new method employed.
The UK NEQAS data indicates that there is a definite move towards the use of the Ventana DDISH method and laboratories introducing either this method or another ISH method should make sure that:

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

Recommendations for Returning FISH Slides for NEQAS Assessments

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

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

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

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

Selected Images showing Optimal and Sub-optimal Immunostaining

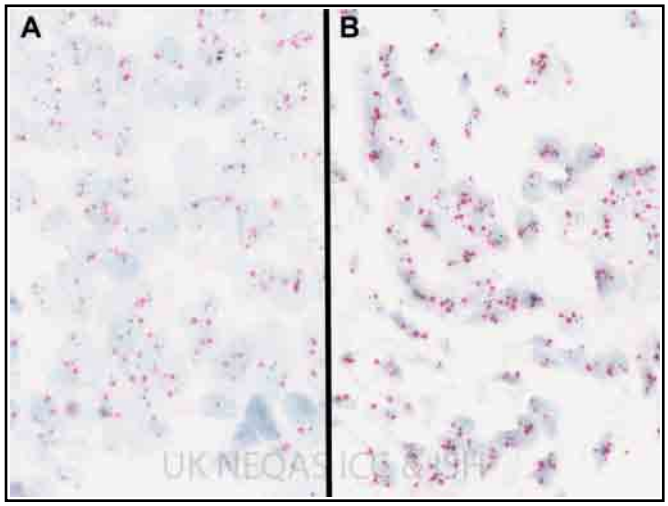


Fig 1. Acceptable Ventana DDISH in the UK NEQAS non-amplified tissue samples A and B. In both cases distinct HER2 signals (black) and Ch17 signals (red) are clearly demonstrated with the expected level of copies per cell.

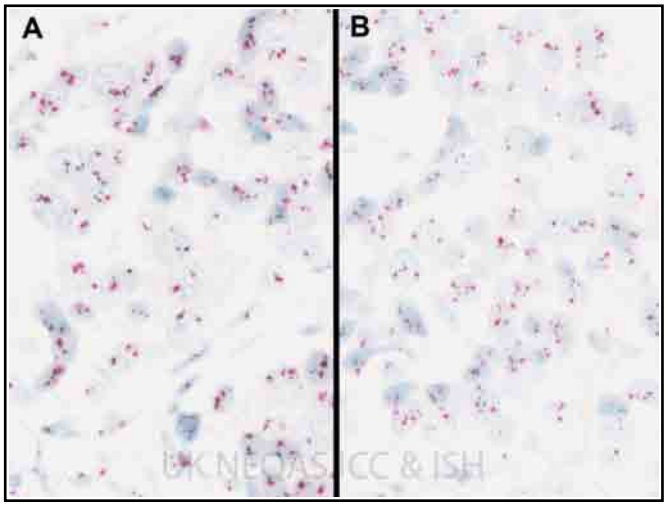


Fig 2. Acceptable Ventana DDISH in the UK NEQAS samples: (A) shows the amplified tissue 'sample C' and (B) the non-amplified tissue 'sample D'. In both examples the HER2 (black) and Ch17 (red) signals are strong and clear and show the expected level of copies per nuclei.

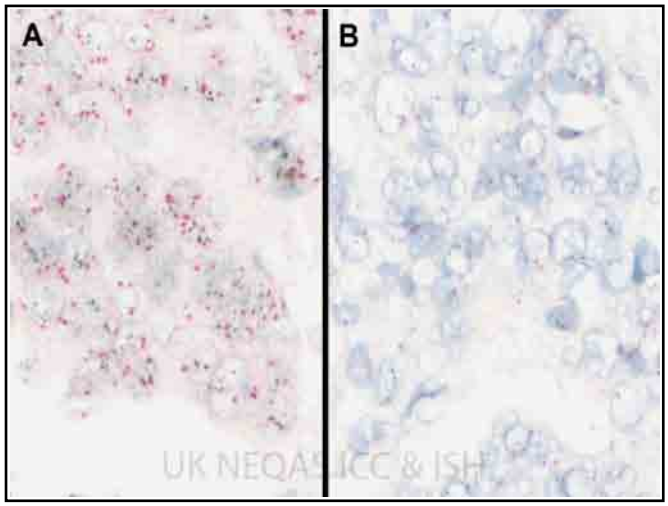


Fig 3. Two examples of slides with unacceptable level of staining. (A), NEQAS 'sample A', shows excessive silver deposit and (B), NEQAS 'sample B', shows 'bubbling' artefact and morphology damage.

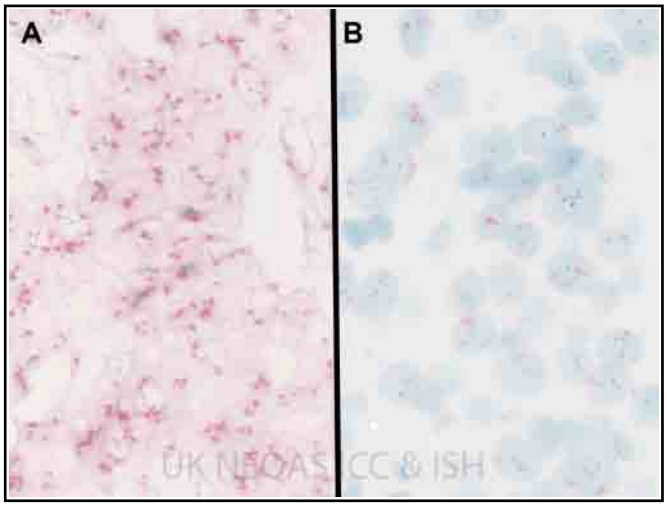


Fig 4. Two examples of slides with unacceptable level of staining. (A), NEQAS 'sample B', shows nuclear merging of borders and excessive red precipitate. (B), NEQAS sample D, shows weak and patch Cep 17 signals.

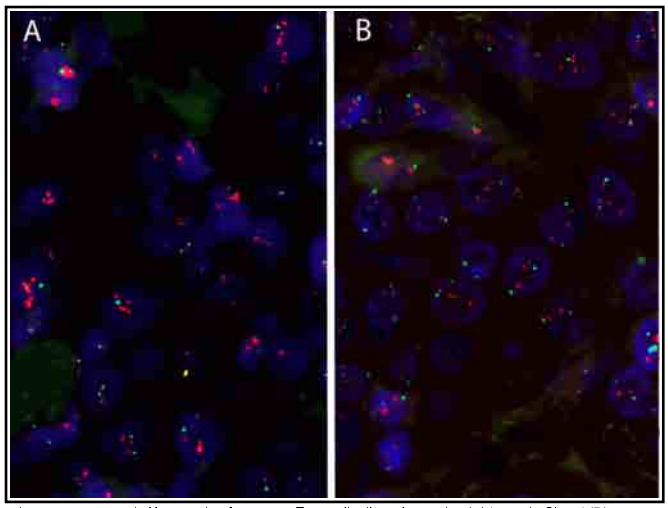


Fig 5. Acceptable FISH examples from UK NEQAS distributed samples (A) 'sample C' and (B) 'sample D'. Both samples shown without dapi, and clearly show distinct HER2 signals (red) and Ch17 signals (green). Stained using (A) Dako pharm DX and (B) Abbott Pathvysion Vysis.

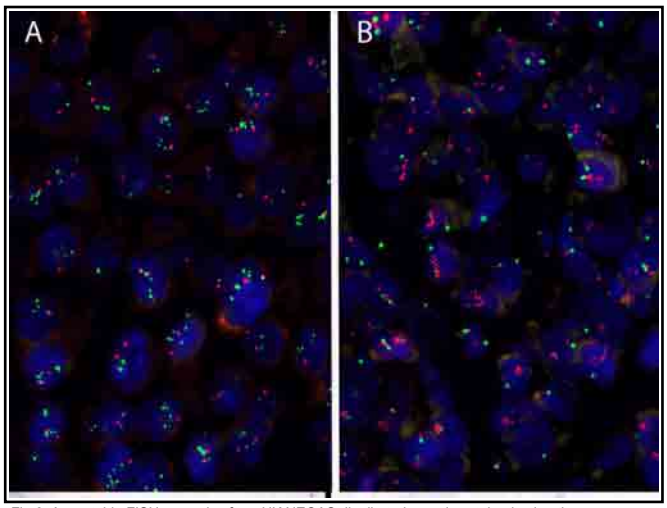
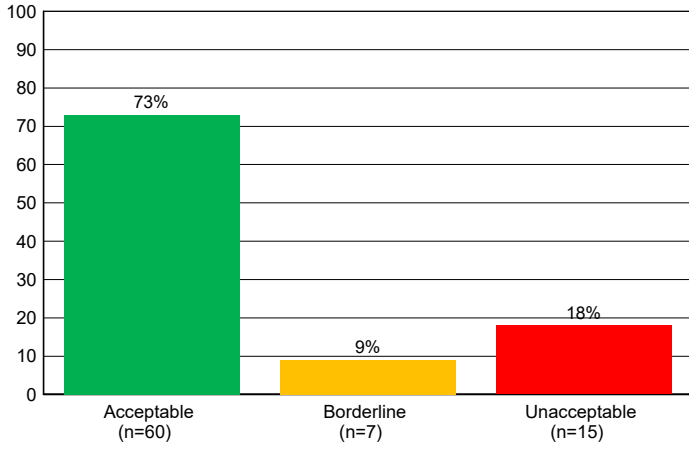


Fig 6. Acceptable FISH examples from UK NEQAS distributed samples stained using the Zytovision ZytoLight (A) 'sample C' and (B) 'sample C' stained with the Leica probes. Note that the Zytolight stains the HER2 signals in green and the Ch17 signals in red.

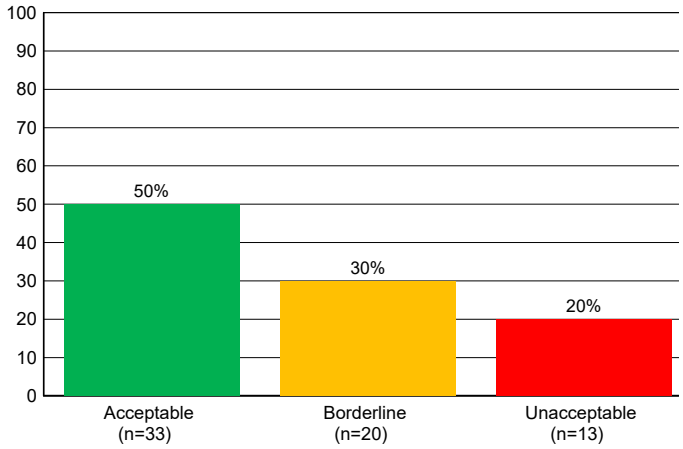
Technical ISH: Pass Rates and Methods

Overall Pass Rates

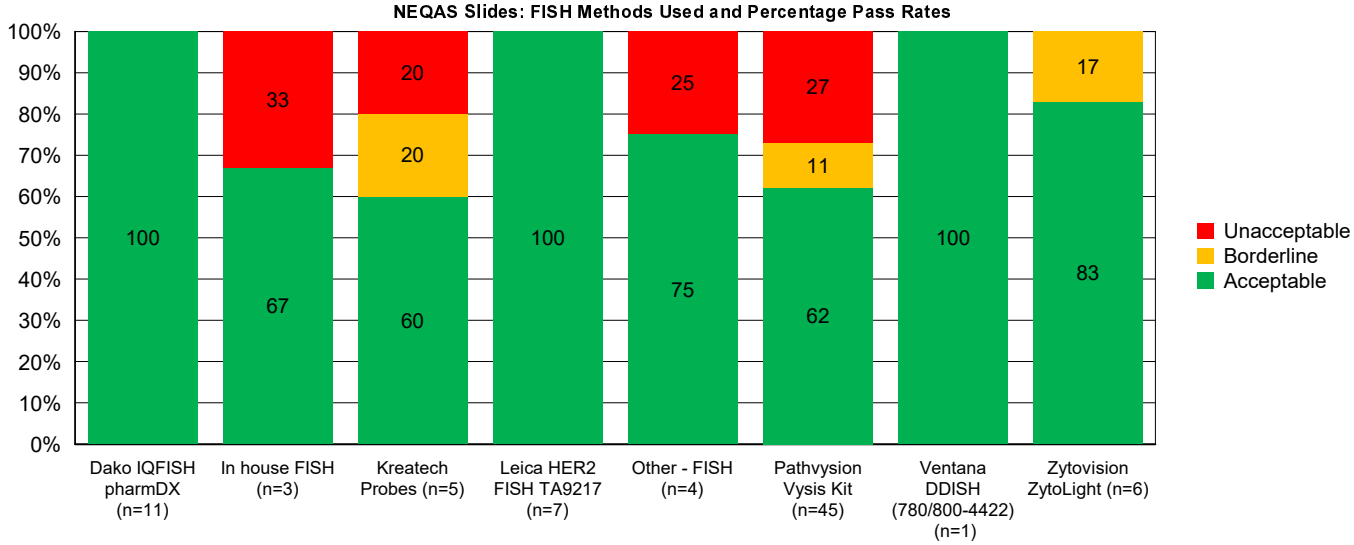
FISH NEQAS slide (n=82)



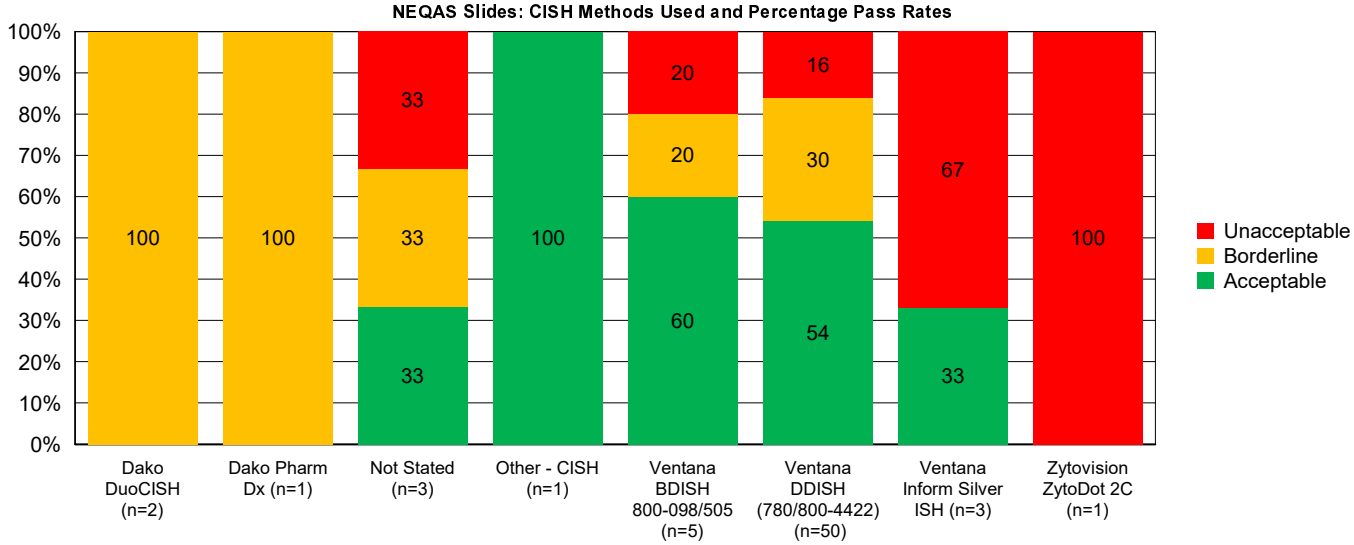
CISH NEQAS Slide (n=66)



NEQAS Slides: FISH Methods Used and Percentage Pass Rates



NEQAS Slides: CISH Methods Used and Percentage Pass Rates



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