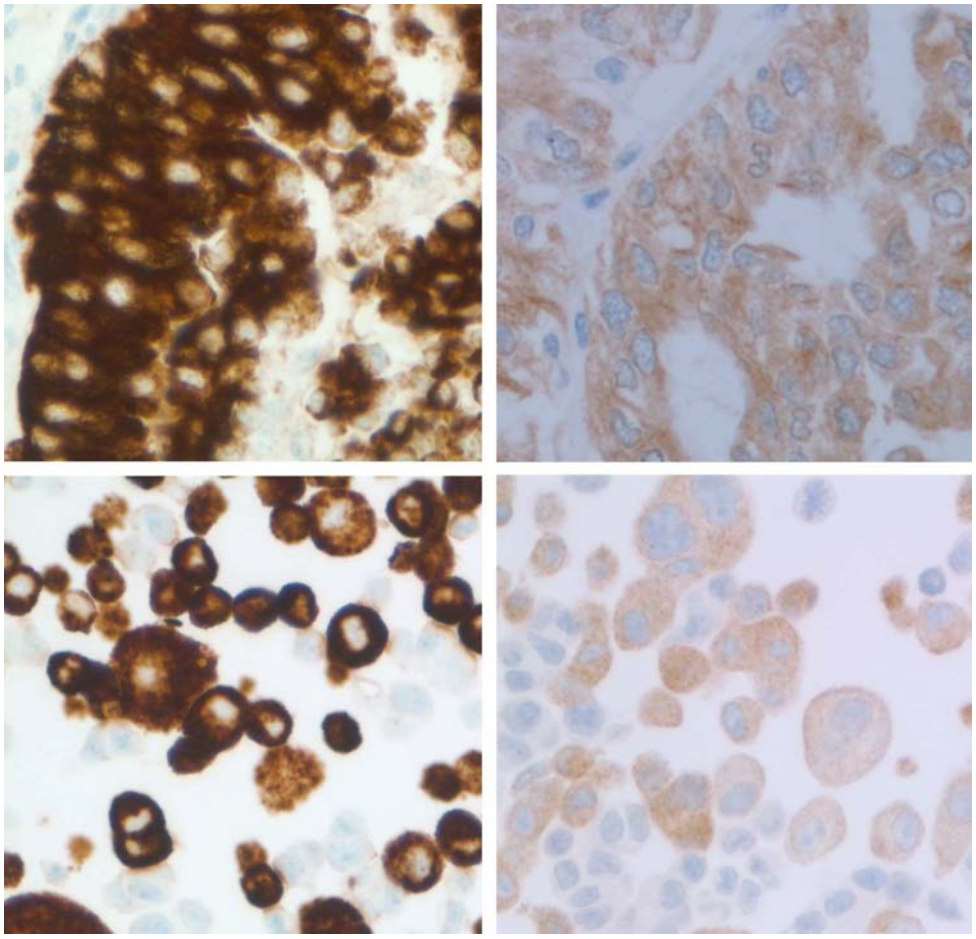
UK NEQAS
International Quality Expertise

Immunocytochemistry

Improving Immunocytochemistry for Over 25 Years

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

Assessment Dates: 5th - 23rd January 2015



Cover Photo: Taken from the NSCLC ALK IHC Module:
Top Left: Optimal ALK IHC staining in NSCLC stained with Roche D5F3
Top Right: Optimal ALK IHC staining in NSCLC stained with Novocastra 5A4
Bottom Left: Optimal ALK IHC staining in NSCLC cell line stained with Roche D5F3
Bottom Right: Optimal ALK IHC staining in NSCLC cell line stained with Novocastra 5A4

Also In This Issue

- NSCLC ALK IHC Pre-pilot Assessment Results
- Scheme Updates: Gastric Her2 and same slide NEQAS & in-house EQA
- Slide stability during transport and storage - A review

Articles / Reports

Immunocytochemistry Modules

In-situ Hybridisation Modules

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

General Information

UK NEQAS International Quality Expertise

CPA Accredited EQA Scheme Reference No: 044

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

UK NEQAS ICC & ISH does not endorse any of the products featured by the commercial sponsors and are placed at the discretion of UK NEQAS ICC & ISH. Furthermore, commercial companies featured do not have any input or influence over the content, including results that are shown.

For further information of the UK NEQAS ICC & ISH scheme, general EQA enquiries, slide returns and advertising opportunities please contact:

Dr Merdol Ibrahim, Scheme Manager
UK NEQAS ICC & ISH
Suite 4/12 Hamilton House
Mabledon Place
London WC1H 9BB, UK
Tel: +44 (2) 07 554 8678
E-mail: merdol.ibrahim@ucl.ac.uk

For enquiries concerning training issues, meetings, or courses, please contact:

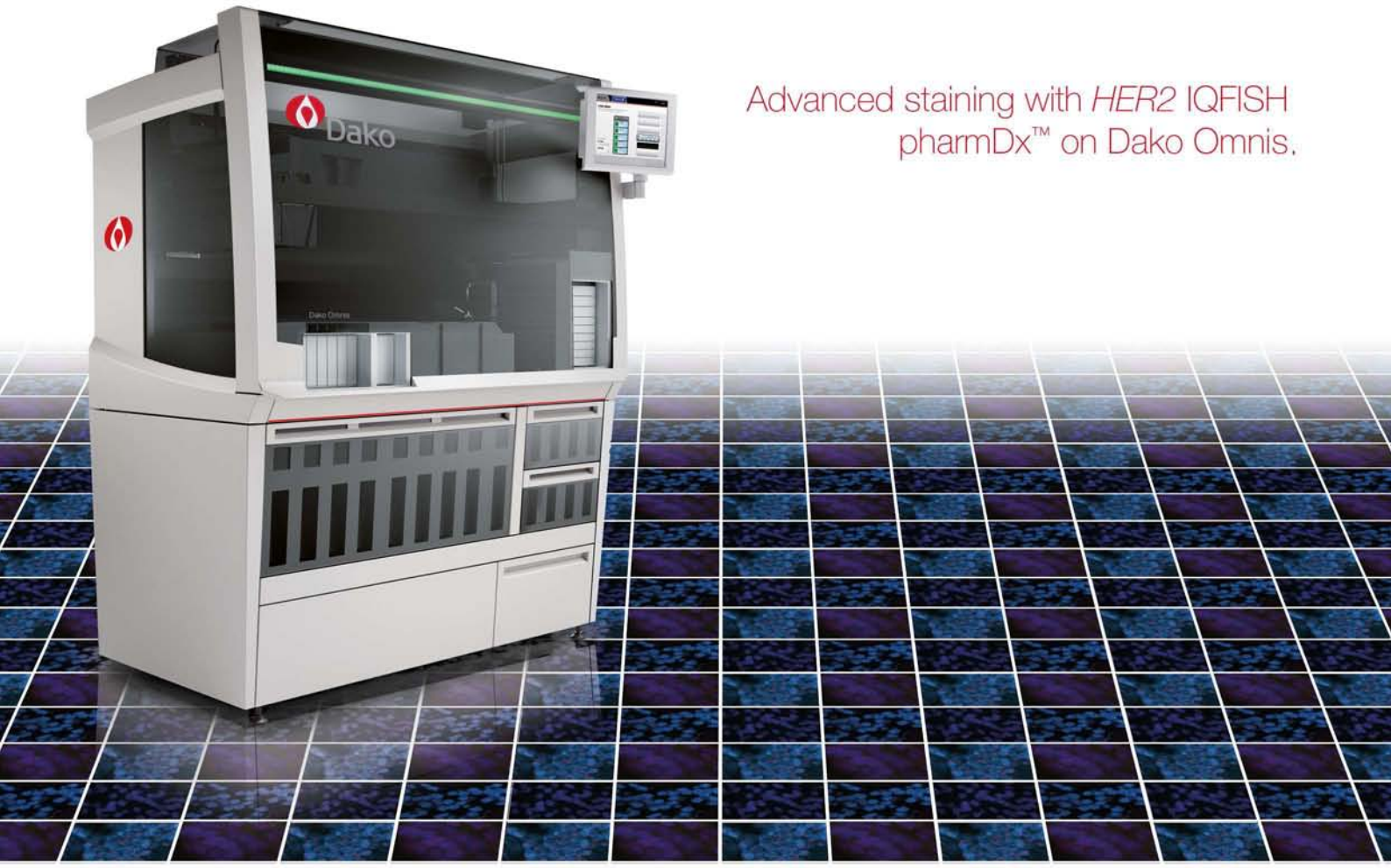
Mr Keith Miller, Scheme Director
UK NEQAS ICC & ISH
Suite 4/12 Hamilton House
Mabledon Place
London WC1H 9BB, UK
Tel: +44 (2) 07 554 8679
E-mail: k.miller@ucl.ac.uk

Journal layout and design prepared by UK NEQAS ICC & ISH

Dako

A Better Path™

Same time FISH and IHC.



Advanced staining with *HER2* IQFISH pharmDx™ on Dako Omnis.

Read more at **www.dako.com**

50238 2015/JAN28

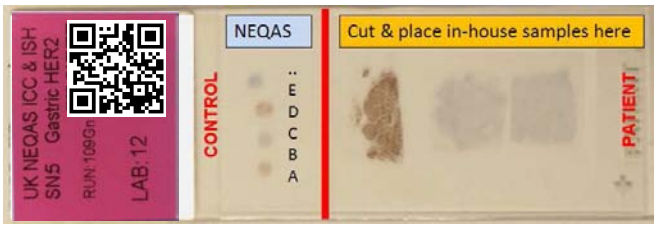
Agilent Technologies

Forthcoming Scheme Updates: Same Slide NEQAS + in-house Control and QR Coding of Distributed Slides

Same slide NEQAS + In-house: 2015/2016

Same slide Controls

- As of **Run 110 (May 2015))** we will be distributing assessment slides as illustrated in the figure below (**Notes: This configuration has already been rolled out for the breast hormonal receptor and gastric HER2 IHC modules)**
- The distributed slides consist of two areas a) containing NEQAS EQA samples and b) area for participants to cut & place their in-house control
- Please cut and place your in-house control below that of the NEQAS samples as shown
- **Important: Distributed slides <u>will be 'unbaked'</u> so please cut your in-house control and place onto the same slide and <u>then</u> bake the slide at either 37°C overnight <u>OR</u> 55-60°C for 1 hour.**
- Once the slides have been baked then carry out your usual staining procedure and send back your slide for assessment



- It is important that participants prepare control samples which are appropriate for the antibody requested and that they fit onto the slide area illustrated in the gastric example above

Breast HER2 ISH Module

- It was initially announced that In-house controls would no longer be required for this module. However, this has now be changed and participants <u>**will be required**</u> to also cut a control section alongside the NEQAS distributed samples
- A composite in-house control is not required for the ISH module and both single or multiple tissue sections are acceptable. This will allow assessors to gauge whether the assay/kit used is staining as expected.
- <u>**Please always return your NEQAS FISH/CISH slides for 'technical' assessment**</u>

- UK NEQAS receives FISH slides from all over the world and if treated carefully, the fluorescence will not deteriorate too quickly.
  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.
  b. Seal the edges of the coverslip onto the slide using clear varnish or another sealing agent, which will stop the slide from drying out during transportation.

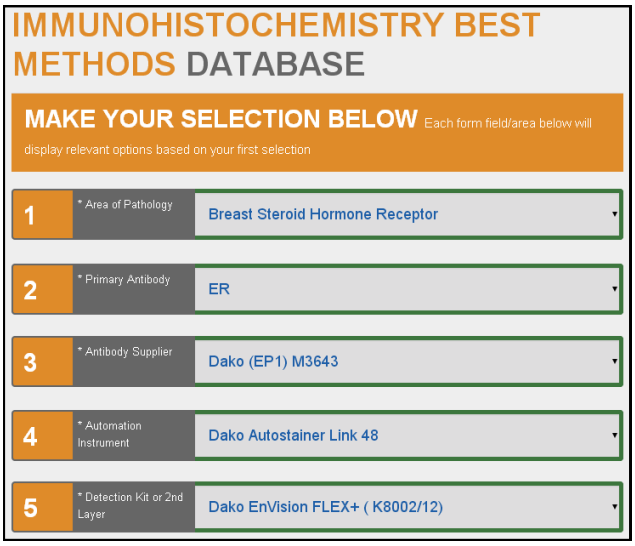
QR coding of NEQAS slides

- As of Run 110 NEQAS distributed slides will all have a QR code (2D barcode), which has information on assessment run numbers and anonymised participant codes
- The QR coded slides will make for easier tracking of slides when they arrive at NEQAS and preparation of slides for assessments
- **Participants must remove their auto generated 'machine labels' or hand written labels from the slides <u>before</u> returning to UK NEQAS ICC & ISH as they will need to be scanned on arrival.**

New UK NEQAS ICC & ISH Website

- We have been working behind the scenes on a completely new website, which will have a 'modern design' and will be available towards the end of April. Some of the new features include:

  * **Searchable 'Best Methods' database** allowing you to search for an antibody and corresponding method which has scored well during the UK NEQAS ICC & ISH assessments.



* Multi language selection

* Calendar, with assessment and meeting dates
* Easy access to journals
* + more

Save the Date: UK NEQAS ICC Meeting in Dublin

Date: Wednesday 24th June 2015
Satellite Symposium: Within the Pathological Society of Great Britain and Ireland
Title: Advanced Diagnostics: Improving testing for the benefit of patient management
Venue: Hilton Doubletree Hotel, Upper Lesson Street, Dublin 4

Further information will be provided soon

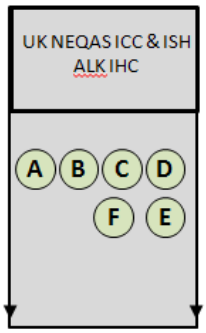
UK NEQAS ICC & ISH: Establishment of a Non-Small Cell Carcinoma (NSCLC) ALK IHC EQA and Results from the Initial Pre-pilot Assessment

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

Sample and Slide Distribution

Antibody assessed	ALK
Samples Circulated	Composite slide (see table below)
Number Registered for the Pre-pilot	46
Number of Returned Slides	36

The Figure and table below illustrates the positioning of the distributed samples along with their pre-tested FISH & IHC



Sample code	Sample	FISH status (Vysis)	IHC status (Roche D5F3)
A	Cell line: 50% knock in + 50% adenocarcinoma	-ve	Approx. 50% +ve & 50% -ve
B	Cell line: 50% isogenic + 50% adenocarcinoma	+ve (Break apart: inversion)	Approx. 50% +ve & 50% -ve
C	Cell line: 50% isogenic + 50% adenocarcinoma	+ve (Break apart: inversion)	Approx. 50% +ve & 50% -ve
D	Cell line : 100% adenocarcinoma	-ve	100% -ve
E	NSCLC tumour	-ve	-ve
F	NSCLC tumour	+ve (Break apart: inversion + deletion)	+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 Criztotonib[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, requiring more sensitive and/or enhanced detection systems.

Aim of the pre-pilot NSCLC ALK IHC Module

The main aim of the pre-pilot assessment was to establish a rigid ALK IHC EQA module and to identify and assist laboratories who may be having technical issues.

Pre-pilot Assessors Meeting

The pre-pilot advisory group meeting took place at UK NEQAS ICC & ISH, Hamilton House on the 17th February 2015 and included:

- **UK NEQAS ICC & ISH**: Mr Keith Miller, Dr Merdol Ibrahim, Ms Suzanne Parry, Mrs Dawn Wilkinson & Mr Neil Bilbe
- **Assessment Panel**: Dr Tony O'Grady (Dublin); Dr Erik Thunnissen (Amsterdam); Dr Phillipe Taniere (Birmingham); Dr Jane Starcynski (Birmingham); Prof John Gosney (Liverpool); Mr Steven Forrest (Liverpool); Dr Perry Maxwell (Belfast) & Mr David Allen (UCL).

- The panel discussed the format that the assessment should take place and whether to use a) A consensus scoring (score out of 20) and feedback method or b) Individually scoring method with individual scores out of 5. The latter individual scoring method out of 5 was the method of choice by the panel.
- The panel also agreed that it was important to provide their own interpretation on each of the samples. Although there are numerous scoring methods used in various publications it was decided for the pre-pilot assessment that a simple '+ve'/'-ve' (positive/negative) would be used, but reviewed during the forthcoming pilot assessments.
- Prior to assessment the panel reviewed some of the slides to make sure that there was not a bias towards a single method/antibody.
- Due to the various antibodies and methodologies submitted the panel did not solely focus on staining intensity of the different methods, but also on the interpretability of the samples.
- 8 assessors (all non-UK NEQAS ICC & ISH personnel) were split into 2 groups of four. Each group comprised of at least one pathologist and at least two scientists. Each group was overseen by a UK NEQAS ICC & ISH microscope lead who presented the slides; in addition, another UK NEQAS ICC & ISH person was on-hand in each assessment room to observe, and provide help with protocols, and to note any salient points for discussion afterwards.
- Each group took half of the slides, assessed them, and slides were then swapped between the two groups, therefore both groups assessed all the slides; the scores between each group were then compared afterwards and a decision made as to the participants' final score.

Results

Features of Acceptable Staining

- ALK IHC positive cases (Sample F) should demonstrate granular cytoplasmic staining in tumour cells
- Cell line samples (sample A-D) were positive for ALK IHC should show more or less homogeneous staining throughout the distributed samples and individual nuclei

(Figs 2A-C, 3A-C, 4A-C and 5A)

- ALK IHC positive tumour samples (Sample F) had in general a heterogeneous level of expression, however the individual nuclei should have a similar level of ALK expression (Figs 2F, 3F, 4F, 5B)
- Macrophages may be stained positive (Fig 2E and 4E)
- Negative samples should show no staining for ALK IHC (Fig 2D, 3D & E and 4D & E)

Features of Unacceptable Staining

- False negative / absence of ALK staining where tumour cells should be staining positive (Fig 5C & D)
- Non-specific staining (Fig 5E & F)

Pass Rates & Methodologies

For the initial pre-pilot assessment 36 participants submitted slides and Graph A shows the distribution of pass rates, with graph B showing the pass rates based on categories of 'Excellent = >16/20 ; Acceptable = 13-15/20 ; Borderline = 10-12/20 and Unacceptable = <9/20'. Overall there was a pass rate of 83% (scores >13/20, n= 30)), with 14% (n=5) having Borderline staining and 3% (n=1) showing unacceptable staining.

A breakdown of the submitted methods showed usage of 5 different antibody clones (D5F3, ALK1, ALK01, 5A4 and A4). The below points highlight the main findings:

- The Roche D5F3 (See Table 1, 2 and fig 2) was the main antibody of choice with 62% (n=21) using this particular clone on a Ventana platform. All those that submitted the D5F3 on the Ventana platform appeared to use the recommended protocol with the enhanced OptiView detection system. Overall this method showed a pass rate of 90% (n=19) with only 2 labs showing a borderline score. The borderline score was due to non-specific staining in both the known negative cell lines and NSCLC tumour (See fig 5E &F).
- The Leica Biosystems 5A4 (See Table 1, 2 & Fig 3) clone was used either prediluted (n=1) or concentrate form (n=4). The ready to use (RTU) form had a 100% pass rate. However the concentrate form had a 25% acceptable pass rate and the images in Figure 3 show what can be achieved along with the staining procedure used. Three participants (75%) using the concentrate form of the 5A4 achieved a borderline pass mark. It was however unfortunate that the antibody dilutions were not collected during this pre-pilot study.
- The Dako ALK1 (See Table 1, 2 & fig 4) although not recommended by Dako for NSCLC was used by 2 participants (Fig 4 and 5 C & D) with one participant achieving an acceptable level of staining (see fig 4) and one participant showing unacceptable staining (Fig 5 C & D; false negative for all samples that were expected to stain positive for AlK).
- The Cell Signalling D5F3 clone (see table 1 & 2) from Cell Signalling was used by 4 participants using a variety of platforms (see table 2) including the Leica Bond III, Ventana Benchmark XT and the Dako Autostainer Link 48, overall there was a 100% pass rate using the D5F3 antibody clone.

An enhanced detection system (see table 2) is recommended for ALK and the majority of participants appear to use the more sensitive detection systems including Dako EnVision FLEX+, Leica Bond Polymer Refine and Ventana Optiview. However some participants are not using a sensitive enough detection kit such as Ventana UltraView and Dako Envision HRP/DAB, which along with antibody dilution (data not collected) may lead to a less sensitive method.

Scoring Systems Employed by Participants

From the participants methodology survey it was also observed that different scoring systems were employed (see table 3), with the main method being a simple positive/ negative result (75% of participants) followed by a 3+,2+,1+ 0 scoring method (17% of participants). There was no correlation with the scoring method and methodology used and the scoring method employed was more than likely due to the personal preference of the laboratory concerned. It was also discussed during the assessment that for future assessments and for better collation of data that UK NEQAS ALK IHC assessments may incorporate a combined positive/ negative and 3+, 2+ 1+ scoring method, such that samples will be may be scored 'Positive (3+)' or 'Positive (2+)', to allow assessors to provide more informative feedback on intensity of staining.

In-house Control Samples

The assessment panel also felt that it was important for participants to submit their in-house controls that they used in NSCLC ALK IHC. For future assessment in-house controls will be requested and participants will be required to cut and place these alongside the NEQAS distributed slides.

Commencement of the Pilot Module

The ALK IHC module will now move to the pilot phase, which will run alongside the other modules offered by NEQAS. The pilot phase will allow further refinement to the ALK IHC module after which the UK NEQAS Steering Committee for Technical Schemes in cellular pathology will be approached to allow the module to be a 'full' UK NEQAS ICC & ISH module, after which ISO 17043 accreditation will then be applied for.

Acknowledgements

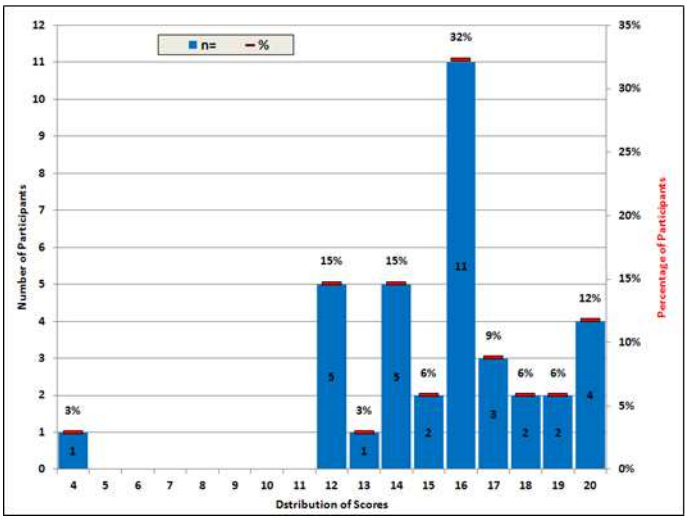
We are grateful for Novartis Pharmaceuticals UK Limited for providing an educational grant which was used to help setup the ALK EQA module. Please note that Novartis are not privy to any data/results until they are publically available.

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.
4. Bang Y, Kwak EL, Shaw AT, et al. Clinical activity of the oral ALK inhibitor PF-02341066 in ALK-positive patients with non-small cell lung cancer (NSCLC). J Clin Oncol 2010;28:18s.
5. Soda M, Choi YL, Enomoto M, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 2007; 448:561–566.
6. Palmer RH, Vernersson E, Grabbe C, et al. Anaplastic lymphoma kinase: signalling in development and disease. Biochem J 2009;420: 345–361.
7. Wong DW, Leung EL, So KK, et al. The EML4-ALK fusion gene is involved in various histologic types of lung cancers from nonsmokers with wild-type EGFR and KRAS. Cancer 2009;115:1723–1733.
8. Inamura K, Takeuchi K, Togashi Y, et al. EML4-ALK lung cancers are characterized by rare other mutations, a TTF-1 cell lineage, an acinar histology, and young onset. Mod Pathol 2009;22:508 –515.
9. Boland JM, Erdogan S, Vasmatzis G, et al. Anaplastic lymphoma kinase immunoreactivity correlates with ALK gene rearrangement and transcriptional up-regulation in non-small cell lung carcinomas. Hum Pathol 2009;40:1152–1158.
10. Takeuchi K, Choi YL, Soda M, et al. Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. Clin Cancer Res 2008;14:6618–6624.
11. Paik JH, Choe G, Kim H, et al. Screening of anaplastic lymphoma kinase rearrangement by immunohistochemistry in nonsmall cell lung cancer: correlation with fluorescence in situ hybridization. J ThoracOncol 2011;6:466-472

Below graphs show (A) the overall distribution of scores (n=36) and (B) Breakdown of data into Unacceptable, Borderline, Acceptable and Excellent categories

Graph A



Graph B

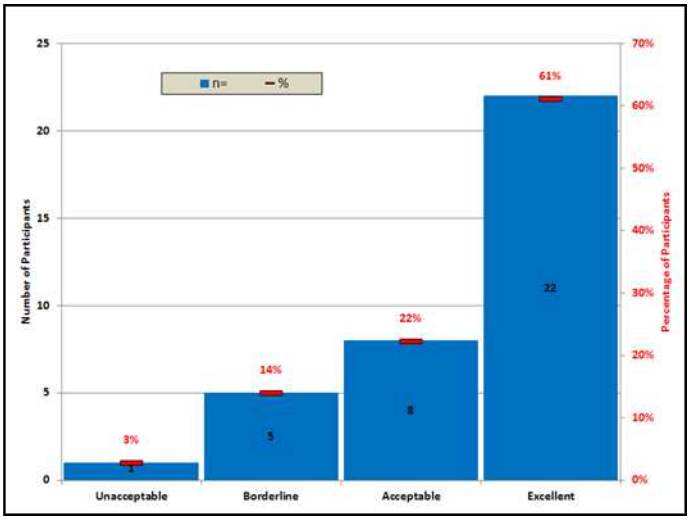


Table 1: Antibodies submitted by participants along with their respective assessment pass rates (n=34)

Antibody	n=	% of total methods submitted	Unacceptable	Borderline	Acceptable	Excellent
Cell Signalling Technology (D5F3)	4	12%	-	-	1 (25%)	3 (75%)
Dako ALK (ALK1)	1	3%	1 (50%)	-	1 (50%)	-
Diag. Biosystems MOB416 (5A4)	1	3%	-	-	1 (100%)	-
Novocastra (5A4)	4	12%	-	3 (75%)	-	1 (25%)
Novocastra RTU PA0306 (5A4)	1	3%	-	-	-	1 (100%)
OriGene Anti Human ALK Clone (1A4)	1	3%	-	-	-	1 (100%)
Ventana Confirm (ALK01)	1	3%	-	-	-	1 (100%)
Ventana/Roche (D5F3)	21	62%	-	2 (10%)	5 (24%)	14 (67%)

N.B. Not all participants returned methodology and protocol details

Table 2: Antibody, associated automation instrument & detection system with pass rates

Antibody	Automation Instrument	Detection Kit	Excellent	Acceptable	Borderline	Unacceptable
Cell Signalling Technology (D5F3)	Dako Autostainer Link 48	Dako EnVision FLEX+ (K8002/12)	1 (100%)			
	Leica Bond-III	Leica Bond Polymer Refine (DS9800)	1 (50%)	1 (50%)		
	Ventana Benchmark ULTRA	Ventana UltraView Kit (760-500)	1 (100%)			
Dako (ALK1)	Dako Autostainer Link 48	DAKO Envision FLEX+ mouse		1 (100%)		
	Ventana Benchmark ULTRA	Ventana UltraView Kit (760-500)				1 (100%)
Diag. Biosystems	Ventana Benchmark XT	Ventana UltraView Kit (760-500)		1 (100%)		
Novocastra (5A4)	LabVision Autostainer	Dako Envision HRP/DAB (K5007)			1 (100%)	
	Leica Bond-III	Leica Bond Polymer Refine (DS9800)	1 (50%)		1 (50%)	
	Ventana Benchmark ULTRA	Ventana OptiView Kit (760-700)			1 (100%)	
Novocastra RTU (5A4)	Leica Bond Max	Leica Bond Polymer Refine (DS9800)	1 (100%)			
OriGene Anti Human	None (Manual)	GBI Labs Polink-2 Broad reagent 1	1 (100%)			
Ventana Confirm	Ventana Benchmark XT	Ventana UltraView Kit (760-500)	1 (100%)			
Ventana/Roche (D5F3)	Roche Ventana Benchmark GX	Ventana OptiView Kit (760-700)			1 (100%)	
	Ventana Benchmark ULTRA	Ventana OptiView Kit (760-700)	1 (100%)			
	Ventana Benchmark XT	OptiView Amplification Kit (860-099)		1 (100%)		
		Ventana OptiView Kit (760-700)	13 (72%)	4 (22%)	1 (6%)	

Fig 1: FISH (Vysis) status of the distributed samples

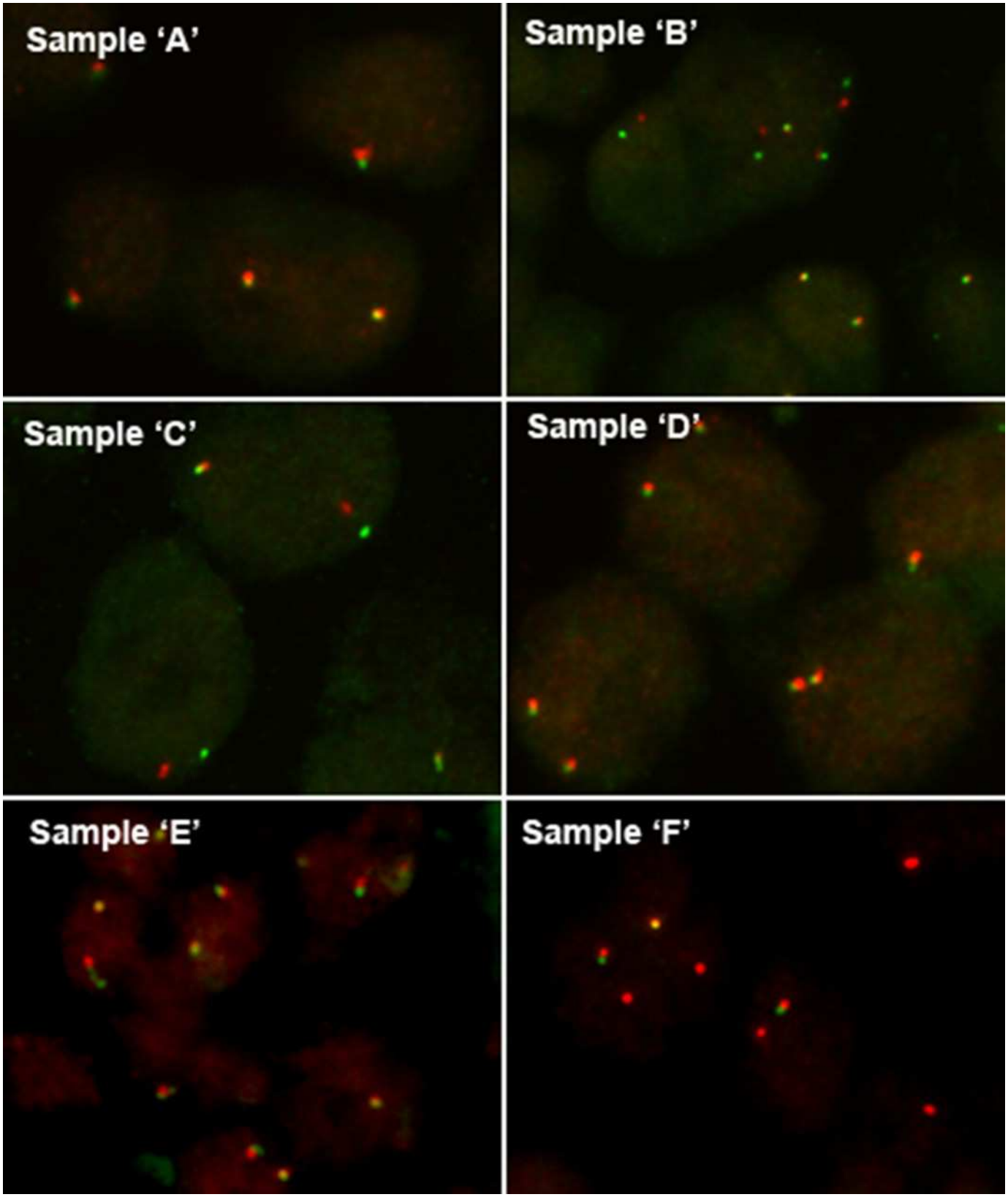


Fig 1. Samples were Fish'd prior to distribution with. The above photographs are shown without DAPI: Sample 'A' = Fish negative; Sample 'B' = Fish positive (inversion); Sample 'C' = Fish positive (inversion); Sample 'D' = Fish negative ; Sample 'E' = Fish negative ; Sample 'F' = Fish positive (inversion with deletion)

Fig 2: Samples stained with the Roche D5F3 assay

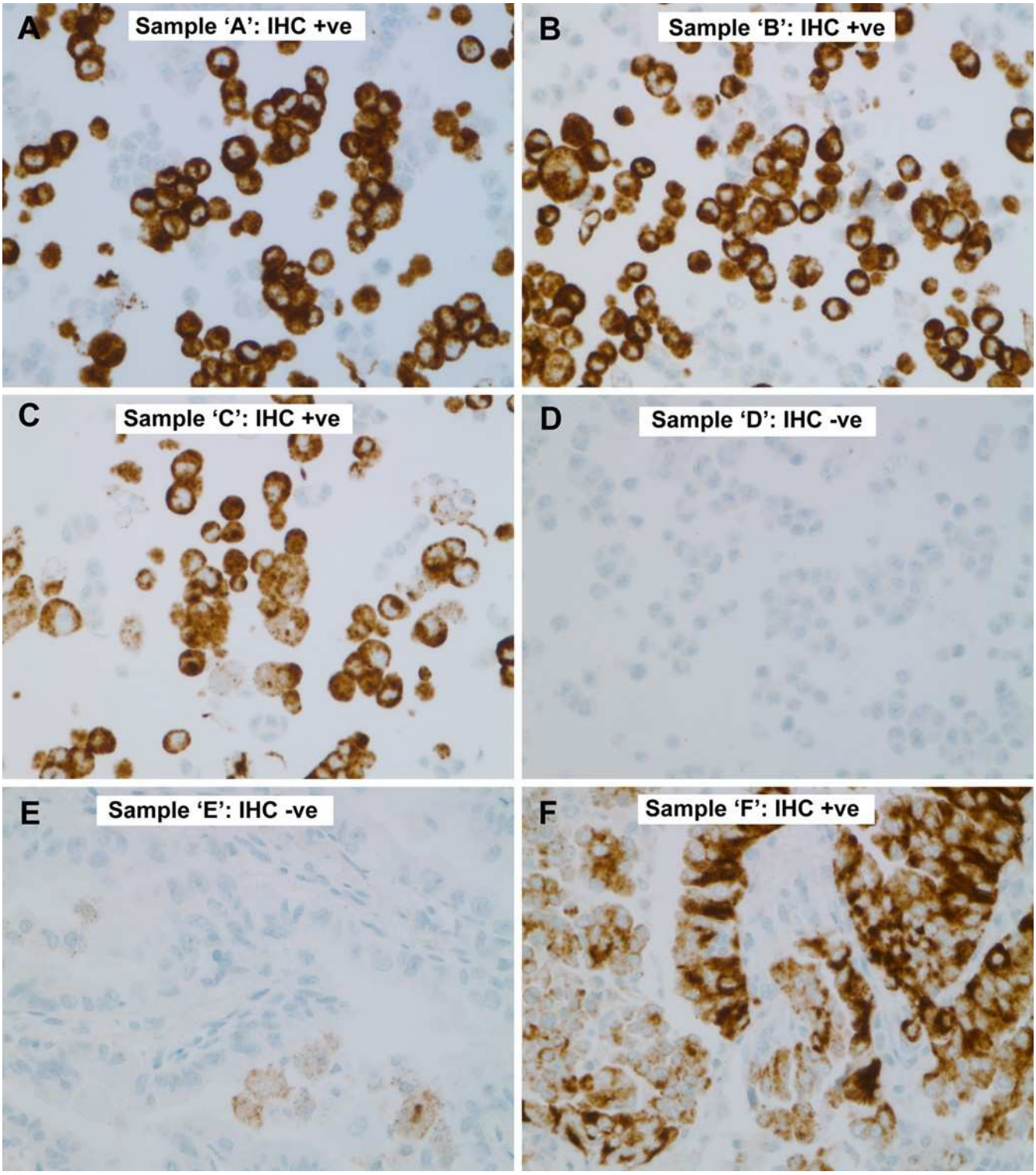


Fig 2. Examples from participants who showed good demonstration of ALK IHC in the distributed Cell lines (A-D) and Tumour samples (E & F) using the Roche D5F3 assay.(A-C) Cell lines show quite uniform staining throughout whereas (F) shows heterogeneous staining with varying cytoplasmic intensity. (E) Tumour is negative although macrophages are staining which can act as a good guide to the sensitivity of the IHC. Samples stained with the Roche D5F3 assay on a Ventana Benchmark using CC1 92 minutes antigen retrieval and Optiview detection

Fig 3: Samples stained with the Leica Biosystems ALK 5A4

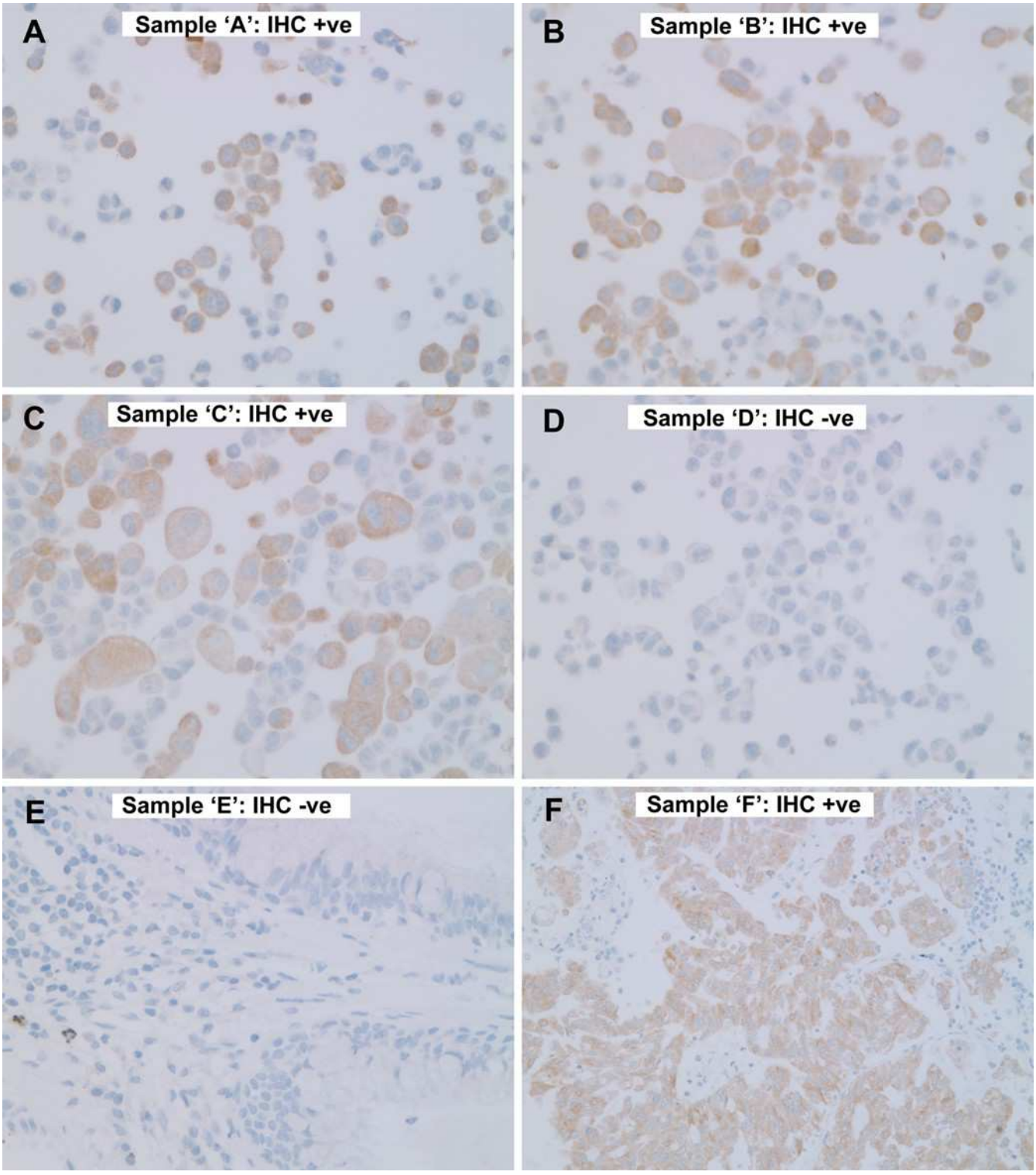


Fig 3. Acceptable demonstration of ALK IHC on the NEQAS distributed (A-D) cell lines and (E &F) Tumour samples using the Leica Biosystems RTU ALK 5A4 clone. Although the intensity of staining is lower than that seen using the Roche D5F3 assay (see Fig 2), the assessment panel found the sensitivity of the method to be sensitive enough for interpretation. Samples stained with the Leica Biosystems ALK 5A4 diluted 1:10 on a Bond III using ER2 for 30 minutes and Leica Bond Polymer Refine detection

Fig 4: Samples stained with the Dako ALK1 antibody

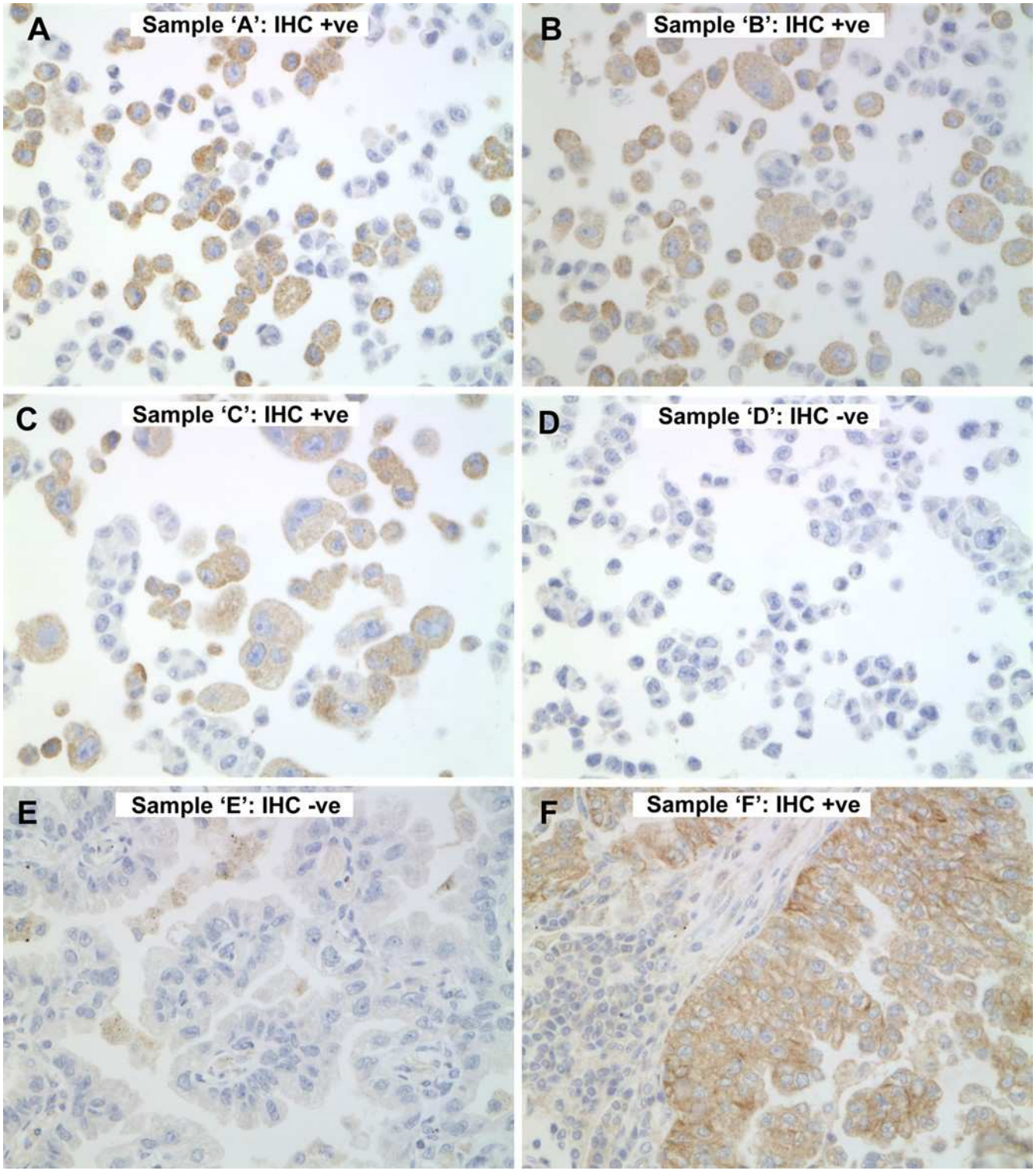


Fig 3. Acceptable demonstration of ALK IHC on the NEQAS distributed (A-D) cell lines and (E &F) Tumour samples using the Dako ALK1 antibody. The assessors found the staining sensitivity to be of acceptable quality and were able to interpret the IHC as expected. Samples stainied using Dako PT link retrieval, Dako Autostainer Link 48 using Dako Envision Flex detection. **Important: The Dako ALK1 antibody is not recommended by Dako for the detection of ALK in NSCLC but the participant appears to have validated their protocol for NSCLC ALK.**

Fig 5. Acceptable and unacceptable examples. See figure legends below

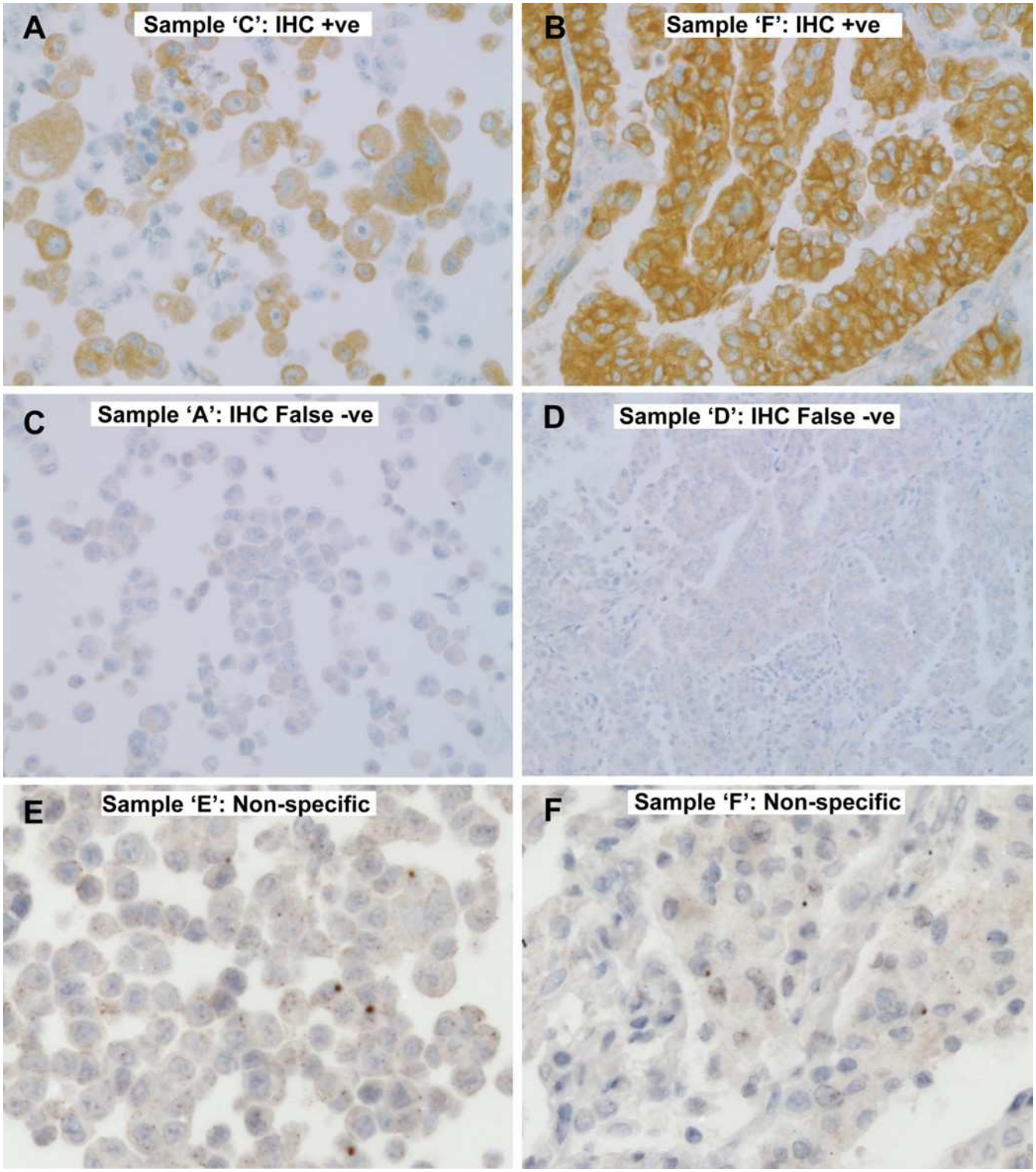


Fig 5

i) Upper 2 images showing acceptable staining from samples 'C' and 'F', from participant who used the Cell signalling D5F3 antibody clone using pressure cooker retrieval and then stained using the Dako Autostainer Link 48 with Dako envision Flex+ detection

ii) Middle 2 images showing unacceptable staining from samples 'A' and 'D'. Both samples should been +ve for ALK IHC indicating a less sensitive method. Samples were stained on a Ventana Benchmark Ultra using CC1 standard antigen retrieval and Ultraview Detection. It would appear that the retreieval may not be long enough and the detection system not sensitive enough.

iii) Lower 2 images showing unacceptable non-specific staining from samples 'E' and 'F', which may be due to excessive tyramide staining. Samples are from the same participant using the Ventana D5F3 assay on the Ventana Benchmark.

A REVIEW OF UK NEQAS ICC & ISH SLIDE STABILITY DURING TRANSPORT AND STORAGE

Dawn Wilkinson, Dr Merdol Ibrahim, Suzanne Parry

Introduction

The scheme has over 600 participants from both the UK and overseas. As a leading provider of EQA services, UK NEQAS ICC & ISH strive to deliver the highest quality service to our participants. We are continually monitoring and re-evaluating our processes and procedures to improve our level of service.
UK NEQAS ICC & ISH distribute 5,000- 6,000 unstained slides per assessment run, equating to over 20,000 unstained slides per year, and it is crucial that all material sent out for assessment is of optimal quality.
To assess the quality, stability, reliability and antigenicity of the slides sent to participants. The UK NEQAS ICC & ISH scheme carries out regular audits.
For this review we evaluated our transport and storage procedures to assess the stability of our unstained slides and to further reassure our participants that the EQA material we provide is robust and of optimal quality.

Background

It is well documented in the literature that the loss of antigenicity in archival formalin fixed paraffin embedded (FFPE) tissue sections negatively impacts on both diagnostic immunohistochemistry (IHC) and molecular studies. The quality of immunohistochemistry results on paraffin sections is affected by numerous factors at the pre-analytical, analytical and post-analytical phase. Some of these factors are listed below, and although the list is not exhaustive it highlights some of the issues that can be encountered in dealing with FFPE material for diagnostic IHC.

- Sample acquisition (e.g. molecular changes before surgery due to the chemical nature and duration of the anaesthetic used and accrue as the tissue is being removed, pre-fixation time. Anoxic changes, (warm/ cold ischaemic time).
- Fixation (type of fixation, concentration, osmolarity, time, volume, temp, pH, size of specimen, slicing of specimen at time of fixation).
- Tissue Processing Time (hydrolysis, exposure of tissues to endogenous water resulting from inadequate tissue processing).
- Decalcification.
- Extended drying.
- Storage of slides (oxidation, time, temperature, humidity thus hydrolysis resulting from exogenous water, light/sun exposure).

Outline

UK NEQAS ICC & ISH use CPA /UKAS accredited suppliers to provide EQA material, slides/blocks for the scheme.
UK NEQAS ICC & ISH carry out regular audits to assess the reliability of our slide storage and transport procedures, including retrospectively assessment of archival material.
3 separate audits were carried out covering transport, refrigeration and storage.

Methods

A. Slide Transport

Two unstained slides were sent out to 13 different countries.
The laboratories were requested to return the slides unstained in the same package when returning their NEQAS slides for the next assessment (time frame 1 month).

On receipt of slides at the NEQAS office the slides were then stained by our host laboratory for four different biomarkers: ER, HER2 (gastric & breast), CD5 & CD10 using their routine validated IHC protocols. Two prognostic/predictive markers ER & HER2, were chosen as they are perceived to be susceptible to transport and storage conditions. The remaining two antibodies chosen (CD5 & CD10) are deemed to be more robust. The slides were given both a NEQAS score and a Reduction in Immuno-reactivity (REDI*) score.

B. Slide Refrigeration

UK NEQAS ICC & ISH recommend our suppliers to cut the sections close to the assessment dispatch date, and to store unstained cut sections at 4 ºC, prior to sending to the NEQAS office. All slides used for the biomarkers (ER, HER2 & Gastric HER2) are stored at 4º C in the fridge prior to dispatch to preserve antigenicity of the sections.

The temperature of the fridge is monitored on a daily basis to ensure optimal conditions are kept for storage of slides. The acceptable temperature range is 4-8º C. If a fluctuation in temperature arises outside the stated acceptable parameters, an audit is carried out to ensure that the sudden fluctuation in temperature has not caused any detrimental effect on the tissue integrity. This is done by retesting a proportion of the slides stored in the fridge at time of fluctuation.

An audit was carried out and unstained sections from the fridge were retested after a logged incident that the temperature of the fridge reached -5 ºC. The slides were re-stained in our host laboratory using the validated routine protocols for 3 modules Breast Hormonal (PR), Lymphoma module (CD5) and General module (CK7).

C. Slide Storage

All routine UK NEQAS ICC and ISH slides are cut and sent out within a tight time frame (4 weeks) for each assessment to ensure quality, stability and reliability of the samples sent to our participants.

Unstained spare slides from previous runs are archived in slide files at room temperature. These slides are used as a good resource to retrospectively evaluate the stability of UK NEQAS ICC unstained slides.

For this review we carried out a stability audit to assess whether there was any loss of antigenicity of the stored unstained slides covering 5 modules over a minimum period of 6 months and maximum period of 15 months. These slides had been stored at room temperature in the dark.

Results

Slides for all of the above 3 audits were scored using the appropriate UK NEQAS ICC & ISH scoring system and compared to the original “GOLD” standard slides. We also used a REDI (reduction in immuno-reactivity) scoring system to assess if there was a substantial loss of immuno-reactivity/ reduction in staining intensity.

*REDI (Reduction in Immuno-reactivity) Scoring Criteria.

Score of:
5 (No REDI)
4 (<10% REDI).
3 (10-25%REDI).
2 (26-50% REDI)
1 (> 50%REDI).
0 (No immuno-reactivity/-ve).

A REVIEW OF UK NEQAS ICC & ISH SLIDE STABILITY DURING TRANSPORT AND STORAGE

Result tables

Slide Refrigeration

Antibody	GOLD Standard NEQAS score	Refrigerated Slide NEQAS score	REDI score
CD5	16	16	5
CK7	16	16	5
PR	16	16	5

Slide Transport

	HER2 (Breast & Gastric)				NEQAS score	ER	Lymphoma		Comments (compared to original GOLD's)	REDI Score
Country	**3+**	**2+**	**1+**	**0**			**CD10**	**CD5**		
UK	3+	2+	1+	0	16(B)	16	N/A	N/A	Her2- same ER - slightly weaker in mid does not affect score	5/4
Malta							16	18	No difference for CD5 or CD10 even though stained in different labs	5/5
Switzerland						16	16	18	No difference for CD5 or CD10, ER slightly weaker mid	5/5/4
Israel	3+	2+	1+	0	16(B)				Slightly weaker but does not affect result.	4
Portugal							16	16	No difference for CD5 or CD10 even though stained in different labs	5/5
Indonesia	3+	2+	1+	0	16(B)	14			Her2 similar staining to GOLD.ER quite a bit weaker mid but still a mid	5/4
USA						16	16	18	No difference for CD5 or CD10. ER slightly weaker mid	5/5/4
Korea	3+	2	1+	1+	15 (G)				Poor mounting. Slightly weaker than GOLD.	4
Hong Kong	3+	NT	0	1+	12 (G)				Slightly weaker and background than expected.	4
Thailand	3+	1+	0	0	10(G)				Slightly weaker than expected.	4
India	3+	2+	1+	0	16(B)				Slightly less crisp, same result.	4

NT = No tumour. (G) = Gastric REDI score (Reduction in immunoreactivity score) (L) = Lymphoma (B) = Breast

Slide Storage

Antibody	Original score	6m NEQAS score	6m REDI Score		Original score	9m NEQAS score	9m REDI Score		Original score	12m NEQAS score	12m REDI Score		Original score	15m NEQAS score	15m REDI Score
ER	12	16	5		20	14	3		-	-	-		-	-	-
CD20	20	16	4		16	18	5		18	16	4		18	20	-
CD117	16	14	4		14	14	5		14	12	4		-	-	-
MIB1	16	14	4		16	12	3		16	12	3		12	12	3
HER2	16	16	4		16	16	5		12	10	3		16	12	4

Discussion

On review of all the slides from the 3 audits covering transport, refrigeration and storage conditions, no non-compliances were raised. The result from the audits provided confidence in the set time limits that UK NEQAS ICC & ISH have for cutting, storage, dispatch and return of slides from participants to the UK NEQAS office.

Our fridge monitoring is carried out on a daily basis and if the temperature is outside the stated range (4-8 ºC) it is reported to the managers and an audit is carried out to ensure slide stability. The results on this occasion showed there to be no detrimental effect or loss of antigenicity to the slides tested. If a problem is recorded with the slides, UK NEQAS ICC & ISH have set procedures and policies in place to resolve this issue.

UK NEQAS ICC & ISH dispatch slides all over the world. An audit was carried out to investigate if there was any major loss of antigenicity/immuno-reactivity to the slides through transportation. We dispatched unstained slides to 13 different countries with instructions. Eleven countries responded and returned the unstained slides after the set period of 4 weeks. On receipt of the slides UK NEQAS ICC & ISH stained the slides for several markers in our 'host' laboratory .

UK NEQAS ICC & ISH takes great care in the packaging and organising of slides fit for transport. However there are some limitations in terms of transport which are out of the scheme's control, such as slides on occasion being delayed in Customs and Excise, local delivery and transport issues, internal organisation postal systems, participant laboratory processes and protocols for dealing with UK NEQAS ICC & ISH slides and environmental issues (increased temperature and humidity).

All slides passed the slide transport review with acceptable scores according to the UK NEQAS ICC & ISH scoring criteria and comparison to the original "GOLD" standard. Overall, there was a slight variation in expression of the mid-expressor for ER, and the expression of the gastric HER2 with a REDI score of 4 (<10%). This highlights the importance of staining the NEQAS slides as soon as they are received by the laboratory. It is well documented in the scientific field that certain biomarkers and antigens are more sensitive than other markers to loss of antigenicity and can be affected by several factors as mentioned above (background).

UK NEQAS ICC & ISH cuts slides locally in-house for the breast and gastric biomarkers to ensure that the turn-around time for cutting to dispatch "2 weeks for biomarkers" is strictly adhered to. We therefore also recommend to all our participants to stain their slides soon after receipt into their laboratory. This is especially important for sensitive biomarkers. We provide recommendations to all our suppliers who provide material for UK NEQAS ICC & ISH.

We retrospectively audited our unstained spare slides from our archives covering a period of up to 15 months. All spare unstained slides after each module assessment are filed in slide storage units at room temperature. We retrieved a variety of slides from several modules for different time periods and stained the stored slides at our host laboratory and compared to the nearest original 'GOLD'.

We never send out old slides to our participants. If a lab requests further slides we always send freshly cut sections.

Interestingly, we discovered that although the slides were stored in the dark at room temperature our slides were reasonably stable for a period of 6 months and showed a maximum reduction in intensity/immuno-reactivity of <10%. This provides further evidence that our UK NEQAS material is well fixed and processed (pre-analytical factors) .This is further backed up by our new RNA scope evidence for the fixation of UK NEQAS ICC & ISH samples. In the future we hope to use digital image analysis to quantify loss of staining intensity.

The staining intensity/antigenicity did decline after the six month time period. However 4 out of the 5 markers still achieved an acceptable NEQAS score. Both ER and MIB-1 received a REDI score of 3 indicating a 10-25% reduction in staining immuno-reactivity. It is well documented that these 2 markers are particularly sensitive to loss of antigenicity and sensitivity in FFPE stored sections over time and our results also suggest this and would explain why both these markers achieved a REDI score of 3.

Unfortunately we did not have slides for all 5 markers over the 12-15 month storage period. Our results mainly showed a decline in staining intensity, particularly those markers known to be sensitive to loss of antigenicity over time. However no entire antigenicity loss was observed.

Conclusion

UK NEQAS ICC & ISH are confident that the quality of the material provided to the participants for EQA is robust.
UK NEQAS ICC & ISH ensure that slides for all assessments are cut and sent out within a 4 week period. Known markers sensitive to loss of antigenicity, such as ER & Gastric HER2, are cut 'in-house' within a two week time frame. UK NEQAS entrusts that their suppliers do cut within this known time frame and treat slides appropriately as stated in their agreement. UK NEQAS never distribute slides which have been cut for more than 4 weeks, so if a laboratory requests extra slides due to a problem they have encountered or for troubleshooting or validation, UK NEQAS will ensure that the slides are no older than this and have been stored in the fridge. If necessary, UK NEQAS ICC & ISH will cut extra fresh sections to distribute if the time has elapsed.

UK NEQAS take great care in storing and distributing slides to all participants, however there are limitations outside of the scheme's control. We do endeavour to continually monitor our processes and procedures and implement quality improvements where necessary.

References

1. Arnold MM, Srivastava S, Fredenburgh J, Stockard CR, Myers RB, Grizzle WE. 1996. Effects of fixation and tissue processing on immunohistochemical demonstration of specific antigens. Biotech Histochem. 71:224–230 [PubMed]
2. Bertheau P, Cazals-Hatem D, Meignin V, de Roquancourt A, Verola O, Lesourd A, Sene C, Brocheiou C, Janin A. 1998. Variability of immunohistochemical reactivity on stored paraffin slides. J Clin Pathol. 51:370–374 [PMC free article] [PubMed]
3. Beitz E, Schultz JE. 1999. The mammalian aquaporin water channel family: a promising new drug target. Curr Med Chem. 6:457–467 [PubMed]
4. Blind C, Koepenik A, Pacyna-Gengelbach M, Fernahl G, Deutschmann N, Dietel M, Krenn V, Petersen I. 2008. Antigenicity testing by immunohistochemistry after tissue oxidation. J Clin Pathol. 61:79–83 [PubMed]
5. Camp RL, Charette LA, Rimm DL. 2000. Validation of tissue microarray technology in breast carcinoma. Lab Invest. 80:1943–1949 [PubMed]
6. Chung JY, Braunschweig T, Williams R, Guerrero N, Hoffmann KM, Kwon M, Song YK, Libutti SK, Hewitt SM. 2008. Factors in tissue handling and processing that impact RNA obtained from formalin-fixed, paraffin-embedded tissue. J Histochem Cytochem. 56:1033–1042 [PMC free article] [PubMed]
7. Chung JY, Lee SJ, Kris Y, Braunschweig T, Traicoff JL, Hewitt SM. 2008. A well-based reverse-phase protein array applicable to extracts from formalin-fixed paraffin-embedded tissue. Proteomics Clin Appls. 2:1539–1547 [PMC free article] [PubMed]
8. Cronin M, Pho M, Dutta D, Stephans JC, Shak S, Kiefer MC, Estban JM, Baker JB. 2004. Measurement of gene expression in archival paraffin-embedded tissues: development and performance of a 92-gene reverse transcriptase-polymerase chain reaction assay. Am J Pathol. 164:35–42 [PMC free article] [PubMed]
9. Dapson RW. 2007. Macromolecular changes caused by formalin fixation and antigen retrieval. Biotech Histochem. 82:133–140 [PubMed]
10. De Marzo AM, Fedor HH, Gage WR, Rubin MA. 2002. Inadequate formalin fixation decreases reliability of p27 immunohistochemical staining: probing optimal fixation time using high-density tissue microarrays. Hum Pathol. 33:756–760 [PubMed]
11. DiVito KA, Charette LA, Rimm DL, Camp RL. 2004. Long-term preservation of antigenicity on tissue microarrays. Lab Invest. 84:1071–1078 [PubMed]
12. Fergenbaum JH, Garcia-Closas M, Hewitt SM, Lissowska J, Sakoda LC, Sherman ME. 2004. Loss of antigenicity in stored sections of breast cancer tissue microarrays. Cancer Epidemiol Biomarkers Prev. 13:667–672 [PubMed]
13. Grizzle WE. 2009. Special symposium: fixation and tissue processing models. Biotech Histochem. 84:185–193 [PMC free article] [PubMed]
14. Henson DE. 1996. Loss of p53-immunostaining intensity in breast cancer. J Natl Cancer Inst. 88:1015–1016 [PubMed]
15. Hewitt SM, Lewis FA, Cao Y, Conrad RC, Cronin M, Danenberg KD, Goralski TJ, Langmore JP, Raja RG, Williams PM, et al. 2008. Tissue handling and specimen preparation in surgical pathology: issues concerning the recovery of nucleic acids from formalin-fixed, paraffin-embedded tissue. Arch Pathol Lab Med. 132:1929–1935 [PubMed]
16. Jacobs TW, Prioleau JE, Stillman IE, Schnitt SJ. 1996. Loss of tumor marker-immunostaining intensity on stored paraffin slides of breast cancer. J Natl Cancer Inst. 88:1054–1059 [PubMed]
17. Leyland-Jones BR, Ambrosone CB, Bartlett J, Ellis MJ, Enos RA, Raji A, Pins MR, Zujewski JA, Hewitt SM, Forbes JF, et al. 2008. Recommendations for collection and handling of specimens from group breast cancer clinical trials. J Clin Oncol. 26:5638–5644 [PMC free article] [PubMed]
18. Mirlacher M, Kasper M, Storz M, Knecht Y, Durmuller U, Simon R, Mihatsche MJ, Sauter G. 2004. Influence of slide aging on results of translational research studies using immunohistochemistry. Modern Pathol. 17:1414–1420 [PubMed]
19. Nirmalan NJ, Harnden P, Selby PJ, Banks RE. 2009. Development and validation of a novel protein extraction methodology for quantitation of protein expression in formalin-fixed paraffin-embedded tissues using Western blotting. J Pathol. 217:497–506 [PubMed]
20. Otali D, Stockard CR, Oelschlager DK, Wan W, Manne E, Watts SA, Grizzle WE. 2009. The combined effects of formalin fixation and individual steps in tissue processing on immunorecognition. Biotech Histochem. 84:223–247 [PMC free article] [PubMed]
21. Sauter G, Mirlacher M. 2002. Tissue microarrays for predictive molecular pathology. J Clin Pathol. 55:575–576 [PMC free article] [PubMed]
22. Specht K, Richter T, Muller U, Walch A, Werner M, Hofler H. 2001. Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. Am J Pathol. 158:419–429 [PMC free article] [PubMed]
23. Srinivasan M, Sedmak D, Jewell S. 2002. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol. 161:1961–1971 [PMC free article] [PubMed]
24. van den Broek LJ, van de Vijver MJ. 2000. Assessment of problems in diagnostic and research immunohistochemistry associated with epitope instability in stored paraffin sections. Appl Immunohistochem Mol Morphol. 8:316–321 [PubMed]
25. Werner M, Chott A, Fabiano A, Battifora H. 2000. Effect of formalin tissue fixation and processing on immunohistochemistry. Am J Surg Pathol. 24:1016–1019 [PubMed]
26. Wester K, Wahlund E, Sundstrom C, Ranefall P, Bengtsson E, Russell PJ, Ow KT, Malmstrom PU, Busch C. 2000. Paraffin section storage and immunohistochemistry: effects of time, temperature, fixation, and retrieval protocol with emphasis on p53 protein and MIB1 antigen. Appl Immunohistochem Mol Morphol. 8:61–70 [PubMed]
27. Ran Xie, Joon-Yong Chung, Kris Ylaya, Reginald L. Williams, Natalie Guerrero, Nathan Nakatsuka, Cortessia Badie, and Stephen M. Hewitt Factors Influencing the Degradation of Archival Formalin-Fixed Paraffin-Embedded Tissue Sections J Histochem Cytochem. Apr 2011; 59 (4): 356–365. [PubMed]

Julie Williams and Suzanne Parry

	Gold Standard	Second Antibody
Antigens Assessed:	CK7	PSA
Tissue Sections circulated:	Normal breast and colon	Normal prostate
Number of Registered Participants:	332	
Number of Participants This Run	321 (96%)	

Introduction

Gold Standard: CK7

Cytokeratin 7 (CK7) is a 54 kDa type II simple cytokeratin which is found in most glandular and transitional epithelia, but not stratified squamous epithelium. Antibodies against CK7 label a variety of cells in normal tissue including subsets of endothelial cells (Chu & Wiess). CK7 can also be demonstrated in a number of different tumours, including adenocarcinoma of the lung, breast, ovary (serous and endometroid), endometrium and in transitional cell carcinoma of the bladder, although the level of staining very much depends on the degree of differentiation of the tumour. It is generally negative in colonic adenocarcinomas, squamous cell carcinomas, hepatomas and renal epithelial tumours. Staining with CK7 should be cytoplasmic, and it is most often used in conjunction with cytokeratin 20 (Dabbs).

Features of Optimal Immunostaining:
Normal Breast: (Figs 1)

- Strong and even cytoplasmic/membranous luminal staining of the glandular epithelial cells
- Weaker staining of the myoepithelial cells
- Clean background with no staining of the stromal cells

- **Normal Colon: (Fig 2)**
- Negative reaction in the colon
- Clean background with no staining of the stromal cells

Features of Suboptimal Immunostaining:

- Weak and/or uneven staining of the glandular epithelial cells of the breast **(Fig 3)**
- Non-specific staining of stromal cells or connective tissue of the breast or colon
- Non-specific staining of the colon epithelial cells
- Excessive background staining

References

1. Chu PG, Wiess LM. Keratin expression in human tissues and neoplasms. Histopathology. 2002 May;40(5):403-39.
2. Dabbs DJ. Carcinomatous differentiation and metastatic carcinoma of unknown primary. In: Diagnostic Immunohistochemistry. Dabbs D (Editor). Churchill Livingstone, Philadelphia 2002; pg. 169.

Second Antigen: PSA

Prostate-Specific Antigen (PSA) is a 34 kDa protein belonging to the kallikrein family of proteases. It is produced by the prostatic epithelium and epithelial lining of the periurethral glands. From the prostatic epithelial cells, PSA is secreted via the prostatic ductal system into the seminal fluid, where it liquefies semen to allow sperm to swim. The main diagnostic use of the PSA antibody is in the identification of metastatic tumours of prostate origin. In benign prostate PSA staining is generally concentrated in the apical portion of the cytoplasm. However, as PSA is strongly expressed in both normal and neoplastic prostatic tissue it cannot be used to differentiate between benign and malignant conditions. Some primary and metastatic tumours of the prostate have been found to be PSA negative, therefore, it is important to use a panel of antibodies when identifying the origin of a tumour, including both PSA and prostatic acid phosphatase (PAP). Both these antibodies are said to be very specific for prostatic epithelium, although some staining in non-prostatic tissue and tumours has been reported (Epstein, Leong), although this appears to be dependent on the type of antibody and pre-treatment used (Varma). PSA expression varies depending on the grade of prostatic tumour, with higher grade carcinomas (Gleeson grade 4 or 5) showing weaker staining with a smaller percentage of positive cells than low grade tumours (Gleeson grade 3 or less) (Varma). Only about 1% of poorly differentiated carcinomas are negative for PSA.

Features of optimal Immunostaining: (Figs 7 & 8)

- Strong, even staining of the prostatic epithelium.
- Minimal background staining (more background is observed when using the polyclonal antibodies)

Features of sub-optimal Immunostaining: (Figs 9-11)

- Weak and/or uneven or no staining in the prostatic epithelium.
- Non-specific staining of lymphocytes (probably due to excessive pre-treatment).
- Excessive background staining and/or nuclear staining, (probably due to the primary antibody being used at a concentration which is too high).

References:

1. JI Epstein. PSA and PAP as immunohistochemical markers in prostate cancer. Urol Clins N Am 1993; 20:757-770.
2. AS-Y Leong, et al. Manual of Diagnostic Antibodies for Immunohistochemistry (1st ed). Oxford University Press, Oxford 1999; 287-288.
3. M Varma, et al. Polyclonal anti-PSA is more sensitive but less specific than monoclonal anti-PSA: Implications for diagnosis prostatic pathology. Am J Clin Pathol 2002; 118: 202-207.

Assessment Summary:

The overall pass rate for the **CK7** assessment was very good, with 94% of labs receiving an acceptable pass, and a further 4% receiving a borderline pass. 2% (7 labs) failed the assessment . The reason for failure was either due to very weak staining or excessive background and non-specific staining or morphology damage caused by excessive pre-treatment. As with previous assessment runs for CK7, the Dako OV-TL-12/30 clone was the most popular choice of antibody used by participants,. This clone worked well on all of the commercially available platforms and detection kits, and showed a pass rate of 87% in this assessment run.

PSA was the second antigen chosen for this assessment run. 82% of labs received an acceptable pass for this, and a further 14% achieved a borderline pass. The main reason for failure was due to weak or uneven staining, which was generally due to the incorrect antibody titre being used. Both the Dako polyclonal and Dako ER-PR8 monoclonal antibodies were favoured by many laboratories, and it was apparent that several labs were using a dilution more appropriate for the polyclonal antibody even though they were using the monoclonal antibody. Weak stromal staining is often seen with the polyclonal antibody, and this is acceptable as long as it does not distract from the strong specific staining in the prostatic epithelium.

Selected Images showing Optimal and Sub-optimal Immunostaining

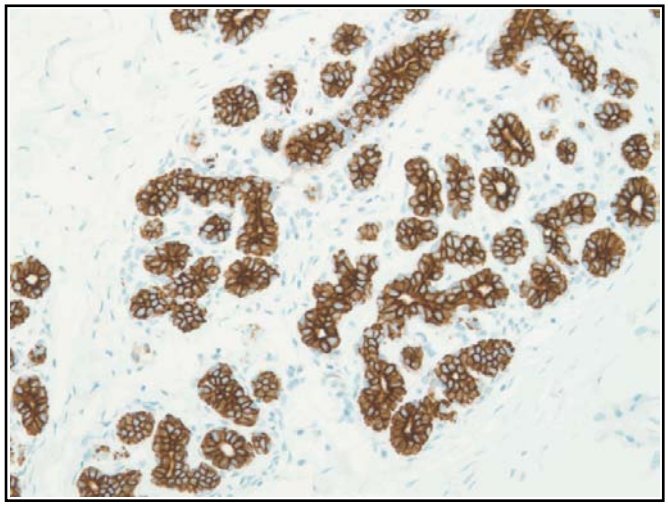


Fig 1. Optimal staining for CK7 in the UK NEQAS distributed normal breast sample. The glandular epithelial cells show cytoplasmic/membranous staining, while the background remains clean. Stained with the Ventana SP52 pre-diluted antibody on the ULTRA machine with CC1 antigen retrieval for 52 minutes.

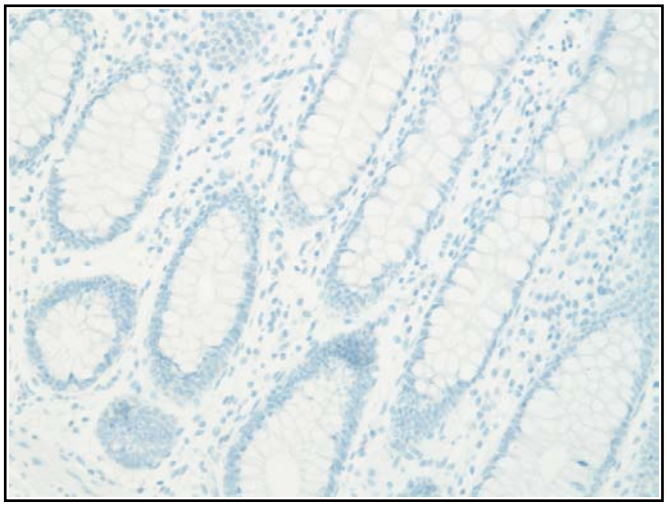


Fig 2. The UK NEQAS distributed colon section stained for CK7. The result is negative as expected. Same protocol as Fig 1.

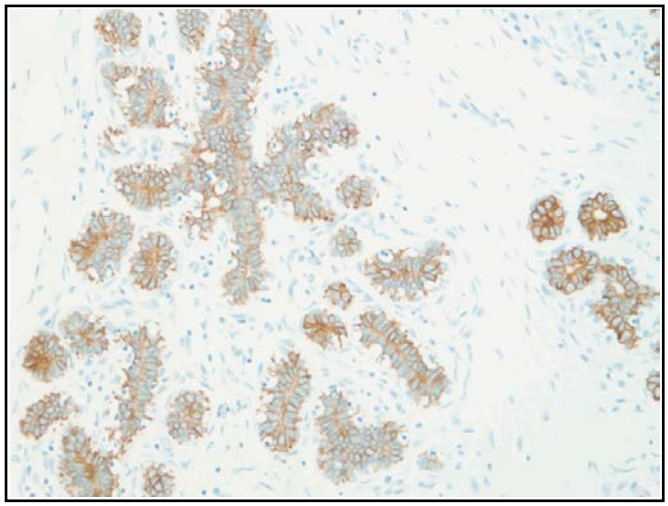


Fig 3. Weak demonstration of CK7 in the UK NEQAS normal breast sample (compare to Fig 1). Staining was carried out using the Dako antibody on the Ventana XT Autostainer, however, no antigen retrieval was used, which is the likely cause of the insufficient staining. The lab received a borderline pass.

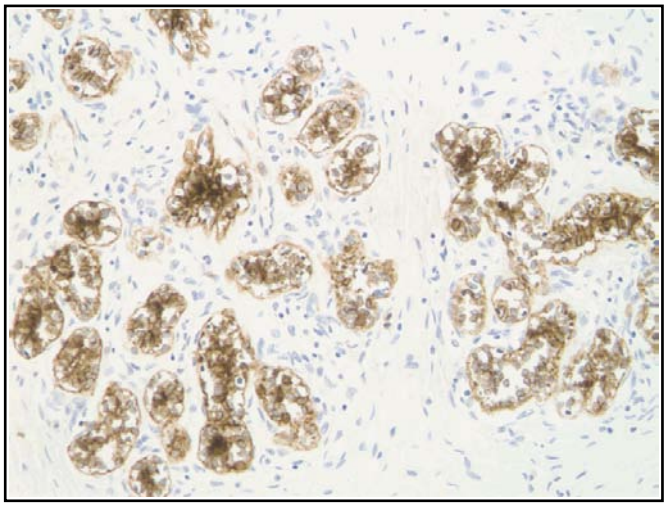


Fig 4. Poor staining for CK7 on the UK NEQAS normal breast sample. The example shows morphology damage in the glands and ducts caused by overdigestion. The section was stained using the Leica OV-TL 12/30 antibody, 1:100, and VBS Bond II Enzyme for 10 minutes on the BondMax machine.

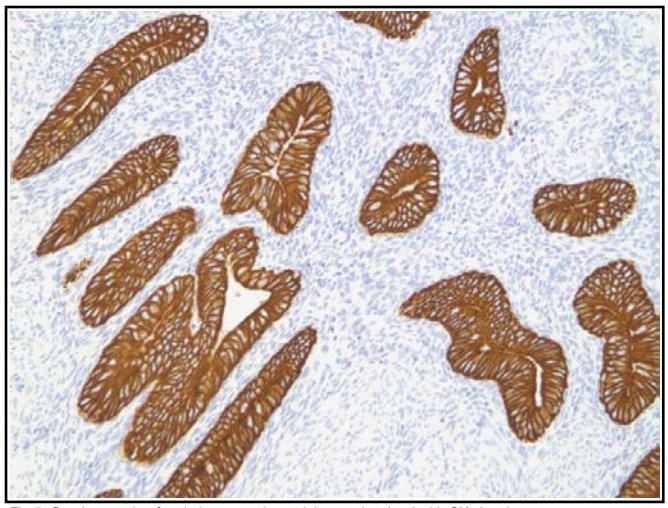


Fig 5. Good example of an in house endometrial control stained with CK showing strong cytoplasmic and membranous staining. Section stained with the Leica OV-TL 12/30 antibody, 1:100, on the Ventana XT, CC1 mild antigen retrieval and UltraView detection.

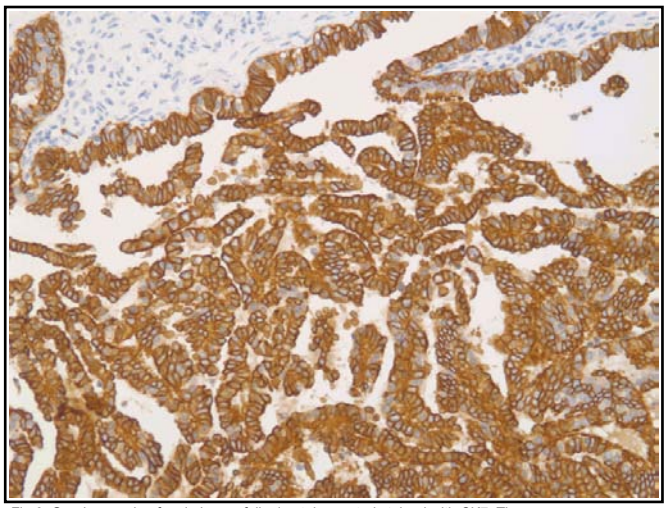


Fig 6. Good example of an in house fallopian tube control stained with CK7. The cytoplasmic/membranous staining is intense while the background remains clean.

Selected Images showing Optimal and Sub-optimal Immunostaining

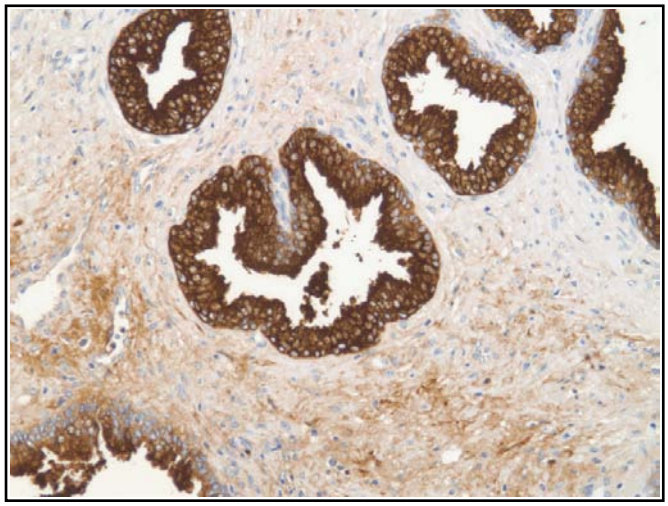


Fig 7. Optimal demonstration of PSA on the UK NEQAS distributed prostate. Weak stromal staining is often seen but does not distract from the strong distinct staining in the prostatic epithelium. Section stained with the Dako polyclonal antibody, no pre-treatment on the Ventana Benchmark XT with UltraView detection kit.

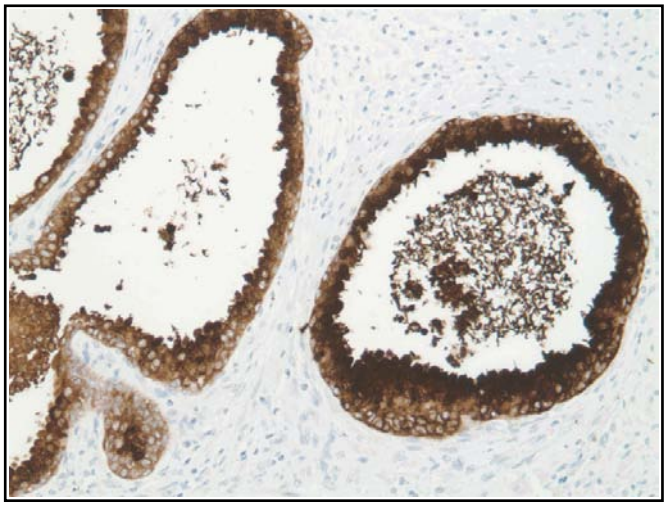


Fig 8. Optimal demonstration of PSA on the UK NEQAS prostate section stained with a monoclonal antibody. The staining is strong and distinct with minimal background staining. Section stained with the Leica 35H9 antibody, 1:100, on the Leica Bond III with ER1 antigen retrieval for 20 minutes.

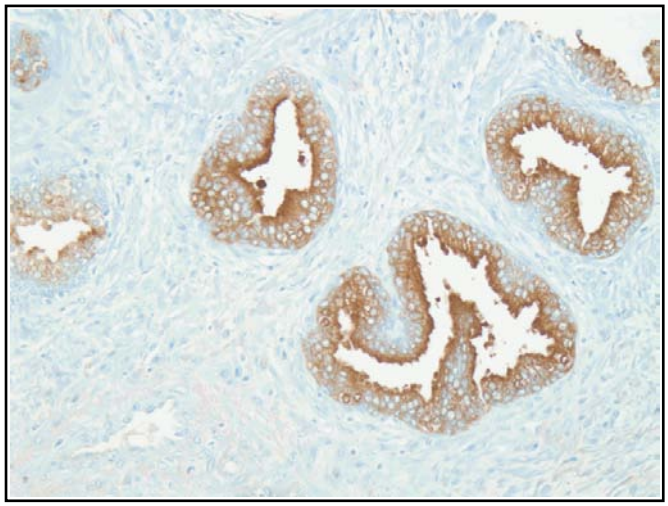


Fig 9. Suboptimal demonstration of PSA on the UK NEQAS prostate section stained using the polyclonal antibody (compare with Fig 7). Although the prostatic epithelium is demonstrated, the staining is weak. Stained using Dako polyclonal, 1:2000 with no pre-treatment on the Ventana Benchmark XT with iView detection kit.

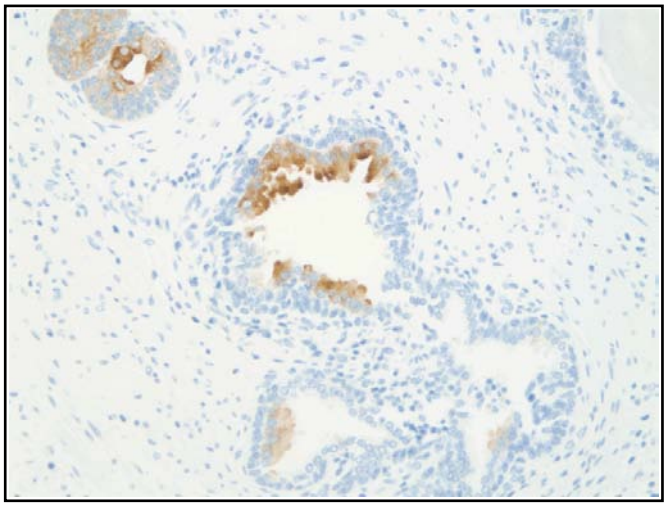


Fig 10. Poor demonstration of PSA on the UK NEQAS prostate section stained using a monoclonal antibody (compare to Fig 8). The reaction is weak with very few of the prostatic epithelium staining at all. Stained with the Leica pre-diluted 35H9 antibody, on the Leica BondMax with ER1 antigen retrieval for 10 minutes.

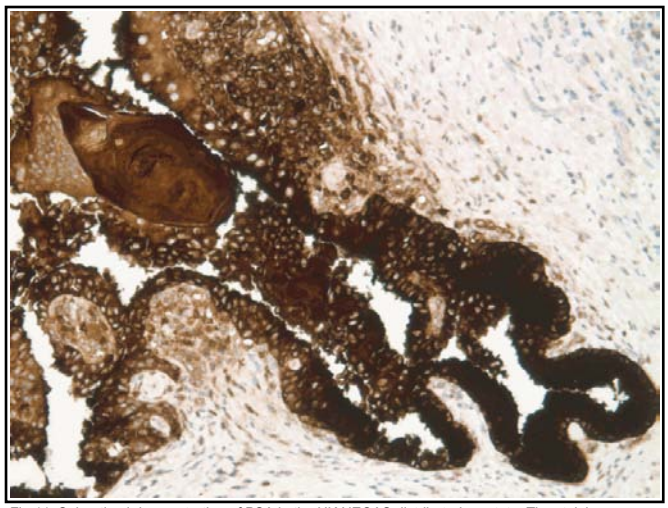


Fig 11. Suboptimal demonstration of PSA in the UK NEQAS distributed prostate. The staining is too strong, also causing excessive background in areas of the section. This is most likely caused by inappropriate antigen retrieval. Section stained using the Dako polyclonal antibody, 1:2000, on the Leica BondMax, ER2 retrieval for 20 minutes.

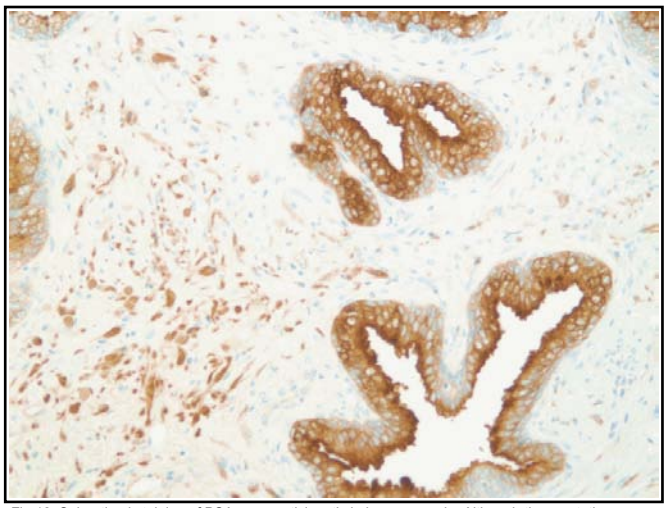
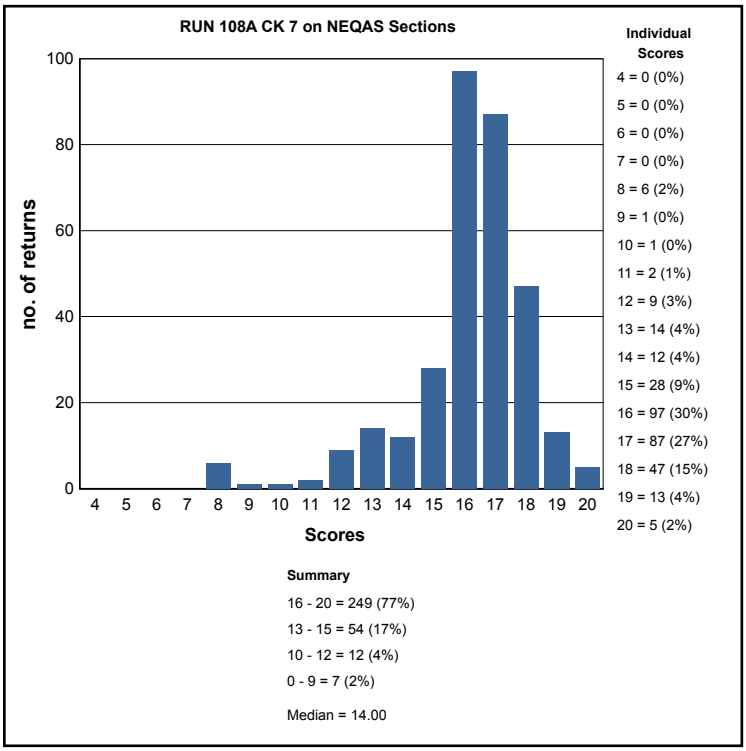
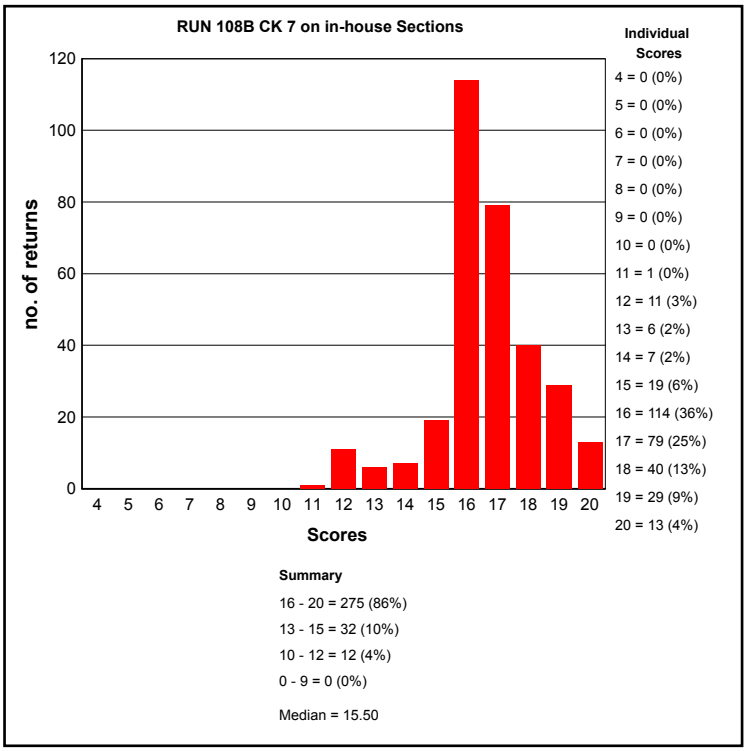
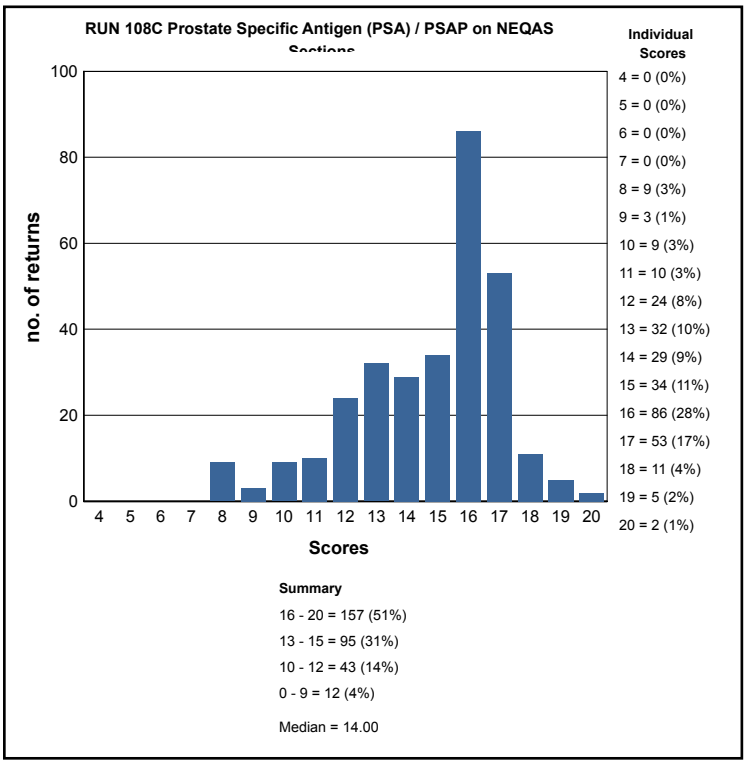


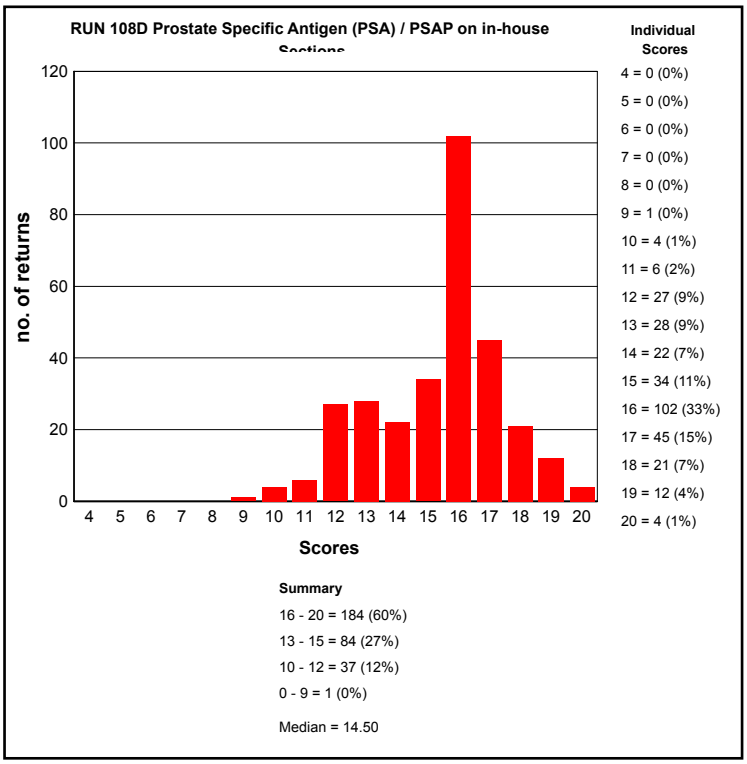
Fig 12. Suboptimal staining of PSA on a participant's in house sample. Although the prostatic epithelium is demonstrated, there is inappropriate non-specific staining in the stroma. Section stained with the Dako polyclonal antibody, 1:4000, and protease digestion on the Ventana ULTRA.

GRAPHICAL REPRESENTATION OF PASS RATES









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

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

General Pathology Run: 108

Primary Antibody : CK 7

Antibody Details	N	%
Biocare CM 061 A,B, C (OV-TL 12/30)	3	33
Cell Marque 307M-96 (OV-TL 12/30)	8	100
Dako M7018 (OV-TL 12/30)	164	96
Dako N1626 (OV-TL 12/30)	2	50
Dako RTU Link IR619 (OV-TL 12/30)	15	100
DAKO RTU Omnis GA619 (OV-TL12/30)	2	100
Epitomics AC-0020 (EP16)	1	100
Linaris (OV-TL 12/30)	1	100
NeoMarkers MS-1352-P (OV-TL 12/30)	4	100
Novcastra NCL-L-CK-560 (RN7)	21	100
Novocastra Bond RTU PA0942 (RN7)	16	94
Novocastra NCL-CK7-OVTL (OV-TL 12/30)	7	71
Novocastra NCL-L-CK7-OVTL (OV-TL 12/30)	17	94
Other	9	67
Vector Labs VPC403 (OV-TL 12/30)	5	100
Ventana 790-4462 (SP52)	42	98
Ventana, 760-2224 (OV-TL 12/30)	2	100
Zymed 18-0234 (OV-TL 12/30)	1	0

General Pathology Run: 108

Primary Antibody : Prostate Specific Antigen (PSA) / PSAP

Antibody Details	N	%
Dako A0562 PSA (polyclonal)	100	89
Dako A0627 PSAP (polyclonal)	1	100
Dako IR514 PSA RTU FLEX Link (polyclonal)	10	100
Dako IS514 PSA RTU FLEX plus (polyclonal)	2	100
Dako M0750 PSA (Clone ER-PR8)	63	68
Dako M0792 PSAP (PASE/4LJ)	6	100
Dako N1517 PSA (polyclonal)	2	50
Leica/Novovcastra NCL-L-PAP (PASE/4LJ)	2	100
Leica/Novovcastra NCL-L-PSA-28A4 (PSA28/A4)	9	56
Leica/Novovcastra NCL-PSA-431 (35H9)	17	76
Leica/Novovcastra PA0006 RTU PSAP (PASE/4LJ)	1	100
Leica/Novovcastra PA0431 PSA RTU (35H9)	31	84
Leica/Novovcastra RTU-PSA-28A4 (PSA28/A4)	1	100
NeoMarker PSA MS260 (Clone ER PR8)	2	50
Other	17	71
Ventana 760 2506	23	87
Ventana 760-4271 PSA (ER-PR8)	14	93

General Pathology Run: 108

Heat Mediated Retrieval	CK 7 N	CK 7 %	Prostate Specific Antigen (PSA) / PSAP N	Prostate Specific Antigen (PSA) / PSAP %
Biocare Decloaking Chamber	4	100	3	100
Dako Omnis	3	100	2	100
Dako Pascal	1	100	1	100
Dako PTLink	51	96	44	84
Lab vision PT Module	4	100	4	75
Leica ER1 10 mins	2	100	18	78
Leica ER1 20 mins	27	93	27	74
Leica ER1 30 mins	9	89	5	60
Leica ER1 40 mins	1	100	0	0
Leica ER2 10 mins	3	100	1	100
Leica ER2 20 mins	36	100	9	100
Leica ER2 30 mins	5	100	3	33
Microwave	5	60	5	60
None	24	96	75	77
Other	3	100	4	50
Pressure Cooker	5	80	4	50
Pressure Cooker in Microwave Oven	1	100	0	0
Steamer	2	100	3	67
Ventana CC1 16mins	5	60	1	100
Ventana CC1 20mins	1	100	3	67
Ventana CC1 24mins	2	50	3	67
Ventana CC1 32mins	12	100	8	88
Ventana CC1 36mins	9	100	4	75
Ventana CC1 40mins	3	100	3	33
Ventana CC1 44mins	1	100	1	100
Ventana CC1 48mins	1	100	0	0
Ventana CC1 52mins	3	100	3	100
Ventana CC1 56mins	3	100	2	100
Ventana CC1 64mins	15	93	7	100
Ventana CC1 88mins	0	0	1	100
Ventana CC1 8mins	0	0	9	89
Ventana CC1 mild	18	94	22	91
Ventana CC1 standard	31	97	17	88
Ventana CC2 16mins	0	0	1	100
Ventana CC2 32mins	1	100	0	0
Water bath 68 OC	1	100	1	100
Water bath 95-98 OC	1	100	2	100

General Pathology Run: 108

Enzyme Mediated Retrieval	CK 7 N	CK 7 %	Prostate Specific Antigen (PSA) N	Prostate Specific Antigen (PSA) %
AS PER KIT	1	100	3	100
NOT APPLICABLE	127	97	139	76
Other	1	0	0	0
Sigma chymotrypsin (C4129)	0	0	1	100
Trypsin	0	0	1	100
VBS Bond Enzyme 1	11	82	4	100
VBS Bond Enzyme 2	1	0	0	0
Ventana Protease	2	100	1	100
Ventana Protease 1 (760-2018)	37	95	8	75

General Pathology Run: 108 Detection	CK 7 N	CK 7 %	Prostate Specific Antigen (PSA) N	Prostate Specific Antigen (PSA) %
A Menerini Polymer (MP-XCP)	0	0	1	100
AS PER KIT	16	81	14	86
BioGenex SS Polymer (QD 420-YIKE)	1	100	1	100
Dako EnVision FLEX (K8000/10)	6	100	7	100
Dako EnVision FLEX+ (K8002/12)	36	97	31	77
Dako Envision HRP/DAB (K5007)	6	83	6	83
Dako Envision+ HRP mouse K4004/5/6/7	2	100	0	0
Dako Envision+ HRP rabbit K4008/9/10/11	0	0	1	0
LabVision UltraVision ONE Polymer (TL-012/5-HDJ/T	1	100	2	100
Leica Bond Intense R Detection (DS9263)	0	0	1	100
Leica Bond Polymer Define (DS9713)	0	0	2	50
Leica Bond Polymer Refine (DS9800)	92	96	80	73
MenaPath X-Cell Plus (MP-XCP)	4	100	2	0
None	2	100	3	100
NOT APPLICABLE	3	100	3	100
Other	11	91	11	82
Vector ImmPRESS Universal (MP-7500)	1	100	1	100
Ventana iView system (760-091)	11	91	13	54
Ventana OptiView Kit (760-700)	31	90	28	89
Ventana UltraView Kit (760-500)	94	96	94	84

General Pathology Run: 108 Automation	CK 7 N	CK 7 %	Prostate Specific Antigen (PSA) / PSAP N	Prostate Specific Antigen (PSA) / PSAP %
BioGenex GenoMX 6000i	2	50	2	100
Dako Autostainer	5	80	5	100
Dako Autostainer Link 48	40	95	42	81
Dako Autostainer plus	7	86	6	83
Dako Autostainer Plus Link	5	100	5	80
Dako Omnis	3	100	1	100
LabVision Autostainer	3	100	3	100
Leica Bond Max	48	90	52	73
Leica Bond-III	49	96	40	78
Menarini - Intellipath FLX	5	100	4	50
None (Manual)	7	100	6	67
Other	1	100	1	100
Shandon Sequenza	4	100	4	75
Ventana Benchmark GX	6	83	7	86
Ventana Benchmark ULTRA	65	97	57	84
Ventana Benchmark XT	72	93	71	85
Ventana NexES	0	0	1	0

General Pathology Run: 108 Chromogen	CK 7 N	CK 7 %	Prostate Specific Antigen (PSA) / N	Prostate Specific Antigen (PSA) / %
AS PER KIT	28	89	30	80
BioGenex Liquid DAB (HK153-5K)	1	100	1	100
BioGenex liquid DBA (HK-124-7K)	1	0	1	100
Dako DAB K3468	0	0	1	100
DAKO DAB+	1	100	2	100
Dako DAB+ Liquid (K3468)	4	100	2	50
Dako DAB+ REAL Detection (K5001)	3	67	0	0
Dako EnVision Plus kits	4	100	2	50
Dako FLEX DAB	41	98	41	85
Dako REAL EnVision K5007 DAB	4	75	6	83
LabVision DAB	0	0	1	100
Leica Bond Polymer Refine kit (DS9800)	90	93	83	75
menapath xcell kit DAB (MP-860)	4	100	4	50
Other	12	92	16	75
Sigma DAB (D5637)	1	100	1	100
Sigma DAB (D5905)	1	100	1	100
Ventana DAB	15	93	18	89
Ventana iview	8	88	9	67
Ventana Ultraview DAB	102	96	88	85

BEST METHODS - Gold Standard Antibody

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

CK 7 - Method 1

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

Primary Antibody: Dako M7018 (OV-TL 12/30) , 36 Mins, 37 ºC Dilution 1: 50

Automation: Ventana Benchmark XT

Method: Ventana iView Kit

Main Buffer: Ventana reaction buffer (950-300)

HMAR:

EAR: Ventana Protease 1 (760-2018), 37 ºC. Digestion Time NEQAS: 8 Mins. In-House: 8 Mins

Chromogen: Ventana iview

Detection: Ventana iView system (760-091) , 37 ºC Prediluted

CK 7 - Method 2

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

Primary Antibody:	Novocastra Bond RTU PA0942 (RN7)
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	AS PER KIT
HMAR:	Leica ER1 20 mins
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	AS PER KIT

CK 7 - Method 3

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

Primary Antibody:	Dako RTU Link IR619 (OV-TL 12/30) , 15 Mins, 23 ºC Prediluted
Automation:	Dako Autostainer Plus Link
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: high ph target retrieval solution
EAR:	
Chromogen:	Dako FLEX DAB, 23 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 30 Mins, 23 ºC Prediluted

CK 7 - Method 4

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

Primary Antibody:	Ventana 790-4462 (SP52) , 60 Mins, 36 ºC
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins
EAR:	
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

BEST METHODS - Secondary Antibody

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

Prostate Specific Antigen (PSA) / PSAP - Method 1

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

Primary Antibody:	Leica/Novovcastra NCL-PSA-431 (35H9) , 20 Mins Dilution 1: 100
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER1 20 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), Time 1: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins

Prostate Specific Antigen (PSA) / PSAP - Method 2

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

Primary Antibody:	Dako A0562 PSA (polyclonal) , 32 Mins, 20 ºC Dilution 1: 1000
Automation:	Ventana Benchmark XT
Method:	Ventana iView Kit
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.5
HMAR:	Ventana CC1 mild
EAR:	NOT APPLICABLE
Chromogen:	Ventana iview, 37 ºC., Time 1: 8 Mins
Detection:	Ventana iView system (760-091) , 8 Mins, 37 ºC Prediluted

Prostate Specific Antigen (PSA) / PSAP - Method 3

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

Primary Antibody:	Dako A0562 PSA (polyclonal) , 30 Mins, 20 ºC Dilution 1: 5000
Automation:	Dako Autostainer Plus Link
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer, PH: 7.6
HMAR:	Dako PTLink, Buffer: HIGH PH TRS, PH: 9
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 20 ºC., Time 1: 10 Mins, Time 2: 10 Mins
Detection:	NOT APPLICABLE

Prostate Specific Antigen (PSA) / PSAP - Method 4

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

Primary Antibody:	Dako M0750 PSA (Clone ER-PR8) , 32 Mins, 36 ºC Dilution 1: 50
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 56mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB, PH: 7.6, 36 ºC., Time 1: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 36 ºC

The Breast Hormonal Receptor Module Run 108

Merdol Ibrahim, Dawn Wilkinson and Keith Miller

Antigen Assessed:	Oestrogen Receptor (ER)
Tissue Sections circulated:	Composite slide consisting of 3 breast carcinomas with different levels of receptor expression. Most slides also included normal breast and normal tonsil.
Number of Registered Participants:	293
Number of Participants This Run	97(%)

Circulated Tissue

The table below shows the staining characteristics of the tissue sections circulated during Run 108. This composed of three infiltrating ductal carcinomas (IDCs) with differing levels of receptor expression, along with sections of normal breast and tonsil. The staining of the breast tumours were characterised using clones Leica 6F11, Ventana SP1 and Dako EP1.

Sections	% Positivity	Expected Staining Intensity	Allred / Quick Score
A. IDC	>95%	High	8
B. IDC	40-60%	Medium	6
C. IDC	0%	Negative	0
D. Normal Breast*	0%	Negative	0
E. Tonsil*	0%	Negative	0

*Normal breast and tonsil samples were also included on most slides but these were not scored in the actual assessment procedure.

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

General Guideline Used in The Assessment of Slides

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

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

In-House Tissue Recommendations

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

The required composite control should consist of the following samples:

1. >80% tumour positivity with high intensity (Allred/ Quick score 7-8)
2. 30-70% tumour positivity with low-moderate intensity (Allred/Quick score 3-6)
3. Negative tumour, ideally with normal positively stained glands (Allred/Quick score '0')

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

Introduction

Oestrogen receptor alpha (ER-α) plays a vital role in both the prognosis and predictive response of patients who may be considered for hormone therapy. Following the work of Harvey and colleagues[1], immunohistochemistry has now become the recognised 'gold standard' for determining patient ER status. It is therefore crucial that not only the antibodies are correctly validated prior to patient-tissue use, but also proper control tissues are used to gauge the sensitivity of the test. An incorrect assay can lead to false ER staining[6,7], which can have a direct impact on patient treatment regime. Furthermore, the UK NHS Breast Screening Programme (www.cancerscreening.nhs.uk/ breastscreen/index.html) recommends using the Quick score (Allred)[1,2] to semi quantify the proportion and intensity of nuclear staining, thus further standardising the scoring criteria.

Choice of Tissue for Assessments

This assessment consisted not only of invasive breast tumours (samples A-C), but most slides also included tonsil and normal breast tissue. These tonsil and normal breast samples were included to help gauge the sensitivity of the assay.

ER Staining Within the Tonsil

The distributed tonsil sections showed ER expression in 2-5% of cells. This has also been indicated in the datasheets from the commercial suppliers, such as the Dako (EP1) and Ventana SP1 clones. Although not noted on the datasheet of the Leica 6F11 clone, staining was also seen in the tonsil of slides stained with this antibody. The staining was observed in some of the epithelial cells, but was mainly seen within the germinal centre lymphocytes.

Quality Control of NEQAS Samples

NEQAS tissue samples were tested by staining every 50th section from the relevant tissue blocks using the Leica clone. This made sure that any heterogeneity in the tissue samples did not result in a participant being penalised. Furthermore, samples were also tested by the relevant commercial companies to further verify the expected level of staining, including the Leica (clone 6F11), Dako (clone EP1) and Ventana (clone SP1). We have also started to investigate the NEQAS distributed breast tumour samples for **mRNA expression** using ACDbio RNAscope technology. The initial results (not shown) indicate a good correlation between protein (immunohistochemistry) and mRNA expression on all of the NEQAS samples.

Assessment Results

Features of Optimal Immunostaining: (Figs 1-6,11,12)

- Staining of the expected proportion of invasive tumour nuclei with the anticipated staining intensity (Figs 1,3,5)
- Intense nuclear staining of the appropriate distribution in normal glands (Figs 2&4)
- Cytoplasmic staining is not excessive
- No background staining of connective tissues or inappropriately localised staining

Features of Sub-Optimal Immunostaining: (Figs 7-10)

- Inappropriate non-specific nuclear staining in the negative tumour
- Weak or lower expression of nuclear staining of the receptor positive tumours (Figs 7,8)
- Excessive cytoplasmic & background staining
- Excessive antigen retrieval

- Inappropriate staining of some cells in the tumour sections e.g. lymphocytes, fibroblasts
- Inappropriate non-specific staining in the normal breast/ tonsil.

NEQAS Slide Results

The overall pass rate on the NEQAS sections was lower 84% compared to the previous ER assessment (Run 106) of 91% (scores of ≥13-20/20). A further 10% received a borderline score (10-12/20) compared to 8% in the previous run, and an increase from (1 %) failure rate to (6%) failure rate for this current assessment. The main reason for failure during this assessment was due to weaker than expected staining within the distributed mid-ER expressing sample .

The most popular antibody used in this assessment run was the Ventana SP1 clone, used by 73 participants with a pass rate of 99%. The Leica/Novocastra 6F11 (not pre-dilute) showed an acceptable pass rate of 70% or over. The 6F11 pre-dilute did not fare so well and achieved a maximum 50% pass rate amongst the participants who used the **pre-dilute** 6F11 clone.

In-House Tissue Results

97% of participants also submitted their in-house controls for assessment. These showed an acceptable pass rate of 81%, and a further 18% of labs received a borderline pass. 2% of participants failed the assessment on their in-house submitted slide. There were various reasons for a failed result, including excessive cytoplasmic staining, excessive pre-treatment, inappropriate non-specific staining, and poor tissue morphology. Several labs that received a borderline pass had not submitted the required composite control including a high, mid/low and a negative expressing tumour.

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, including the Novocastra/Leica (clone 6F11), Dako (clone EP1) and the Ventana (clone SP1) antibodies.
- The **In-house** samples are scored on the staining quality, readability of the samples, the correct choice of tissue, and good morphological preservation. If the in-house control samples do not comprise of the required composite breast tissue, containing a high (Allred 7-8), mid- (Allred 3-6) and a negative (Allred 0) ER-expressing tumour, participants are given a maximum score of 12/20. The presence of normal staining glands is also encouraged.

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

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

Assessment Summary

The overall pass rate for ER (84% for NEQAS and 81% for in -house sections) has decreased compared to the previous ER run (106) with pass rates of 91% NEQAS and 92% in-house respectively. The main observation noted by the assessors for the NEQAS samples was a weaker than expected mid-expressor. And this was also reflected in the in -house samples.

It was both refreshing and reassuring to see, that with the new method of a one slide approach for both the NEQAS and in-house samples, that the majority of laboratories received similar scores for both their in-house and NEQAS samples. Some laboratories even achieving higher scores with the NEQAS samples than with their own in-house samples. This may be due to the fact that some laboratories find it difficult to find a mid-expressor. These laboratories that score highly on their NEQAS sample and maybe only received a borderline pass due to not having a decent mid-expressor will have the reassurance that their NEQAS score is indicative that the level of sensitivity of their assay is acceptable.

A few laboratories received much lower scores with their NEQAS sections than with their own in-house sections. The main reason being lower level of expression in the mid-expressor than expected. This may explain the lower pass rates for this run, there could be a number of reasons for this:

(1) The slides are now sent out un-baked with an instruction sheet. We advise to cut your own in-house slide onto the slides as soon as you receive the slide into the laboratory. To drain well and bake in the oven for 1 hour 55-60º C or at 37ºC overnight. We do not advocate drying slides directly on a hotplate. And we advise to stain the slides as soon as possible on receipt into the laboratory.

(2) If the slides are stored at room temperature and not stained for a couple of weeks this can affect the antigenicity of this particular biomarker. E.g. If the NEQAS slide has been sitting around at room temperature before the in-house section is cut onto the slides, and then stained this could explain the lower score for the NEQAS section.

(3) The sensitivity of your assay may be acceptable, in that it is acceptable for your tissue sections locally and you have validated your assay, However it can indicate the sensitivity of your assay may not be optimal.

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

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.
2. Robin Leake, Diana Barnes, Sarah Pinder, Ian Ellis, Liz Anderson, Tom Anderson, Ruth Adamson, Tony Rhodes, Keith Miller and Rosemary Walker (2000) J. Clin. Pathol. 2000: 634-635.
3. Anderson E. The role of oestrogen and progesterone receptors in human mammary development and tumorigenesis. Breast Cancer Res. 2002; 4:197-201.
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.
5. Ciocca DR and Elledge R. Molecular markers for predicting response to tamoxifen in breast cancer patients. Endocrine. 2000;13: 1-10.
6. Rhodes A, Jasani B, Balaton AJ, Barnes DM, Anderson E, Bobrow LG, Miller KD (2001). Study of inter-laboratory reliability and reproducibility of estrogen and progesterone receptor assays in Europe. Documentation of poor reliability and identification of insufficient microwave antigen retrieval time as a major contributory element of unreliable assays. Am J Clin Pathol. 2001 Jan;115(1):44-58.
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.

Selected Images showing Optimal and Sub-optimal Immunostaining

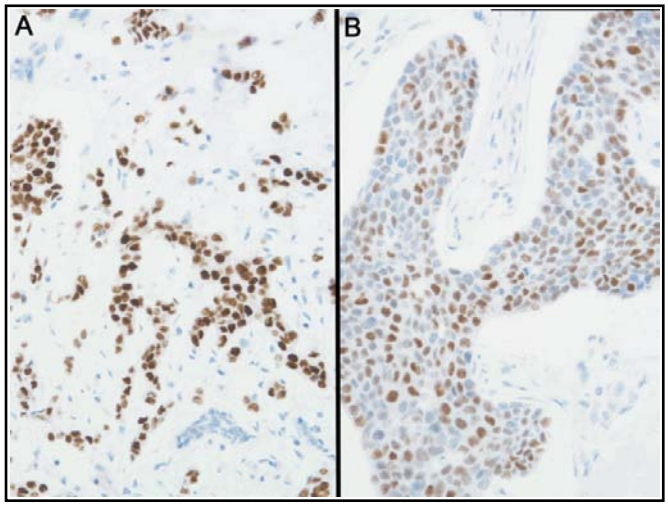


Fig 1. Optimally stained UK NEQAS distributed samples: (A) High expressing ER tumour with intense staining in >90% of neoplastic cells, while (B) mid-expressing tumour (B) shows varying intensity of positive staining in >60% of neoplastic cells. Stained with the Dako EP1 pre-diluted antibody on the Dako Autostainer with high pH buffer retrieval in the PT link.

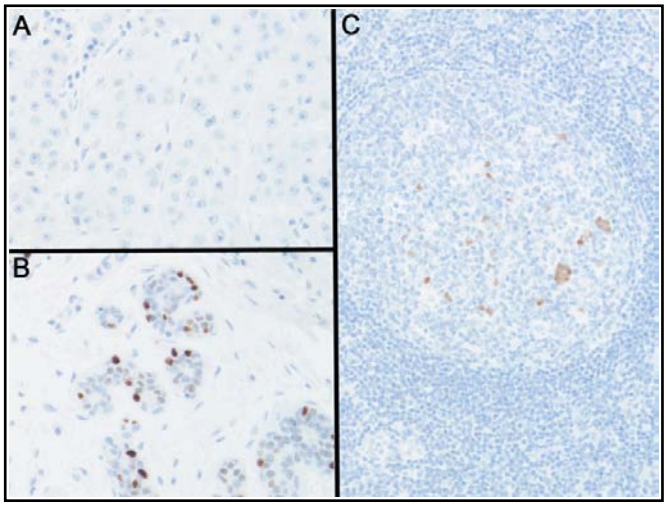


Fig 2. Optimal demonstration of ER in the UK NEQAS distributed samples. The ER negative tumour (A) remains unstained; only a percentage of the nuclei in the normal glands are positive (B), and as expected, the tonsil (C) shows staining in less than 5% of the germinal centre lymphocytes (same protocol as Fig 1).

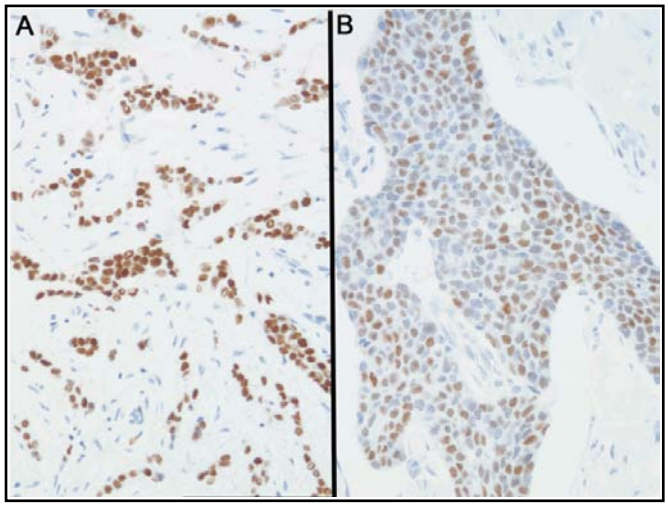


Fig 3. Good demonstration of ER in the UK NEQAS high (A) and mid (B)-expressing tumours. Both examples show the expected level of staining. Sections stained with the Leica 6F11 antibody, 1:50, on the Dako Autostainer with antigen retrieval carried out in the PT link with high pH buffer.

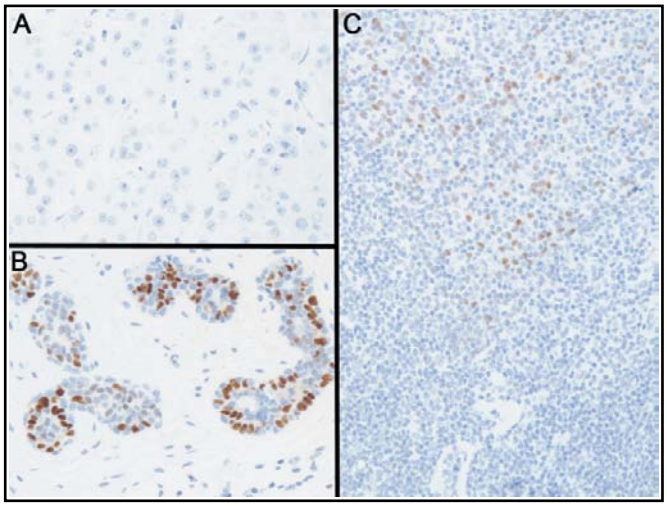


Fig 4. Good staining in the UK NEQAS ICC samples: (A) Negative ER tumour, (B) normal glands show the expected percentage of positive nuclei, and (C) tonsil section shows the expected level of less than 5% of lymphocytes staining. (Same protocol as Fig 3).

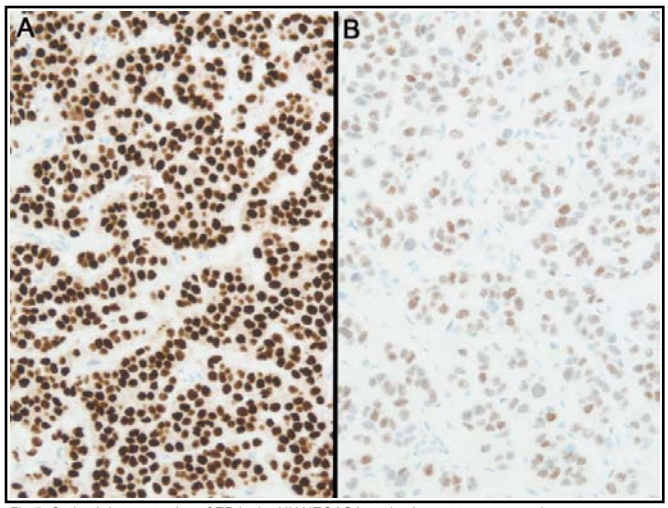


Fig 5. Optimal demonstration of ER in the UK NEQAS invasive breast tumour samples (Block2). (A) High expressor showing strong staining in over 90% of the tumour cells, (B) mid-expressor, showing varying intensity of positive neoplastic cells. Section stained with the Ventana SP1 pre-diluted antibody on the Benchmark XT with CC1 standard antigen retrieval.

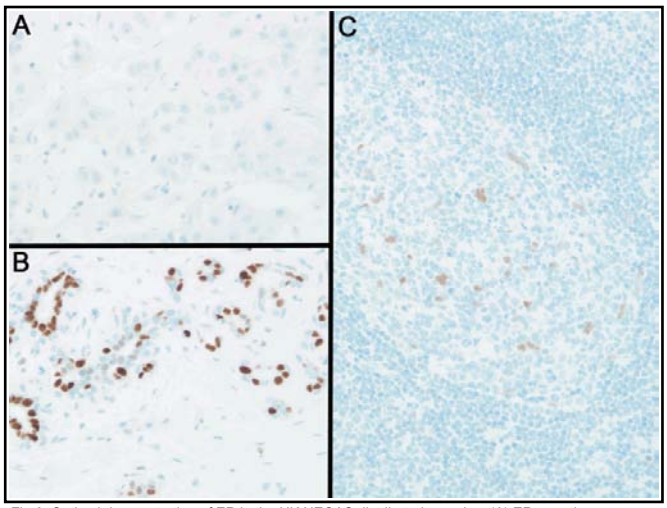


Fig 6. Optimal demonstration of ER in the UK NEQAS distributed samples. (A) ER-negative tumour. (B) Normal glands show the expected proportion of staining, and (C) tonsil shows less than 5% of positive lymphocytes staining in the germinal centre (same protocol as Fig 1).

Selected Images showing Optimal and Sub-optimal Immunostaining

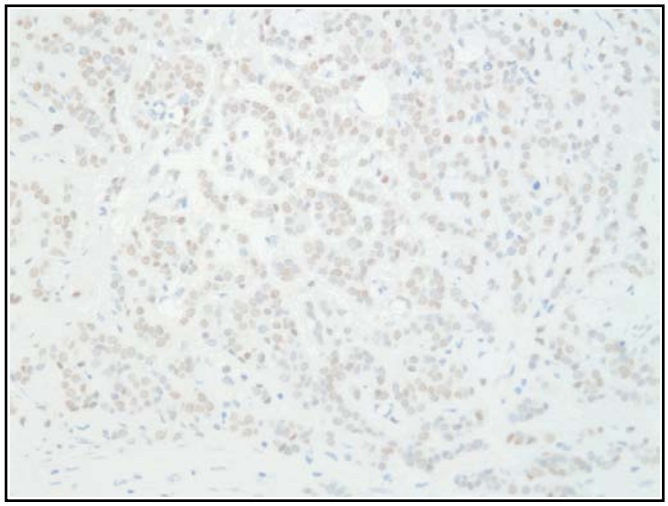


Fig 7. Unacceptable demonstration of ER in the UK NEQAS high expressing tumour: The staining is weak with less tumour nuclei staining than expected. Section stained with the Leica 6F11 pre-dilute antibody on the BondMax with ER2 antigen retrieval.

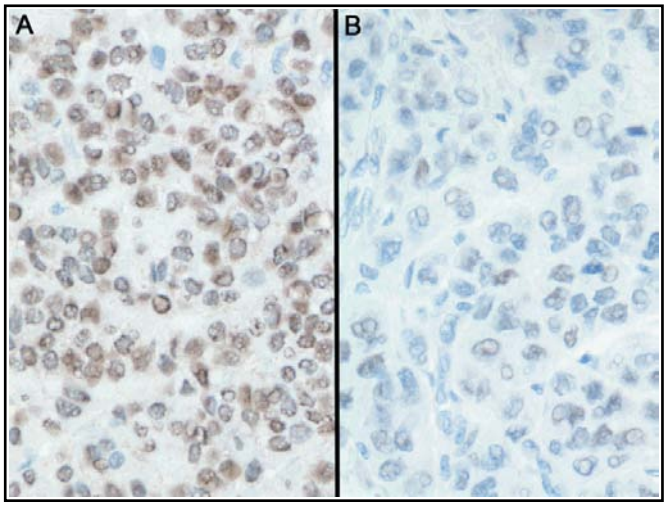


Fig 8. 2 examples showing poor staining with ER in the UK NEQAS samples: Both the high expresser (A) and mid expresser (B) show weak staining and morphology damage caused by excessive pretreatment. Stained with the Neomarkers antibody on the Dako autostainer with 40 minutes antigen retrieval in the PT link (high pH buffer).

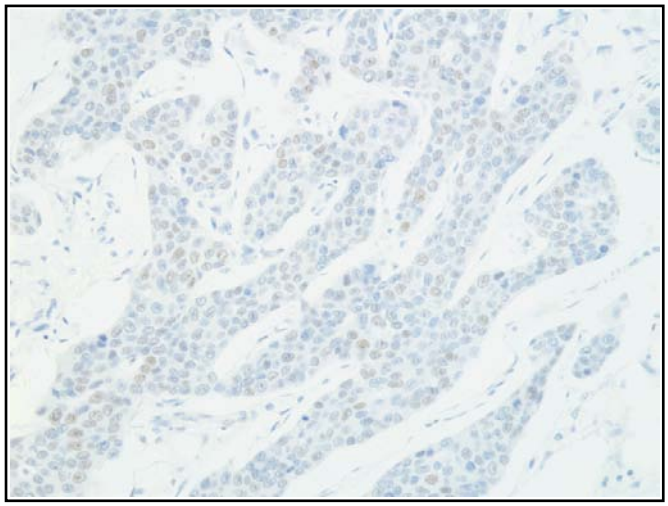


Fig 9. Unacceptable staining of the UK NEQAS mid expressing tumour. The staining is very weak with less nuclei demonstrated than expected. Section stained with the Leica 6F11 antibody, 1:40 on the Bond III with ER1 antigen retrieval for 20 minutes.

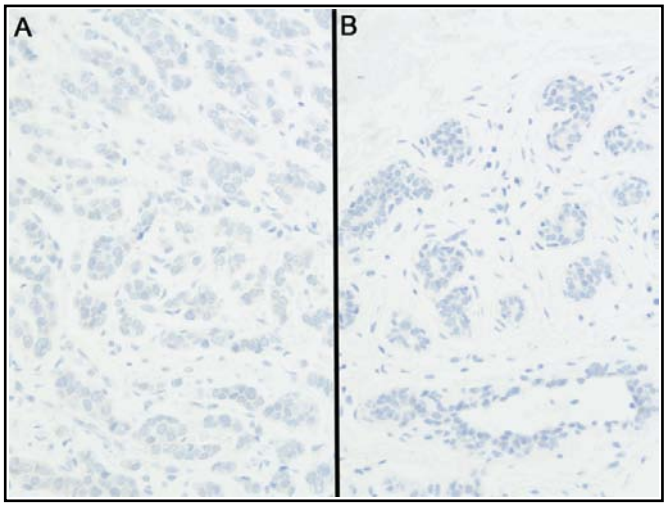


Fig 10.2 examples of unacceptable staining in the UK NEQAS samples. (A) mid-expresser, but is actually falsely negative, and (B) no staining in the any of the normal glands.

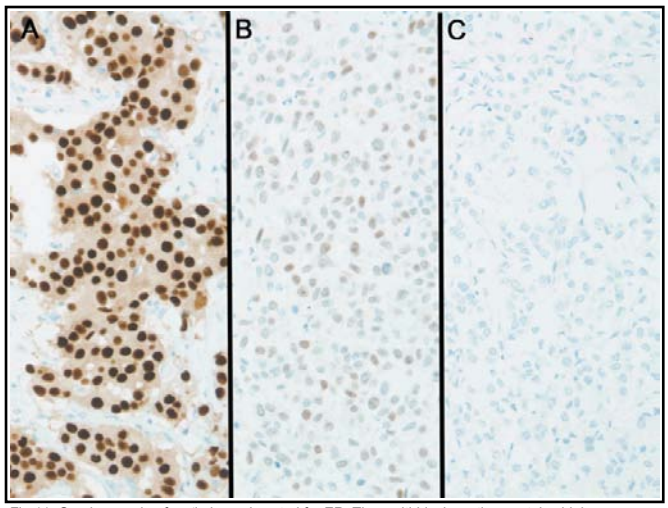


Fig 11. Good example of an 'in house' control for ER. The multi-block section contains high, mid, and negative expressing tumours (A-C respectively). The section was stained with the Leica 6F11 on the Bond III with ER1 antigen retrieval for 30 minutes.

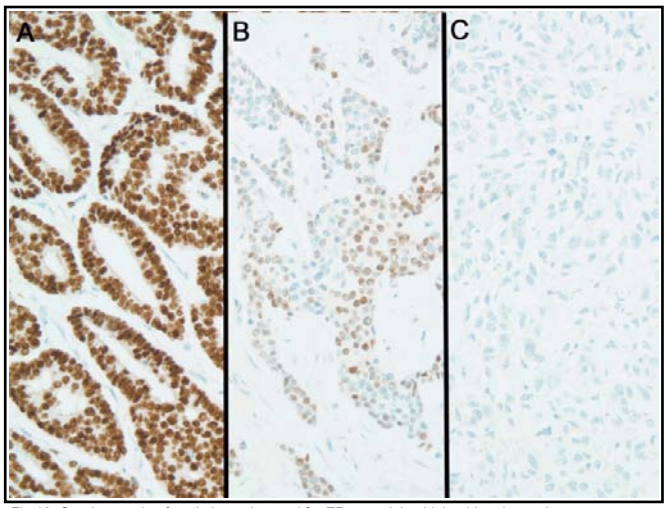
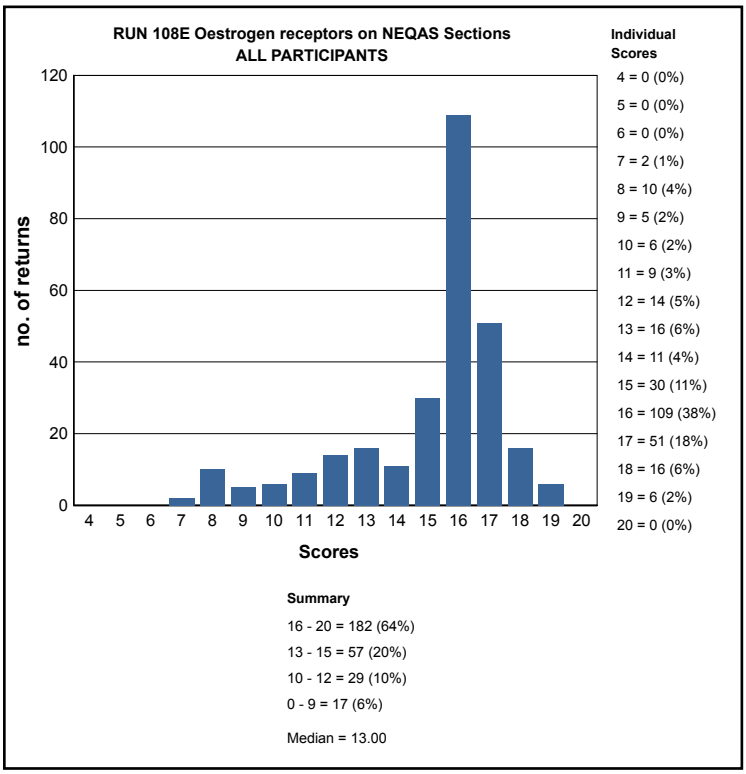
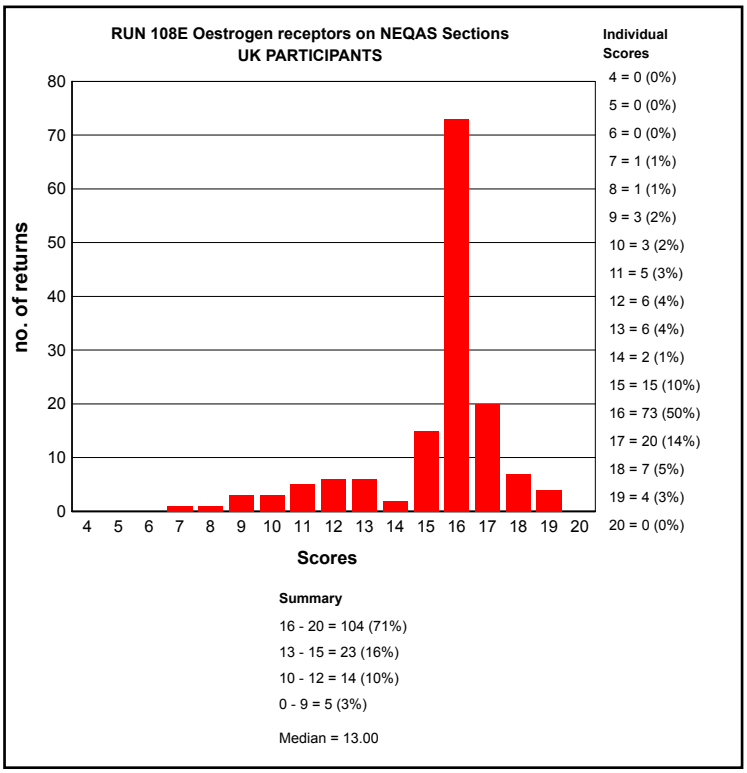
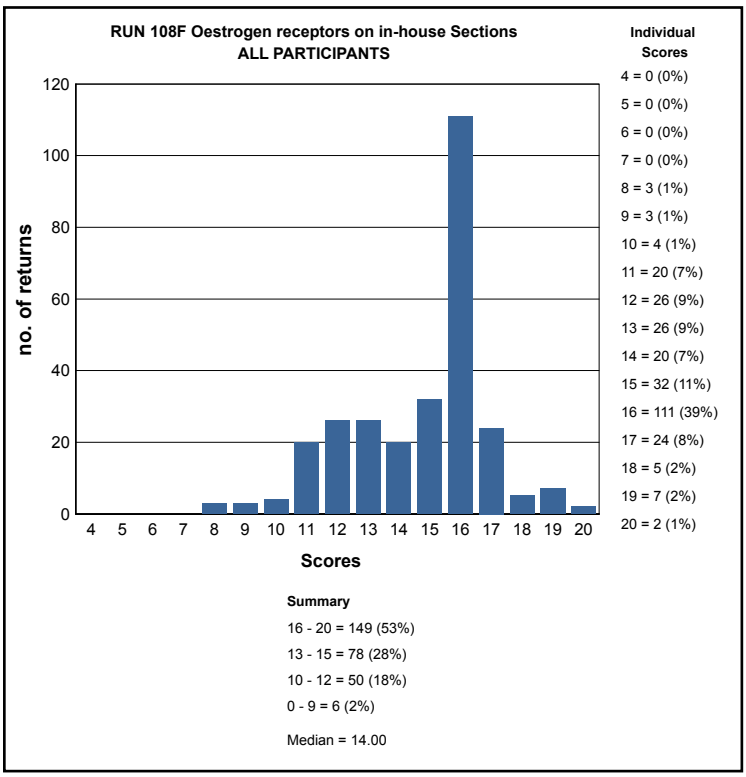


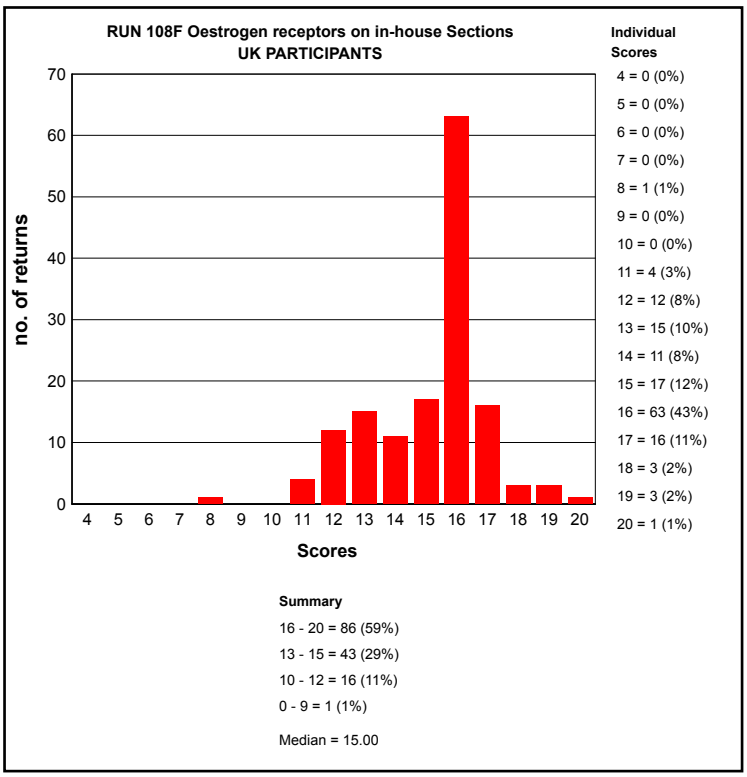
Fig 12. Good example of an 'in house' control for ER, containing high mid and negative expressing tumours to help gauge the sensitivity of the assay.

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: 108

Primary Antibody : Oestrogen receptors

Antibody Details	N	%
Dako (EP1) M3643	23	96
Dako (EP1) RTU Auto Plus IS084	3	100
Dako (EP1) RTU FLEX IR084	19	89
Dako FLEX (1D5) IR/IS657	2	0
Dako IR151 Autostainer Link (SP1)	1	0
Dako M3634 (SP1)	7	100
Dako M7047 ER (1D5)	8	50
Dako PharmDx (Autostainer kit) K4071	1	100
Dako RTU IR151 (SP1)	1	100
Leica Bond PA0151 (6F11)	5	40
Leica/Novocastra NCL-ER-6F11 (6F11)	18	72
Leica/Novocastra NCL-ER-6F11/2	10	70
Leica/Novocastra NCL-L-ER- 6F11	34	71
Leica/Novocastra RTU-ER-6F11	4	50
Other	9	67
Thermo Fisher/ Neomarkers RM 9101-S (SP1)	15	73
Vector VP-E613/4 (6F11)	7	86
Ventana 250- 2596 ER (6F11)	3	67
Ventana 790-4324 (SP1)	73	99
Ventana 790-4325 (SP1)	37	97

Breast Steroid Hormone Receptor Run: 108

Automation	Oestrogen receptors N	Oestrogen receptors %
BioGenex GenoMX 6000i	1	0
Dako Autostainer	3	67
Dako Autostainer Link 48	39	79
Dako Autostainer plus	6	100
Dako Autostainer Plus Link	5	60
LabVision Autostainer	3	67
Leica Bond Max	34	65
Leica Bond-III	40	75
Menarini - Intellipath FLX	2	0
None (Manual)	3	67
Other	1	100
Shandon Sequenza	3	100
Ventana Benchmark GX	6	83
Ventana Benchmark ULTRA	72	100
Ventana Benchmark XT	66	89

Breast Steroid Hormone Receptor Run: 108

Heat Mediated Retrieval	Oestrogen receptors N	Oestrogen receptors %
Biocare Decloaking Chamber	2	50
Dako Pascal	2	100
Dako PTLink	45	76
Lab vision PT Module	5	60
Leica ER1 20 mins	17	53
Leica ER1 30 mins	16	75
Leica ER1 40 mins	7	43
Leica ER2 10 mins	1	100
Leica ER2 20 mins	23	78
Leica ER2 30 mins	7	86
Leica ER2 40 mins	3	100
Microwave	2	50
Other	3	100
Pressure Cooker	5	100
Ventana CC1 16mins	2	50
Ventana CC1 20mins	2	50
Ventana CC1 24mins	2	100
Ventana CC1 32mins	7	86
Ventana CC1 36mins	16	100
Ventana CC1 48mins	1	100
Ventana CC1 52mins	5	100
Ventana CC1 56mins	1	100
Ventana CC1 64mins	30	93
Ventana CC1 76mins	1	100
Ventana CC1 88mins	1	100
Ventana CC1 8mins	1	0
Ventana CC1 extended	5	80
Ventana CC1 mild	19	95
Ventana CC1 standard	47	98
Ventana CC2 mild	1	100
Water bath 95-98 OC	3	100

Breast Steroid Hormone Receptor Run: 108

Enzyme Mediated Retrieval	Oestrogen receptors N	Oestrogen receptors %
AS PER KIT	6	83
NOT APPLICABLE	153	84
Ventana Protease 1 (760-2018)	1	100

Breast Steroid Hormone Receptor Run: 108

Detection	Oestrogen receptors N	%
AS PER KIT	10	70
Dako EnVision FLEX (K8000/10)	9	89
Dako EnVision FLEX+ (K8002/12)	28	79
Dako Envision HRP/DAB (K5007)	3	100
Dako REAL HRP/DAB (K5001)	2	50
LabVision UltraVision ONE Polymer (TL-012/5-HDJ/T	1	0
Leica Bond Polymer Refine (DS9800)	71	69
MenaPath X-Cell Plus (MP-XCP)	1	0
None	3	100
Novocastra Novolink PDS (RE7-140/150/280/290-K)	2	100
Other	10	80
Vector ImmPRESS Universal (MP-7500)	1	100
Ventana iView system (760-091)	8	100
Ventana OptiView Kit (760-700)	12	83
Ventana UltraView Kit (760-500)	122	94

Breast Steroid Hormone Receptor Run: 108

Chromogen	Oestrogen receptors N	%
AS PER KIT	19	89
BioGenex liquid DBA (HK-124-7K)	1	0
Dako DAB K3468	1	0
DAKO DAB+	1	100
Dako DAB+ Liquid (K3468)	3	100
Dako DAB+ REAL Detection (K5001)	2	100
Dako EnVision Plus kits	4	50
Dako FLEX DAB	32	78
Dako REAL EnVision K5007 DAB	3	67
Dako REAL K5001 DAB	1	100
Leica Bond Polymer Refine kit (DS9800)	69	68
menapath xcell kit DAB (MP-860)	1	0
Other	8	88
Sigma DAB (D5905)	1	100
Ventana DAB	6	100
Ventana Enhanced Alk. Phos. Red Detection Kit	1	100
Ventana iview	8	100
Ventana Ultraview DAB	123	93
Vision BioSystems Bond X DAB	1	100

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 16/20 (In House slide) using this method.

Primary Antibody: Dako (EP1) RTU FLEX IR084 , 20 Mins, ambient ºC Prediluted
Automation: Dako Autostainer Link 48
Method: Dako FLEX kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako PTLink, Buffer: DAKO target retrieval high pH
EAR:
Chromogen: Dako FLEX DAB, ambie ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection: Dako EnVision FLEX (K8000/10) , 20 Mins, ambient ºC Prediluted

Oestrogen receptors - Method 2

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

Primary Antibody: Leica/Novocastra NCL-ER-6F11 (6F11) , 30 Mins, rt ºC Dilution 1: 1/100
Automation: Leica Bond Max
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590), PH: 7.6
HMAR: Leica ER1 20 mins
EAR: NOT APPLICABLE
Chromogen: Leica Bond Polymer Refine kit (DS9800), rt ºC., Time 1: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 15 Mins, rt ºC Prediluted

Oestrogen receptors - Method 3

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

Primary Antibody: Ventana 790-4325 (SP1) , 48 Mins, 21 ºC Prediluted
Automation: Ventana Benchmark XT
Method: Ventana UltraView DAB
Main Buffer: Ventana reaction buffer (950-300)
HMAR: Ventana CC1 standard
EAR: NOT APPLICABLE
Chromogen: Ventana Ultraview DAB
Detection: Ventana UltraView Kit (760-500)

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 Auto Plus IS084 , 15 Mins
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

The Breast HER2 ICC Module Run 108

Keith Miller and Suzanne Parry

Antigen Assessed:	HER2
Sections Circulated:	Composite slide consisting of 4 breast carcinoma cell lines (see table below)
Number of Registered Participants:	415
Number of Participants This Run	393 (95%)

Specific Guidelines Used in The Assessment of Slides:

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

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

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

Validation of Distributed UK NEQAS ICC & ISH Cell Lines

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

Updated Assessment and Scoring Procedure (As of Run 106)

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 additional technical feedback. As well as taking into account the expected range (30-90% see above) of cell line membrane staining, 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.

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

Introduction

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

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

Two recommended HER2 testing guidelines are: 1. The ASCO/ CAP guidelines by Wolff et al.(2007), and 2. the UK guidelines by Walker et al., (2008). Please note, the ASCO/CAP guidelines have recently been updated (Wolff et al., 2013). Both publications provide invaluable guidelines covering interpretation, tissue fixation and antibody validation. The article by Walker et. al., also provides 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 that is 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 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 of the tumour they have scored otherwise the invasive component (if present) will be assessed.

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

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.

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.

Assessment Summary:

Pass rates for the assessment are shown in the subsequent summary graphs. As with previous assessments the most popular antibody was the Ventana 4B5, used by 60% (N=235) of participants and showed an acceptable pass rate of 82%. 31 labs are using the Dako HercepTest. This showed a pass rate of 44%. The Leica Oracle kit is used by 23 labs, and showed a pass rate of 83%. The rest of the labs are using lab devised methods with a variety of antigen retrieval methods and platforms.

Selected Images showing Optimal and Sub-optimal Immunostaining

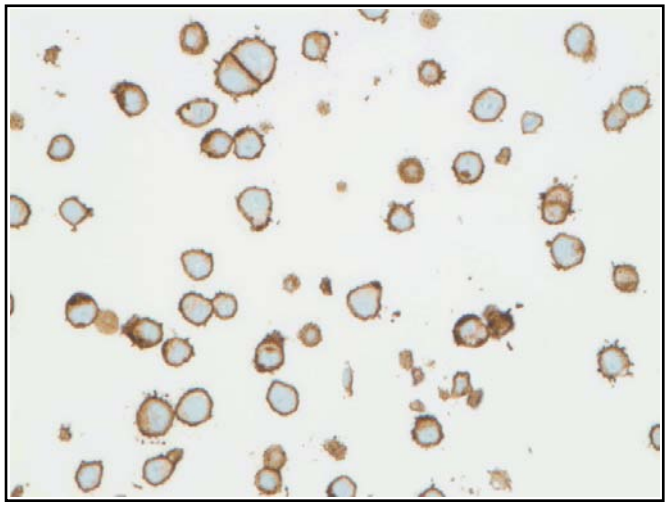


Fig 1. Appropriate level of staining of the UK NEQAS SK-BR3 (3+) cell line. The example shows strong membrane staining, which is completely circumferential and there is minimal cytoplasmic staining. Section stained with the Ventana 4B5 kit with the recommended protocol of CC1 mild on the Benchmark Autostainer.

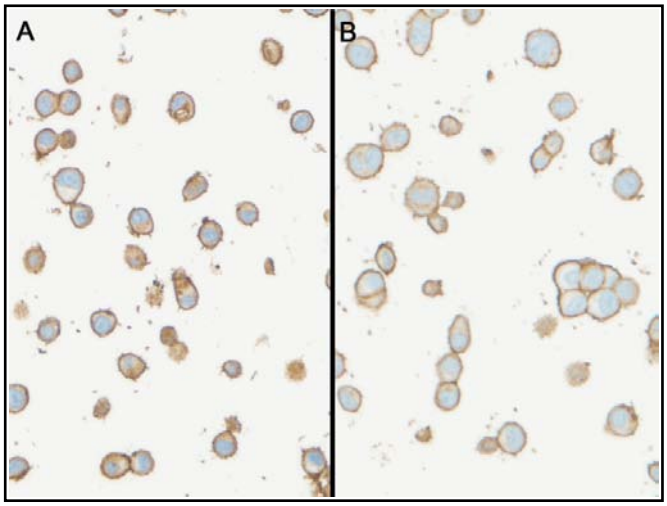


Fig 2. Two examples showing the appropriate level of staining of the UK NEQAS SK-BR3 (3+) cell line. Both sections show strong complete membranous staining. (A) Stained with the Leica Oracle kit, and (B) with the Dako HercepTest.

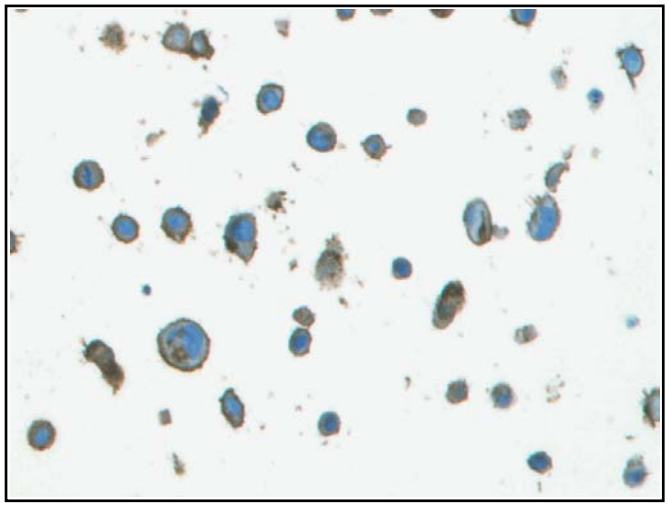


Fig 3. Poor staining of the UK NEQAS distributed SK-BR3 (3+) cell line. The example shows excessive morphology damage, most likely caused by excessive antigen retrieval. The counterstain is also too strong. Section stained using a lab devised method with the Genemed HER2 antibody.

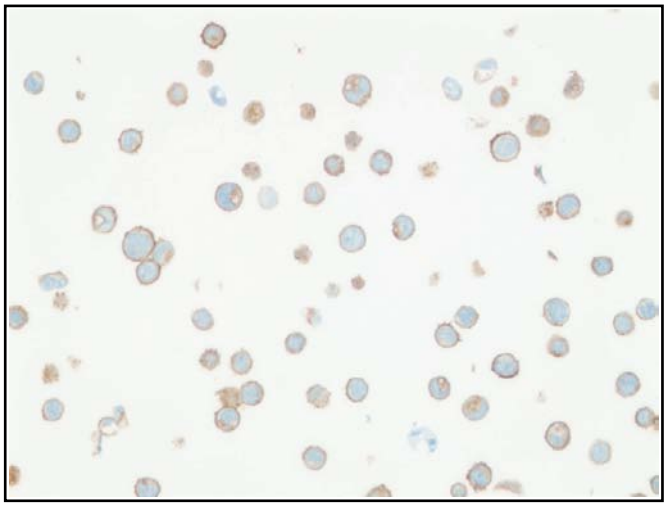


Fig 4. Optimal HER2 staining of the MDA-MB-453 (2+) cell line: The majority of cells show complete membrane staining which is less intensive than that seen with the 3+ cell line. Section stained with the Dako HercepTest.

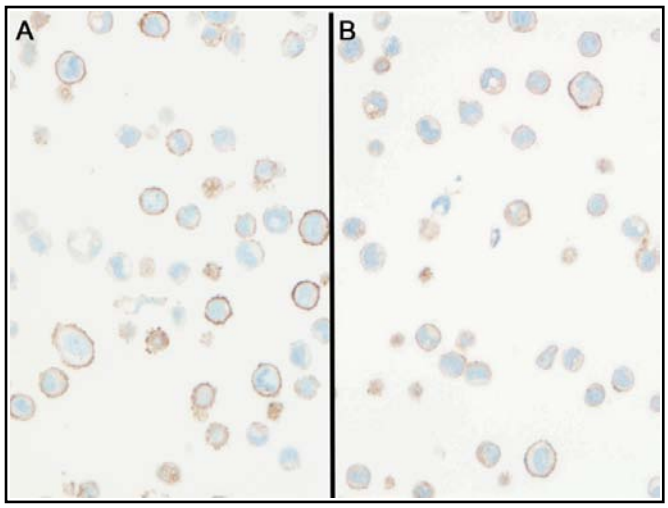


Fig 5. Two examples showing the appropriate level of HER2 staining of the MDA-MB-453 (2+) cell line: Both sections show the expected weak to moderate complete membrane staining. (A) Stained with the Ventana 4B5 Pathway kit and (B) stained with the Leica Oracle kit.

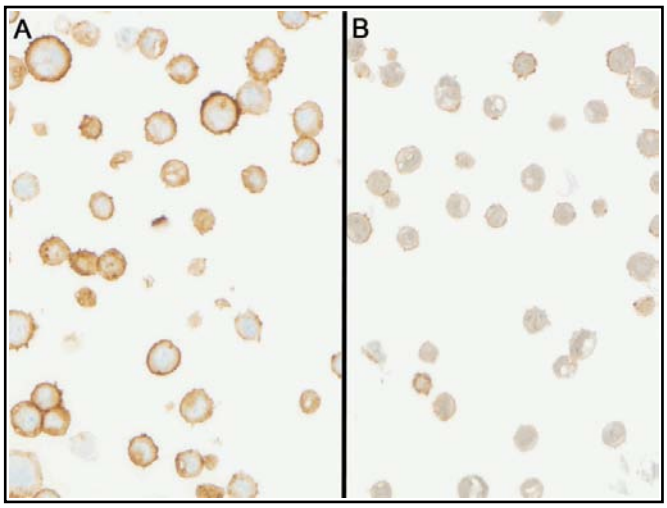


Fig 6. Two examples of inappropriate levels of staining of the UK NEQAS MDA-MB-453 (2+) cell line. The staining in (A) is too strong and more representative of a 3+, while the staining in (B) shows excessive background, making interpretation difficult.

Selected Images showing Optimal and Sub-optimal Immunostaining

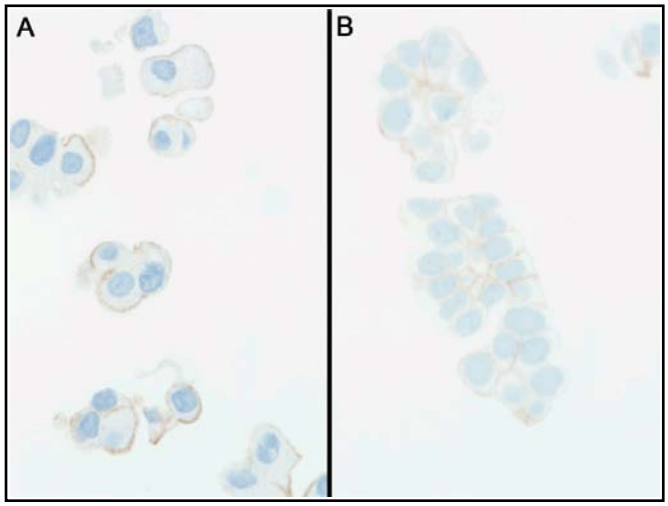


Fig 7. Two examples showing the expected level of staining of the UK NEQAS 1+ cell line (MDA-MB-175). Over 10% of the tumour cells show fine incomplete membrane staining. (A) Stained with the Dako HercepTest, and (B) stained with the Ventana 4B5 Pathway.

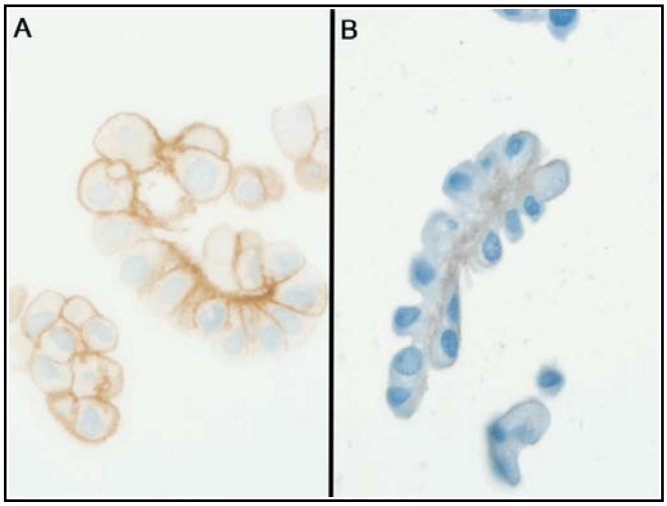


Fig 8. Inappropriate levels of staining of the UK NEQAS MDA-MB-175 (1+) cell line. (A) Staining is too strong and more representative of a 2+, while (B) shows excessive counterstain masking the membranes, therefore making the section uninterpretable.

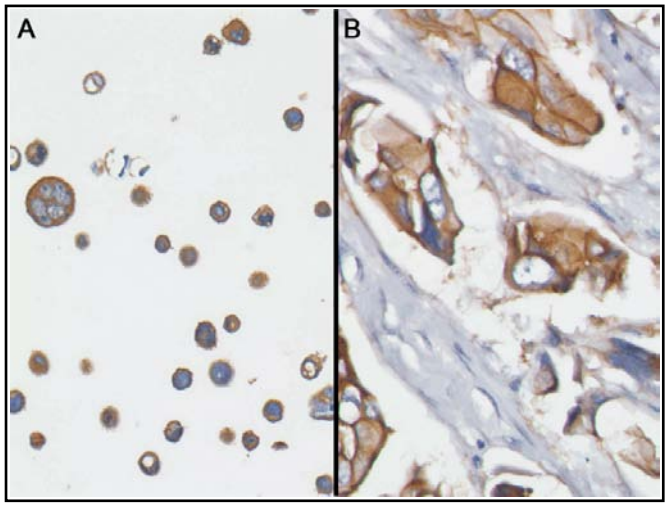


Fig 9. (A) Unacceptable staining of the UK NEQAS MDA-MB-453 (2+) cell line. Similarly to the NEQAS sample, the participant's own in house 2+ control (B) also shows excessive cytoplasmic staining and a heavy counterstain. The in house tissue control is also poorly fixed and therefore causing retraction artefact.

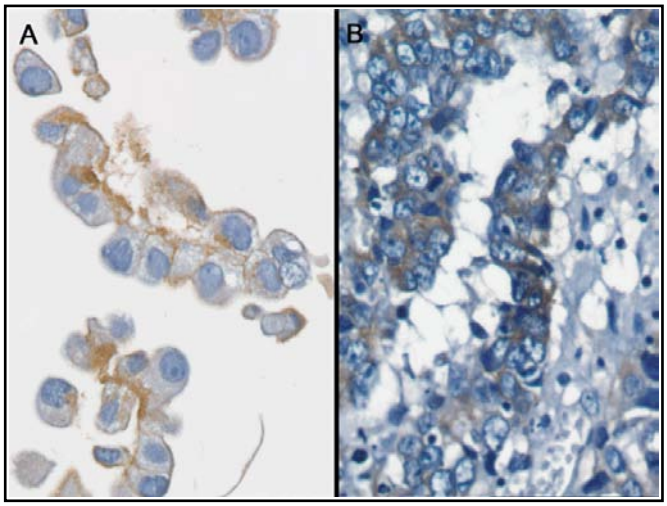


Fig 10. (A) Unacceptable staining of the UK NEQAS MDA-MB-175 1+ cell line. The cells show morphology damage caused by excessive antigen retrieval. (B) The 1+ in house tissue from the same laboratory, also showed morphology damage and poor preservation of tissue.

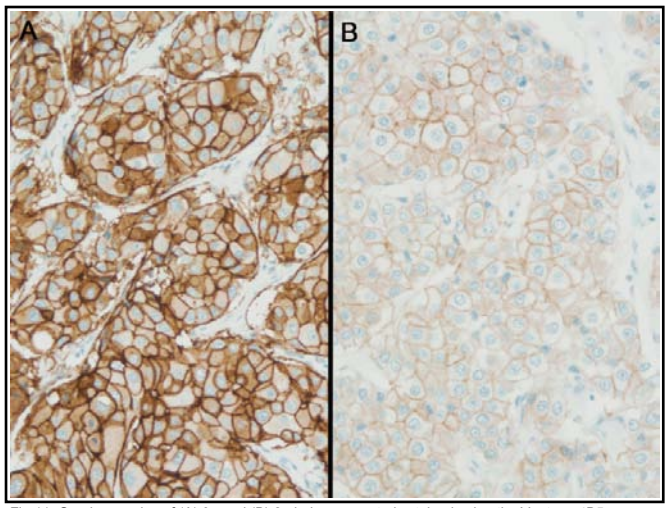


Fig 11. Good examples of (A) 3+ and (B) 2+ in-house controls stained using the Ventana 4B5 Pathway kit on the Benchmark XT with CC1 mild antigen retrieval (see also Fig 12).

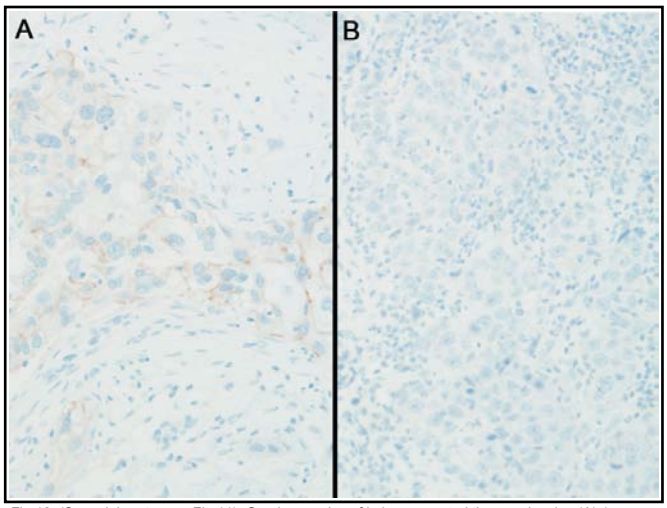
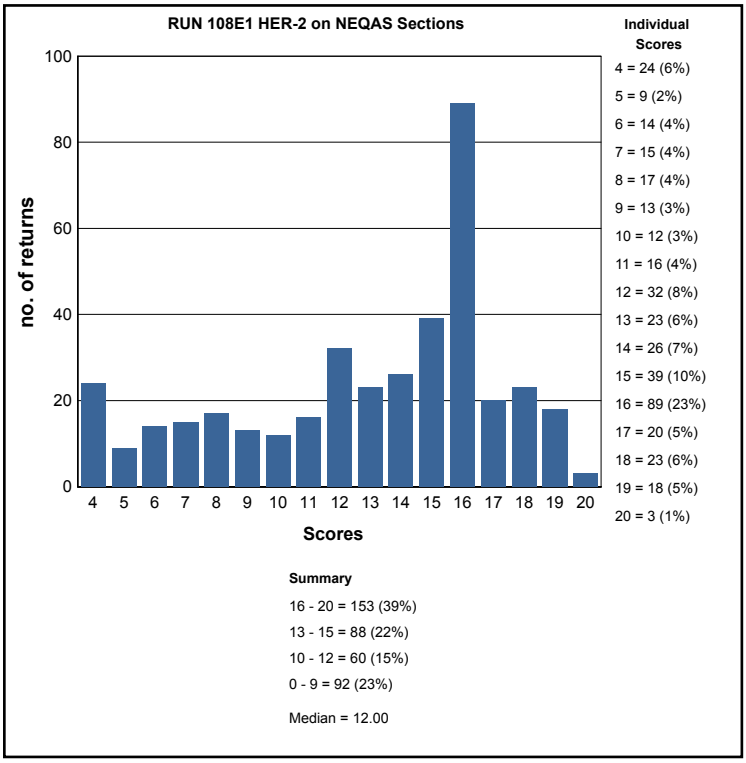
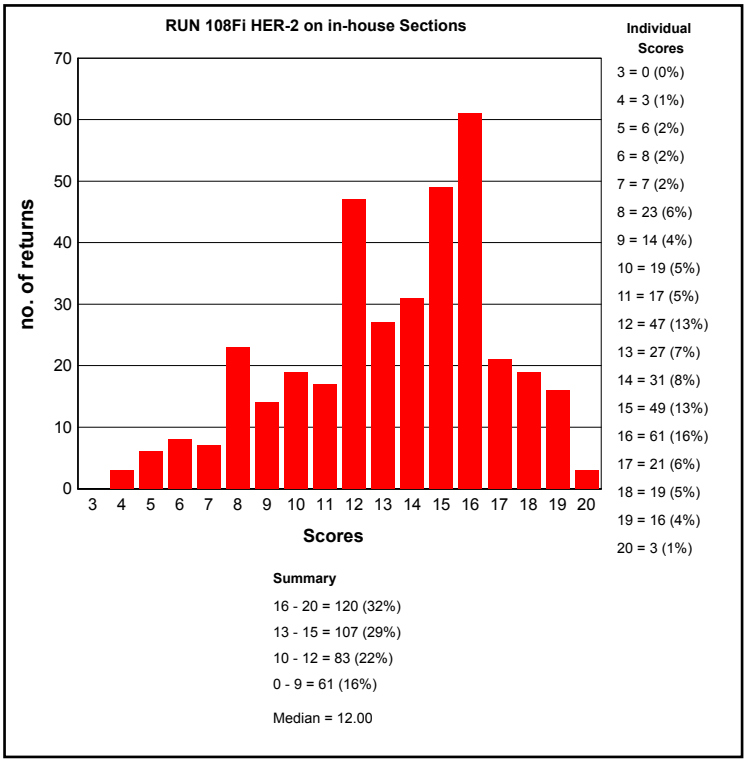


Fig 12. (Same laboratory as Fig 11). Good examples of in house control tissues showing (A) 1+ and (B) negative levels of HER2 expression.

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: 108

Primary Antibody	N	%
Biocare CME 342 A,B (EP1045Y)	5	40
BioGenex (EP1045Y) rb mono	4	25
Biogenex AM134-5M (CB11)	2	0
Cell Marque CMA 601 (CB11)	5	0
Dako A0485 C-erB-2 (poly)	45	33
Dako HercepTest K5204 (poly)	6	33
Dako HercepTest K5207 (poly)	9	44
Dako Link HercepTest SK001 (poly)	16	56
Labvision / Neomarkers RM-9103 (SP3)	7	14
Leica Oracle HER2 Bond IHC (CB11)	23	83
Novocastra NCL-L-CB11 (CB11)	4	25
Novocastra NCL-L-CBE356 (10A7)	2	0
Novocastra RTU-CB11 (CB11)	3	0
Novocastra RTU-CBE-356 (10A7)	1	0
Other	12	8
Ventana Confirm 790-4493 (4B5)	90	69
Ventana pathway 760-2694 (CB11)	1	100
Ventana Pathway 790-100 (4B5)	12	75
Ventana Pathway 790-2991 (4B5)	133	83

Breast HER2 ICC Run: 108

Automation	N	%
BioGenex GenoMX 6000i	4	0
Dako Autostainer	5	20
Dako Autostainer Link 48	27	48
Dako Autostainer plus	6	17
Dako Autostainer Plus Link	5	60
LabVision Autostainer	2	0
Leica Bond Max	22	45
Leica Bond-III	19	79
None (Manual)	40	15
Other	4	25
Shandon Sequenza	2	50
Ventana Benchmark GX	20	65
Ventana Benchmark ULTRA	74	84
Ventana Benchmark XT	149	74

Breast HER2 ICC Run: 108

Heat Mediated Retrieval	N	%
Biocare Decloaking Chamber	6	0
Dako Pascal	1	0
Dako PTLink	33	45
Lab vision PT Module	3	0
Leica ER1 10 mins	4	25
Leica ER1 20 mins	11	36
Leica ER1 25 mins	18	83
Leica ER1 30 mins	2	100
Leica ER2 20 mins	2	0
Microwave	17	6
None	1	100
Other	7	71
Pressure Cooker	12	17
Steamer	1	0
Ventana CC1 16mins	4	100
Ventana CC1 20mins	4	75
Ventana CC1 32mins	19	47
Ventana CC1 36mins	37	78
Ventana CC1 40mins	2	100
Ventana CC1 48mins	1	0
Ventana CC1 52mins	4	100
Ventana CC1 56mins	4	100
Ventana CC1 64mins	5	80
Ventana CC1 76mins	2	100
Ventana CC1 8mins	3	33
Ventana CC1 mild	114	77
Ventana CC1 standard	23	87
Ventana CC2 16mins	1	100
Ventana CC2 32mins	1	100
Ventana CC2 mild	1	0
Water bath 95-98 OC	16	31

Breast HER2 ICC Run: 108

Detection	N	%
AS PER KIT	32	69
Biocare polymer (M4U534)	1	0
Biocare SLAB (STU HRP 700H,L10)	1	0
BioGenex HRP (HK 519-06K)	1	0
BioGenex SS Polymer (QD 420-YIKE)	1	0
BioGenex SS Polymer (QD 430-XAKE)	2	0
Dako HerCep Test (K5204)	4	25
Dako EnVision FLEX (K8000/10)	15	20
Dako EnVision FLEX+ (K8002/12)	5	60
Dako Envision HRP/DAB (K5007)	10	20
Dako HerCep Test Autor (K5207)	5	60
Dako HerCep Test Autor (SK001)	8	63
Dako REAL HRP/DAB (K5001)	2	0
LabVision UltraVision LP HRP (TL 125 HLJ)	1	0
LabVision UltraVision ONE Polymer (TL-012/5-HDJ/T	1	0
Leica Bond Polymer Refine (DS9800)	23	48
None	1	100
Novocastra Novolink PDS (RE7-140/150/280/290-K)	2	0
Other	20	20
Ventana iView system (760-091)	11	64
Ventana OptiView Kit (760-700)	7	86
Ventana UltraView Kit (760-500)	207	75

Breast HER2 ICC Run: 108

Enzyme Retrieval	N	%
AS PER KIT	14	50
Enzyme digestion + HIER	1	0
NOT APPLICABLE	159	62
Ventana Protease	2	50
Ventana Protease 1 (760-2018)	1	100

Breast HER2 ICC Run: 108

Chromogen	N	%
AS PER KIT	56	64
BioGenex DAB (QD430)	1	0
BioGenex Liquid DAB (HK153-5K)	1	0
BioGenex liquid DBA (HK-124-7K)	2	0
DAKO DAB+	2	50
Dako DAB+ Liquid (K3468)	4	0
Dako DAB+ REAL Detection (K5001)	3	0
Dako EnVision Plus kits	4	75
Dako FLEX DAB	25	36
Dako REAL EnVision K5007 DAB	9	22
LabVision DAB	2	0
Leica Bond Polymer Refine kit (DS9800)	25	44
NOT APPLICABLE	1	0
Other	24	33
Sigma DAB (D5905)	1	0
Ventana DAB	6	83
Ventana iview	9	67
Ventana Ultraview DAB	204	76

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)
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)

HER-2 - Method 2

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

Primary Antibody:	Ventana Pathway 790-2991 (4B5) , 12 Mins, 37 ºC Prediluted
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) Prediluted

HER-2 - Method 3

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

Primary Antibody:	Dako Link HercepTest SK001 (poly) , 30 Mins, 25 ºC Prediluted
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: HERCEPTEST EPITOPE RETRIEVAL SOLUTION
EAR:	NOT APPLICABLE
Chromogen:	Dako EnVision Plus kits, 25 ºC., Time 1: 10 Mins
Detection:	Dako HerCep Test Autor (SK001) , 30 Mins, 25 ºC Prediluted

HER-2 - Method 4

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

Primary Antibody:	Leica Oracle HER2 Bond IHC (CB11)
Automation:	Leica Bond Max
Method:	AS PER KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER1 20 mins
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	AS PER KIT

See BRAF V600E differently

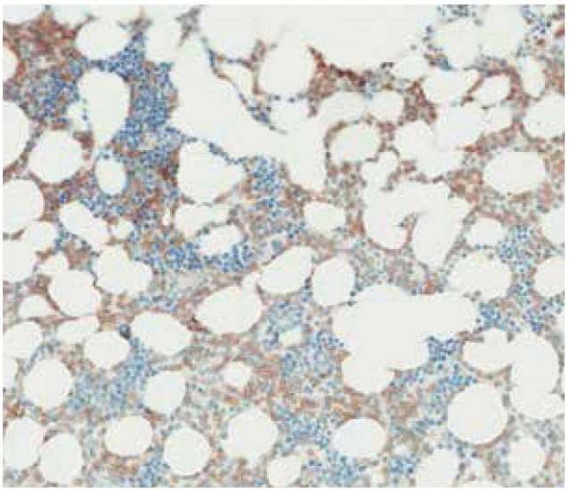
Now with IHC

Empower your laboratory with state-of-the-art IHC testing for the BRAF V600E mutation in a wide variety of cancers, including hairy cell leukemia. Introducing the BRAF V600E (VE1) antibody, only from Ventana Medical Systems, Inc.

Our exclusive BRAF V600E (VE1) Mouse Monoclonal Primary Antibody is optimized for use with the BenchMark IHC/ISH platforms, and is ready-to-use, so you can provide timely, accurate results.

Discover how the BRAF V600E (VE1) antibody can enhance your workflow and help inform your diagnostic decisions.

To learn more, stop by the VENTANA booth or visit www.ventana.com.



Hairy cell leukemia with BRAF V600E (VE1) IHC with OptiView DAB IHC detection

VENTANA

Empowering | Cancer Diagnostics

www.roche.com
www.ventana.com

E4695-15 0314A

The Gastric HER2 ICC Module Run 108

Merdol Ibrahim and Suzanne Parry

Antigen Assessed:	**HER2 for IHC**
The NEQAS distributed sections consisted of a composite slide containing 4 surgical /resections of intestinal gastric carcinoma:	The expected expression levels of each tissue sample were: **A.** 3+ (Please note: A few distributed sections did not have any tumour in section A, and therefore labs who received these serial sections were not scored on section A) **B**. 2+ **C**. 2+, 1+ or 0 depending on the serial section received.* **D**. 1+ or 0 depending on the serial section received.* * Due to the heterogeneous nature of the samples, the HER2 expression level changed throughout the tissue blocks used. This was taken into consideration during the assessment by comparing the staining to the Gold standard slides at the closest serial level.
Number of Registered Participants	155
Number of Participants This Run	136 (88%)

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

Score	Surgical / resections As used in NEQAS assessments	Biopsies
0 (negative)	No staining in < 10% of tumour cells	No staining in any of the tumour cells
1+ (negative)	Faint barely perceptible incomplete membrane staining in >10% of cells staining	Tumour cells with faint barely perceptible incomplete membrane staining, irrespective of percentage of tumour cells stained
2+ (equivocal*)	Weak/ moderate complete, basolateral or lateral membrane reactivity in ≥ 10% of tumour cells	Tumour cells with weak/ moderate complete, basolateral or lateral membrane reactivity irrespective of % tumour cells stained
3+ (positive)	Strong complete, basolateral or lateral membrane reactivity in ≥ 10% of tumour cells	Tumour cells with strong, basolateral or lateral membrane reactivity irrespective of percentage of tumour cells stained

*** Equivocal cases should be refluxed to ISH testing. Note: in the UK, NICE guidelines recommend that only 3+ cases are put forward for Trastuzumab treatment, see http://guidance.nice.org.uk/TA208**

Validation of Distributed Samples

IHC Validation of Distributed Samples

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

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

Table 2:

Section Label From slide Label End	Staining Pattern with IHC	HER2 status by ISH*
A	**3+**	Amplified
B	**2+**	Non-Amplified
C	**2+, 1+ or 0**	Non-Amplified or Amplified depending on the serial section received by the laboratory
D	**1+ or 0**	Non-Amplified

Assessment Procedure

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

Table 3: UK NEQAS Specific Membrane Scoring Criteria

UK NEQAS ICC & ISH uses an EQA specific scoring criteria when scoring the tissue sections, so as to provide participants additional technical feedback, which is illustrated below.

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) 2+/3+ or 3+/2+: Staining is slightly weaker than expected with membrane showing more 2+ compared to 3+ (2+/3+) or 3+ membrane staining is present but also showing 2+ staining (3+/2+).
1+	i) 0/1+: Staining is more towards the weaker end of 1+ staining but still acceptable. ii) 1+/0: Staining is more towards the weaker end of 1+ staining but still acceptable.
Neg.	0/1+ or 1+/0 = Staining starting to show very weak membrane staining

'**U' = Uninterpretable:** Assessors may also give a score of **'U'** which indicates that the cell lines / tissue sections were 'uninterpretable' due to the reasons set out below.
U/x = Borderline interpretable. A score of U/x e.g. U/3+ or U/2+ or U/1+ or U/0 indicates that the staining is just about readable and further improvements are required.
Any other membrane score other that assigned for each of the expected scores are deemed as unacceptable

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

Individual Assessor Score Out of 5 Marks	Combined Assessor Scores Out of 20 Marks	Overall Score Interpretation
0	**0**	Slide not submitted for assessment
1 & 2	**4-9 = Unacceptable**	**Overall the samples are of unacceptable quality for clinical interpretation and technical improvements need to be made**. Marks may have been deducted due to: • False positive / negative membrane staining • Excessive cytoplasmic staining • Excessive morphological damage • Excessive staining of normal glands
3	**10-12 = Borderline**	**Overall the samples are borderline interpretable indicating that technical improvements need to be made**. Marks may have been deducted due to: • Weaker / stronger than expected membrane staining • Some cytoplasmic staining • Morphological damage
4 & 5	**13-15 = Acceptable** **16-20 = Acceptable/ Excellent Standard**	**Overall the samples show acceptable membrane staining and are suitable for interpretation.**

Further comments are also provided on individual participant reports. Other factors which may lead to a low score include: excessive/ insufficient haematoxylin staining; poor quality of in-house control tissue; poor/inadequate choice of control tissue; poor/inadequate fixation; excessive antigen retrieval etc.

Introduction

Immunohistochemical testing of HER2 status is now routinely used in breast cancer testing and is recognised as a prognostic and predictive marker, generally used alongside breast hormonal receptor markers ER/PR. Patients who are HER2 positive (IHC 3+ and IHC 2+/ISH+) have been shown to benefit from Herceptin (Trastuzumab) therapy and increased overall survival rate. More recently the Trastuzumab for Gastric Cancer (ToGA) study, which investigated Trastuzumab in HER2 positive advanced gastric cancer (Bang et al., 2010) showed overall median survival of nearly 3 months. Similar to breast cancer, the ToGA trial showed an increased benefit from Trastuzumab treatment for patients showing 3+ IHC and IHC2+/FISH+ expression. The initial development of the HER2 scoring criteria was developed as a precursor to the ToGA trial (Hoffman et al., 2008) with the study group modifying the breast HER2 IHC scoring algorithm to compensate for the incomplete membrane staining and greater tumour heterogeneity seen in gastric cancers. A different scoring system was also established for resection and core biopsies as illustrated in table 1. A more recent article by Rüschoff et al., (2010) has validated the scoring procedure further with a detailed approach to 'stepwise' HER2 IHC scoring in gastric cancers. The article also compared the Ventana 4B5 assay with the HercepTest and indicated "a

tendency towards higher sensitivity for 4B5 detecting positive HER2 amplification" but "there was no difference between both test platforms with respect to the ISH-negative cases, with the one equivocal case testing negative at some sites and as positive at others". Rüschoff and colleagues also used both fluorescent (Dako PharmDX or PathVysion Vysis) and chromogen (Ventana DISH) to confirm their IHC findings.

Assessment Results

Features Of Acceptable Staining: (Figs 1-7)

- 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:

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

Additional Comment:

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

Pass Rates

The pass rates for the UK NEQAS distributed samples were much higher than the previous assessment Run (107), with 80% of lab receiving an acceptable pass, and a further 9% of labs achieving a borderline pass. 15 labs (11%) failed the assessment, and this was predominantly due to weaker membrane staining and therefore a lower HER2 expression level than expected. Other failed labs showed excessive cytoplasmic or inappropriate non-specific staining. Most of these problematic issues were seen on slides that had been stained using lab devised methods or using inappropriate antigen retrieval techniques, including the pressure cooker and microwave.

Of the 136 participants who submitted their NEQAS slides, 119 labs (88%) also submitted their in-house controls. The pass rates for these were slightly better with an acceptable pass rate of 75%, and a further 20% of labs achieving a borderline pass. Only 6 labs (5%) failed on their in house controls. This was mostly due to cytoplasmic and non-specific inappropriate staining, which again was most likely due to inappropriate or excessive antigen retrieval methods. Poor tissue quality was also another reason for scores being marked down. Several of the laboratories that received a borderline did not submit the required composite control material, consisting of a 3+, 2+ and 1+/0 expressing gastric/ breast control tumour sample, and therefore these labs were given a maximum score of 12/20, i.e. 3/5 from each assessor.

Methodologies

As we have seen with previous assessment runs, most labs are using the Ventana 4B5 standardised kit for their gastric HER2 testing. This assay was used by 79% (N=107) of participants and showed an acceptable pass rate of 83% on the UK NEQAS distributed sections. 8 labs are using the standardised Dako HercepTest, which showed a pass rate of 50%. The rest of the labs that participated in the assessment are using lab devised methods. Of these 11 labs, 56% achieved an acceptable level of staining.

Control Tissue and Recommendations

While UK NEQAS recommends that gastric tissue samples are used as an ideal in–house control for gastric HER2 testing, laboratories were given the choice to submit either gastric or breast control material for their in-house control assessment. Laboratories were therefore not penalised if they submitted breast carcinomas.

Whether gastric or breast tissue is used, laboratories should still submit in-house controls showing 3+, 2+ and 1+/0 levels of membrane expression. Due to the heterogenic nature of many gastric tumours, it is acceptable for labs to submit 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.

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

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

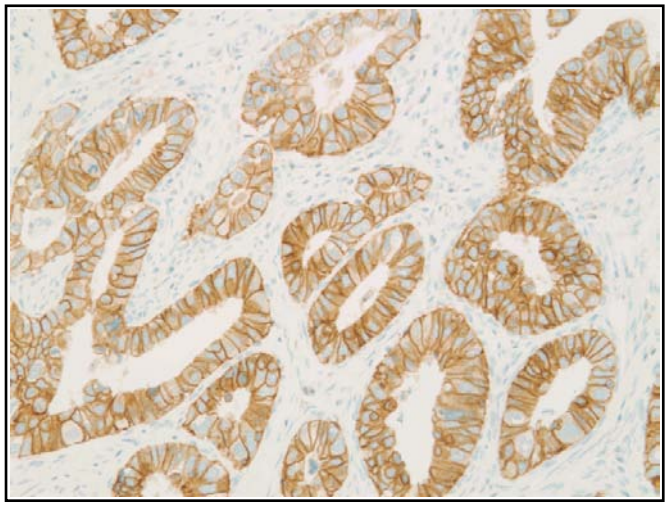


Fig 1. Optimal staining in the UK NEQAS distributed 3+ gastric tumours 'sample A': The membrane staining is strong and completely circumferential with minimal cytoplasmic staining. Stained with the Ventana 4B5 on the ULTRA and 36 minutes pre-treatment with CC1 buffer.

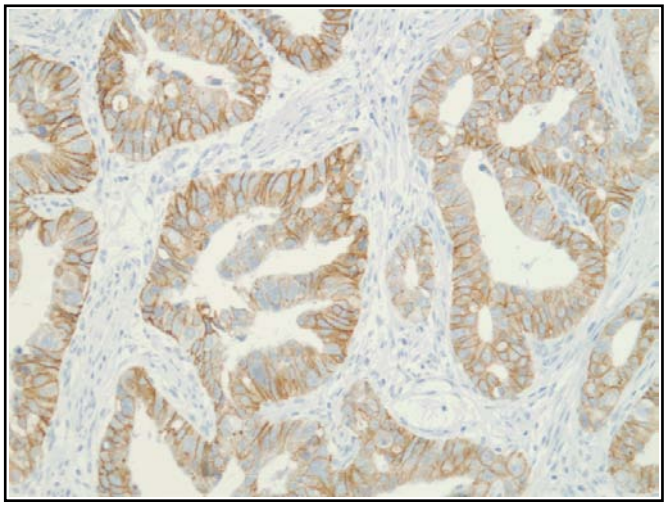


Fig 2. Good demonstration of HER2 staining in the gastric 3+ tumour 'sample A'. The example shows strong complete membranous staining in over 10% of the tumour cells. Section stained with the HercepTest on the Autostainer machine with antigen retrieval in the PT Link.

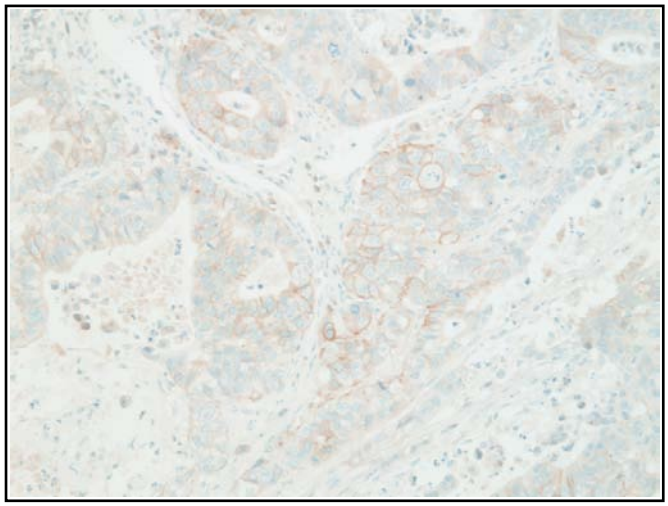


Fig 3. Optimal staining in the UK NEQAS distributed 2+ gastric tumour 'sample B': The majority of cells show weak to moderate complete membrane staining. Same protocol as Fig 1.

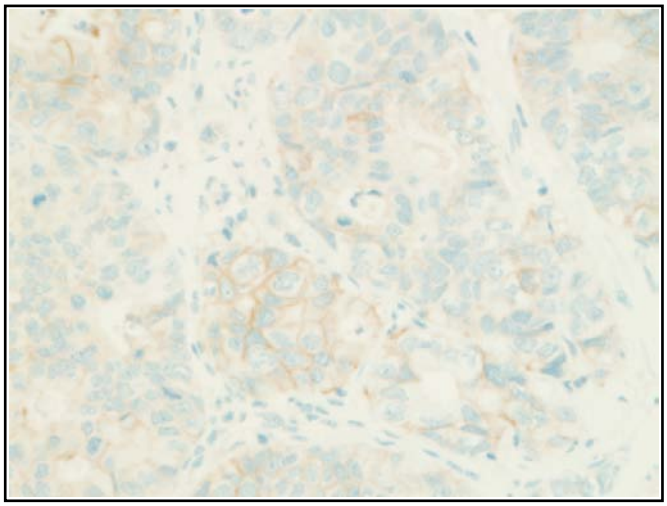


Fig 4. Good demonstration of HER2 in the 2+ gastric tumour 'sample B', showing complete membrane staining which is less intensive than that seen with the 3+ cell line. Same protocol as Fig 2.

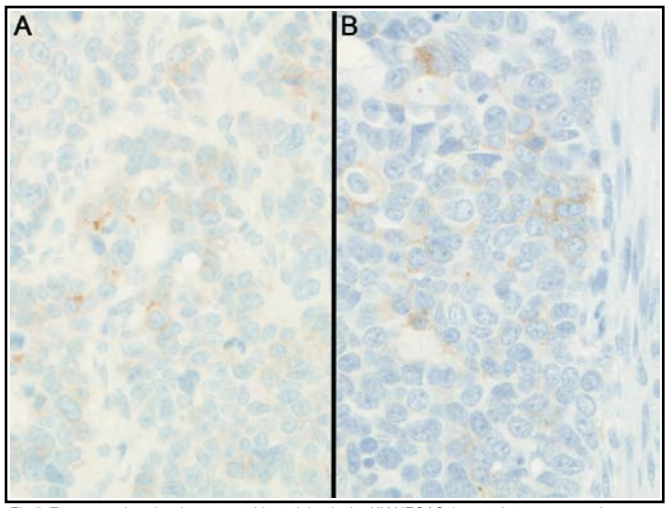


Fig 5. Two examples showing acceptable staining in the UK NEQAS 1+ gastric tumour sample C: The membrane staining is incomplete and barely perceptible. (A) Stained with the Ventana 4B5 and (B) with the Dako HercepTest.

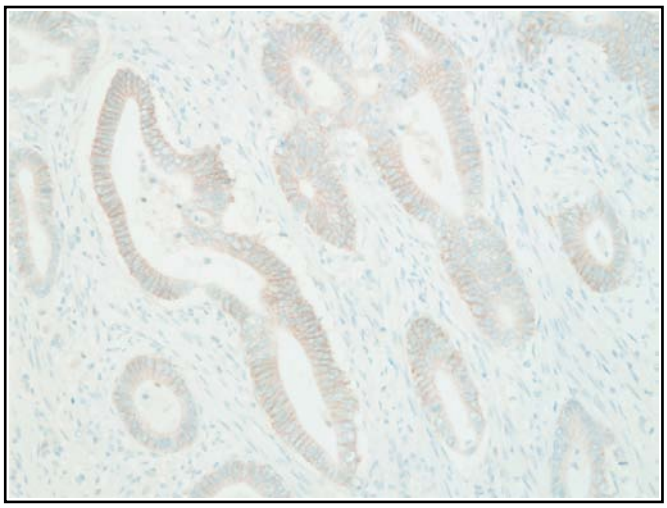


Fig 6. Optimal demonstration of HER2 staining in the UK NEQAS Gold standard 2+ gastric tumour 'sample C' (block 2). Stained with the Ventana 4B5 on the Benchmark XT with CC1 mild antigen retrieval.

Selected Images showing Optimal and Sub-optimal Immunostaining

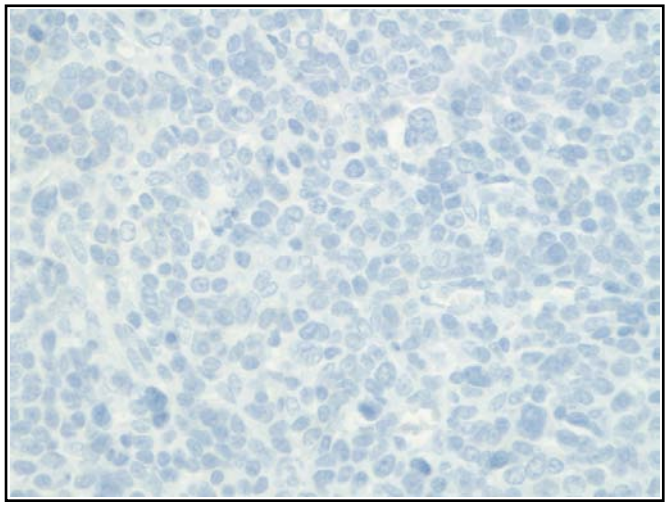


Fig 7. As expected no staining is demonstrated in the UK NEQAS negative 'sample D'. Staining carried out using the Dako HercepTest.

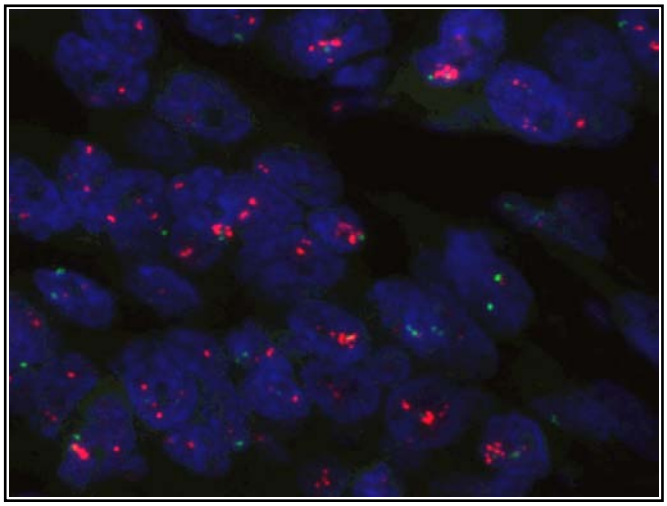


Fig 8. FISH staining on the UK NEQAS IHC 3+ 'sample A'. As expected, the sample shows amplification of HER2. Section was stained by Dako using their IQ FISH PharmDX kit.

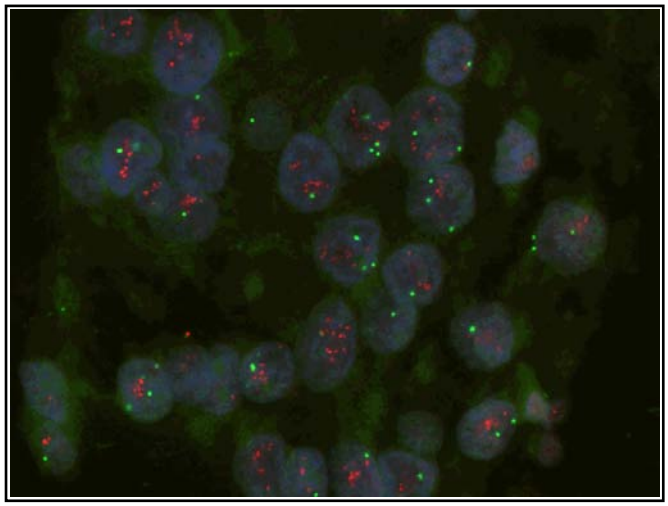


Fig 9. FISH staining on the UK NEQAS IHC HER2 2+ 'sample B'. The ISH counts confirmed that the sample had HER2 amplification. Section stained by Dako with the IQ FISH PharmDX kit.

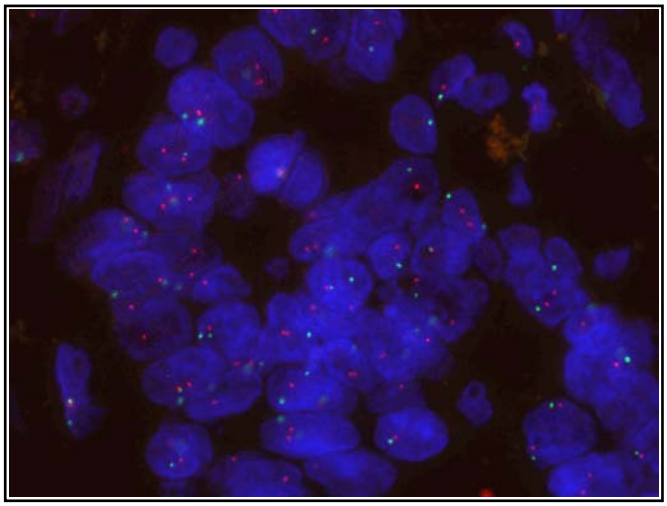


Fig 10. The UK NEQAS 'sample C' (block 1) stained by FISH: The image and ISH counts show that this sample was not amplification for HER2. Section stained by Dako with the IQ FISH PharmDX kit.

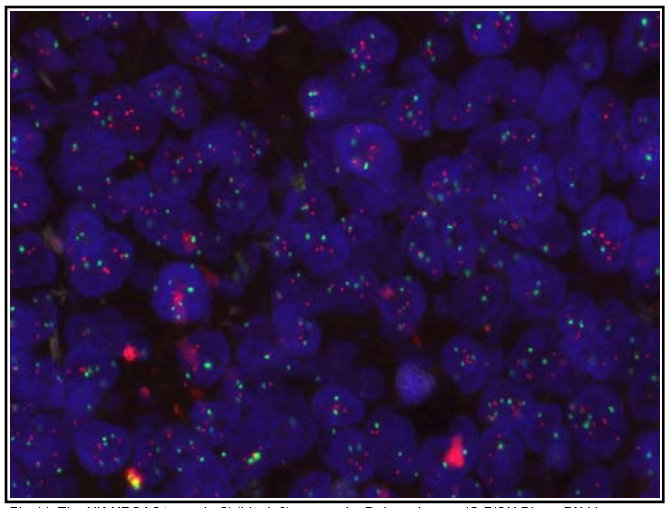


Fig 11. The UK NEQAS 'sample C' (block 2) stained by Dako using the IQ FISH PharmDX kit. This block/sample showed amplification of HER2. This sample showed 1+ or 2+ IHC expression depending on the serial section received.

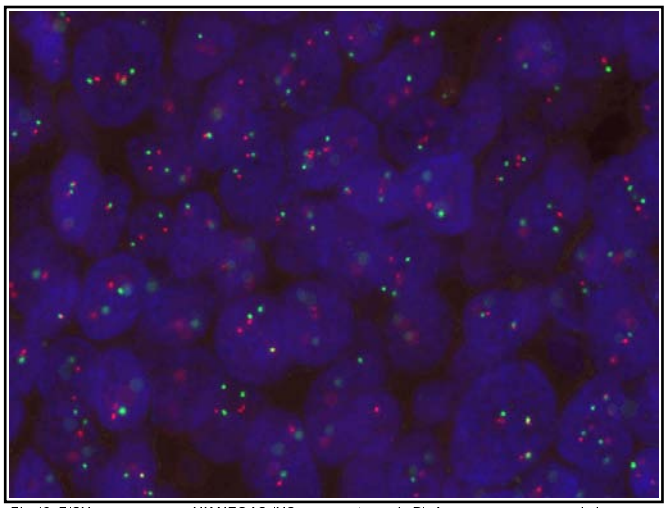
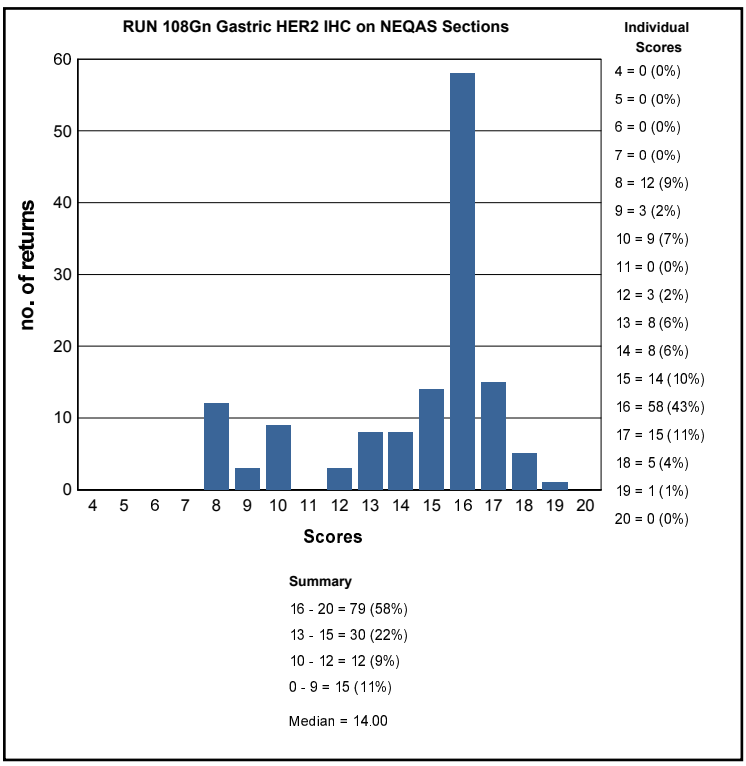
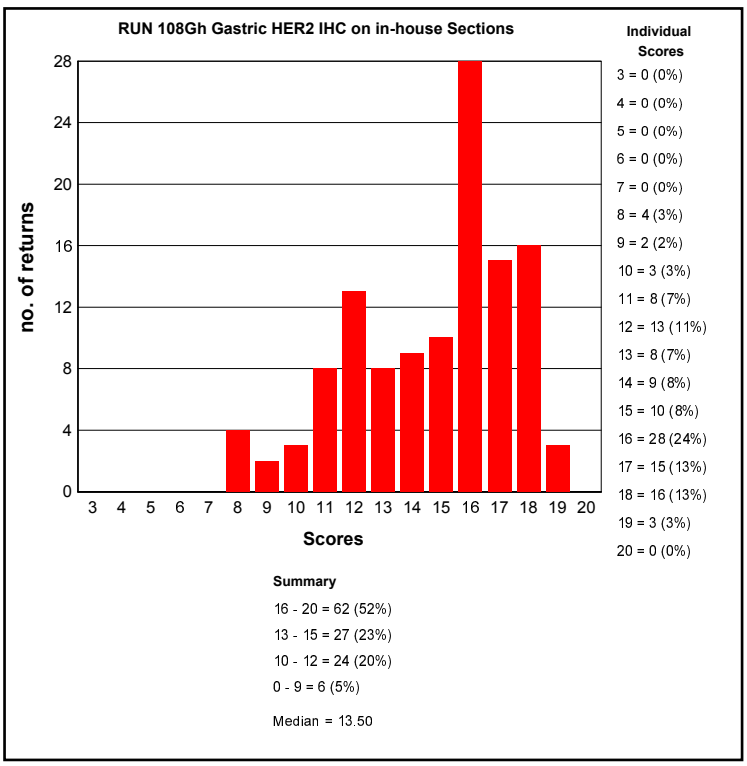


Fig 12. FISH staining on the UK NEQAS IHC negative 'sample D'. As expected, the sample is not amplified for HER2. Section was stained by Dako using their IQ FISH PharmDX kit.

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: 108

Primary Antibody	N	%
Biocare CME 342 A,B (EP1045Y)	1	0
Dako A0485 C-erB-2 (poly)	5	60
Dako HercepTest K5204 (poly)	1	0
Dako HercepTest K5206 (poly)	1	100
Dako HercepTest K5207 (poly)	1	0
Dako Link HercepTest SK001 (poly)	5	100
Labvision / Neomarkers RM-9103 (SP3)	1	100
Other	9	67
Ventana Confirm 790-4493 (4B5)	28	96
Ventana Confirm 790/800-2996 (4B5)	36	81
Ventana Confirm 790/800-2996 (4B5)	1	100
Ventana pathway 760-2694 (CB11)	1	0
Ventana Pathway 790-100 (4B5)	5	60
Ventana Pathway 790-2991 (4B5)	36	81

Gastric HER2 ICC Run: 108

Automation	N	%
Dako Autostainer	1	100
Dako Autostainer Link 48	5	80
Dako Autostainer plus	2	50
Dako Autostainer Plus Link	2	100
Leica Bond Max	4	25
None (Manual)	7	43
Ventana Benchmark GX	6	83
Ventana Benchmark ULTRA	27	74
Ventana Benchmark XT	79	89

Gastric HER2 ICC Run: 108

Heat Mediated Retrieval	N	%
Dako PTLink	8	75
Lab vision PT Module	1	100
Leica ER1 25 mins	2	0
Leica ER2 20 mins	1	0
Microwave	1	100
Other	1	100
Pressure Cooker	6	33
Ventana CC1 16mins	2	50
Ventana CC1 24mins	3	100
Ventana CC1 32mins	10	80
Ventana CC1 36mins	11	64
Ventana CC1 52mins	2	50
Ventana CC1 56mins	1	0
Ventana CC1 64mins	4	100
Ventana CC1 72mins	1	100
Ventana CC1 8mins	1	100
Ventana CC1 mild	53	87
Ventana CC1 standard	13	100

Gastric HER2 ICC Run: 108

Detection	N	%
AS PER KIT	8	63
Dako EnVision FLEX (K8000/10)	2	50
Dako Envision HRP/DAB (K5007)	1	100
Dako HerCep Test Autor (SK001)	3	100
Dako REAL HRP/DAB (K5001)	1	0
Leica Bond Polymer Refine (DS9800)	3	33
None	1	100
Other	5	40
Ventana iView system (760-091)	4	100
Ventana OptiView Kit (760-700)	2	100
Ventana UltraView Kit (760-500)	97	87

Gastric HER2 ICC Run: 108

Enzyme Retrieval	N	%
AS PER KIT	1	100
NOT APPLICABLE	54	76
Ventana Protease 1 (760-2018)	1	100

Gastric HER2 ICC Run: 108

Chromogen	N	%
AS PER KIT	10	70
DAKO DAB+	2	50
Dako DAB+ REAL Detection (K5001)	1	0
Dako FLEX DAB	5	80
Dako REAL EnVision K5007 DAB	1	100
Leica Bond Polymer Refine kit (DS9800)	4	25
Other	4	50
Ventana iview	3	100
Ventana Ultraview DAB	102	85

BEST METHODS

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

Gastric HER2 IHC - Method 1

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

Primary Antibody:	Dako Link HercepTest SK001 (poly) , 30 Mins, 25 ºC Prediluted
Automation:	Dako Autostainer Plus Link
Method:	Dako FLEX kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: Dako citrate
EAR:	
Chromogen:	Dako FLEX DAB, 25 ºC., Time 1: 10 Mins, Time 2: 10 Mins
Detection:	Dako HerCep Test Autor (SK001) , 30 Mins, 25 ºC Prediluted

Gastric HER2 IHC - Method 2

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

Primary Antibody:	Ventana Pathway 790-2991 (4B5)
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 36mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	AS PER KIT

Gastric HER2 IHC - Method 3

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

Primary Antibody:	Ventana Confirm 790/800-2996 (4B5) , 37 ºC Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.6
HMAR:	Ventana CC1 32mins, PH: 8.5
EAR:	
Chromogen:	Ventana Ultraview DAB, 37 ºC., Time 1: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC Prediluted

David Blythe

	Gold Standard	Second Antibody
Antigens Assessed:	CD5	CD4
Tissue Sections circulated:	Reactive tonsil & lymph node (MCL)	Reactive lymph node, Hodgkin's & NHL
Number of Registered Participants:	211	
Number of Participants This Run	207 (98%)	

Introduction

Gold Standard: CD5

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

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

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

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

Features of Optimal Immunostaining (Figs 1, 2 & 4)
- Strong, predominantly membranous staining of the majority of the T-cells in the inter-follicular area of the tonsil
- Moderate staining of the scattered B-cells in the mantle zone of the tonsil
- Strong, predominantly membranous staining of the tumour cells of the MCL
- Darker staining of any normal scattered T-cells within the MCL
- Clean background

Features of Suboptimal Immunostaining (Figs 3, 5 & 6)
- Weak staining of cells expected to stain, especially T-cells in the inter-follicular areas.
- Poor localisation of staining to cell membranes.
- Disruption of normal cellular detail, due to excessive or incorrect antigen retrieval.

References

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

Second Antigen: CD4

The CD4 molecule (T4) is a single chain transmembrane glycoprotein with a molecular weight of 59 kD. It is expressed on a T cell subset (helper/inducer) representing 45 percent of peripheral blood lymphocytes and at a lower level on monocytes and dendritic cells. CD4 recognises the MHC class II antigen. Most cases of cutaneous T cell lymphoma, including mycosis fungoides, express the CD4 antigen. HTLV-1 associated adult T cell leukaemia/lymphoma is also generally CD4 positive.

Features of Optimal Immunostaining (Figs 7, 8 & 12)
- Strong and crisp membrane staining of T cells in the tonsil
- Weak to moderate staining of the macrophages in the germinal centre of the tonsil
- Minimal background staining

Features of Suboptimal Immunostaining (Figs 9, 10 & 11)
- Very little, weak or uneven staining of T-cells
- Diffuse staining
- Background or inappropriate non-specific staining

References

1. Hodak E, David M, Maron L et al. CD4/CD8 double-negative epidermotropic cutaneous T-cell lymphoma: an immunohistochemical variant of mycosis fungoides. J. Am. Acad. Dermatol. 2006; 55; 276–284.
2. Harvell JD, Nowfar-Rad M, Sundram U. An immunohistochemical study of CD4, CD8, TIA-1 and CD56 subsets in inflammatory skin disease. J. Cutan. Pathol. 2003; 30; 108–113
3. Rakozy CK, Mohamed AN, Vo TD *et al.* CD56+/CD4+ lymphomas and leukaemias are morphologically, immunophenotypically, cytogenetically and clinically diverse. *Am. J. Clin. Pathol.* 2001*;* **116***;* 168–176
4. Macon WR, Salhany KE. T-cell subset analysis of peripheral T-cell lymphomas by paraffin section immunohistology and correlation of CD4/CD8 results with flow cytometry. Am J Clin Pathol. 1998 May; 109(5):610-7.

Assessment Summary:

At the start of the run there were 211 registered labs (UK : N=108, Non-UK: N=103). Four labs did not submit slides for any of the antigens (CD5 or CD4). 24 participants did not stock/submit any slides for CD4, and several labs only returned their NEQAS slides, with no corresponding in-house controls, resulting in 60 non-submitted slides, and so left a total of 796 slides for assessment.

The overall pass rate (N=655) was 82%, with 4% of slides assessed as borderline (N=108) and 4% (N=33) failing.

CD5 (L & M):

Overall the results were very good - 73% of participants scoring between 16-20 on the NEQAS sections (**L**) and 75% achieving 16-20 on the in-house material (**M**). All antibody clones used demonstrated both the mantle cell tumour cells and both the reactive T-cells and mantle zone B-cells in the reactive tonsil.

No one single automated platform outscored the others, as very good staining was seen on all the different systems. However, sub-optimal staining could also be seen on each of the major platforms. Variation between participants using similar machines and even similar staining protocols still exist.

David Blythe

Antigen retrieval protocols remain a key factor in the demonstration of the mantle zone B-cells in the tonsil. Insufficient retrieval resulted in only the T-cells (in the tonsil) being well demonstrated. The use of the water bath failed to give good B-cell demonstration and localisation was poor. The most frequently used assessor comment related to over retrieval of the sections, surprisingly this was for both NEQAS and in-house material. The favoured retrieval for Leica bond users was ER2 for 20 / 30 mins and for the Ventana users CC1 standard - this was the case for both CD5 and CD4 assessments.

The in-house sections (**M**) mainly consisted of reactive tonsil, some of which also contained mantle cell lymphoma - these were ideal assessment material. Some participants just used tumour as their control material and this made assessment of the mantle zone B-cells difficult to assess.

CD4 (N & P):

This was the first time we have assessed the antigen and results were again overall very good. A composite lymphoid block was used as the NEQAS material (**N**), on the recommendation of the assessing pathologist, we restricted the assessment to the reactive lymph node (this was the central piece of tissue on all the sections), it was felt we could most accurately assess the staining quality on the cells expected to stain.

Surprisingly the number of laboratories scoring between 16-20 was much higher on the NEQAS sections than on their own in-house material (**P**) (70% compared to 49%).

With regards to antibody clone choice the best performing clone for the majority of participants was 4B12, although some users of clones 1F6 and SP35 also scored highly.

The use of the water bath for the antigen retrieval stage cannot be recommended for this antigen (and many lymphoid antigens), users using this method showed poor antigen demonstration and localisation. As with the CD5 the most frequent assessor comment related to over retrieval of the tissue section. The use of amplification steps often resulted in granular staining.

It was encouraging to see that the majority of participants used reactive tonsil as their control material

Selected Images showing Optimal and Sub-optimal Immunostaining

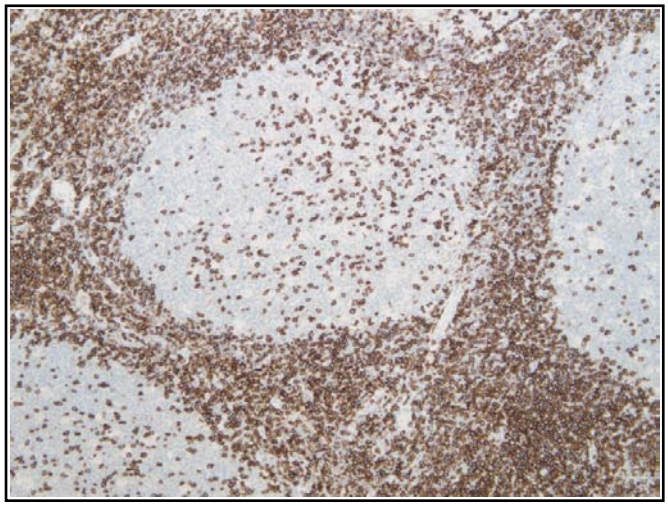


Fig 1. Optimal demonstration of CD5 in the UK NEQAS reactive tonsil. The section shows strong predominantly membranous staining of T-cells within the inter-follicular areas and some T-cells in the germinal centre. Section was stained with the Novacastra 4C7 antibody, 1:100, on the Bond III with ER2 antigen retrieval for 30 minutes.

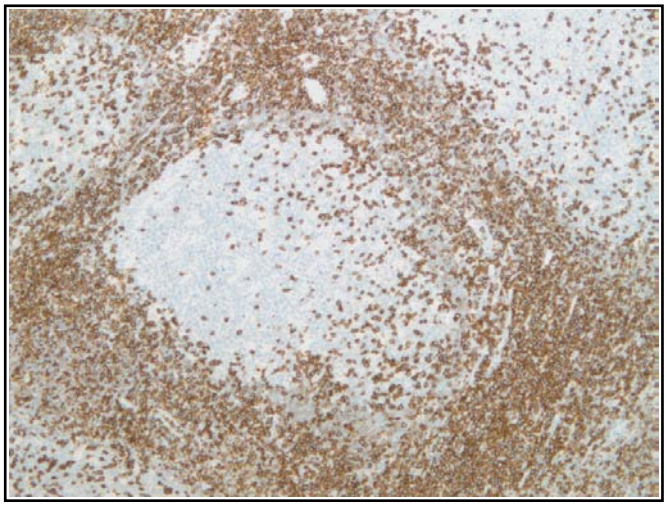


Fig 2. Good example of CD5 staining in the UK NEQAS reactive tonsil section. The image shows strong staining of the T-cells around the T-zone and some T-cells within the germinal centre. Section stained with the Dako 4C7 antibody, 1:50, on the Dako Autostainer with pretreatment in the PT link for 20 minutes with high pH buffer solution.

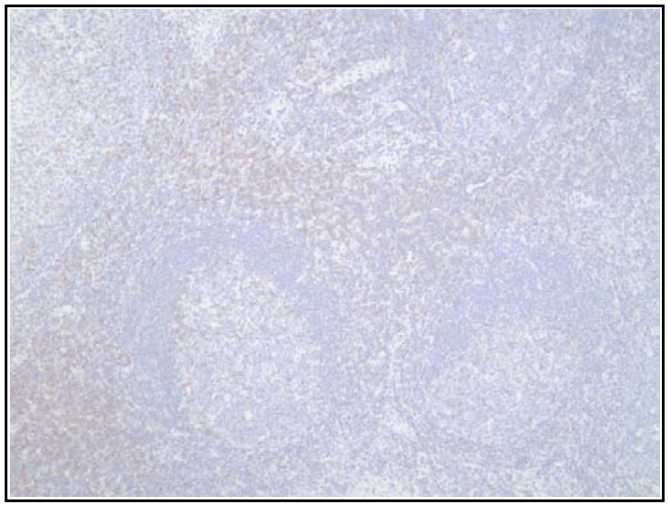


Fig 3. Suboptimal demonstration of CD5 in the UK NEQAS reactive tonsil. The reaction is weak with less cells staining than expected (compare to Figs 1&2). The section was stained using the Dako SP19 antibody with antigen retrieval in the Dako Pascal and manually stained with the Dako Advance detection kit.

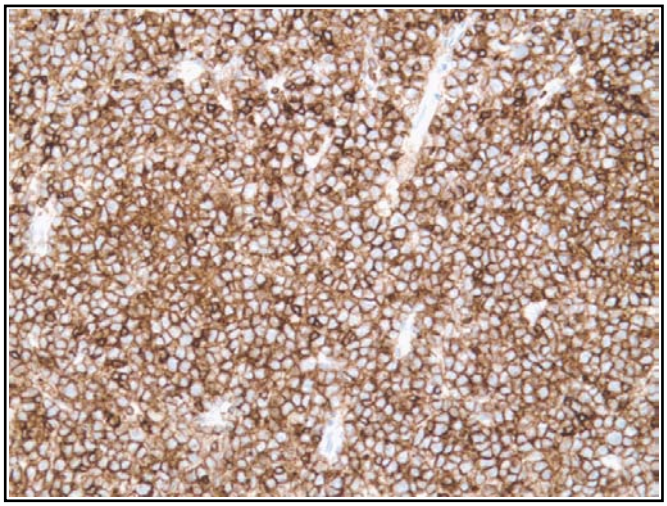


Fig 4. Optimal demonstration of CD5 in the UK NEQAS distributed Mantle Cell Lymphoma (MCL) tissue. The example shows strong membranous staining in the neoplastic and scattered normal T-cells (same protocol as Fig 1).

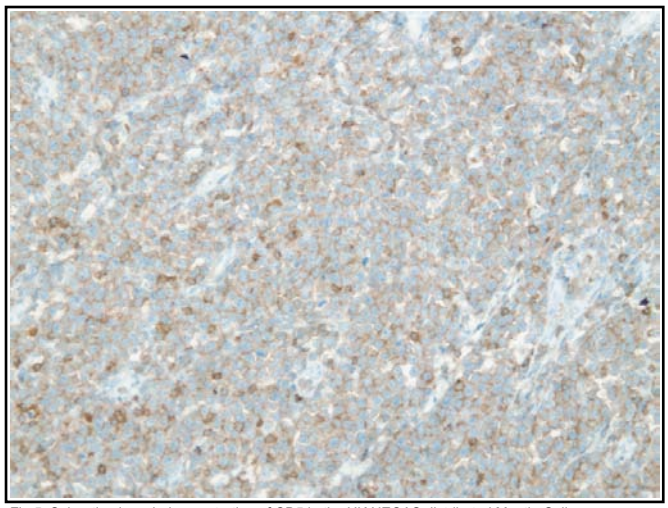


Fig 5. Suboptimal weak demonstration of CD5 in the UK NEQAS distributed Mantle Cell Lymphoma. The section was stained with the Dako 4C7 pre-diluted antibody, with pressure cooker antigen retrieval and visualized with the BioGenex SS Polymer-HRP detection kit.

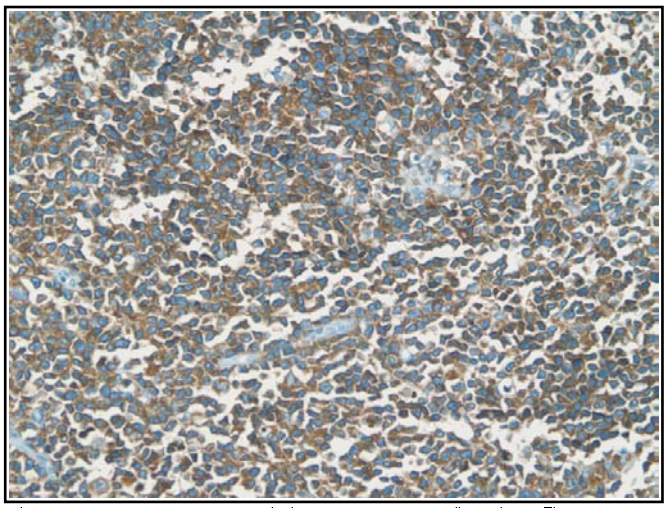


Fig 6. Suboptimal demonstration of CD5 in the UK NEQAS Mantle Cell Lymphoma. The example shows morphology damage caused by excessive antigen retrieval. Section stained with the Labvision SP19 antibody, 1:25, on the Menarini Intellipath Autostainer, waterbath pretreatment and detection with the Dako REAL kit.

Selected Images showing Optimal and Sub-optimal Immunostaining

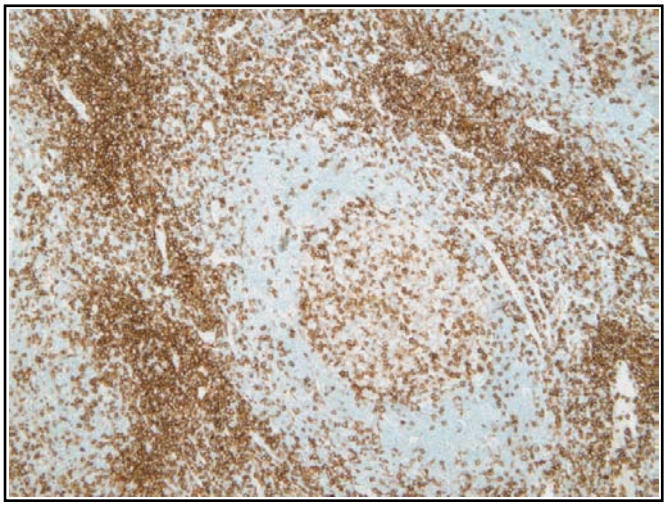


Fig 7. Optimal demonstration of CD4 in the UK NEQAS reactive lymph node. The majority of T-cells show strong membranous staining, while the macrophages in the germinal centre show a weak to moderate reaction. Section stained with the Novocastra 1F6 antibody, 1:25, on the BondMax with ER2 antigen retrieval for 20 minutes.

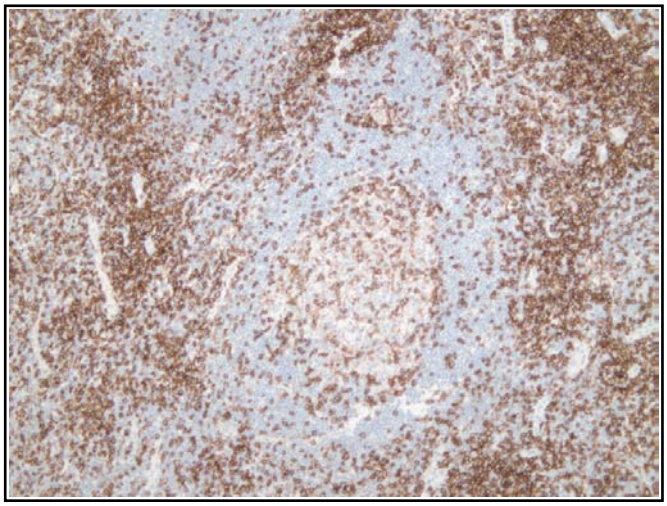


Fig 8. Good staining of CD4 in the UK NEQAS reactive lymph node, showing strong staining of T-cells and moderate staining of macrophages. The section was stained with the Vector Laboratories 4C7 antibody, 1:25, on the Ventana Benchmark XT with CC1 mild antigen retrieval.

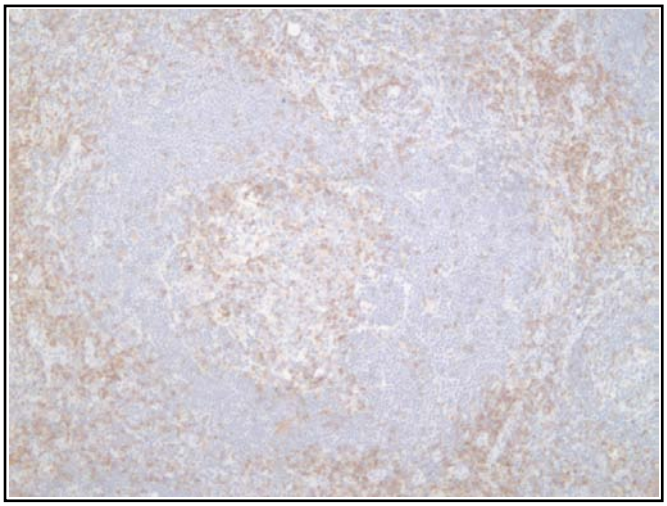


Fig 9. Suboptimal demonstration of CD4 in the UK NEQAS distributed reactive lymph node. The example shows weak staining (compare to Figs 7&8). Section stained with the Novocastra 4C7 antibody, 1:50, on the Menarini Intellipath with antigen retrieval carried out in the Biocare decloaking chamber.

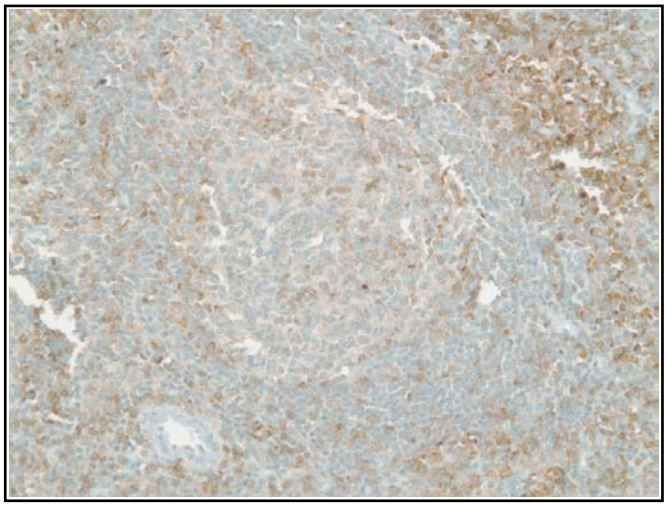


Fig 10. Poor demonstration of CD4 in the UK NEQAS ICC reactive lymph node. The staining is weak and diffuse. Section stained with the Novocastra 4C7 antibody, 1:50, on the Ventana ULTRA with CC1 antigen retrieval for 40 minutes.

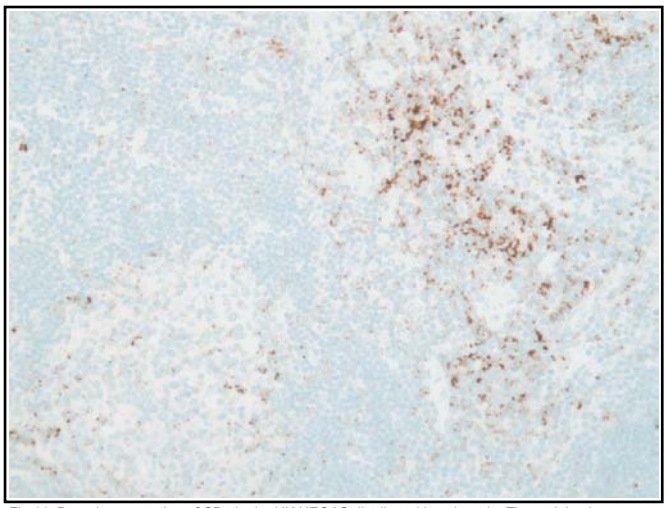


Fig 11. Poor demonstration of CD4 in the UK NEQAS distributed lymph node. The staining is weak and granular, and many of the T-cells expected to stain are not demonstrated. This is most likely due to the antibody titre being too dilute. Section stained with the Novocastra antibody, 1:100 on the Ventana ULTRA with a CC1 standard protocol

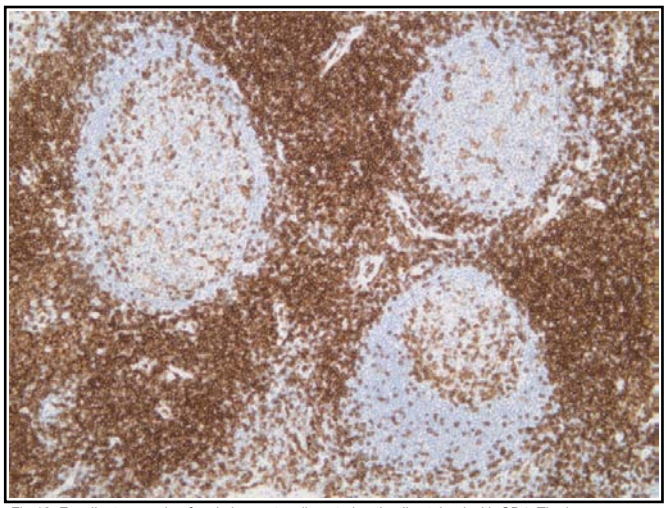
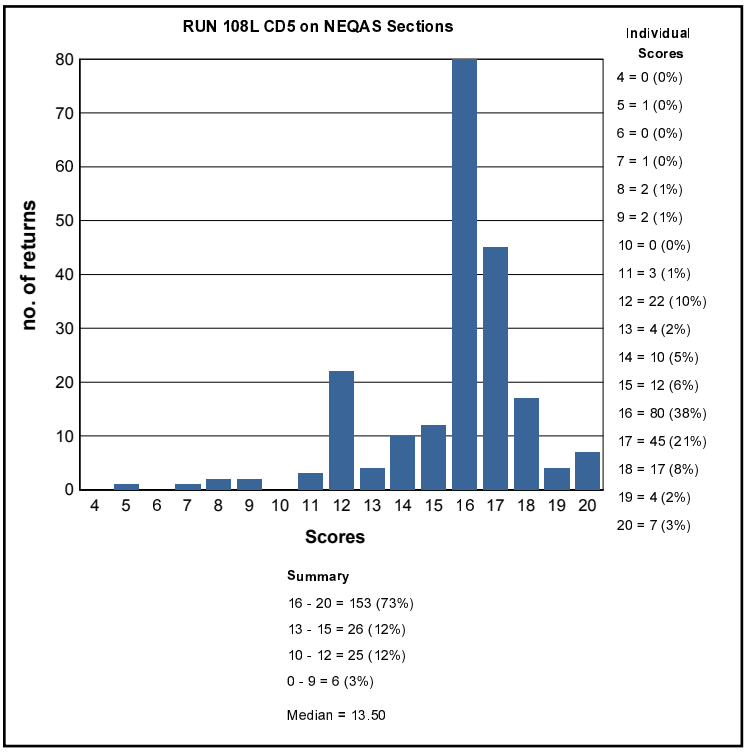
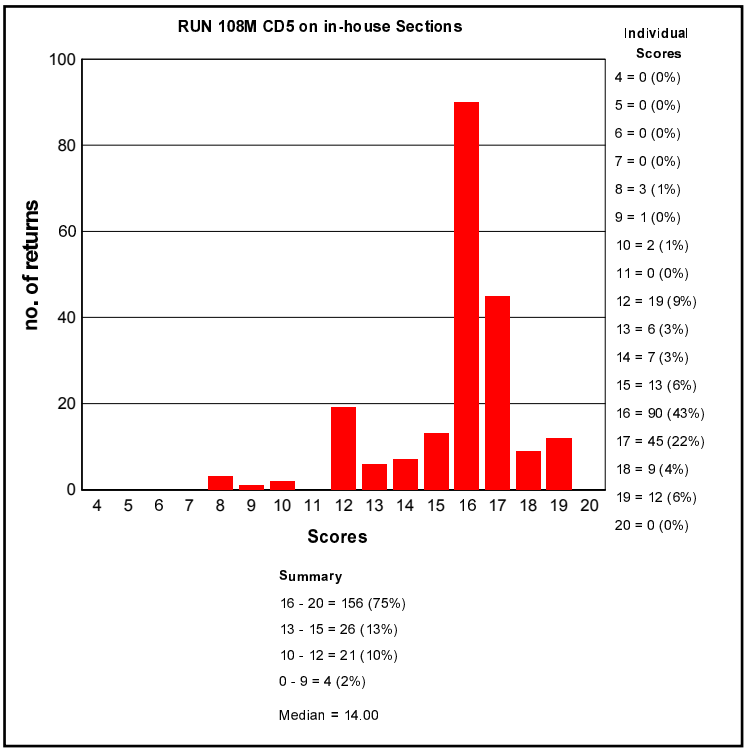
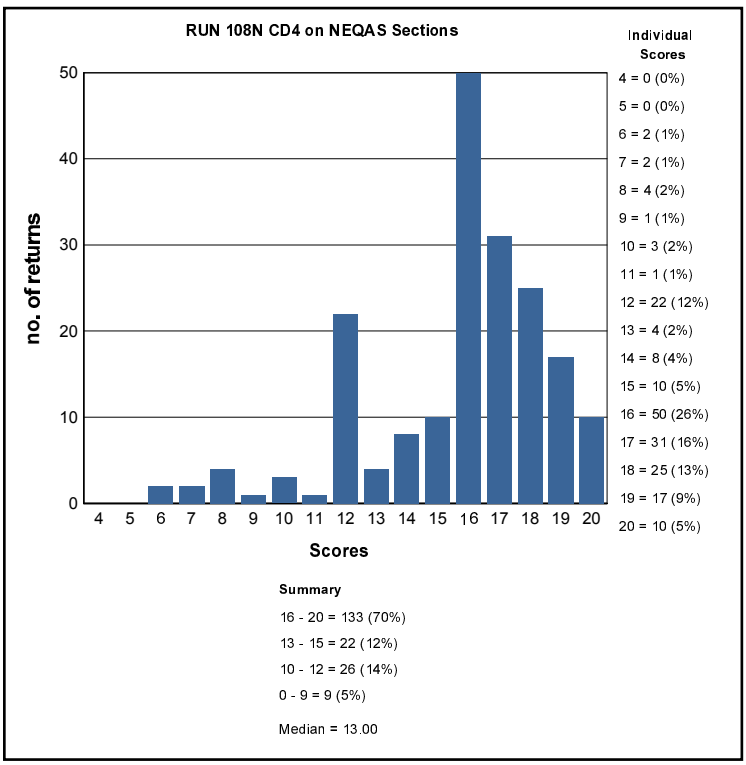


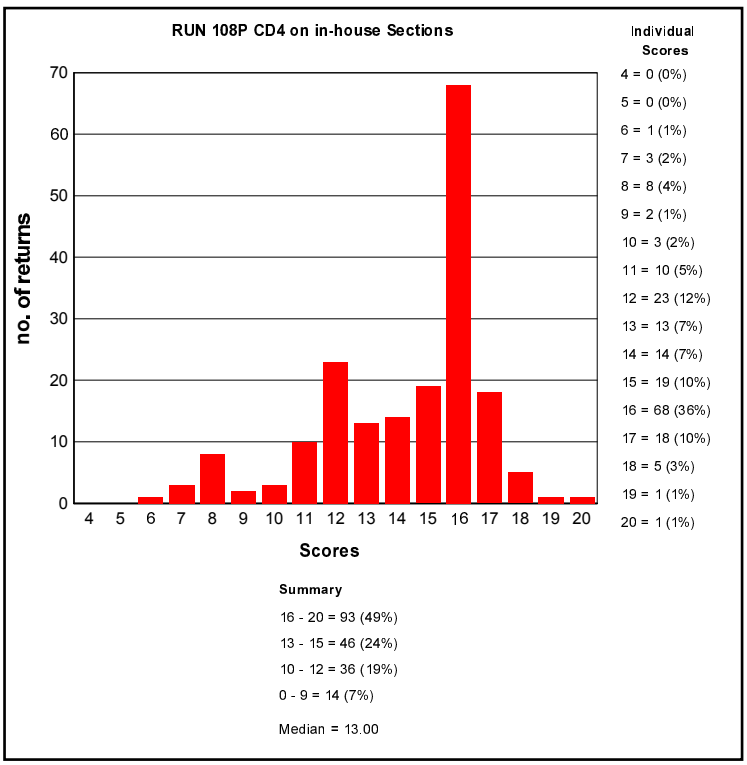
Fig 12. Excellent example of an in house tonsil control optimally stained with CD4. The image shows strong membranous staining in the T-cells and moderate staining in the germinal centre macrophages. The section was stained using the Cell Marque SP35 antibody, 1:30, on the Leica Bond III autostainer with ER2 antigen retrieval for 20 minutes.

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: 108

Primary Antibody : CD5

Antibody Details	N	%
Biogenex AM430-5M (4C7)	1	100
Biogenex MU430-UC (4C7)	1	100
Cell Marque 205M/S-x (4C7)	3	33
Cell Marque 760-4280 (SP19)	2	0
Dako IR081 (SP19)	1	100
Dako IR082 RTU FLEX Link (4C7)	10	80
Dako IS082 RTU Auto Plus (4C7)	2	100
Dako M3633 (SP19)	1	0
Dako M3641 (4C7)	15	87
Gennova AP10123C	1	0
Labvision MS-393-S (4C7)	1	100
Labvision RM-9119 (SP19)	3	33
Leica Bond RTU PA0168 (4C7)	11	91
NeoMarkers MS-393-S (4C7)	1	100
Novocastra Bond PA0168 (4C7)	7	100
Novocastra NCL-CD5-4C7 (4C7)	36	83
Novocastra NCL-L- CD5-4C7 (4C7)	63	86
Novocastra RTU-CD5-4C7 (4C7)	1	100
Other	2	50
Vector VP-C322 (4C7)	8	100
Ventana 760-4280 (SP19)	7	86
Ventana CD5 790-4451 (SP19)	32	97

Lymphoma Run: 108

Primary Antibody : CD4

Antibody Details	N	%
Biogenex A403B	1	100
Cell Marque 104R (SP35)	7	57
Dako M0716	8	88
Dako M7310 (4B12)	7	100
Dako RTU FLEX IR649 (4B12)	7	71
Epitomics AC-0173 (EP204)	1	100
Leica Bond RTU PA0427 (4B12)	3	100
Neomarkers MS 392 (Clone 1F6)	1	100
Novocastra NCL-CD4 368 (Clone 4B12)	54	83
Novocastra NCL-CD4-1F6 (1F6)	11	55
Other	41	73
Serotec MCAP547 (Clone 1F6)	1	100
Thermo MS-1528 R/S (Ab-8)	2	0
Vector VP C318 (Clone 1F6)	1	100
Ventana 250 2712 (Clone 1F6)	4	75
Ventana Confirm 790-4423 (SP35)	28	100

Lymphoma Run: 108

Heat Mediated Retrieval	CD4 N	CD4 %	CD5 N	CD5 %
Biocare Decloaking Chamber	3	67	3	67
Dako Omnis	2	0	2	50
Dako Pascal	0	0	1	0
Dako PTLink	28	86	33	91
Lab vision PT Module	1	100	1	100
Leica ER1 10 mins	1	0	2	0
Leica ER1 20 mins	2	100	2	100
Leica ER1 30 mins	2	50	6	83
Leica ER2 20 mins	32	97	44	98
Leica ER2 30 mins	22	91	13	92
Leica ER2 40 mins	3	100	0	0
Microwave	3	67	3	67
Other	0	0	1	100
Pressure Cooker	2	50	2	0
Steamer	0	0	1	100
Ventana CC1 16mins	0	0	1	100
Ventana CC1 24mins	4	100	2	50
Ventana CC1 32mins	7	100	12	75
Ventana CC1 36mins	2	0	3	67
Ventana CC1 40mins	4	100	7	100
Ventana CC1 48mins	2	50	2	100
Ventana CC1 52mins	4	50	4	50
Ventana CC1 56mins	2	100	3	100
Ventana CC1 64mins	16	56	14	86
Ventana CC1 76mins	3	33	1	100
Ventana CC1 88mins	1	100	1	100
Ventana CC1 extended	1	100	2	100
Ventana CC1 mild	5	100	5	80
Ventana CC1 standard	23	74	34	88
Ventana CC2 32mins	0	0	1	100
Water bath 95-98 OC	1	0	2	0

Lymphoma Run: 108

Enzyme Mediated Retrieval	CD4 N	CD4 %	CD5 N	CD5 %
AS PER KIT	0	0	1	100
Dako Protease (S2019)	1	0	0	0
NOT APPLICABLE	64	75	110	86
Sigma chymotrypsin (C4129)	1	100	0	0

Lymphoma Run: 108	CD4		CD5	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	12	75	10	100
BioGenex SS Polymer (QD 420-YIKE)	1	100	0	0
BioGenex SS Polymer (QD 430-XAKE)	0	0	1	0
Dako ADVANCE HRP (K4068/9)	0	0	1	0
Dako EnVision FLEX (K8000/10)	2	50	7	57
Dako EnVision FLEX+ (K8002/12)	19	89	21	95
Dako Envision HRP/DAB (K5007)	1	100	1	100
Dako Envision+ HRP mouse K4004/5/6/7	2	50	0	0
Dako REAL HRP/DAB (K5001)	1	0	1	0
Leica Bond Polymer Define (DS9713)	2	50	0	0
Leica Bond Polymer Refine (DS9800)	51	94	62	92
MenaPath X-Cell Plus (MP-XCP)	0	0	3	67
None	0	0	3	67
Other	7	43	6	67
Vector ImmPRESS Universal (MP-7500)	1	100	1	100
Ventana iView system (760-091)	2	100	5	40
Ventana OptiView Kit (760-700)	27	78	30	90
Ventana UltraView Kit (760-500)	38	74	56	84

Lymphoma Run: 108	CD4		CD5	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	2	100	2	50
Dako Autostainer	1	100	1	100
Dako Autostainer Link 48	29	86	30	87
Dako Autostainer plus	1	100	1	100
Dako Autostainer Plus Link	1	100	4	100
Dako Omnis	2	0	2	50
LabVision Autostainer	1	100	2	100
Leica Bond Max	23	87	28	86
Leica Bond-III	38	95	39	97
Menarini - Intellipath FLX	3	33	4	50
None (Manual)	1	0	1	0
Other	1	100	2	50
Shandon Sequenza	1	0	1	0
Ventana Benchmark GX	2	100	3	100
Ventana Benchmark ULTRA	37	68	47	81
Ventana Benchmark XT	34	76	42	86

Lymphoma Run: 108	CD4		CD5	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	25	84	19	95
BioGenex Liquid DAB (HK153-5K)	1	100	1	0
BioGenex liquid DBA (HK-124-7K)	1	100	1	100
Dako DAB K3468	1	100	1	0
DAKO DAB+	1	0	0	0
Dako DAB+ Liquid (K3468)	0	0	1	100
Dako DAB+ REAL Detection (K5001)	0	0	1	100
Dako EnVision Plus kits	4	100	3	100
Dako FLEX DAB	20	75	27	85
Dako REAL EnVision K5007 DAB	1	100	2	100
Dako REAL K5001 DAB	2	50	1	0
Leica Bond Polymer Refine kit (DS9800)	51	94	61	92
menapath xcell kit DAB (MP-860)	2	50	3	67
Other	9	78	10	80
Sigma DAB (D5905)	1	0	1	0
Ventana DAB	16	75	13	85
Ventana Enhanced Alk. Phos. Red Detection Kit	1	100	0	0
Ventana iview	2	50	4	50
Ventana Ultraview DAB	40	70	60	83

BEST METHODS - Gold Standard Antibody

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

CD5 - Method 1

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

Primary Antibody: Novocastra NCL-L- CD5-4C7 (4C7) , 20 Mins Dilution 1: 100
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 30 mins
EAR: NOT APPLICABLE
Chromogen: Leica Bond Polymer Refine kit (DS9800), Time 1: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins

CD5 - Method 2

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

Primary Antibody: Dako M3641 (4C7) , 30 Mins, 21 ºC Dilution 1: 50
Automation: Dako Autostainer Link 48
Method: Dako FLEX kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako PTLink, Buffer: DAKO HIGH PH TARGET RETRIEVAL SOLUTION, PH: 9
EAR: NOT APPLICABLE
Chromogen: Dako FLEX DAB, 21 ºC., Time 1: 10 Mins
Detection: Dako EnVision FLEX (K8000/10) , 30 Mins, 21 ºC Prediluted

CD5 - Method 3

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

Primary Antibody:	Novocastra NCL-CD5-4C7 (4C7) , 30 Mins, 21 ºC Dilution 1: 50
Automation:	Dako Autostainer Plus Link
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink
EAR:	NOT APPLICABLE
Chromogen:	Dako EnVision Plus kits, 21 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	None

CD5 - Method 4

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

Primary Antibody:	Ventana CD5 790-4451 (SP19) , 16 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 64mins, Buffer: 0
EAR:	
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700) Prediluted

BEST METHODS - Secondary Antibody

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

CD4 - Method 1

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

Primary Antibody:	Ventana Confirm 790-4423 (SP35) , 12 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 standard
EAR:	
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500) Prediluted

CD4 - Method 2

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

Primary Antibody:	Ventana 250 2712 (Clone 1F6) Prediluted
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800) Prediluted

CD4 - Method 3

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

Primary Antibody:	Dako M0716 , 30 Mins, 21 ºC Prediluted
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink
EAR:	
Chromogen:	Dako EnVision Plus kits, 21 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako Envision+ HRP mouse K4004/5/6/7 , 20 Mins, 21 ºC Prediluted

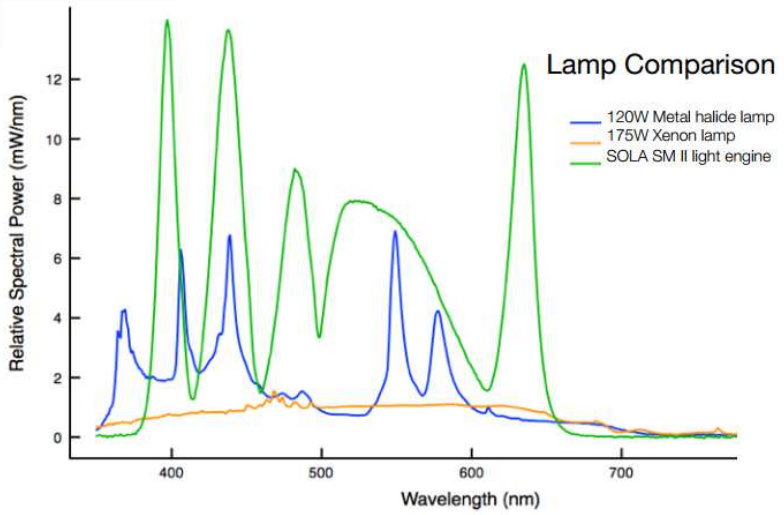
CD4 - Method 4

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

Primary Antibody:	Novocastra NCL-CD4 368 (Clone 4B12) , 40 Mins, 21 ºC Prediluted Dilution 1: 10
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer, PH: 7.6
HMAR:	Dako PTLink, Buffer: HIGH PH TARGET RETRIEVAL SOLUTION, PH: 9
EAR:	
Chromogen:	Dako FLEX DAB, 21 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 30 Mins, 21 ºC Prediluted

Mercury FREE Microscopy

The safer, cheaper solution for your lab



All mercury and metal halide bulbs contain potentially dangerous levels of Mercury. On top of that they are inefficient, difficult to dispose of and expensive to run. Lumencor's light engines from Imsol are unique, solid state illuminators, ideally suited for use with fluorescence microscopes and bioanalytical instruments without the need for mercury.

Performance Metrics: Mercury-based light sources vs. light engines in a 72h microscopy experiment		
Measured	**130w metal halide**	**Light engine**
On time	72 hours (shuttered)	72 seconds (shutterless)
Energy used	11.5 KWh	0.0001 KWh
CO_2	23lbs	0.0002lbs
Unit lifetime used	4%	0.0001%
Experiment cost	£15.40	£0.01

If you are interested in reducing your costs AND your carbon footprint by upgrading to Solid State Illumination, contact Image Solutions UK Ltd. for a free consultation.

imsol Image Solutions
expertise in scientific imaging

Imsol House, 2 Cable Court, Fulwood, Lancs, PR2 9YW Phone: 01772 663140
Fax: 01772 663150
Web: www.imsol.co.uk
Email: info@imsol.co.uk

Neil Bilbe

	Gold Standard	Second Antibody
Antigens Assessed:	MIB-1	Synaptophysin
Tissue Sections circulated:	Meningioma and glioblastoma	Cerebellum and metastatic lung tumour
Number of Registered Participants:	67	
Number of Participants This Run	66 (99%)	

Introduction

Gold Standard: MIB-1

Antigen Ki-67 is a nuclear protein that is associated with and may be necessary for cellular proliferation. Furthermore it is associated with ribosomal RNA transcription. Inactivation of antigen Ki-67 leads to inhibition of ribosomal RNA synthesis.
Ki-67 is an excellent marker to determine the growth fraction of a given cell population. The fraction of Ki-67-positive tumour cells (the *Ki-67 labelling index*) is often correlated with the clinical course of cancer. The best-studied examples in this context are carcinomas of the prostate, brain, breast and nephroblastoma . For these types of tumours, the prognostic value for survival and tumour recurrence have repeatedly been proven in uni- and multivariate analysis.
Ki-67 and MIB-1 monoclonal antibodies are directed against different epitopes of the same proliferation-related antigen. Ki-67 and MIB1 may be used on fixed sections. MIB-1 is used in clinical applications to determine the *Ki-67 labelling index*. One of its primary advantages over the original Ki-67 antibody (and the reason why it has essentially supplanted the original antibody for clinical use) is that it can be used on formalin-fixed paraffin-embedded sections, after heat-mediated antigen retrieval.

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

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

References

1. Hsu DW et al, Use of MIB-1 (Ki-67) immunoreactivity in differentiating grade II and grade III gliomas. J Neuropathol Exp Neurol. 1997 Aug;56 (8):857-65.
2. Ralte AM, et al, Clinicopathological features, MIB-1 labeling index and apoptotic index in recurrent astrocytic tumours. Pathol Oncol Res. 2001; 7(4):267-78.
3. S. H Torp. Proliferative activity in human glioblastomas: evaluation of different Ki-67 equivalent antibodies. Mol Pathol 1997;50:198-200.

Second Antigen: Synaptophysin

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

Features of Optimal Immunostaining:
- Intense, punctate staining around the neuron perikarya.
- Intense staining in normal cerebellum.
- Clean background.

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

References

1. Wiedenmann B, et al. Synaptophysin: A marker protein for neuroendocrine cells and neoplasms. Proc Natl Acad Sci 1986; 83:3500-4.
2. Gould VE et al. Synaptophysin expression in neuroendocrine neoplasms as determined by immunocytochemistry. Am J Pathol 1987; 126:243-57.

Assessment Summary:

There were 67 registered labs at the start of the run; only one lab did not submit any slides at all for assessment; a further 2 labs did not submit any in-house controls **(H & K).** One participant returned only a MIB-1 slide (**G**), a total of 259 slides. Overall, 90% of all slides passed (scored ≥ 11/20), 18 (7%) were borderline, and 7 (3%) failed the assessment. All failed scores were on NEQAS slides **(G & J),** divided 4 (6%) and 3 (5%) respectively for MIB-1 and synaptophysin.

The four failed MIB-1 slides (**G**) all showed uniformly weak tumour staining, although in addition one also had an unacceptable amount of background staining. This lab employed the Dako monoclonal antibody (M7240) on a Leica Bond instrument. Of the other 3 labs, one also used this marker, but on the Ventana UltraView; the other 2 labs both used prediluted (RTU) solutions, one a Dako and one a Ventana. Interestingly, all four labs used a different type of automation. The 8 (12%) NEQAS (**G**) borderline scores again showed weak staining, but usually in only one or two isolated areas. Several labs had section lifting problems on the NEQAS (**G**) slides, although this was not seen in the Golds viewed prior to assessment.

The three labs which failed on the NEQAS synaptophysin slides (**J**) all used different antibodies (Novacastra, Biogenex, and Dako) and automation (Ventana UltraView, Dako Flex, and a manual method using a microwave). Two were very weakly stained and one was completely unstained, with only the counterstain visible; this was 'stained' using the Dako monoclonal SY38 clone, on the Dako Autostainer, using a Dako PT link. 7 labs (11%) were scored as borderline on their NEQAS synaptophysin slides (**J**), 5 for overall weak staining; of the other two, one was using an over-diluted primary (1:1000); and the other had stained the metastatic lung portion of the section adequately, but, uncharacteristically, the cerebellum was far too weakly stained to be able to give the slide an overall pass score.

The assessors felt that the participants performed better on the NEQAS synaptophysin (**J**) slides than with the MIB-1 (**G**) sections.

In-house slides (**H & K**) were rather more straightforward; there were no failed slides, and only 3 were borderline, two (3%) for MIB-1 (**H**) and one (2%) for synaptophysin (**K**), all due to the weak demonstration of the antigens. For the type of control material submitted, 13 (20%) for MIB-1 (**H)** and 34 (53%) for synaptophysin (**K)** were from tissue considered to be primarily of neurological origin.

Selected Images showing Optimal and Sub-optimal Immunostaining

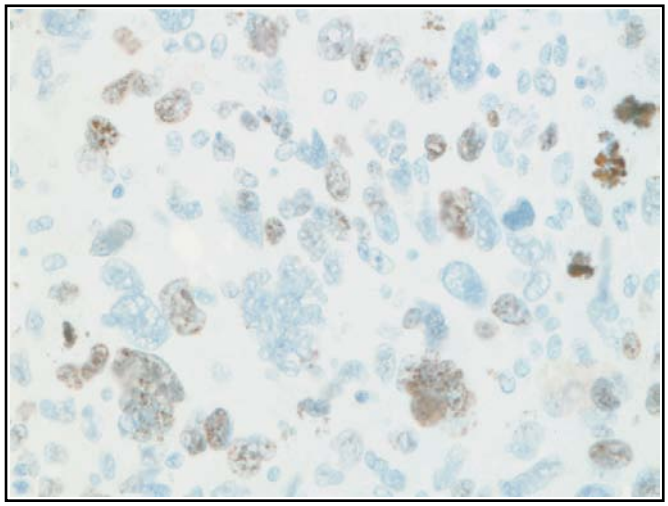


Fig 1. Poor demonstration of MIB-1 on the NEQAS slide; staining is far too weak to be diagnostically acceptable, with only the occasional tumour cell visible. This slide failed the assessment. Dako M7240 (MIB1), 1:200, on the Ventana Benchmark XT, and using the Ventana CC1 32mins.

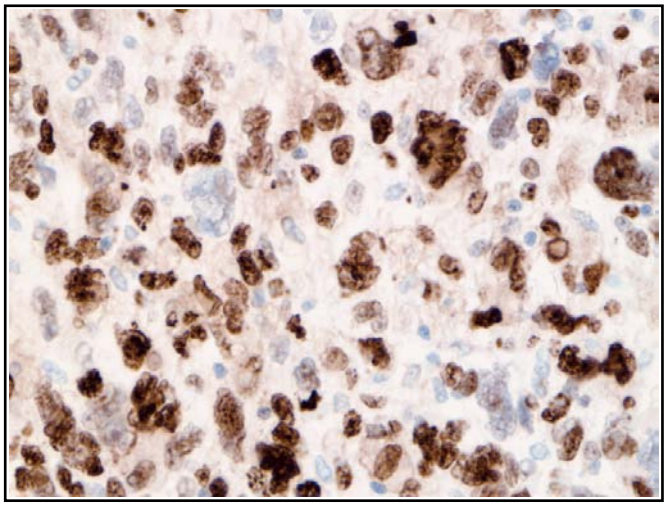


Fig 2. Sub-optimal MIB-1 staining of the glioblastoma in the NEQAS Gold section. Although the tumour is nicely demonstrated, there is a background 'wash' which detracts from the overall quality of the slide. Dako M7187 (Ki-67), 1:120, Leica Bond-III, Leica ER2 20 mins, and Leica Bond Refine.

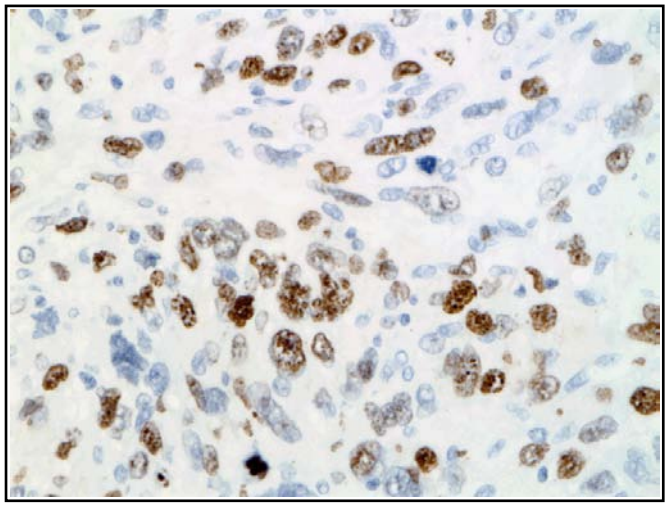


Fig 3. Borderline result on a NEQAS MIB-1 slide. The staining is slightly weak, and some of the tumour is not adequately demonstrated; in addition the nuclei have a vacuolar appearence to them. Dako M7240 (MIB1), 1:100, on the Ventana Benchmark XT, with Ventana CC1 mild.

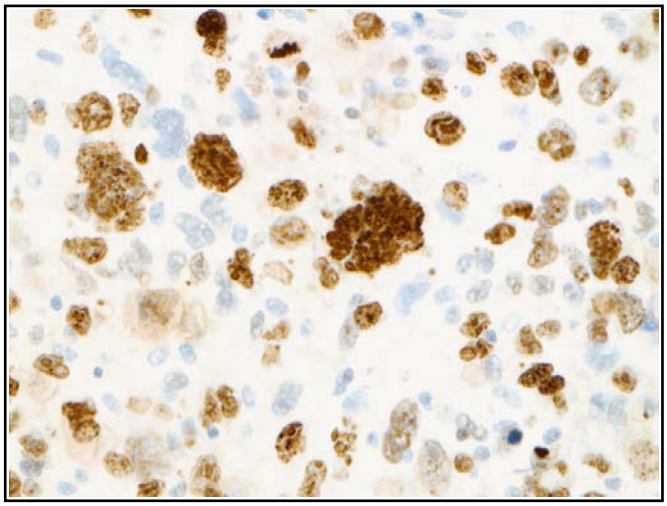


Fig 4. Sub-optimal MIB-1 staining of the glioblastoma on the NEQAS Gold (G) slide; staining intensity is low and there is some background. This was assessed as a high borderline score. Novocastra (MM1) RTU, on the Leica Bond Max, with the Leica ER2 20 mins, and the Bond Polymer Refine kit.

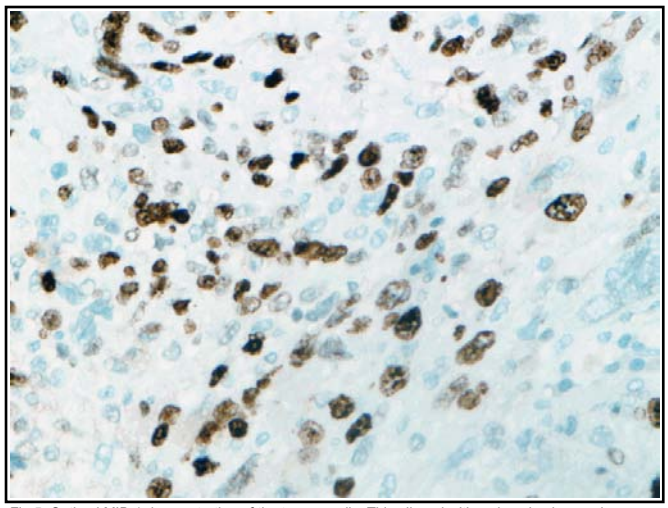


Fig 5. Optimal MIB-1 demonstration of the tumour cells. This aligned with a clean background, gives an excellent assessment result. Ventana RTU (30-9) 790-4286 for 24 mins, on the Ventana Benchmark ULTRA, with a Ventana CC1 36 mins, and the Ventana UltraView Kit (760-500).

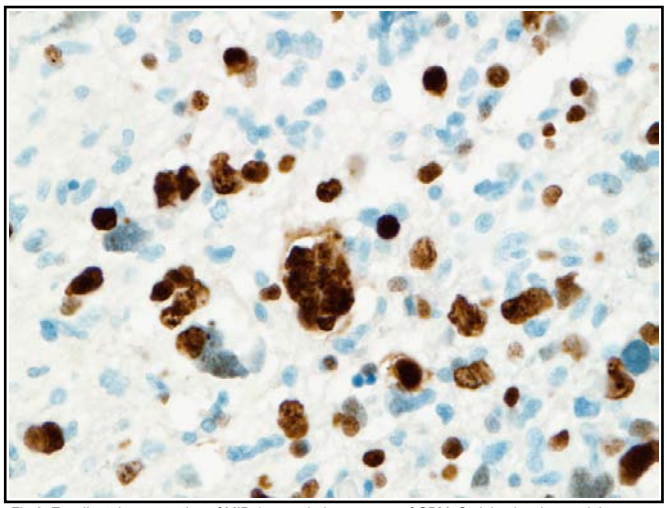


Fig 6. Excellent demonstration of MIB-1 on an in-house case of GBM. Staining is crisp, and the background is clean. Ventana RTU (30-9), for 16 mins, on the Ventana Benchmark ULTRA, with Ventana CC1 at pH8 for 52 mins, and a prediluted Ventana UltraView Kit (760-500) for 8 mins.

Selected Images showing Optimal and Sub-optimal Immunostaining

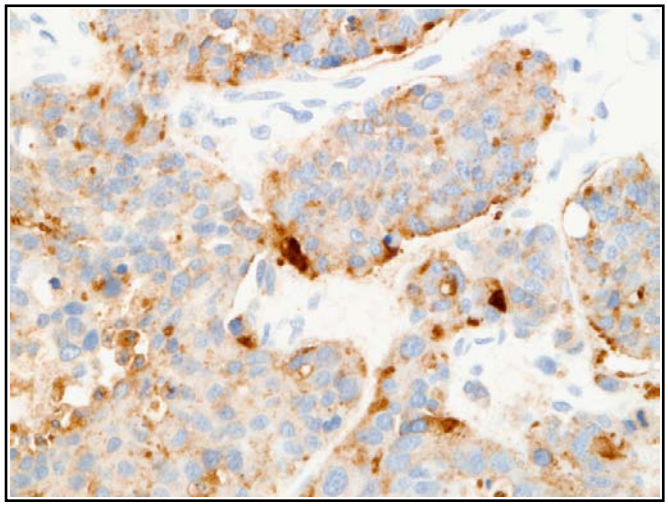


Fig 7. Sub-optimal staining of synaptophysin on the metastatic lung portion of the NEQAS slide (J). The tumour is weakly stained, although adequate for diagnostic purposes. Dako M0776 (clone SY38) 1:50, 40 mins, on a Dako Autostainer plus, and a low pH Dako PT Link for 20 mins.

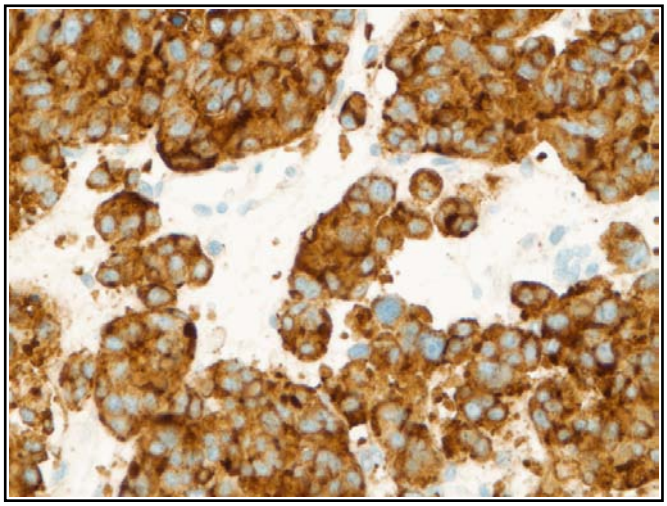


Fig 8. Optimal staining of the tumour, showing the characteristic granular staining seen with demonstration of synaptophysin. Dako M7315, 1:50, 20 mins, on a Dako Autostainer Link 48, with the Dako PT Link and a high pH Flex kit.

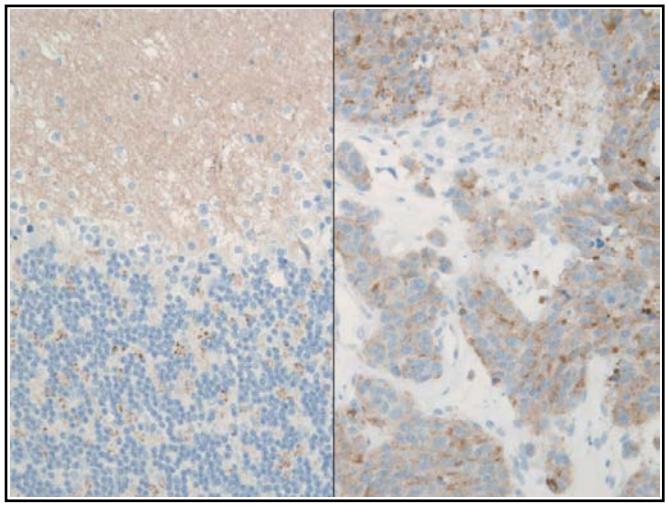


Fig 9. Very weak staining of both the tumour component (R), and uncharacteristically the cerebellum (L) in the NEQAS slide. This slide was assessed as low borderline. Prediluted Dako A0010 (polyclonal), 15 mins, on a Leica Bond Max, with Leica ER2 20 mins, and a Leica BondMAx Refine KIT.

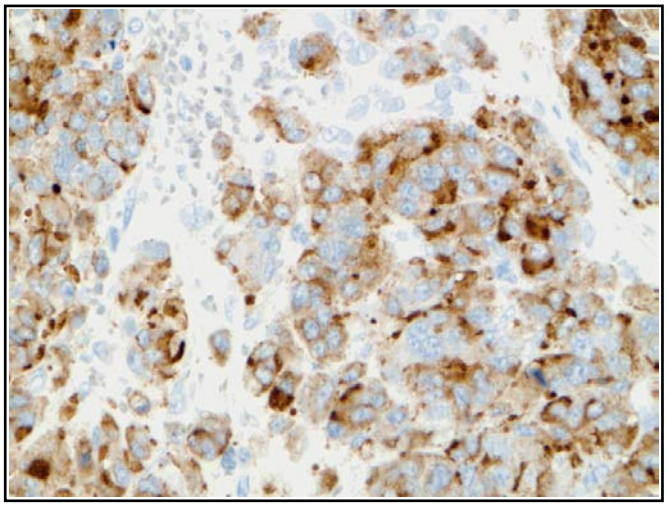


Fig 10. Sub-optimal demonstration of the metastatic lung tumour; staining is patchy, although perfectly adequate for diagnostic interpretation. Novocastra (27G12), no dilution given (some are RTU), on a Menarini - Intellipath FLX, with a Biocare Decloaking Chamber and Biocare polymer kit.

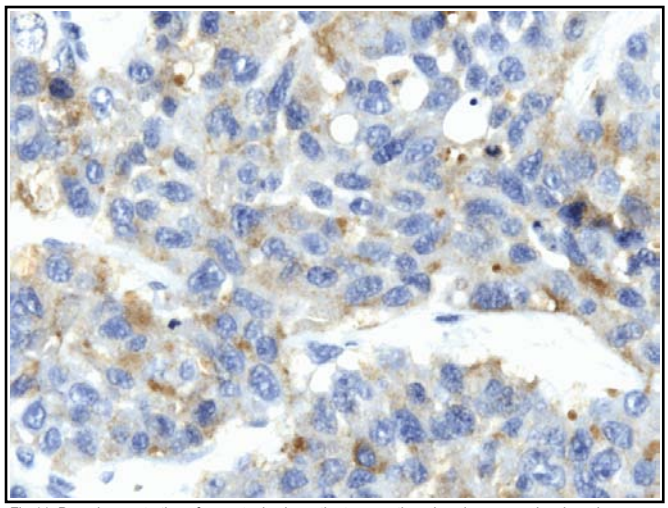


Fig 11. Poor demonstration of synaptophysin on the tumour, there is only an occasional weak reaction (see image) with much of the tissue unstained. This slide failed the assessment. Dako M0776 (clone SY38), 1:50, 30 mins, on Dako Autostainer Plus Link, with Dako PTLink pH9 for 45 min.

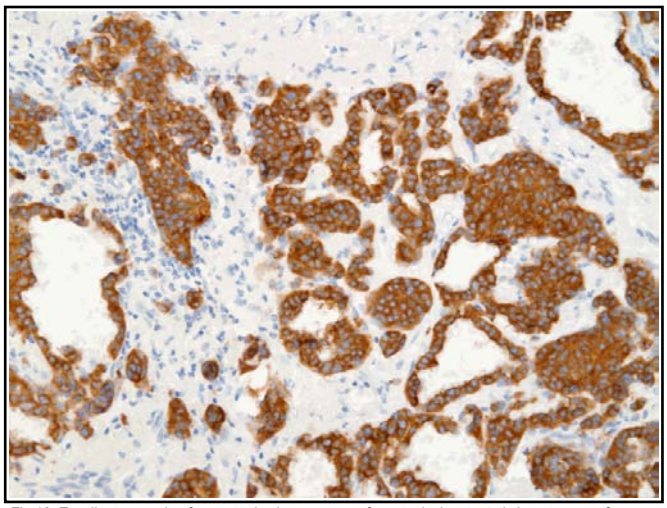
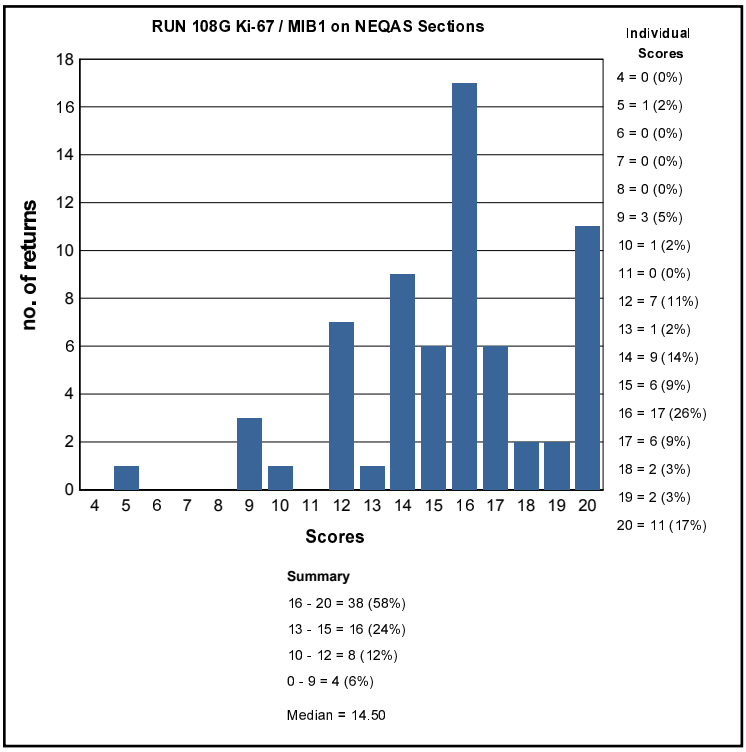
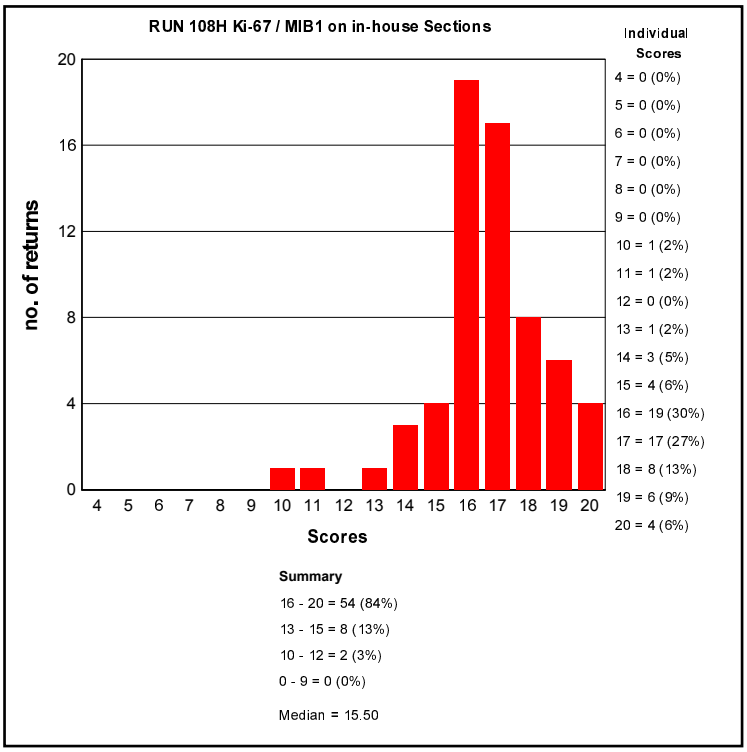
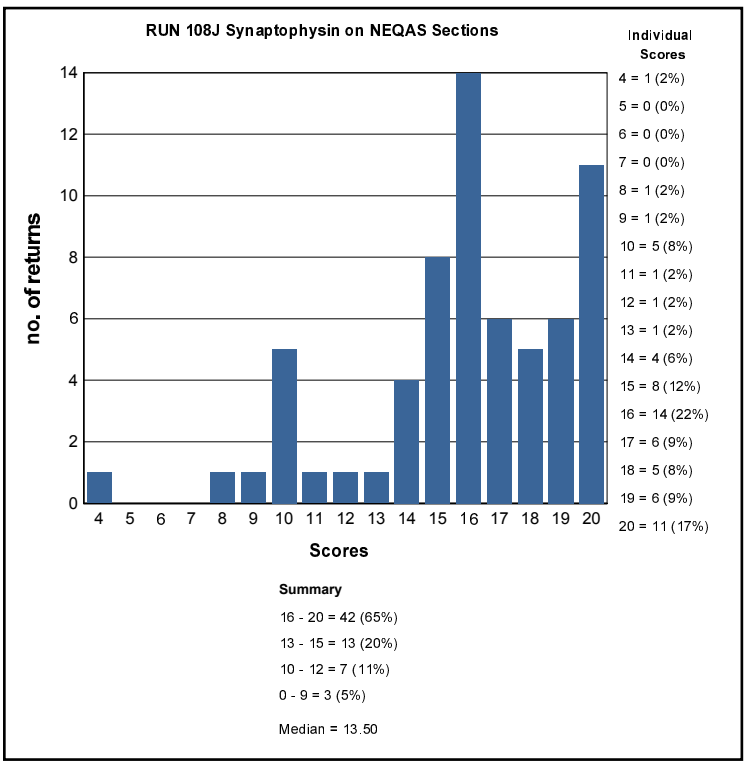


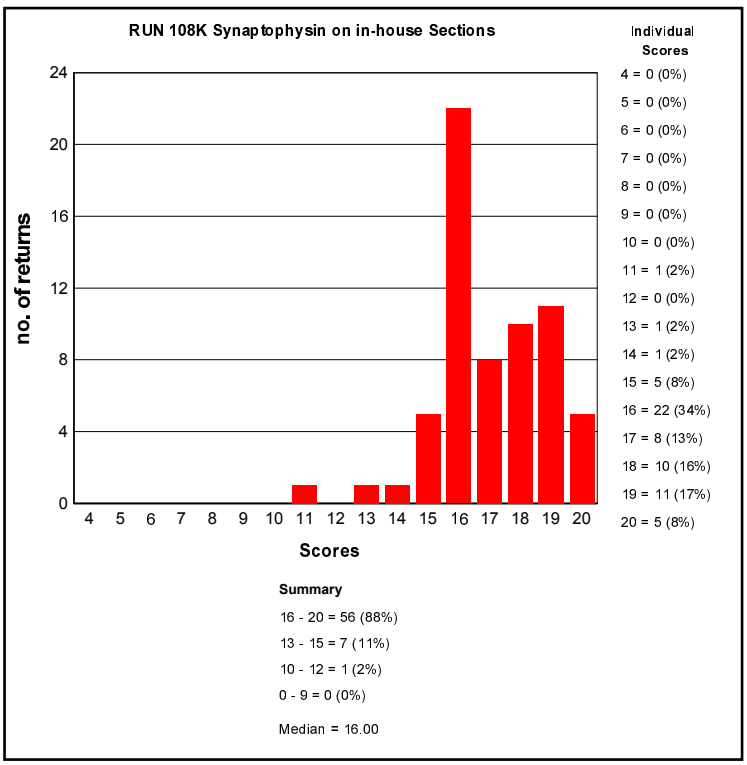
Fig 12. Excellent example of synaptophysin on a case of an atypical metastatic lung tumour of neuroendocrine origin from an in-house control (K). Ventana CONFIRM 790-4407 (SP-11), 44 mins, on the UltraView with Ventana CC1 for 44mins, and the Ventana UltraView Kit (760-500).

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: 108

Primary Antibody : Ki-67 / MIB1

Antibody Details	N	%
Dako FLEX RTU (MIB1) IR626	5	40
DAKO FLEX RTU Omnis (MIB1) GA626	2	50
Dako M7187 (Ki-67)	1	100
Dako M7240 (MIB1)	39	87
Dako N1574 (Ki67)	1	0
Gennova AP10244C (SP6)	1	100
Leica/Novocastra (MM1) NCL-Ki67-CE	4	100
Leica/Novocastra RTU (K2) PA0230	1	100
Leica/Novocastra RTU (MM1) PA0118	3	67
Other	1	100
Ventana RTU (30-9) 790-4286	8	88

Neuropathology Run: 108

Primary Antibody : Synaptophysin

Antibody Details	N	%
BioGenex AM 6 (clone SNP)	1	0
Biogenex MU 363 UC	1	100
Dako A0010 (polyclonal)	1	0
DAKO FLEX IR776 (SY38)	1	100
Dako M0776 (clone SY38)	12	75
NeoMarkers SP11	1	100
Novocastra Bond RTU PA0299 (rb poly)	2	100
Novocastra NCL-L-SYNAP-299 (27G12)	12	92
Novocastra NCL-SYNAP-299 (27G12)	9	89
Other	12	92
Ventana 760-4595 (MRQ-40)	5	100
Ventana CONFIRM 790-4407 (SP-11)	8	75

Neuropathology Run: 108

Heat Mediated Retrieval	Ki-67 / MIB1 N	Ki-67 / MIB1 %	Synaptophysin N	Synaptophysin %
_Leica BondMax ER2	0	0	1	100
Biocare Decloaking Chamber	1	100	1	100
Dako Omnis	2	50	0	0
Dako PTLink	10	60	12	83
Leica ER1 20 mins	0	0	2	100
Leica ER1 30 mins	0	0	4	100
Leica ER1 40 mins	0	0	1	100
Leica ER2 20 mins	14	79	9	56
Leica ER2 30 mins	6	100	3	100
Leica ER2 40 mins	2	100	1	100
Microwave	1	100	2	50
Other	1	100	0	0
Ventana CC1 32mins	4	75	4	75
Ventana CC1 36mins	1	100	0	0
Ventana CC1 40mins	1	100	0	0
Ventana CC1 44mins	0	0	1	0
Ventana CC1 52mins	0	0	2	100
Ventana CC1 56mins	1	100	1	100
Ventana CC1 64mins	7	100	4	100
Ventana CC1 76mins	0	0	1	100
Ventana CC1 8mins	0	0	1	100
Ventana CC1 mild	4	75	4	75
Ventana CC1 standard	8	75	7	100
Ventana CC2 52mins	0	0	1	0
Ventana CC2 64mins	1	100	0	0
Ventana CC2 mild	1	100	0	0
Water bath 95-98 OC	1	100	1	100

Neuropathology Run: 108

Enzyme Mediated Retrieval	Ki-67 / MIB1 N	Ki-67 / MIB1 %	Synaptophysin N	Synaptophysin %
AS PER KIT	0	0	1	100
NOT APPLICABLE	37	78	34	88

Neuropathology Run: 108	Ki-67 / MIB1		Synaptophysin	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	7	86	9	100
Biocare polymer (M4U534)	1	100	1	100
Dako EnVision FLEX (K8000/10)	2	50	3	67
Dako EnVision FLEX+ (K8002/12)	6	67	5	80
Dako Envision HRP/DAB (K5007)	2	100	1	0
Leica Bond Polymer Refine (DS9800)	20	85	18	83
NOT APPLICABLE	1	0	2	100
Other	2	50	0	0
Ventana iView system (760-091)	2	100	2	100
Ventana OptiView Kit (760-700)	4	75	4	100
Ventana UltraView Kit (760-500)	19	89	19	79

Neuropathology Run: 108	Ki-67 / MIB1		Synaptophysin	
Automation	**N**	**%**	**N**	**%**
Dako Autostainer Link 48	8	63	10	90
Dako Autostainer plus	0	0	1	100
Dako Autostainer Plus Link	3	67	2	50
Dako Omnis	2	50	0	0
Leica Bond Max	8	88	10	70
Leica Bond-III	14	86	13	92
Menarini - Intellipath FLX	1	100	1	100
None (Manual)	2	100	2	50
Ventana Benchmark GX	2	50	1	100
Ventana Benchmark ULTRA	13	100	11	91
Ventana Benchmark XT	13	77	14	86

Neuropathology Run: 108	Ki-67 / MIB1		Synaptophysin	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	8	75	14	93
Dako DAB+ REAL Detection (K5001)	1	100	1	100
Dako EnVision Plus kits	2	100	1	100
Dako FLEX DAB	9	56	6	83
Dako REAL EnVision K5007 DAB	1	100	1	0
Leica Bond Polymer Refine kit (DS9800)	21	86	20	80
Other	1	100	1	100
Ventana DAB	2	50	1	100
Ventana iview	1	100	1	100
Ventana Ultraview DAB	20	90	20	80

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

Primary Antibody: Dako M7240 (MIB1) , 30 Mins, 23 ºC Dilution 1: 800
Automation: Dako Autostainer Link 48
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako PTLink, Buffer: target retrieval solution, PH: 9
EAR:
Chromogen: Dako EnVision Plus kits, 23 ºC., Time 1: 10 Mins
Detection: Dako EnVision FLEX+ (K8002/12) , 15 Mins, 23 ºC Prediluted

Ki-67 / MIB1 - Method 2

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

Primary Antibody: Ventana RTU (30-9) 790-4286 , 15 Mins Prediluted
Automation: Leica Bond Max
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 20 mins
EAR:
Chromogen: Leica Bond Polymer Refine kit (DS9800), Time 1: 10 Mins
Detection: Leica Bond Polymer Refine (DS9800) , 8 Mins Prediluted

Ki-67 / MIB1 - Method 3

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

Primary Antibody:	Dako M7240 (MIB1)
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)

Ki-67 / MIB1 - Method 4

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

Primary Antibody:	Ventana RTU (30-9) 790-4286 , 16 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.4
HMAR:	Ventana CC1 64mins, Buffer: Ultra CC1 (cat 950-224), PH: 8
EAR:	
Chromogen:	Ventana Ultraview DAB, PH: 7, 36 ºC., Time 1: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 36 ºC Prediluted

BEST METHODS - Secondary Antibody

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

Synaptophysin - Method 1

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

Primary Antibody:	Novocastra NCL-L-SYNAP-299 (27G12) , 15 Mins, 25 ºC Dilution 1: 100
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER1 20 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, 25 ºC

Synaptophysin - Method 2

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

Primary Antibody:	Dako M0776 (clone SY38)
Automation:	Leica Bond Max
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800)

Synaptophysin - Method 3

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

Primary Antibody:	Ventana 760-4595 (MRQ-40) , 32 Mins, 36 ºC Prediluted
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 56mins
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB, 36 ºC., Time 1: 8 Mins, Time 2: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 36 ºC Prediluted

Synaptophysin - Method 4

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

Primary Antibody:	Novocastra NCL-L-SYNAP-299 (27G12) , 32 Mins, 37 ºC Dilution 1: 1:50
Automation:	Ventana Benchmark XT
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.4
HMAR:	Ventana CC1 32mins
EAR:	NOT APPLICABLE
Chromogen:	AS PER KIT
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 37 ºC Prediluted

Neil Bilbe and Irena Srebotnik Kirbis

	Gold Standard	Second Antibody
Antigens Assessed:	CD45	Melanoma markers
Tissue Sections circulated:	FNA of LN, melanoma cell line, effusion with mixed cell population	Melanoma cell line, FNA of LN, effusion with mixed cell population
Number of Registered Participants:	78	
Number of Participants This Run	78 (100%)	

Introduction

Gold Standard: 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.

Second Antigen: Melanoma Markers

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

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

The antibody **Melan-A** labels melanocytes and is a useful tool for the identification of melanomas, and if melanoma is ruled out, for adrenocortical carcinomas. Melan-A, isolated as a melanoma-specific antigen, is a transmembrane protein composed of 118 amino acids with uncertain function. Melan-A is expressed in skin, retina and the majority of cultured melanocytes and melanomas, whereas a vast variety of other tissues and cancers do not express Melan-A.

Features of Optimal Immunostaining:

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

Features of Sub-optimal Immunostaining:

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

References

1. Liu K, Dodge R, Glasgow BJ, Layfield LJ. Fine-needle aspiration: Comparison of smear, cytospin, and cell block preparations in diagnostic and cost effectiveness. Diagn Cytopathol. 1998;19:70–4.
2. Dalquen P, Sauter G, Epper R, Kleiber B, Feichter G, Gudat F. Immunocytochemistry in diagnostic cytology. Recent Results Cancer Res. 1993;133:47–80
3. Leung SW, Bedard YC. Immunocytochemical staining on ThinPrep processed smears. Mod Pathol 1996; 9(3): 304–6.
4. Beaty M, Fetsch PA, Wilder AM, Marincola FM, Abati A. Effusion cytology of malignant melanoma. Cancer (Cancer Cytopathol) 1997; 81(1): 57–63.

References (cell blocks in cytology)

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

Assessment Summary:

At the start of the run there were 78 registered participants; all labs submitted slides for both the NEQAS Gold (**R**) and 2nd antigen (**T**). Six labs failed to return one, or both of their in-house controls, a total of 9 non-submitted slides (6%), 3 were unreturned for the in-house CD45 (**S**) and 6 for the in-house melanoma markers (**U**). A total of **303** slides were viewed and scored by the assessors: **R & T** = 78 each, **S** = 75, **U** = 72.

Neil Bilbe and Irena Srebotnik Kirbis

Run 108 was the second where labs were given the choice of either cytospins or cell block sections for the NEQAS samples. For this circulation, 28 labs requested/returned cytospins (**36%**) and 50 submitted cell block sections (**64%**), which represented a 3% *increase* in cell blocks from run 107.

7 slides **failed** the assessment (2.3%); two NEQAS slides (1·3%) and five in-house (3·4%); the breakdown was as follows:

- 1/78 NEQAS CD45 slides (R) failed = 1·3%
- 3/75 In-house CD45 slides (S) failed = 4·0%
- 1/78 NEQAS Melanoma slides (T) failed = 1·3%
- 2/72 In-house Melanoma slides (U) failed = 2·8%

Both failed NEQAS slides were on cytospins; the CD45 used a Dako monoclonal (no dilution given), Envision method on the Dako Autostainer Link 48. The melanoma slide used a monoclonal Dako Melan A, diluted 1:100, employing an APAAP method, with a Levamisol endogenous block, and also used a Dako Autostainer Link 48. No antigen retrieval was used, which is normal for cytospins.

For the in-house slides, the three CD45 slides (**S**) consisted of: a cell block, a cytospin and a liquid based preparation; whereas both the failed in-house melanoma controls (**U**) were on FFPE sections. The trend is for the non FFPE controls (**S**) to fail because of excessive background and/or non-specific staining, whereas, the FFPE controls (**U**) were usually of poor section quality or the choice of control was inappropriate.

27 (8·9%) of all slides were assessed as being **borderline**. The spread for the four slide types, (**R, S, T, U**) varied considerably though:

- 12/78 NEQAS CD45 slides borderline (R) = 15·4%
- 6/75 In-house CD45 slides borderline (S) = 8·0%
- 1/78 NEQAS Melanoma slides borderline (T) = 1·3%
- 8/72 In-house Melanoma slides borderline (U) = 11·1%

The interesting statistic was the fact that 12 slides were assessed as borderline for the NEQAS CD45 slides (**R**), 6 each for cell block sections and cytospins, as a percentage this represents 12% (CB) and 21% (CS) respectively. This also backed up the impression formed by the NEQAS assessors, that in general, the melanoma marker slides were more straightforward, in comparison to those for CD45.

Analysing the methods used by these 12 participants scoring between 10-12 for their NEQAS Gold slide (**R**) shows that 10 out of the 12 used the Dako CD45 monoclonal antibody*; all of the cytospin labs (6) and 4/6 of the cell block labs. For the other two cell block labs, one used a Novacastra marker, the 2nd selected 'other'. It appears that this reagent was supplied by a large company, albeit not one known primarily as a supplier of antibodies; the clones though were the same as the Dako marker: 2B11+PD7/26. *This was far and away the most popular marker anyway, used by 56/78 of labs (72%).

The use of pre-treatment was variable, and difficult to accurately gauge; e.g. only 3 out of the 6 participants using **cell blocks** had ticked the box: Antigen Retrieval on NEQAS section 'R' - Yes *but* 5 had entered a PT method: CC1 (3), ER1 (1) and ER2 (1). As the default for this is No one suspects that this may be an oversight by the person entering the protocol details.

For the 6 labs who had stained the **cytospin** preparation, 4 had ticked No Antigen retrieval, and had correspondingly *not* entered any PT details. Another had ticked Yes and used a Dako PT link, the other had ticked No but entered a waterbath method. Coincidentally, both these 2 labs submitted a liquid based cytology in-house sample (**S**).

Automation employed was the usual range of instrumentation, primarily Leica: Bond and Autostainer; Ventana: UltraView and Benchmark, plus a single manual method.

The in-house slides (**S, U**) were from a variety of sample types (cell blocks, smears, cytospins, liquid based) roughly in line with the overall number of samples submitted (see below). There was only one borderline slide for the NEQAS melanoma markers (**T**) which was a cytospin, stained with a Cell Marque Melan A antibody.

Lab self- assessment of *borderline* CD45 (**R**) slides (n=12)

It is always of interest to compare the scores allocated by the NEQAS assessors with those entered by the laboratories themselves, i.e. one 'technical' and one from a pathologist.

23/24 scores were entered, a single pathologists' score was not submitted from one lab. Of these scores, only **one** was entered as a borderline (12/20), *all* the rest ranged from a low of 14/20 to a high of 18/20. The average and median scores from both the technologists and the pathologists was 16/20.

There are discrepancies between the two sets of marks in 5 out of the 11 slides (one did not qualify), in four of those the *technologists'* score was higher, in one the *pathologists'* score was higher, and for 5 of the slides both were in agreement.

All average scores between both the technologists and the pathologists, and between cell block sections and cytospin scores were within 0.5 of a mark, with the greatest difference being between technologists (high) and pathologists (low) scores for the cell block sections of 16.0 and 15.5 respectively.

Type of in-house samples submitted (**S**: n=75,& **U**: n=72)

As with previous runs, the sample type submitted for the in-house controls recorded at the assessment, are as follows:

S (CD45)	No	%	U (Mel)	No	%	Total	Total %
FFPE	33	44%	FFPE	38	53%	71	48%
CS	15	20%	CS	12	17%	27	18%
CB	20	27%	CB	15	21%	35	24%
Smear	2	3%	Smear	2	3%	4	3%
Thin Prep	5	7%	Thin Prep	5	7%	10	7%
	75	100%		72	100%	147	100%

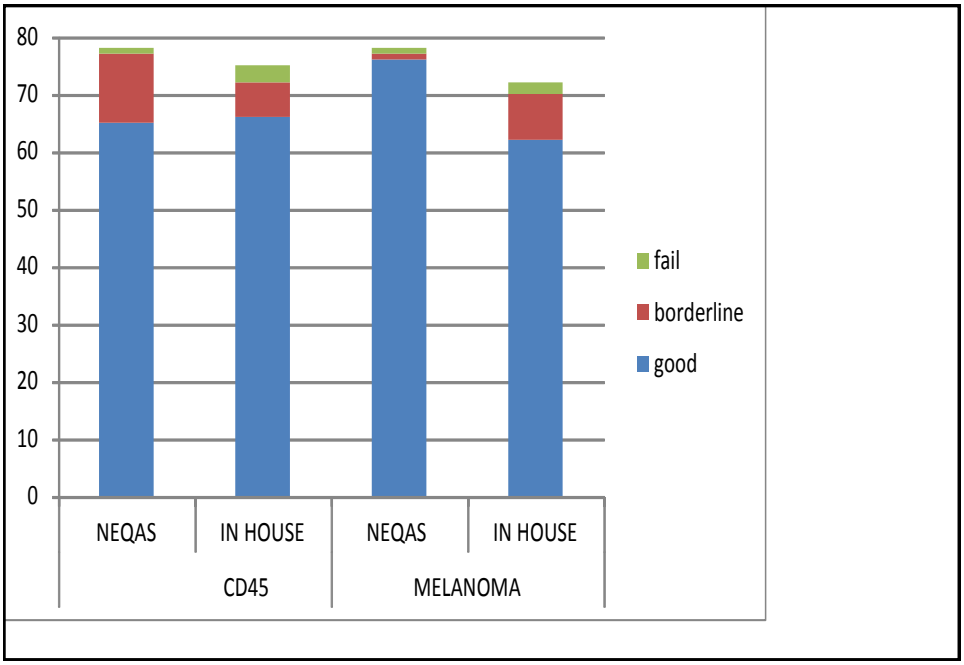
These averages are almost identical to those from Run 107 with only very slight differences, where there are any. Where a direct comparison can be made; e.g. for the CD45 in-house controls (**S**) the differences from runs 107 to 108 are:

- FFPE ↓ 1%
- Cytospins ↑ 2%
- Cell blocks ↑ 1%
- Smears unchanged
- Thin preps ↓ 1%.

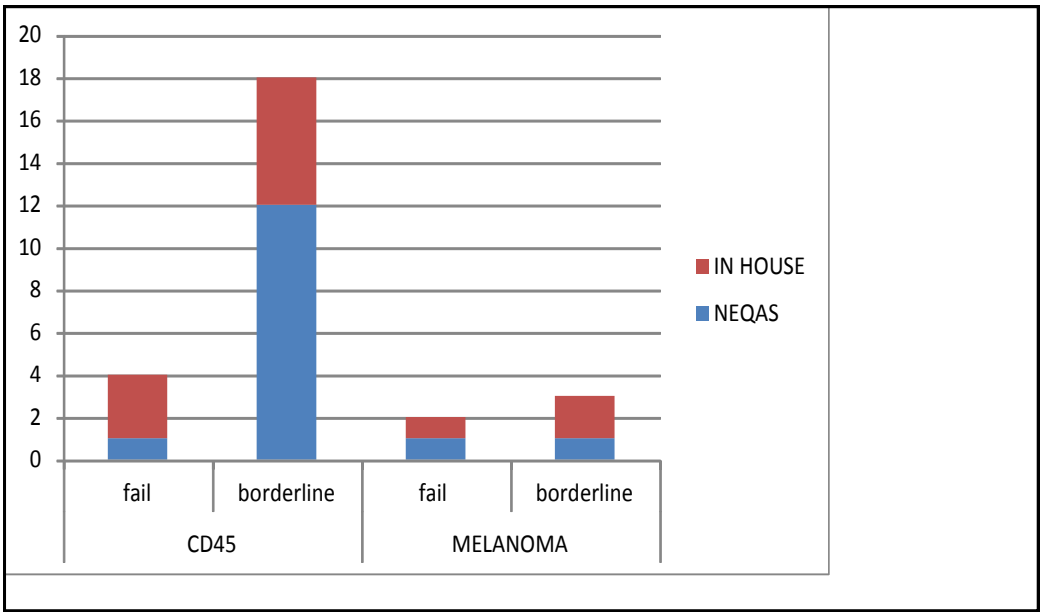
Neil Bilbe and Irena Srebotnik Kirbis

Summary of Assessment outcomes

Distribution of ICC assessment categories for Run 108



Distribution of slides assessed as failed and borderline for NEQAS and in house slides CD45 & Melanoma



Please see the graphs, images and tables on the following pages, or available online using your login to the participant website.

Selected Images showing Optimal and Sub-optimal Immunostaining

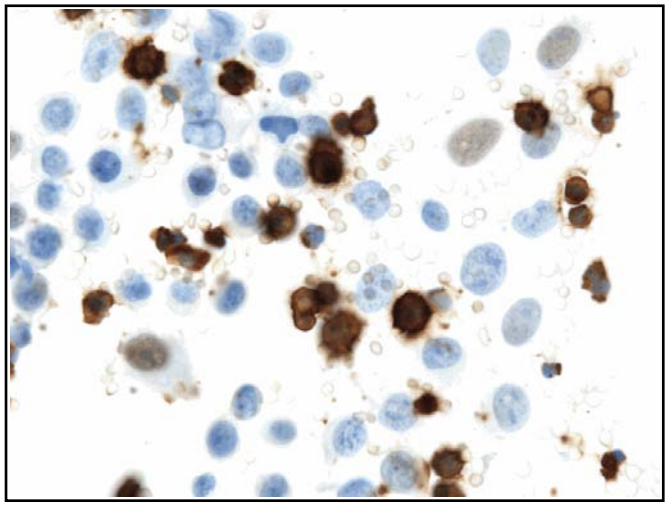


Fig 1. Sub-optimal CD45 demonstration on a NEQAS cytospin (CS) slide, there is non-specific nuclear staining, though not detracting from the overall lymphocytic staining, and assessed as a low pass. Dako (2B11+PD7/26), 1:250, on Ventana Benchmark ULTRA, and the UltraView Kit. No retrieval.

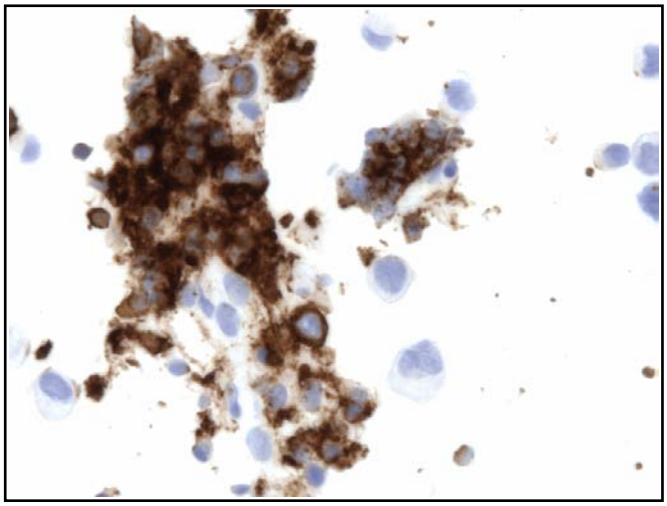


Fig 2. Excellent CD45 staining of a cluster of cells in a NEQAS cell block (CB) slide. The background and other cellular components are clean. Dako (2B11+PD7/26), 1:100, on the Ventana Benchmark ULTRA, with Ventana CC1 standard for 64 mins, and the Ventana UltraView Kit.

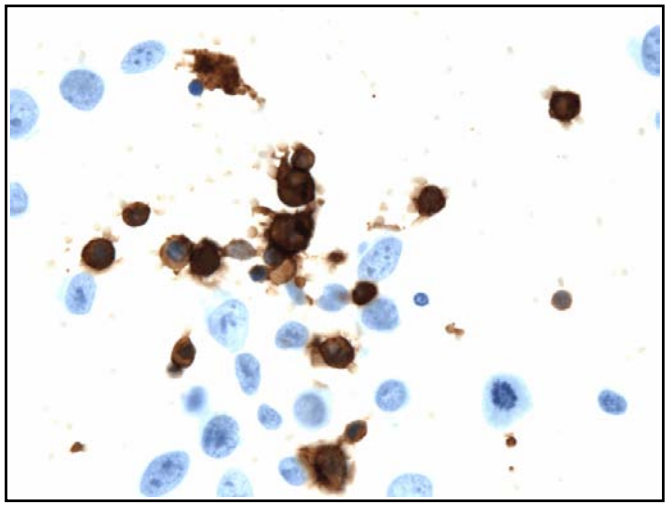


Fig 3. Optimal CD45 staining on a NEQAS cytospin, there is no inappropriate staining of cells, and the counterstain intensity is also optimal. Ventana CONFIRM RTU (RP2/18), for 32 mins, on the Ventana Benchmark ULTRA, and the Ventana UltraView Kit (760-500), again with no retrieval (see fig 1).

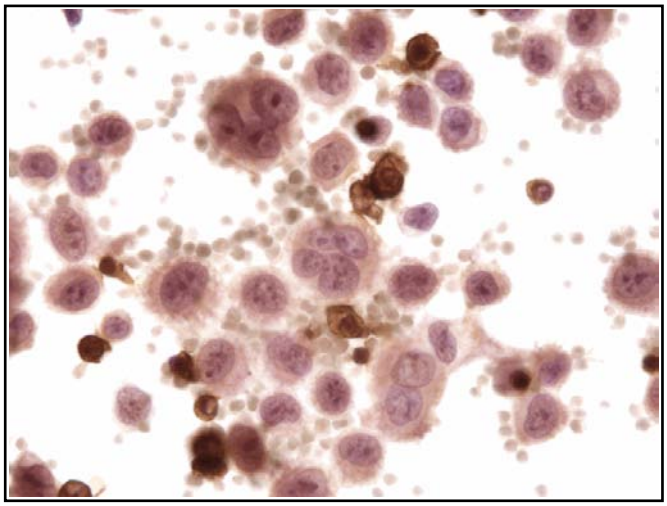


Fig 4. Poor staining (fail) for CD45 on a NEQAS cytospin. Although some lymphocytes are demonstrated, the overall quality of staining is not sufficient to merit a pass if this was a diagnostic sample. Dako monoclonal, 30 mins, (dilution not given), Dako Autostainer Link 48 and Dako Envision.

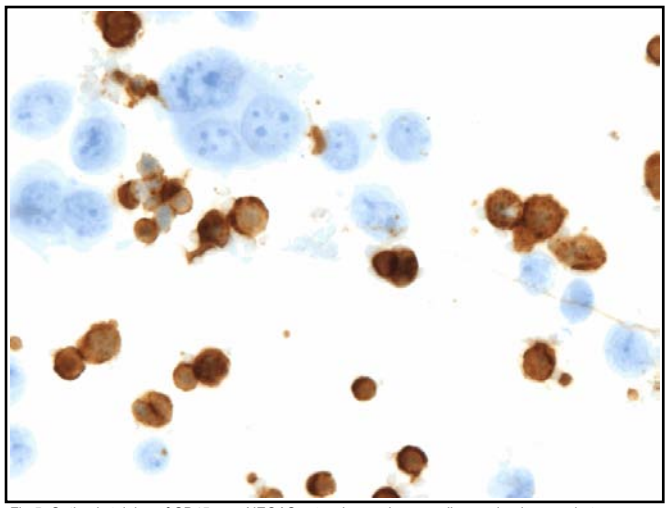


Fig 5. Optimal staining of CD45 on a NEQAS cytospin; carcinoma cells are clearly seen, but completely unstained. Dako monoclonal, 1:150, 20 mins, on the Ventana Benchmark ULTRA. Ventana CC1 for 48 mins is given, but states that this has not been performed on the NEQAS slide.

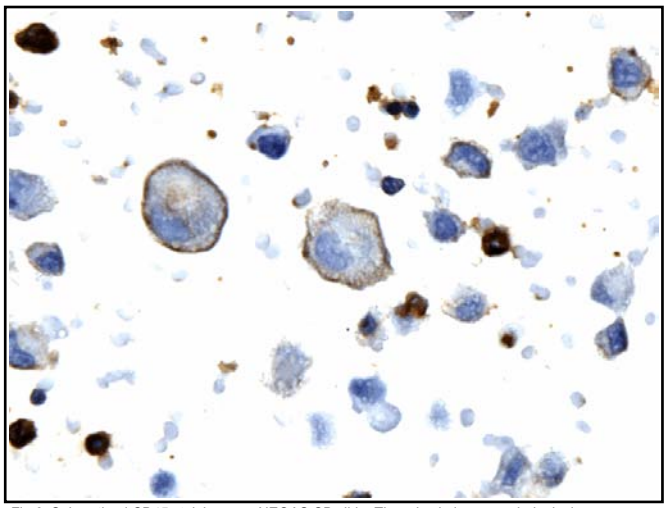


Fig 6. Sub-optimal CD45 staining on a NEQAS CB slide. There is obvious morphological damage to many of the cells present, albeit lymphocytes are clearly demonstrated in some areas. Dako RTU, 30 min, Dako Autostainer Plus Link, Dako PTLink (hot), 45 mins, and a prediluted Dako EnVision FLEX+.

Selected Images showing Optimal and Sub-optimal Immunostaining

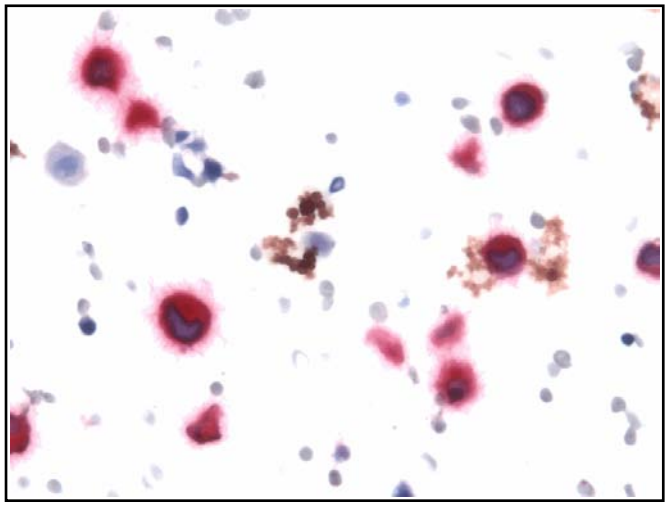


Fig 7. Staining of melanoma cells in a NEQAS cell block. The presence of a brownish contaminant reduced the overall score to a good pass. There is also a slight pinkish tinge. But perfectly adequate for diagnosis. Novocastra Melan A, Leica Bond Max, APAAP method, few other details were given.

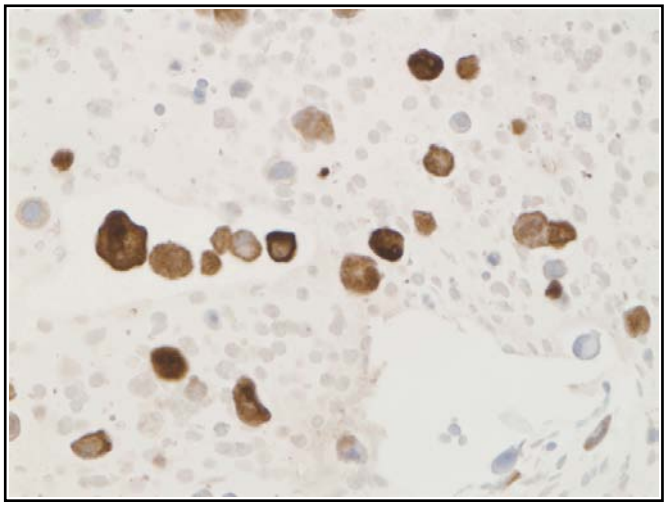


Fig 8. Sub-optimal staining using the Dako polyclonal S100 on a NEQAS CB. The background protein is 'stained', a frequent finding with this antibody. Slide is still acceptable, and passed the assessment. Applied at 1:500, 20 min, on a Leica Bond-III, without retrieval, Leica Bond Polymer Refine 8 mins.

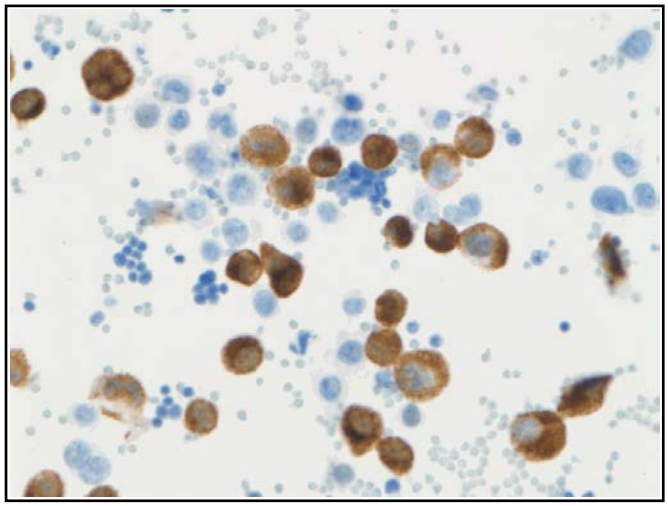


Fig 9. Optimal demonstration of melanoma cells on a NEQAS cytospin. There is no discernible inappropriate staining, and tumour cells are easily and nicely visualised. Novocastra Melan A, at 1:50, on a Leica Bond-III, without retrieval, and the Leica Bond Polymer Refine kit.

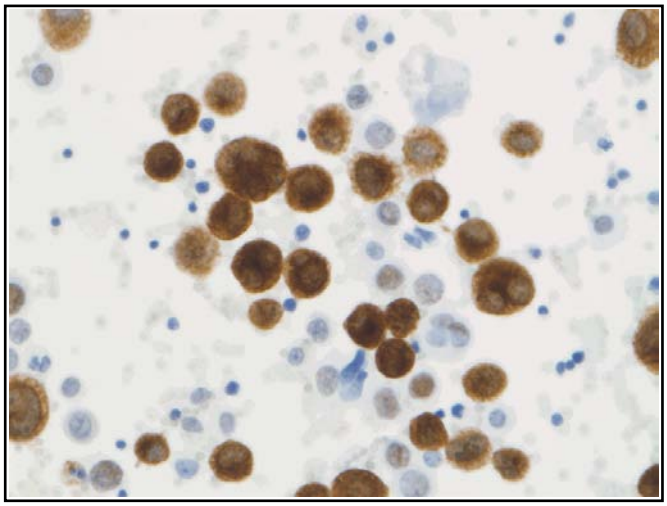


Fig 10. Sub-optimal melanoma demonstration on a NEQAS CS. There is some non-specific nuclear staining (compare with fig 9), but still perfectly adequate for diagnostic use. Dako A 103 Melan A, 1:50, on a Leica Bond-III, and the Leica Bond Polymer Refine (no time given). No retrieval used.

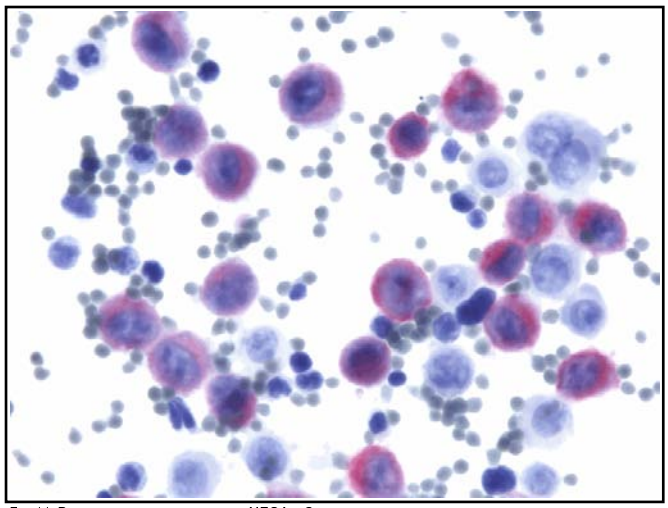


Fig 11. Poor melanoma staining on a NEQAS CS; it is difficult to confidently assess possible cellular components on the slide; this failed the assessment. Dako A103 Melan A (as above), 1:100, 30 min, on a Dako Autostainer Link 48, and the Dako REAL K5005 APAAP for 2 x 10 min.

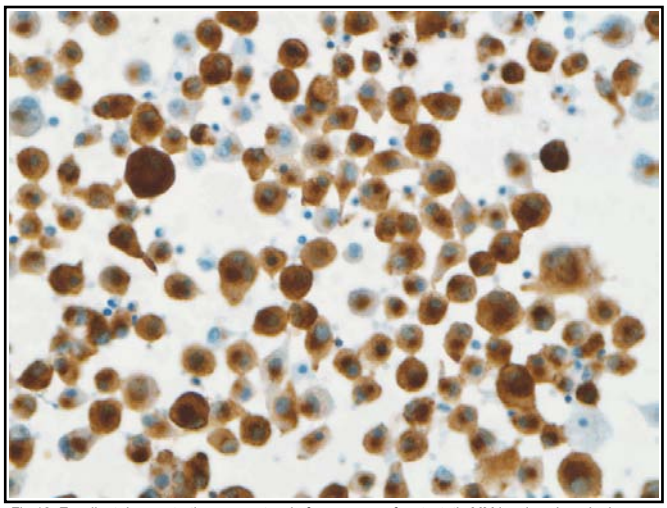
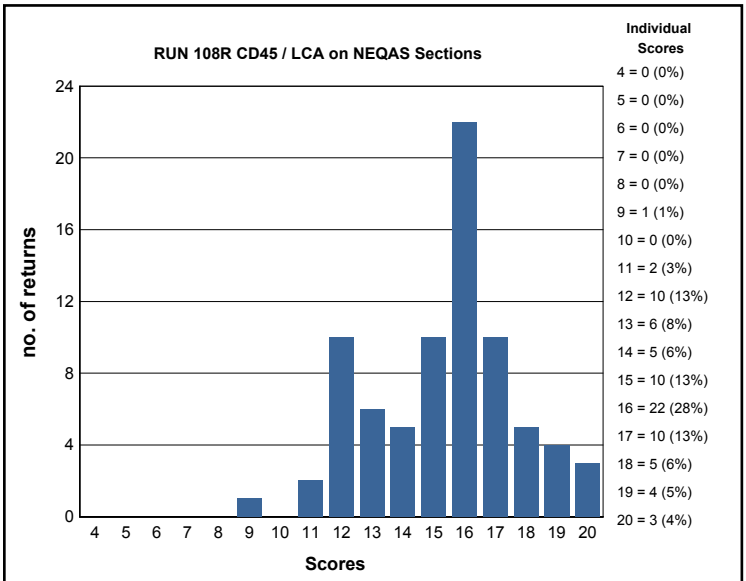


Fig 12. Excellent demonstration on a cytospin from a case of metastatic MM in a lymph node, in an in-house control slide (U). Tumour, and non-tumour cells are easily distinguished. Dako M0634 (HMB45), 1:500, for 32 min, on a Ventana Benchmark GX, and the Ventana iView system (760-091).

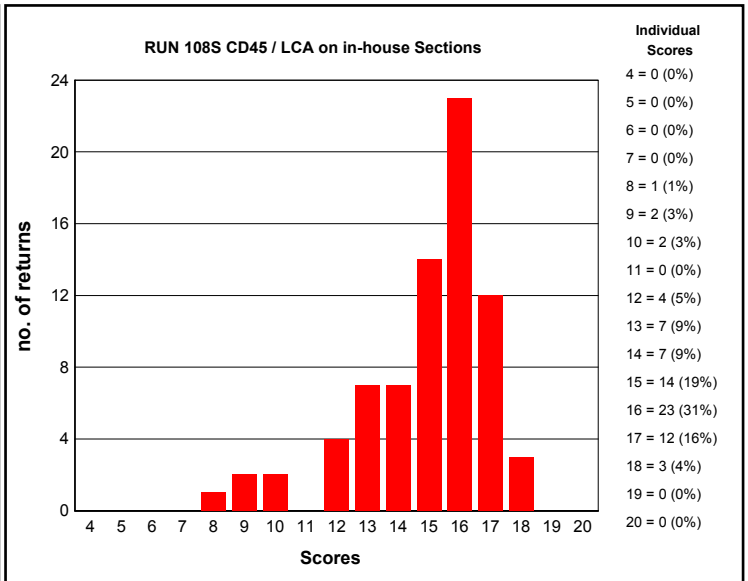
GRAPHICAL REPRESENTATION OF PASS RATES



Summary

16 - 20 = 44 (56%)
13 - 15 = 21 (27%)
10 - 12 = 12 (15%)
0 - 9 = 1 (1%)

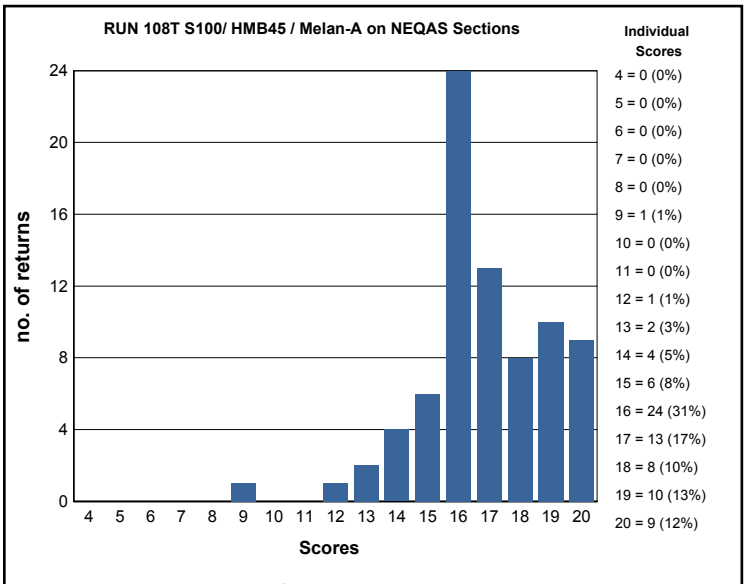
Median = 15.00



Summary

16 - 20 = 38 (51%)
13 - 15 = 28 (37%)
10 - 12 = 6 (8%)
0 - 9 = 3 (4%)

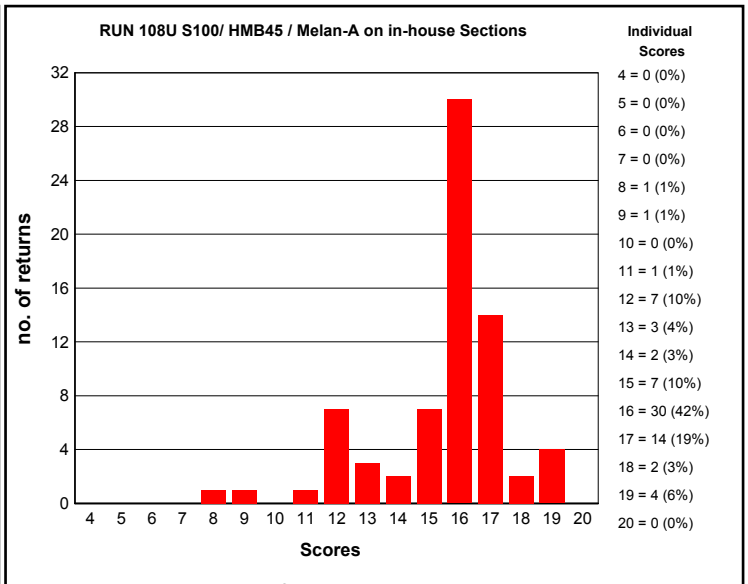
Median = 13.50



Summary

16 - 20 = 64 (82%)
13 - 15 = 12 (15%)
10 - 12 = 1 (1%)
0 - 9 = 1 (1%)

Median = 15.50



Summary

16 - 20 = 50 (69%)
13 - 15 = 12 (17%)
10 - 12 = 8 (11%)
0 - 9 = 2 (3%)

Median = 14.00

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: 108

Primary Antibody : CD45 / LCA

Antibody Details	N	%
Cell Marque 145M-97	1	100
Dako M00742 (UCHL1) CD45RO	1	100
Dako M0701 (2B11+PD7/26)	56	80
Dako Omnis RTU GA751 (2B11+PD7/26)	1	100
Dako RTU FLEX LINK IR751 (2B11 + PD7/26)	5	100
Leica/Novocastra Bond RTU PA0042 (X16/99)	2	50
Leica/Novocastra NCL-L-LCA (X16/99)	2	100
Leica/Novocastra RTU-LCA-RP (RP2/18 + 2/22)	1	100
Other	2	50
Ventana 760-4279 (2B11 & PD7/26)	1	100
Ventana CONFIRM 760-2505 (RP2/18)	6	100

Cytology Run: 108

Primary Antibody : S100/ HMB45 / Melan-A

Antibody Details	N	%
Biogenex Melan A (MART-1) (A103) AM361/MU361	1	100
Cell Marque CMA710 (HMB45)	1	100
Dako FLEX RTU Melan A (A103) IR633	3	100
Dako M0634 (HMB45)	13	100
Dako M7196 (A103 Melan A)	27	96
Dako U7025 (clone HMB45)	1	100
Dako Z0311 (S100)	4	100
Novocastra/Leica NCL-MELAN A (Melan A)	9	100
Novocastra/Leica NCL-RTU-MelanA(A103) PA0233	2	100
Other	8	88
Ventana Melan A (MART-1) 790-2990	7	100
Ventana S100 (4C4.9) 790-2914	2	100

Cytology Run: 108

Primary Antibody : CD45 / LCA

Antigen Retrieval	N	%
YES	36	46
NO	42	54
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	36	
Not Specified	0	

Cytology Run: 108

Primary Antibody : S100/ HMB45 / Melan-A

Antigen Retrieval	N	%
YES	34	44
NO	44	56
Breakdown of participants reporting YES	**N**	
Heat Mediated	0	
Enzyme	0	
Both	34	
Not Specified	0	

Cytology Run: 108

Heat Mediated Retrieval

Cytology Run: 108

Heat Mediated Retrieval

Cytology Run: 108

Enzyme Mediated Retrieval

Cytology Run: 108

Enzyme Mediated Retrieval

Cytology Run: 108

Detection	CD45 / LCA		S100/ HMB45 / Melan-A	
	N	%	N	%
AS PER KIT	6	83	5	100
Dako EnVision FLEX+ (K8002/12)	9	89	9	100
Dako Envision+ HRP mouse K4004/5/6/7	1	0	1	100
Dako REAL (K5005)	1	100	1	0
Dako REAL HRP/DAB (K5001)	1	100	0	0
Leica Bond Polymer Define (DS9713)	1	0	0	0
Leica Bond Polymer Refine (DS9800)	21	76	22	95
None	1	100	1	100
NOT APPLICABLE	1	100	1	100
Other	5	60	5	100
Ventana iView system (760-091)	3	100	4	100
Ventana OptiView Kit (760-700)	7	100	8	100
Ventana UltraView Kit (760-500)	19	89	17	100

Cytology Run: 108

Automation	CD45 / LCA		S100/ HMB45 /	
	N	%	N	%
Dako Autostainer	1	0	2	100
Dako Autostainer Link 48	11	82	12	92
Dako Autostainer Plus Link	4	100	1	100
Dako Omnis	1	100	1	100
Leica Bond Max	13	85	12	92
Leica Bond-III	12	67	12	100
None (Manual)	2	50	3	100
Other	1	0	1	100
Ventana Benchmark GX	3	100	2	100
Ventana Benchmark ULTRA	17	94	17	100
Ventana Benchmark XT	13	92	15	100

Cytology Run: 108

Chromogen	CD45 / LCA		S100/ HMB45 /	
	N	%	N	%
AS PER KIT	6	100	8	100
DAKO DAB+	2	50	0	0
Dako DAB+ Liquid (K3468)	0	0	1	100
Dako DAB+ REAL Detection (K5001)	0	0	1	100
Dako FLEX DAB	11	91	12	100
Dako REAL EnVision K5007 DAB	2	50	1	100
Dako REAL K5005 Alkaline phosphatase 1	1	100	1	0
Leica Bond Polymer Refine kit (DS9800)	23	74	22	95
Other	2	50	3	100
Sigma DAB (D5637)	1	0	0	0
Ventana DAB	4	100	5	100
Ventana Enhanced Alk. Phos. Red Detection Kit	1	100	1	100
Ventana iview	3	100	4	100
Ventana Ultraview DAB	22	91	19	100

BEST METHODS - Gold Standard Antibody

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

CD45 / LCA - Method 1

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

Primary Antibody:	Dako M0701 (2B11+PD7/26) , 15 Mins, RT ºC Dilution 1: 500
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

CD45 / LCA - Method 2

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

Primary Antibody:	Dako RTU FLEX LINK IR751 (2B11 + PD7/26)
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	AS PER KIT
HMAR:	Dako PTLink, Buffer: dako high pH TRS
EAR:	
Chromogen:	AS PER KIT, 21 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection:	, 20 Mins, 21 ºC Prediluted

CD45 / LCA - Method 3

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

Primary Antibody:	Leica/Novocastra NCL-L-LCA (X16/99) , 30 Mins, RT ºC Dilution 1: 1:400
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	_Leica Bond III ER1
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	

CD45 / LCA - Method 4

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

Primary Antibody:	Dako M0701 (2B11+PD7/26) , 20 Mins Dilution 1: 150
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 48mins
EAR:	
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana OptiView Kit (760-700)

BEST METHODS - Secondary Antibody

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

S100/ HMB45 / Melan-A - Method 1

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

Primary Antibody:	Dako M7196 (A103 Melan A) , 25 Mins, 21 ºC Dilution 1: 50
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: FLEX TRS HIGH PH
EAR:	NOT APPLICABLE
Chromogen:	Dako FLEX DAB, 21 ºC., Time 1: 10 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 20 Mins, 21 ºC Prediluted

S100/ HMB45 / Melan-A - Method 2

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

Primary Antibody:	Novocastra/Leica NCL-RTU-MelanA(A103) PA0233 , 15 Mins, 25 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)
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, 25 ºC

S100/ HMB45 / Melan-A - Method 3

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

Primary Antibody:	Novocastra/Leica NCL-MELAN A (Melan A) , 40 Mins, 37 ºC Dilution 1: 10
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, 37 ºC., Time 1: 12 Mins, Time 2: 12 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC Prediluted

S100/ HMB45 / Melan-A - Method 4

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

Primary Antibody:	Dako M0634 (HMB45) , 32 Mins, 37 ºC Dilution 1: 500
Automation:	Ventana Benchmark GX
Method:	Ventana iView Kit
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	
EAR:	
Chromogen:	Ventana iview
Detection:	Ventana iView system (760-091)

The Alimentary Tract Module: GIST Run 108

Merdol Ibrahim

	First Antibody	Second Antibody
Antigens Assessed:	CD117	DOG-1
Tissue Sections circulated:	GIST, Appendix & Desmoid	GIST, Appendix & Desmoid
Number of Registered Participants:	109	
Number of Participants This Run	108 (99%)	

Introduction

Gold Standard: CD117

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

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

- Good localisation of CD117 to cells of the GIST (Fig 1)
- Good localisation of CD117 to mast cells in the appendix and desmoid sections (Figs 3 & 5)
- Good localisation of CD117 to interstitial cells of Cajal
- No staining of the desmoid tumour (Fig 3)

Features of Suboptimal Immunostaining: **(See Figs 2 & 4)**

- Weak and/or patchy staining of the tumour cells of the GIST (Fig 2)
- Little or no staining of the mast cells
- Excessive background / non specific staining (Fig 4)
- 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. Molecular analysis should be considered[6].

Features of Optimal Immunostaining (See Figs 7, 8, 9A,10 &11)

- Good localisation of DOG-1 to cells of the GIST (Figs 7, 8 & 9A)
- Good localisation of DOG-1- to interstitial cells of Cajal (Fig 11)
- No staining of desmoid tumour

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

- Weak and/or patchy staining of the tumour cells of the GIST (Fig 9B)
- Excessive background or non specific staining (Fig 10)
- 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.

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.

Assessment Summary:

Results from the **CD117** assessment were similar to the previous assessment run (107), and showed an acceptable pass rate of 83%. A further 16% of labs received a borderline pass, and only 2 labs (2%) failed the assessment. Weak staining, particularly noticeable in the GIST, was the main reason for receiving a borderline score, and the 2 labs that failed the assessment showed barely any staining on the NEQAS sections. As with previous CD117 assessment Runs the Dako polyclonal antibody was the most popular antibody choice, which worked well on all the commercially available platforms. A high pH antigen retrieval buffer is recommended for the pre-treatment with this antibody, and this was carried out by most labs.

The **DOG-1** assessment results showed a higher acceptable pass rate of 90%. A further 9% receiving a borderline result. Again, weak staining was the main reason for failure or a borderline score.

The majority of labs are now using in house controls comprising of both normal appendix and a GIST, or a GIST which also contains normal epithelium: The use of both positive tumour and normal tissue features is a good control to help gauge the level of staining expression.

Selected Images showing Optimal and Sub-optimal Immunostaining

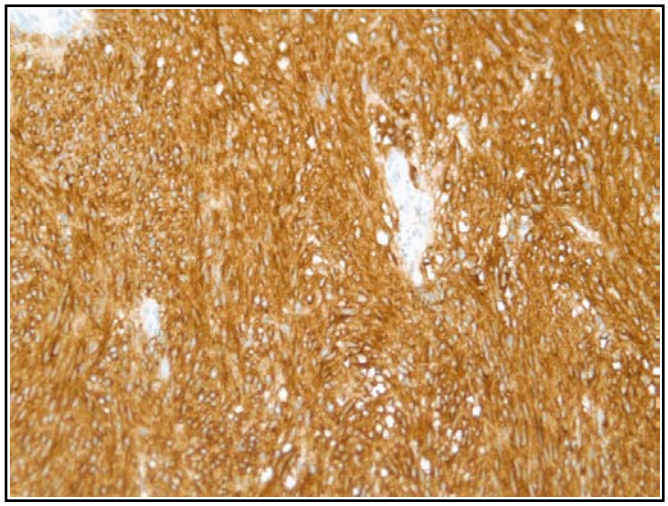


Fig 1. Optimal demonstration of CD117 in the UK NEQAS ICC distributed GIST. The section shows strong and well localised cytoplasmic and membranous staining in the tumour cells. Section stained with the Dako polyclonal antibody, 1:100, on the Dako Autostainer with pre-treatment in the PT link using high pH buffer for 20 mins. With FLEX kit detection.

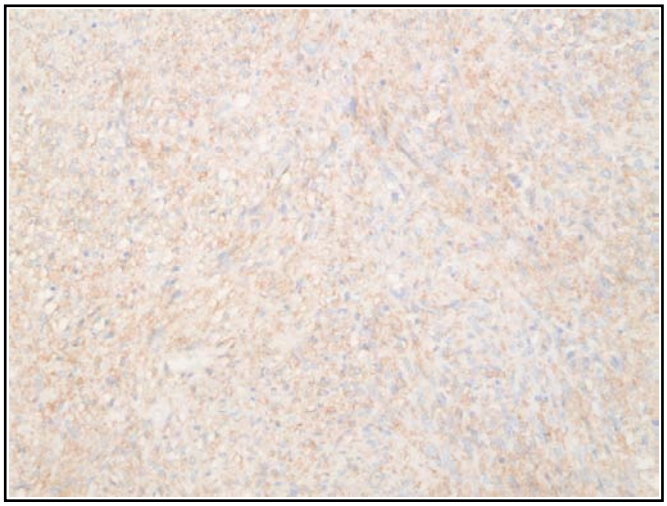


Fig 2. Suboptimal weak demonstration of CD117 in the UK NEQAS ICC distributed GIST (compare to Fig 1). The laboratory used a similar protocol to that in Fig 1, but with no antigen retrieval, which is most likely the reason for the weak staining.

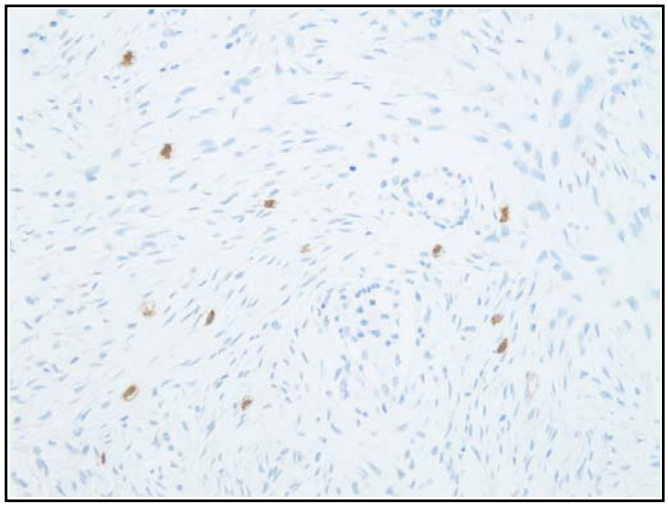


Fig 3. Optimal staining for CD117 in the UK NEQAS ICC distributed desmoid tumour. As expected, the tumour is negative but the mast cells show distinct predominantly membranous staining and act as a good internal control. Section stained with the Dako polyclonal antibody, 1:40, on the Leica BondMax with ER1 antigen retrieval for 30 mins.

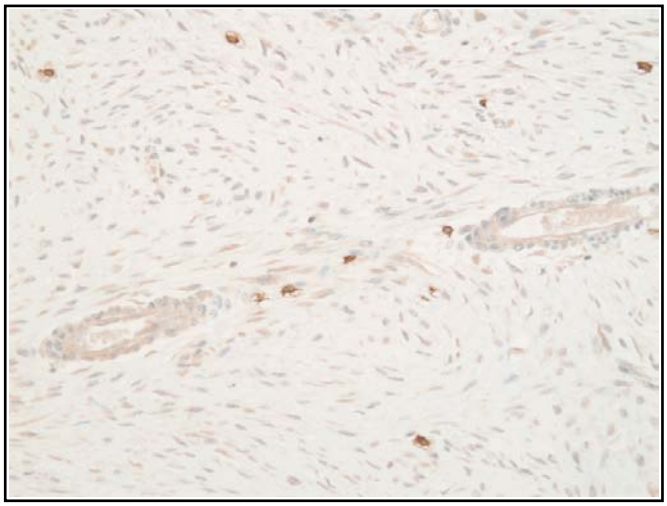


Fig 4. Suboptimal CD117 staining in the UK NEQAS distributed desmoid tumour (compare to Fig 3). As expected the mast cells are demonstrated, but the section also shows excessive non-specific background staining. Section stained with the Dako polyclonal antibody, 1:100, with the Menarini antigen access unit and Intellipath X-Cell system.

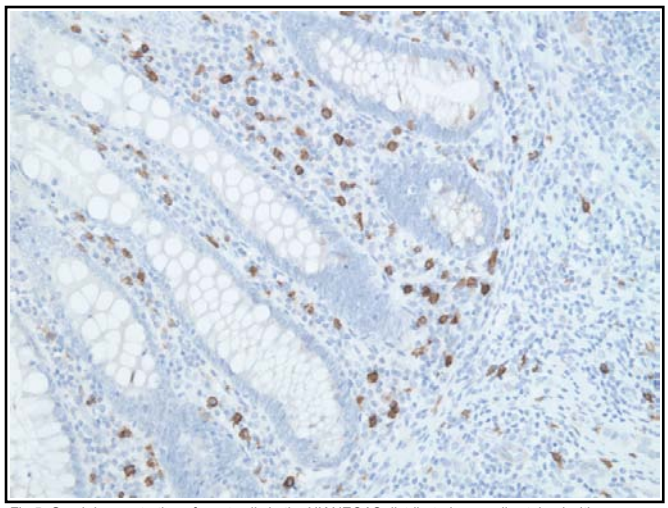


Fig 5. Good demonstration of mast cells in the UK NEQAS distributed appendix stained with CD117. Section stained with the Dako polyclonal antibody, 1:100, on the Ventana Benchmark XT and CC1 mild antigen retrieval.

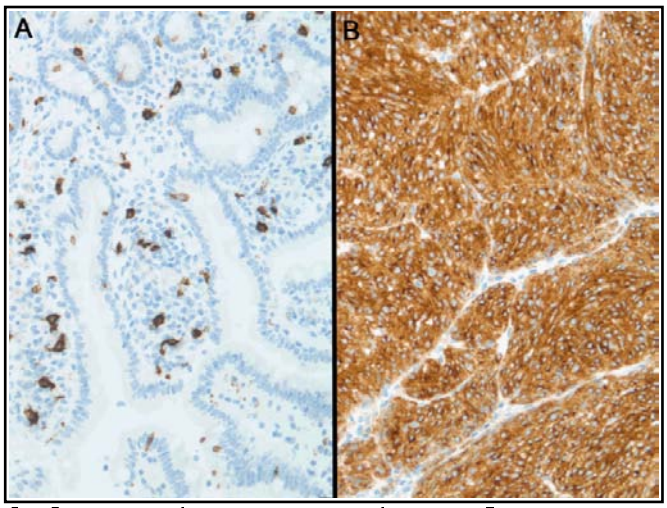


Fig 6. Excellent example of an in house control submitted for assessment. The section included an appendix (A) and a GIST (B). The lab received a score of 20/20 for providing ideal control material with optimal staining.

Selected Images showing Optimal and Sub-optimal Immunostaining

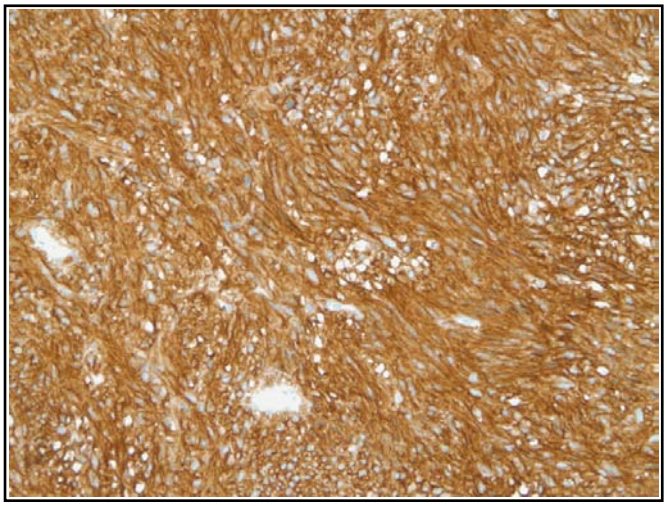


Fig 7. Optimal demonstration of DOG-1 in the UK NEQAS GIST. The tumour cells show strong crisp staining, while the non-tumour endothelial cells remain unstained. Section stained with the Leica K9 antibody, 1:50, on the Ventana Benchmark XT with CC1 standard pre-treatment.

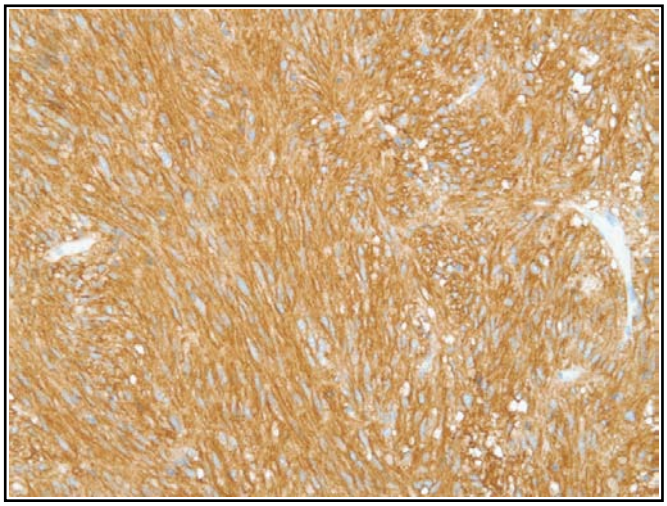


Fig 8. Good demonstration of DOG-1 in the UK NEQAS GIST, showing strong and well localised staining of the tumour cells. Section stained with the Leica RTU K9 antibody, on the BondMax Autostainer with ER2 antigen retrieval for 20 minutes.

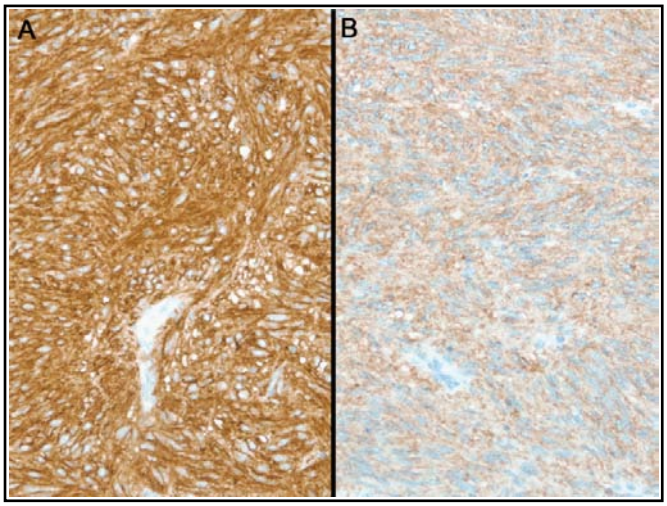


Fig 9. 2 examples of DOG-1 staining in the UK NEQAS GIST sample. Both were stained using the Ventana SP31 antibody and carried out on the Ventana Benchmark ULTRA. In (A) the staining is optimal and was carried out using a protocol of CC1 standard antigen retrieval. In (B) the staining is weak and was carried out with CC1 for 20 minutes antigen retrieval.

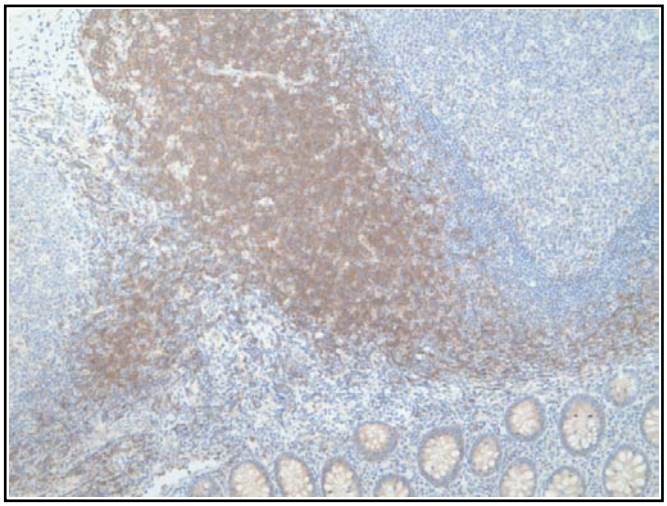


Fig 10. Poor demonstration of DOG-1 in the UK NEQAS distributed appendix. The section shows non-specific inappropriate staining of lymphocytes. Stained using the Dako polyclonal antibody on the Leica Bond III with ER2 antigen retrieval for 20 minutes.

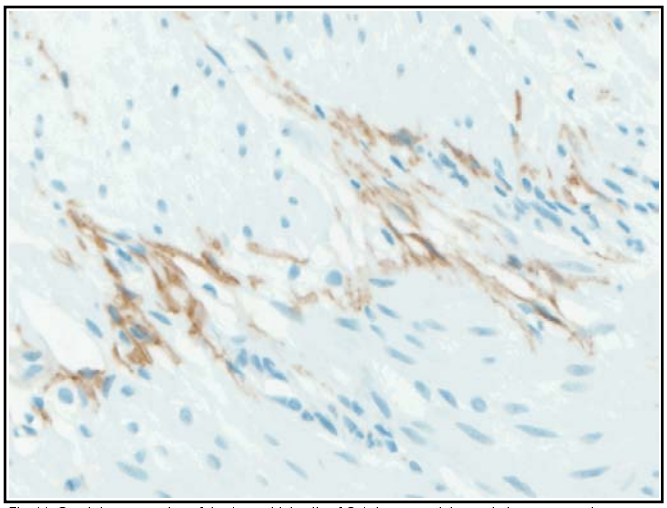


Fig 11. Good demonstration of the Interstitial cells of Cajal on a participants in house control section. Stained with the Leica K9 antibody on the Ventana Benchmark ULTRA with CC2 antigen retrieval for 44 minutes.

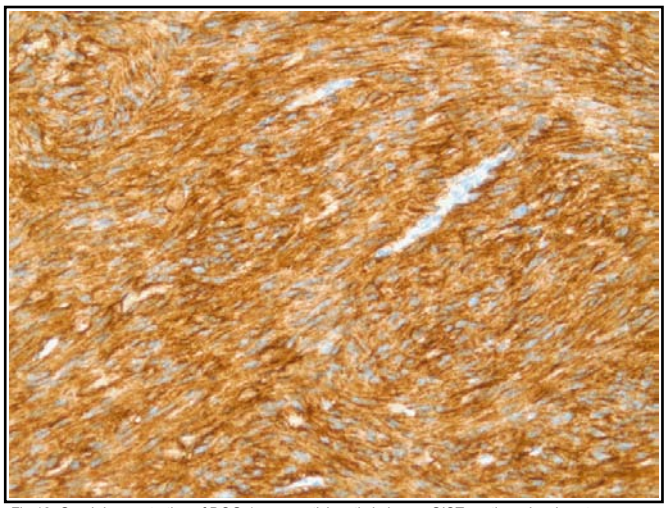
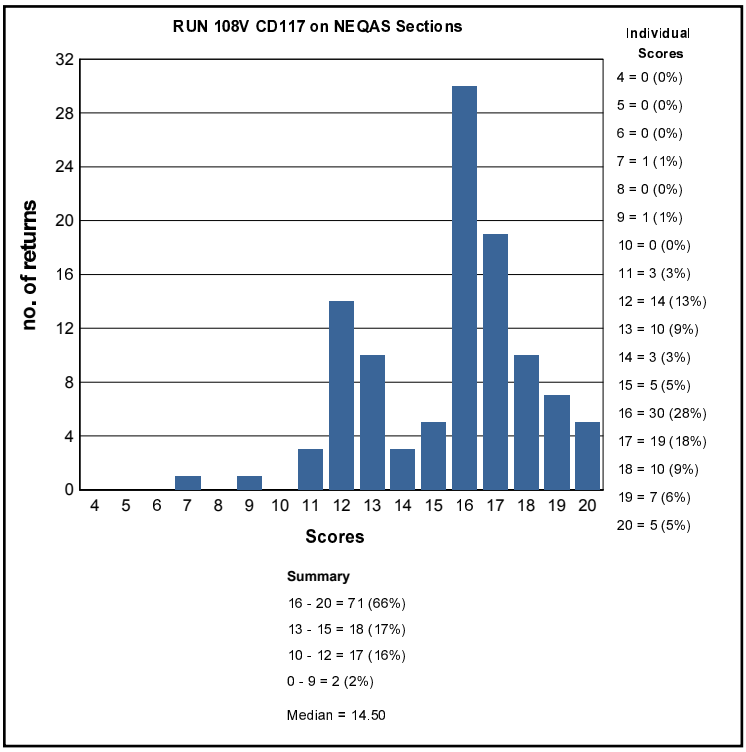
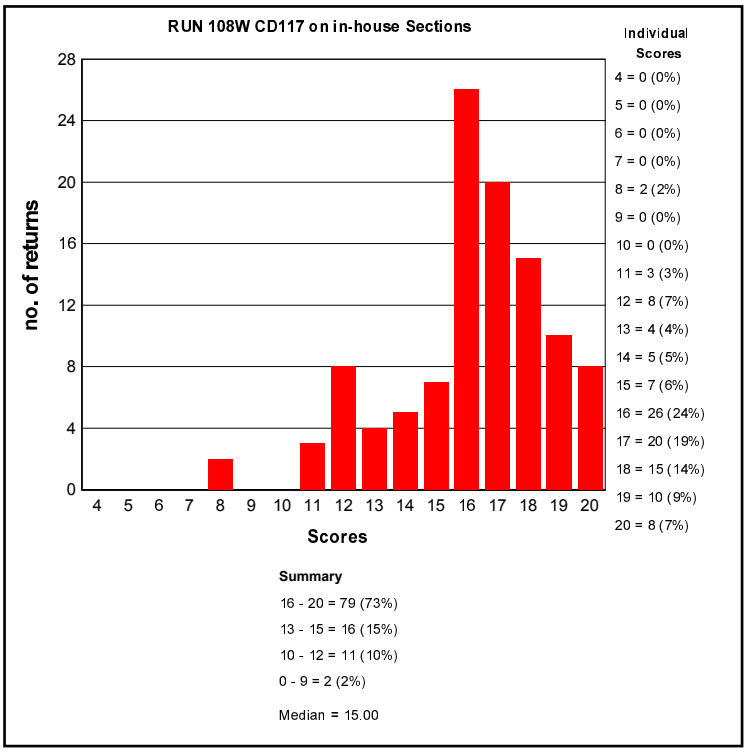
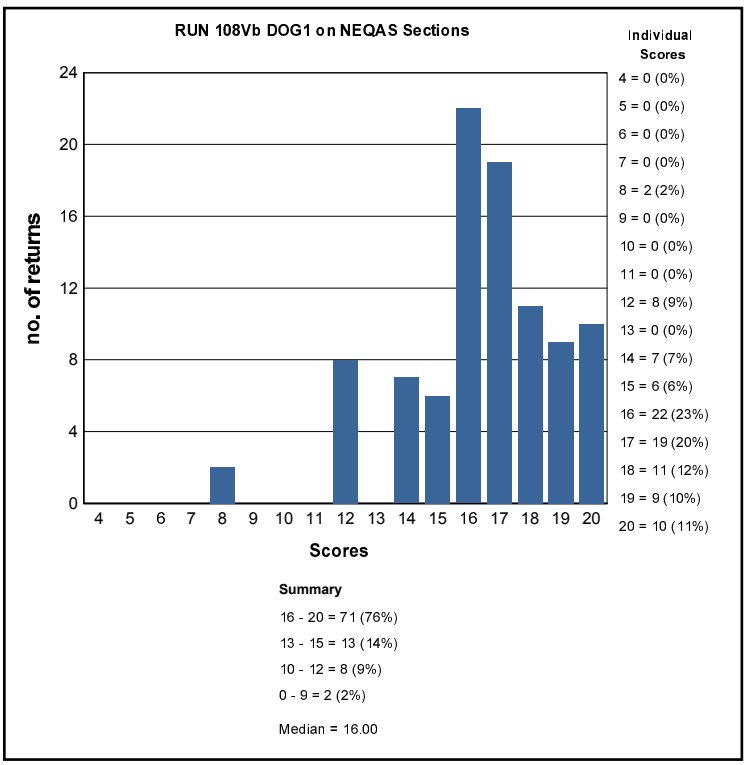


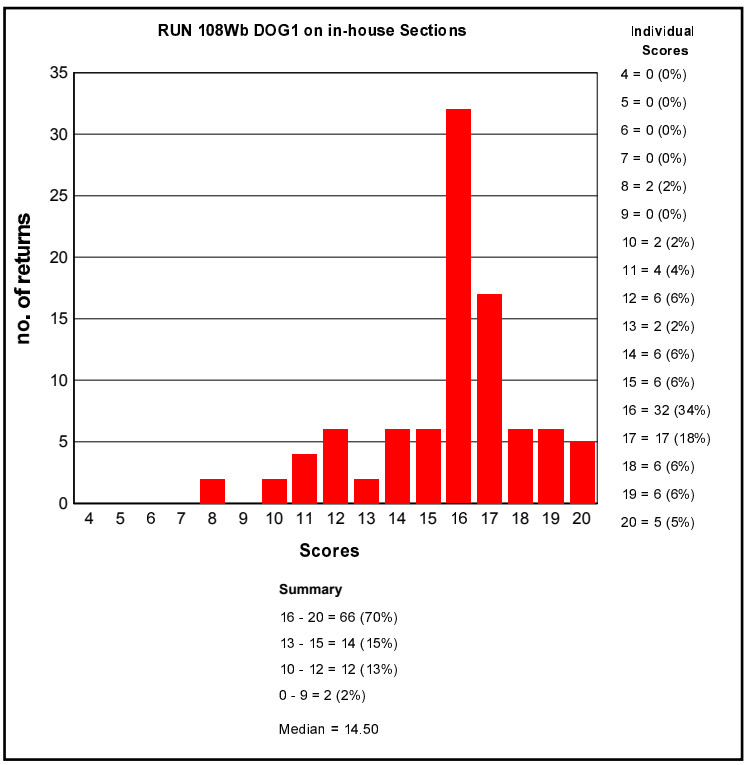
Fig 12. Good demonstration of DOG-1 on a participant's in house GIST section, showing strong and well localised staining of the tumour cells. Section stained with the Leica K9 antibody, 1:100, on the Dako Autostainer.

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: 108

Primary Antibody : CD117

Antibody Details	N	%
Cell Marque 117R/S-xx (YR145)	2	100
Dako A4502 (rb poly)	96	80
Epitomics AC-0029 (EP10)	1	100
Leica/Novocastra NCL-CD117 (T595)	1	100
NeoMarker RB 9038 (rb poly)	1	100
Ventana 790-2951 (9.7)	7	100

Alimentary Tract Pathology Run: 108

Primary Antibody : DOG1

Antibody Details	N	%
Abcam TMEM16A (ab53212)	1	100
Biocare CM 385 (1.1)	1	100
Cell Marque 244R-14/15/16 (SP31)	3	67
Cell Marque 244R-17/18 (SP31)	3	0
Diagomics 516-16714 (rb poly)	1	100
Leica NCL-L-DOG-1 (K9)	53	96
Leica PA0219 (K9)	16	100
Menarini MP-385-CM01/1	1	100
Other	2	50
Thermo RM-9132-R7 (SP31)	1	100
Ventana (SP31) 760-4590	12	75

Alimentary Tract Pathology Run: 108

Heat Mediated Retrieval	CD117 N	CD117 %	DOG1 N	DOG1 %
Dako Omnis	0	0	1	100
Dako PTLink	15	93	13	100
Leica ER1 10 mins	1	100	1	100
Leica ER1 20 mins	5	60	6	100
Leica ER1 30 mins	4	100	1	100
Leica ER1 40 mins	1	100	0	0
Leica ER2 10 mins	1	100	0	0
Leica ER2 20 mins	13	92	21	95
Leica ER2 30 mins	9	89	7	86
Microwave	0	0	1	100
None	7	43	0	0
Other	1	0	1	100
Pressure Cooker	2	50	1	100
Pressure Cooker in Microwave Oven	0	0	1	100
Steamer	1	100	1	100
Ventana CC1 16mins	1	100	2	50
Ventana CC1 20mins	0	0	1	100
Ventana CC1 24mins	0	0	1	100
Ventana CC1 32mins	5	100	4	50
Ventana CC1 36mins	5	80	1	100
Ventana CC1 40mins	0	0	1	100
Ventana CC1 48mins	1	100	1	100
Ventana CC1 52mins	2	50	2	100
Ventana CC1 56mins	3	100	0	0
Ventana CC1 64mins	7	71	6	83
Ventana CC1 mild	10	70	5	100
Ventana CC1 standard	15	87	13	77
Ventana CC2 44mins	0	0	1	100
Ventana CC2 standard	0	0	1	0

Alimentary Tract Pathology Run: 108

Enzyme Mediated Retrieval	CD117 N	CD117 %	DOG1 N	DOG1 %
NOT APPLICABLE	65	88	50	90

Alimentary Tract Pathology Run: 108	CD117		DOG1	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	8	75	7	100
BioGenex SS Polymer (QD 420-YIKE)	0	0	1	100
BioGenex SS Polymer (QD 430-XAKE)	1	100	0	0
Dako EnVision FLEX (K8000/10)	1	100	0	0
Dako EnVision FLEX+ (K8002/12)	9	89	8	100
Dako Envision HRP/DAB (K5007)	1	100	2	100
Dako Envision+ HRP mouse K4004/5/6/7	0	0	1	100
Dako Envision+ HRP rabbit K4008/9/10/11	1	100	0	0
Leica Bond Intense R Detection (DS9263)	0	0	1	100
Leica Bond Polymer Refine (DS9800)	34	85	33	94
MenaPath X-Cell Plus (MP-XCP)	1	0	1	100
Other	3	67	3	100
Ventana iView system (760-091)	3	67	3	100
Ventana OptiView Kit (760-700)	15	87	10	80
Ventana UltraView Kit (760-500)	31	77	23	74

Alimentary Tract Pathology Run: 108	CD117		DOG1	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	1	100	1	100
Dako Autostainer Link 48	13	92	11	100
Dako Autostainer plus	2	100	1	100
Dako Autostainer Plus Link	2	50	2	100
Dako Omnis	0	0	1	100
LabVision Autostainer	1	100	2	100
Leica Bond Max	14	93	13	100
Leica Bond-III	23	83	22	91
Menarini - Intellipath FLX	1	0	1	100
None (Manual)	1	0	0	0
Ventana Benchmark GX	2	100	1	0
Ventana Benchmark ULTRA	20	70	17	76
Ventana Benchmark XT	29	83	20	85

Alimentary Tract Pathology Run: 108	CD117		DOG1	
Chromogen	**N**	**%**	**N**	**%**
A. Menarini Liquid Stable DAB kit	1	100	0	0
AS PER KIT	12	92	12	100
BioGenex liquid DBA (HK-124-7K)	0	0	1	100
Dako EnVision Plus kits	2	100	2	100
Dako FLEX DAB	11	82	9	100
Dako REAL EnVision K5007 DAB	1	100	2	100
Leica Bond Polymer Refine kit (DS9800)	33	85	32	94
menapath xcell kit DAB (MP-860)	1	0	1	100
Other	1	100	2	50
Ventana DAB	10	80	5	60
Ventana iview	3	67	3	100
Ventana Ultraview DAB	34	76	25	80

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

Primary Antibody: Dako A4502 (rb poly) , 15 Mins Dilution 1: 250
Automation: Leica Bond Max
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 30 mins
EAR: NOT APPLICABLE
Chromogen: Leica Bond Polymer Refine kit (DS9800)
Detection: Leica Bond Polymer Refine (DS9800)

CD117 - Method 2

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

Primary Antibody: Dako A4502 (rb poly) , 25 Mins, 21 ºC Dilution 1: 100
Automation: Dako Autostainer Link 48
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako PTLink, Buffer: HIGH FLEX SOLUTION
EAR:
Chromogen: Dako FLEX DAB, 21 ºC., Time 1: 5 Mins, Time 2: 5 Mins
Detection: Dako Envision+ HRP rabbit K4008/9/10/11 , 25 Mins, 21 ºC Prediluted

CD117 - Method 3

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

Primary Antibody:	Dako A4502 (rb poly) , 32 Mins, RT ºC Dilution 1: 200
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 standard
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

CD117 - Method 4

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

Primary Antibody:	Dako A4502 (rb poly) , 32 Mins, 37 ºC Dilution 1: 400
Automation:	Ventana Benchmark ULTRA
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.6
HMAR:	Ventana CC1 32mins, Buffer: Ultra CC1 - TRIS, PH: 7
EAR:	NOT APPLICABLE
Chromogen:	Ventana DAB
Detection:	Ventana OptiView Kit (760-700) , as per kit ºC Prediluted

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 16/20 (In House slide) using this method.

Primary Antibody:	Leica PA0219 (K9) 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)
Detection:	Leica Bond Polymer Refine (DS9800) Prediluted

DOG1 - Method 2

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, 20 ºC Dilution 1: 100
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)

DOG1 - Method 3

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

Primary Antibody:	Ventana (SP31) 760-4590 , 32 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 standard
EAR:	NOT APPLICABLE
Chromogen:	Ventana Ultraview DAB
Detection:	Ventana UltraView Kit (760-500)

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) , 60 Mins, 37 ºC Dilution 1: 1/50
Automation:	Ventana Benchmark XT
Method:	Ventana UltraView DAB
Main Buffer:	Ventana reaction buffer (950-300), PH: 7.6
HMAR:	Ventana CC1 standard
EAR:	
Chromogen:	Ventana Ultraview DAB, 37 ºC., Time 1: 8 Mins, Time 2: 8 Mins
Detection:	Ventana UltraView Kit (760-500) , 8 Mins, 37 ºC

Lynch Syndrome/HNPCC Run 108

Suzanne Parry

	Gold Standard	Second Antibody
Antigens Assessed:	MLH1	PMS2
Tissue Sections circulated:	Normal Appendix & Colonic Tumours	Normal Appendix, & Colonic Tumours
Number of Registered Participants:	74	
Number of Participants This Run:	72 (97%)	

General Introduction

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

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

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

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

Mismatch Repair Markers

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

Clinical Interpretation & Reporting Guidelines

We recommend reporting MMR IHC findings in tumours as either:

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

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

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

Reports

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

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

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

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

Further Discussion on MMR proteins

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

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

Assessment Procedure:

Composite slides comprising of a normal appendix and a colonic tumour (with loss of MMR protein), were distributed to all participants for them to stain with **MLH1** (1st Antibody) and **PMS2** (2nd antibody) using their routine protocol. Assessment was carried out around a multi-header microscope with each slide being assessed by 4 independent assessors and a microscope lead. Both 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:
Appendix: (Figs 1 & 7)

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

Tumour without loss of MMR protein: (Figs 3 & 9)

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

Tumour with loss of MMR protein: (Figs 5 & 11)

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

Features of Suboptimal Immunostaining:
Appendix: (Figs 2 & 8)

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

Tumour without loss of MMR protein: (Figs 4 & 10)

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

Tumour with loss of MMR protein: (Figs 6 & 12)

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

Assessment Summary:

The overall acceptable pass rate (score of ≥13/20) for **MLH1** was lower than the last time that this antibody was tested . i.e. from 71% (Run 106) to 63% in the current Run (108). However, a further 25% of labs received a borderline pass, and therefore the overall pass rate (88%) was similar to that of the previous Run (90%). 8 labs (11%) failed the assessment, and this was mainly due to either weak or inappropriate non-specific staining.

The Ventana M1 and the Leica ES05 clones were the most popular choices of antibodies. These showed pass rates of 55% and 68% respectively.

The **PMS2** assessment showed a lower acceptable pass rate of 57% (N=41). A further 26% (N=19) received a borderline pass. The fail rate was quite a bit higher than the last time PMS2 was assessed (Run 106), with 17% (N=12) failing Run 108, compared to just 4 labs (6%) at Run 106.
Similarly to the MSH2 assessment results, weak staining was the main reason for failure or borderline scores (depending on the severity). One lab showed false positive staining in the tumour known to be negative for PMS2.
The Ventana EPR3947 antibody was the most popular choice of PMS2 antibody used by 27 labs. However, the acceptable pass rate for this clone was only 33%. Weak staining was the reason for the low pass rate with this antibody. The next most popular antibody was the BD Pharmingen A16-4 clone. This was by 16 labs and showed an acceptable pass rate of 75%.

In-House Control Tissue Recommendations

It was encouraging to see that the majority of labs are using an appendix section along with a colon tumour for their in house controls. Better still were the labs that had both a positive and negative tumour along with the appendix. Tonsil was used by several labs, and although this does stain positivity for both MLH1 and PMS2, the nuclear staining is very intense in both the lymphoid follicles and the overlying squamous epithelium, making it difficult to gauge the sensitivity of the assay. The assessors therefore recommend the use of appendix and normal colon as more appropriate tissues to use for control purposes for immunohistochemistry of all 4 MMR proteins.

References

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

Selected Images showing Optimal and Sub-optimal Immunostaining

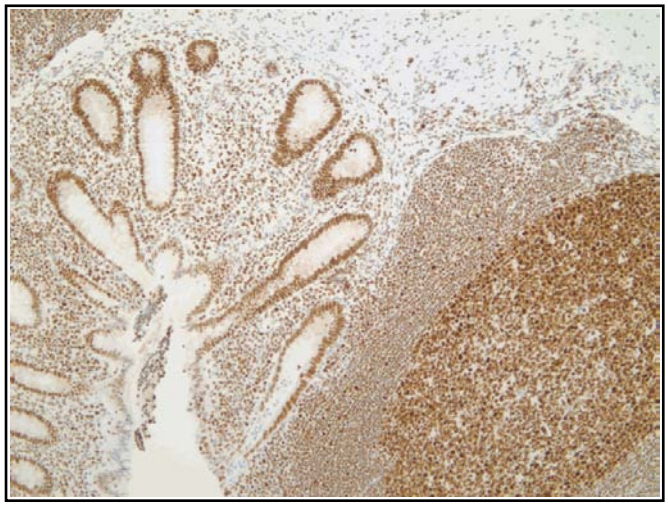


Fig 1. Optimal demonstration of MLH1 in the UK NEQAS distributed appendix. The epithelial and stromal cells show strong to moderate nuclear staining, which fades in intensity towards the luminal surface of the epithelial crypts. Stained with the Dako RTU polyclonal antibody, on the Autostainer with PT link antigen retrieval in high pH buffer for 20 mins.

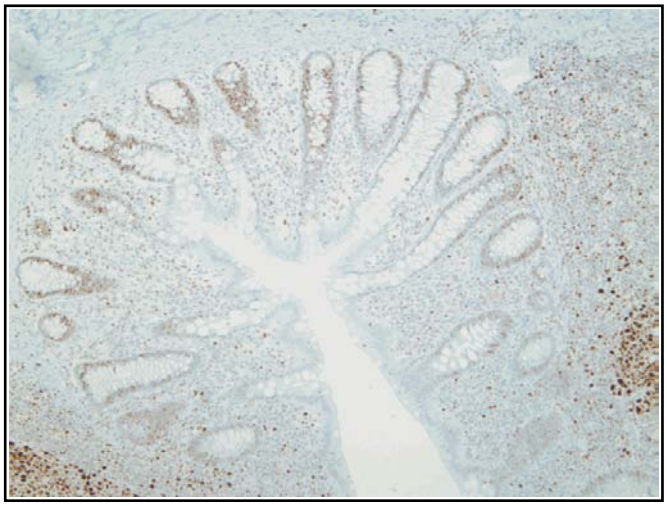


Fig 2. Suboptimal demonstration of MLH1 in the UK NEQAS appendix section. The staining intensity and proportion of cells stained is significantly reduced (compare to Fig 1). Section stained with the Leica ES05 antibody, 1:100, on the Ventana Benchmark XT with CC1 antigen retrieval for 64 minutes.

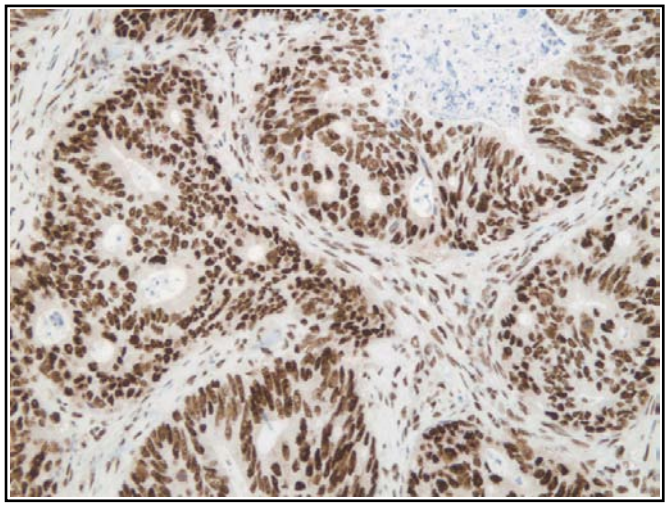


Fig 3. Optimal staining in the UK NEQAS distributed colonic tumour with normal MLH1 expression. Virtually all the tumour cells show a strong nuclear reaction. There is also distinct staining in the lymphocytes and stromal cells. Section stained with the Leica ES05 RTU antibody, on the Bond III with 20 minutes pretreatment in ER2 buffer.

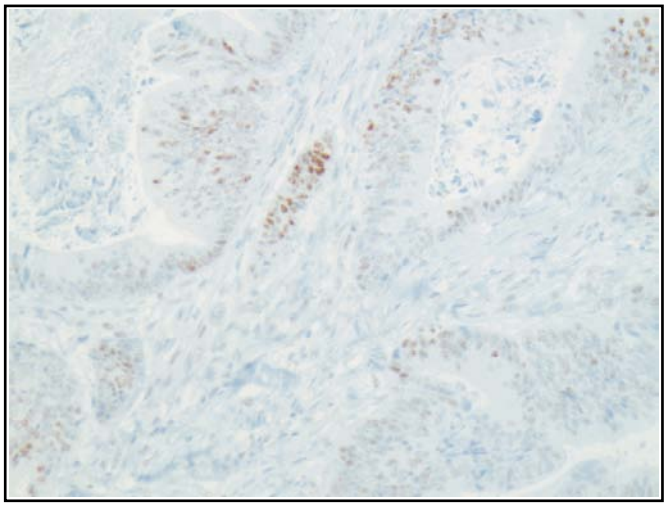


Fig 4. Suboptimal demonstration in the UK NEQAS colonic tumour known to have normal expression of MLH1. The staining is very weak in the tumour nuclei and there is no staining at all in the stromal cells (compare to Fig 3). This slide received a fail mark at the UK NEQAS assessment. Same protocol as Fig 2.

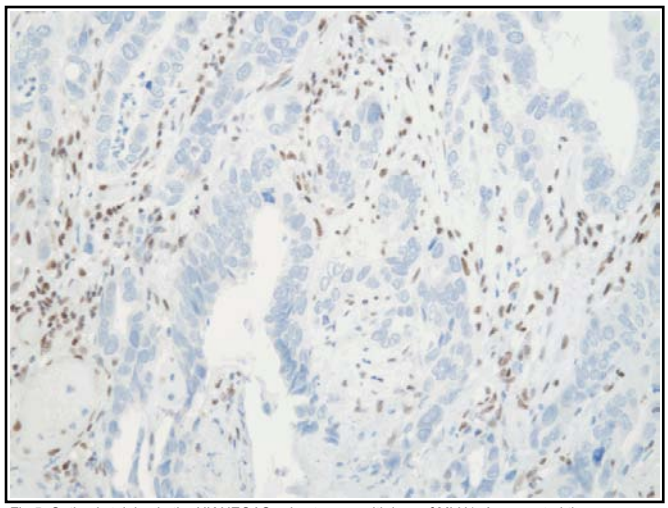


Fig 5. Optimal staining in the UK NEQAS colon tumour with loss of MLH1. As expected the neoplastic cells are negative, while the stromal and lymphocyte cells show distinct nuclear staining and therefore act as an internal positive control. Same protocol as Fig 3.

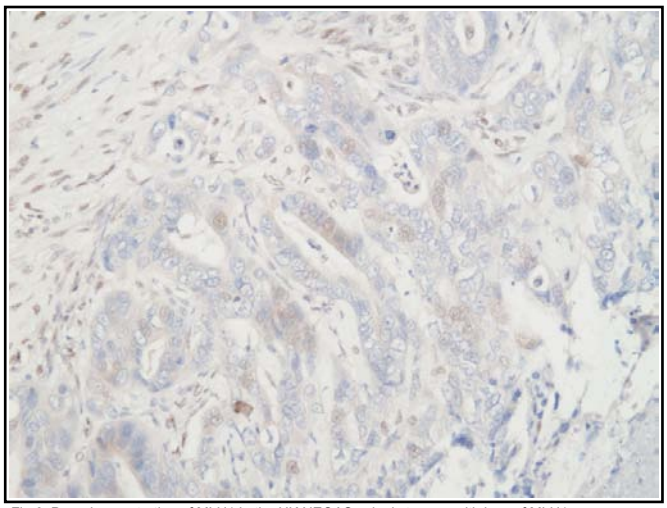


Fig 6. Poor demonstration of MLH1 in the UK NEQAS colonic tumour with loss of MLH1. section shows inappropriate background staining, and may lead to a false positive diagnosis. Excessive antigen retrieval is the most likely the cause of this inappropriate staining. Antigen retrieval carried out was ER1 for 40 minutes on the Leica BondMax machine.

Selected Images showing Optimal and Sub-optimal Immunostaining

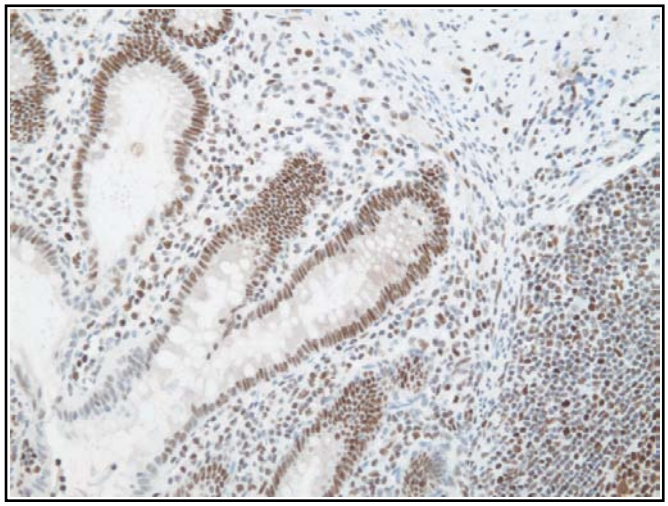


Fig 7. Optimal demonstration of PMS2 in the UK NEQAS distributed appendix. The section shows strong to moderate nuclear staining in the basal and lower half of the epithelial crypts, which fades towards the luminal surface. Section stained with the Dako EP51 antibody, 1:50, on the Leica BondMax, pre-treatment with ER2 for 20 minutes.

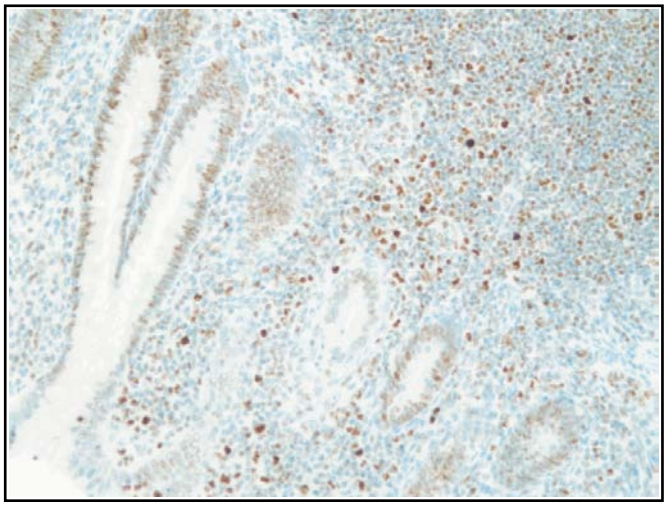


Fig 8. Suboptimal demonstration of PMS2 in the UK NEQAS distributed appendix. Although most of the cells expected to stain are demonstrated, the staining is weak (compare to Fig 7). Section stained with the Ventana EPR3947 prediluted antibody on the Benchmark ULTRA with CC1 pretreatment for 64 minutes and Optiview detection kit.

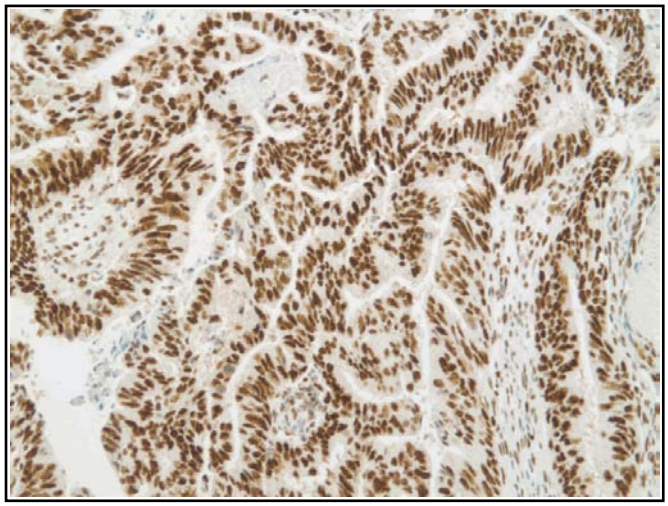


Fig 9. Good demonstration of PMS2 in the colon tumour with normal expression of PMS2. The section shows moderate to strong nuclear staining of the tumour cells and distinct staining in the lymphocytes and stromal cells. Section stained using the Cell Marque MRQ28 antibody on the Dako Autostainer with pretreatment in the PT Link for 20 minutes.

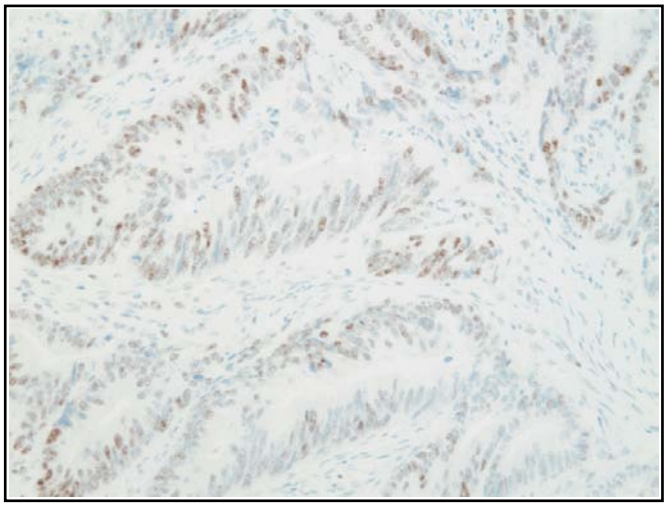


Fig 10. Suboptimal demonstration of PMS2 in the colon tumour with normal expression of PMS2 (compare to Fig 9). The proportion of positive cells and the staining intensity is much weaker than expected and none of the expected stromal cells are staining, therefore the staining result is unreliable.

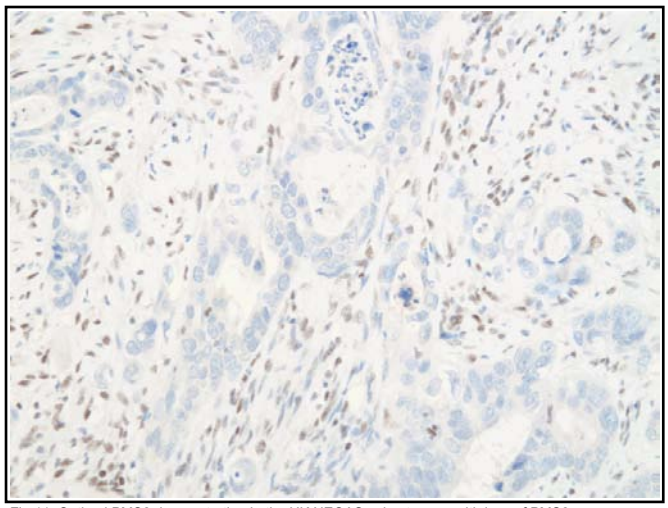


Fig 11. Optimal PMS2 demonstration in the UK NEQAS colon tumour with loss of PMS2 expression. The tumour is negative as expected, while the stromal and lymphocyte cells show nuclear staining, thereby acting as an internal positive control. Same protocol as Fig 7.

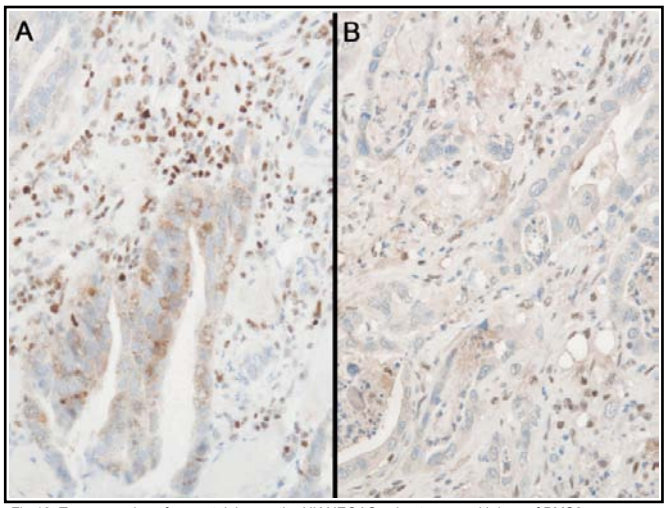
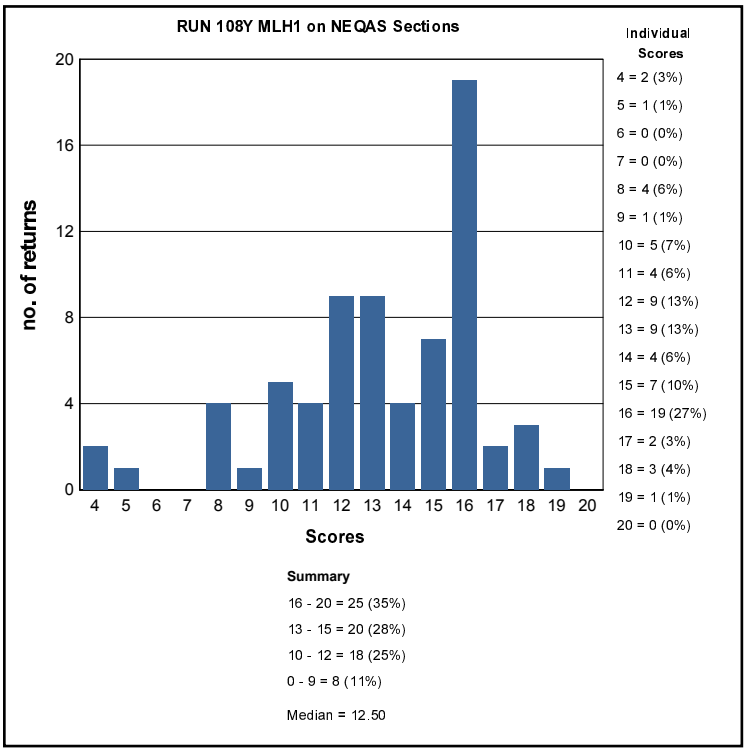
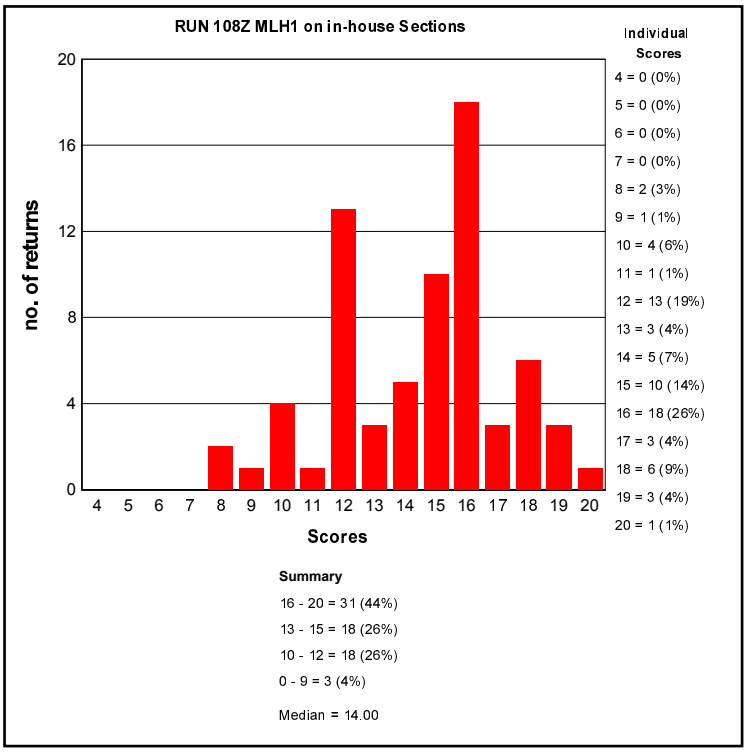
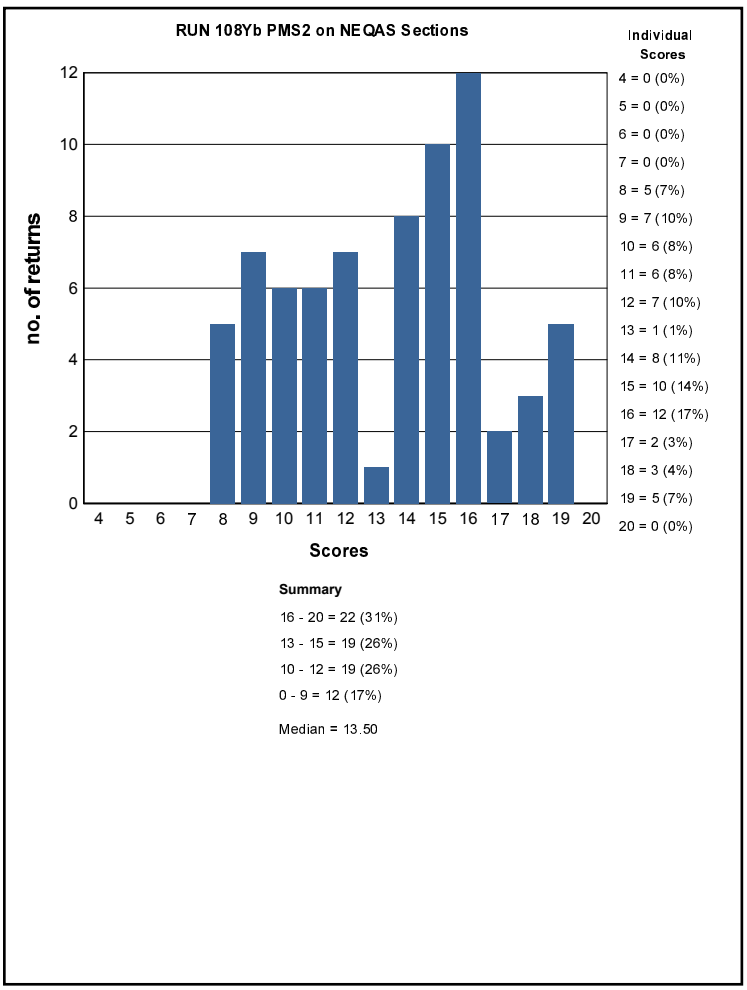


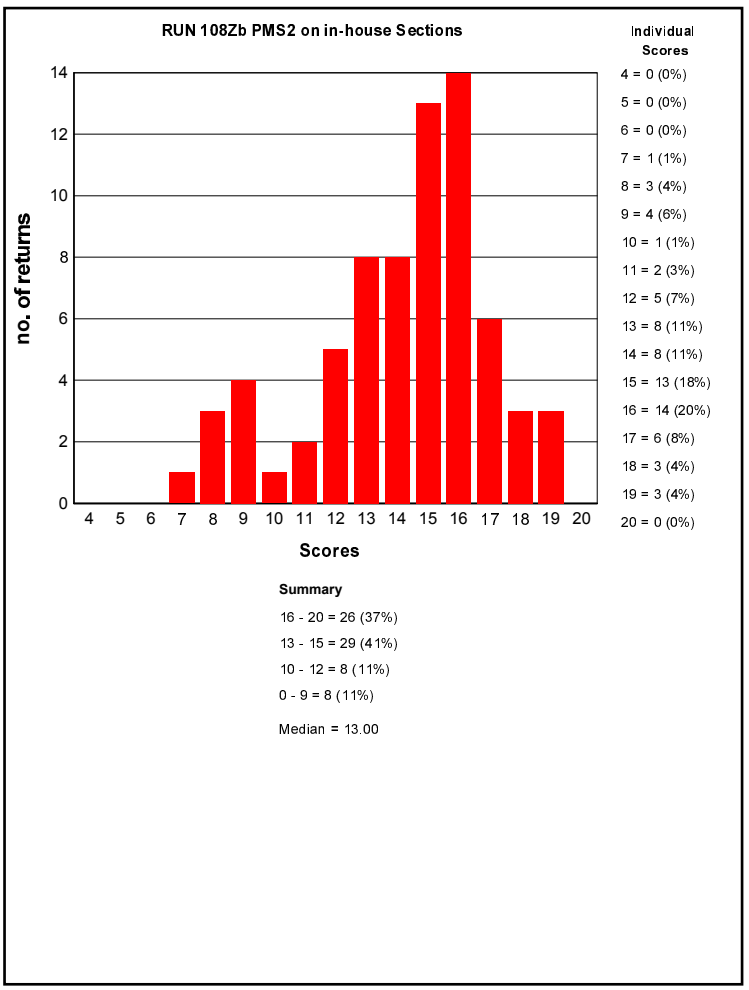
Fig 12. Two examples of poor staining on the UK NEQAS colon tumour with loss of PMS2 expression. (A) shows non-specific and cytoplasmic staining in the tumour cells, and (B) shows excessive background staining throughout the section.

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: 108

Primary Antibody : MLH1

Antibody Details	N	%
BD Pharmingen (G168-15)	9	56
BD Pharmingen (G168-728)	1	100
Biocare medical CM/PM 220 (G168-15)	1	0
Dako Flex RTU IR079/IS079 (ES05)	8	88
Dako M3640 (ES05)	4	50
Leica Bond RTU PA0610 (ES05)	3	67
Novocastra NCL-L-MLH1 (ES05)	19	68
Other	3	67
Ventana 760-4264 (G168-728)	1	100
Ventana 790-4535 (M1)	22	55

Primary Antibody : PMS2

Antibody Details	N	%
BD Bio/Pharmingen 556415 (A16-4)	16	75
Cell Marque 288M -16 (MRQ28)	2	50
Cell Marque 288R -17/18 (EPR3947)	1	0
Dako M3647 (EP51)	13	62
Dako RTU FLEX IR087 (EP51)	7	86
Leica/Novoca NCL-L-PMS2 (MOR4G)	3	100
Other	3	67
Ventana 760-4531 (EPR3947)	27	33

HNPCC Run: 108

Heat Mediated Retrieval	MLH1 N	MLH1 %	PMS2 N	PMS2 %
Biocare Decloaking Chamber	1	0	1	0
Dako PTLink	8	88	8	100
Leica ER1 20 mins	2	50	0	0
Leica ER1 30 mins	2	50	0	0
Leica ER1 40 mins	1	0	0	0
Leica ER2 20 mins	8	75	11	91
Leica ER2 30 mins	7	71	10	60
Leica ER2 40 mins	9	67	7	86
Pressure Cooker in Microwave Oven	0	0	1	0
Steamer	1	100	1	0
Ventana CC1 20mins	1	0	0	0
Ventana CC1 32mins	3	67	0	0
Ventana CC1 40mins	4	50	2	50
Ventana CC1 48mins	4	50	4	25
Ventana CC1 56mins	3	67	2	0
Ventana CC1 64mins	8	50	7	29
Ventana CC1 72mins	0	0	1	100
Ventana CC1 80mins	2	50	2	0
Ventana CC1 88mins	1	100	1	100
Ventana CC1 92mins	1	0	6	33
Ventana CC1 extended	1	100	1	100
Ventana CC1 standard	3	67	4	25
Ventana CC2 72mins	0	0	1	0
Ventana CC2 standard	1	0	1	0

HNPCC Run: 108

Enzyme Mediated Retrieval	MLH1 N	MLH1 %	PMS2 N	PMS2 %
AS PER KIT	2	100	1	100
Enzyme digestion + HIER	1	0	0	0
NOT APPLICABLE	32	69	33	58
Ventana Protease	1	100	0	0
Ventana Protease 1 (760-2018)	0	0	1	100

HNPCC Run: 108	MLH1		PMS2	
Detection	**N**	**%**	**N**	**%**
AS PER KIT	4	100	6	83
Biocare polymer (M4U534)	1	0	1	0
Dako EnVision FLEX (K8000/10)	1	100	1	0
Dako EnVision FLEX+ (K8002/12)	4	75	3	100
Dako Envision HRP/DAB (K5007)	0	0	1	0
Dako Envision+ HRP mouse K4004/5/6/7	1	100	1	100
Leica Bond Polymer Define (DS9713)	0	0	1	100
Leica Bond Polymer Refine (DS9800)	27	63	24	83
Other	2	100	2	50
Ventana OptiView Kit (760-700)	25	60	27	37
Ventana UltraView Kit (760-500)	7	29	5	0

HNPCC Run: 108	MLH1		PMS2	
Automation	**N**	**%**	**N**	**%**
BioGenex GenoMX 6000i	0	0	1	100
Dako Autostainer Link 48	6	83	6	100
Dako Autostainer plus	1	100	1	100
Dako Autostainer Plus Link	1	100	1	100
LabVision Autostainer	1	100	2	0
Leica Bond Max	9	78	8	75
Leica Bond-III	19	63	19	84
Menarini - Intellipath FLX	1	0	1	0
None (Manual)	1	0	1	0
Ventana Benchmark ULTRA	17	53	17	18
Ventana Benchmark XT	16	56	15	47

HNPCC Run: 108	MLH1		PMS2	
Chromogen	**N**	**%**	**N**	**%**
AS PER KIT	12	67	12	42
Dako DAB K3468	1	100	0	0
DAKO DAB+	1	100	1	100
Dako DAB+ Liquid (K3468)	0	0	1	100
Dako EnVision Plus kits	2	50	2	100
Dako FLEX DAB	3	100	3	67
Dako REAL EnVision K5007 DAB	0	0	1	0
Leica Bond Polymer Refine kit (DS9800)	27	67	25	84
Other	6	67	7	43
Ventana DAB	7	57	9	44
Ventana Ultraview DAB	13	38	11	18

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 16/20 (In House slide) using this method.

Primary Antibody: Dako Flex RTU IR079/IS079 (ES05) , 20 Mins, 20 ºC Prediluted
Automation: Dako Autostainer Link 48
Method: Dako FLEX+ kit
Main Buffer: Dako FLEX wash buffer
HMAR: Dako PTLink
EAR: NOT APPLICABLE
Chromogen: Dako EnVision Plus kits
Detection: Dako EnVision FLEX+ (K8002/12)

MLH1 - Method 2

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

Primary Antibody: Leica Bond RTU PA0610 (ES05) , 25 Mins, 20 ºC Prediluted
Automation: Leica Bond-III
Method: Leica BondMAx Refine KIT
Main Buffer: Bond Wash Buffer (AR9590)
HMAR: Leica ER2 20 mins
EAR:
Chromogen: Leica Bond Polymer Refine kit (DS9800)
Detection: Leica Bond Polymer Refine (DS9800) Prediluted

MLH1 - Method 3

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

Primary Antibody:	BD Pharmingen (G168-15) , 15 Mins Dilution 1: 30
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 40 mins
EAR:	NOT APPLICABLE
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins

MLH1 - Method 4

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

Primary Antibody:	Ventana 790-4535 (M1) , 32 Mins, 37 ºC Prediluted
Automation:	Ventana Benchmark XT
Method:	Ventana Optiview
Main Buffer:	Ventana reaction buffer (950-300)
HMAR:	Ventana CC1 48mins, Buffer: CC1
EAR:	NOT APPLICABLE
Chromogen:	Other, 37 ºC., Time 1: 8 Mins, Time 2: 8 Mins
Detection:	Ventana OptiView Kit (760-700) , 8 Mins, 37 ºC Prediluted

BEST METHODS - Secondary Antibody

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

PMS2 - Method 1

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

Primary Antibody:	Dako M3647 (EP51) , 30 Mins, 37 ºC Dilution 1: 1:40
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 40 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800), RT ºC., Time 1: 10 Mins
Detection:	Leica Bond Polymer Refine (DS9800) , 8 Mins, RT ºC Prediluted

PMS2 - Method 2

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

Primary Antibody:	Dako RTU FLEX IR087 (EP51) , 25 Mins, 20 ºC Prediluted
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica ER2 20 mins
EAR:	
Chromogen:	Leica Bond Polymer Refine kit (DS9800)
Detection:	Leica Bond Polymer Refine (DS9800) Prediluted

PMS2 - Method 3

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

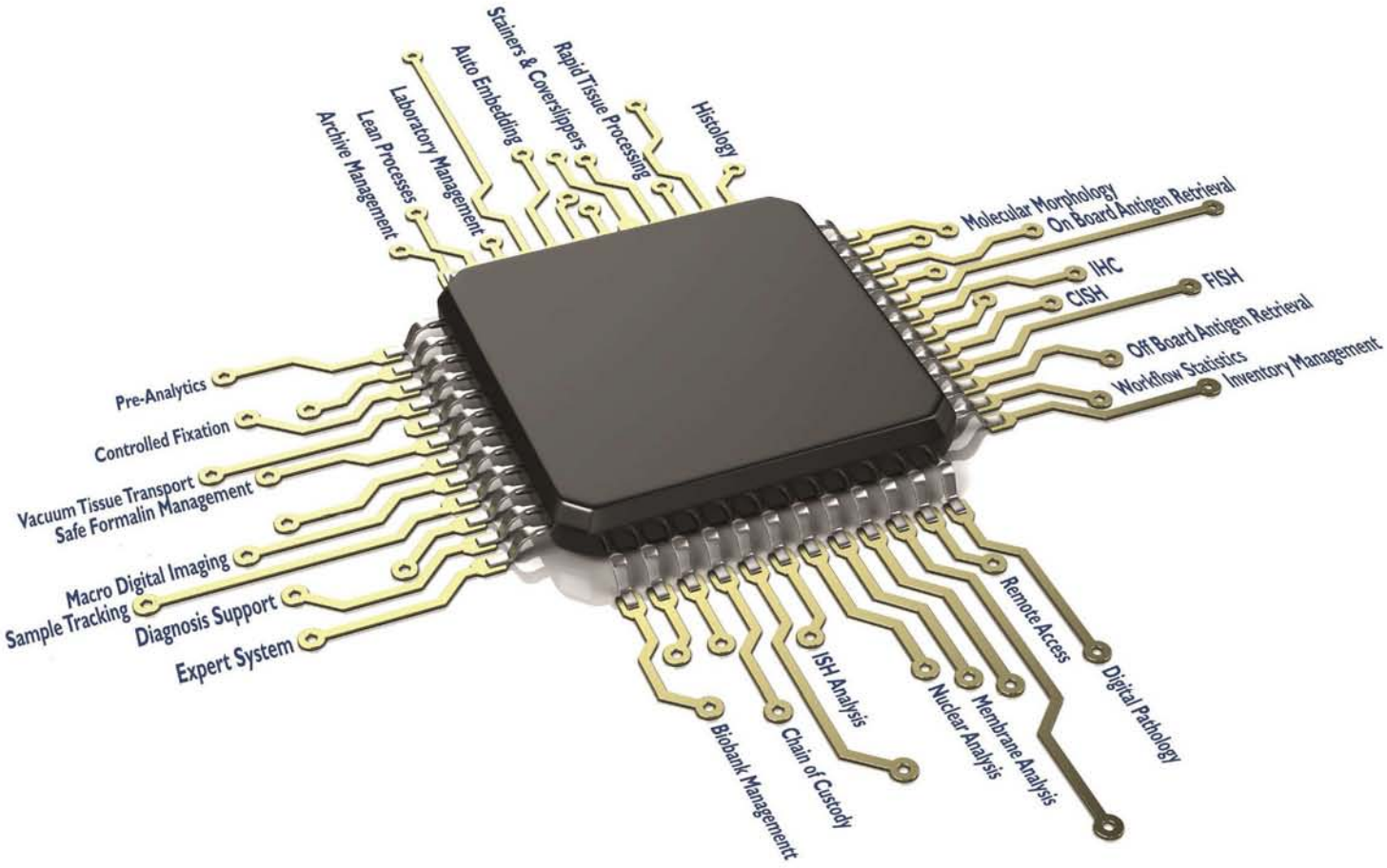
Primary Antibody:	BD Bio/Pharmingen 556415 (A16-4) , 40 Mins, 23 ºC Dilution 1: 100
Automation:	Dako Autostainer Link 48
Method:	Dako FLEX+ kit
Main Buffer:	Dako FLEX wash buffer
HMAR:	Dako PTLink, Buffer: Target retrieval solution, PH: 9
EAR:	
Chromogen:	Dako EnVision Plus kits, 23 ºC., Time 1: 6 Mins, Time 2: 6 Mins
Detection:	Dako EnVision FLEX+ (K8002/12) , 20 Mins, 23 ºC Prediluted

PMS2 - Method 4

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

Primary Antibody:	Cell Marque 288M -16 (MRQ28) , RT ºC Prediluted Dilution 1: 30
Automation:	Dako Autostainer plus
Method:	Dako FLEX+ kit
Main Buffer:	AS PER KIT
HMAR:	Dako PTLink
EAR:	AS PER KIT
Chromogen:	AS PER KIT, PH: 9
Detection:	AS PER KIT

Cellular Pathology is changing...



TOTAL TISSUE DIAGNOSTICS

A Fully Integrated Laboratory Solution

From surgical specimen to digital slide and diagnosis

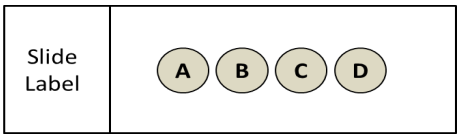
A.MENARINI diagnostics

The HER2 ISH Module - Interpretive Run 37

Merdol Ibrahim and Suzanne Parry

Gene Assessed:	HER2
Sections Circulated:	Composite slide consisting of 4 breast carcinoma sections (see table below)
Number of Registered Participants:	226
Number of Participants This Run	196 (87%)

Tissue Section Positioning:
Tissue sections were positioned on microscope slides as illustrated below.



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	HER2 status by IHC	HER2 status by FISH
A	**3+**	Amplified
B	**2+**	Non-Amplified
C	**2+**	Non-Amplified
D	**2+**	Non-Amplified

Introduction

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

Recommendations

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

Updated Assessment Procedure

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

Assessment Results

Similarly to the previous assessments, all participants taking part in the module are using a dual probe (ratio) method. 60% of labs are using a fluoescence ISH method. This is slightly lower than the number of labs that used a fluorescent ISH technique in the previous run (65%) . The Pathvysion Vysis kit still remains the most popular, and this was used by 60 labs (51% of FISH method users). The Ventana DDISH was the most favoured chromogenic ISH technique, which was used by 48 labs (75% of CISH method users).
Overall the acceptable pass rate for all participants was 70% (≥30/36). A further 25% received a borderline pass (24-29/36), and 11 labs (6%) failed the assessment (scores of <24/36). Interestingly, the pass rate for the FISH users was 82% compared to 40% for those labs using the chromogenic method. The Ventana DDISH method alone showed a pass rate of 58%, which was a slight improvement on the previous run where only 49% of participants using this kit passed the assessment. In comparison the FISH Pathvysion Vysis kit showed a 73% pass rate. The China Medical Technologies probes (N=15), Dako IQFISH Pharm DX (n=6) and the Leica FISH (N=8) also shoed good pass rates of 73%, 83% and 100% respectively. Please see graphical representation and methods used for a detailed breakdown of results.

Frequency Histograms

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

HER2 ISH Method and Probe Enumeration

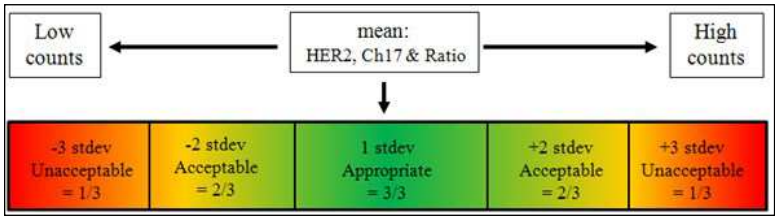
The table below (table 1) shows the various ISH methods employed along with mean HER2, Cen17 counts and ratio calculations.

The Ventana DDISH method showed on average lower cen17 counts when compared to the two other main methodologies submitted, namely the Pathvysion vysis and China Medical technologies. This was quite evident in 'sample D', which resulted in a higher average ratio for those using the Ventana DDISH kit.

Further analysis of 'sample D' submitted ratio calculations showed that 27% (13/48) of submitted results had a an average ratio ≥2.00; whereas 0% of Vysis or china Medical ISH users had average ratios ≥2.00.

Table: Statistical Approach to the Interpretive ISH Scoring System

1. All participant data is initially evaluated to exclude '**outliers**'. An outlier is defined as those scores that are 1.5 times the interquartile range (IQR) outside of the lower and upper quartiles.
2. A mean score is then generated for the submitted HER2 copy, chromosome 17 and ratios.
3. Counts for HER2 copy, CEN17 and ratio are scored individually such that results within +/- 1 standard deviation (stdev) of the mean are score 3/3, +/- 2 stdev = 2/3 and ≥ +/- 3 stdev = 1/3. A non submission is scored 0/3.
4. For each of the samples there is therefore a possible score out of 9 (those using a ratio method) and 3 (those using a single copy method). As NEQAS ICC & ISH distributes 4 samples there is a possible score out of 36 (those using a ratio method) and 12 (those using a single copy method). (score = 3).



Dual Probe: Ratio Scoring Method

- Marks are awarded for each criteria (HER2 copy, cen17 and Ratio) with a possible score out of 9 for each sample (A,B,C & D). For the 4 samples, the marks are added together to give a possible score out of 36.
- Marks are awarded for each criteria (HER2 copy, cen17 & Ratio) by comparing your score to standard deviation (stdev) from the mean score, such that: (a) 3/3 = within 1 stdev from the mean, (b) 2/3 = within 2 stdev from the mean (c) 1/3 = > 3 stdev from the mean.
- Score interpretation: 36/36 = Excellent ; 30-35/36 = Acceptable ; 24-29/36 = Borderline ; <24/36 = Unacceptable

Single Probe: HER2 Copy Scoring Method

- Marks are awarded for HER2 copy counts, with a possible score out of 3 for each samples (A,B,C & D). For the 4 samples, the marks are summed together to give a possible score out of 12.
- Marks are awarded for HER2 copy counts by comparing your counts with the standard deviation (stdev) from the mean score, such that: (a) 3/3 = within 1 stdev from the mean, (b) 2/3 = within 2 stdev from the mean (c) 1/3 = > 3 stdev from the mean.
- Score interpretation: 12/12 = Excellent ; 10-11/12 = Acceptable ; 8-9/12 = Borderline ; <8/12 = Unacceptable

The low cen17 counts was again noted during the technical assessment (see write-up on technical assessment module).

How these observations translate into the clinical setting is not clear, but participants should make sure, that whichever method they are using, it is reproducible and quality controlled, and should include making a note of batch numbers, auditing cases on a regular basis, including control material of known expression, and monitoring 'normal cells' within the clinical sample being tested to make sure that the hybridisation is optimal.

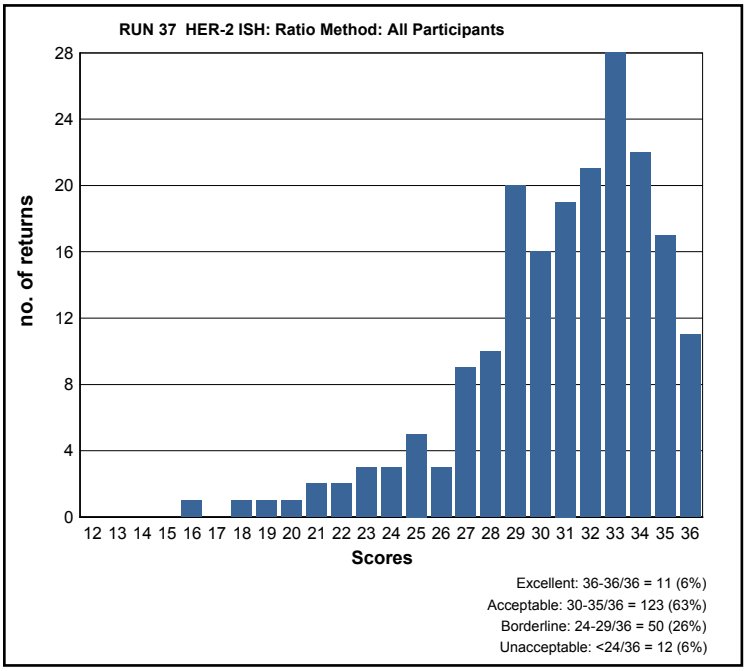
References

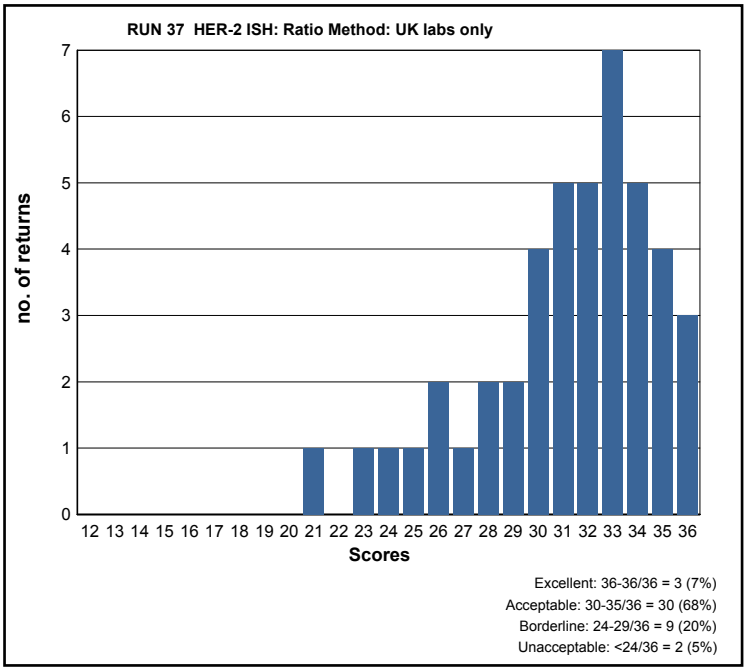
1. Walker RA, Bartlett JM, Dowsett M, Ellis IO, Hanby AM, Jasani B, Miller K. Pinder SE. (2008) HER2 testing in the UK: further update recommendations. J Clin Pathol;61818-824.
2. Bartlett JM, Starczynski J, Atkey N, Kay E, O'Grady A, Gandy M, Ibrahim M, Jasani B, Ellis IO, Pinder SE, Walker RA. (2011)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

Table 1: Methods and mean counts/ratios (n/a= not applicable ; n/d = not done):

		Sample A: mean (stdev)			Sample B: mean (stdev)			Sample C: mean (stdev)			Sample D: mean (stdev)		
METHOD	n=	Copy No	cen17	Ratio	Copy No	cen17	Ratio	Copy No	cen17	Ratio	Copy No	cen17	Ratio
Ratio: Pathvysion Vysis Kit	64	19.48 (5.58)	2.34 (0.46)	8.33 (2.05)	1.98 (0.52)	1.77 (0.33)	1.13 (0.27)	2.9 (0.79)	2.35 (0.6)	1.22 (0.15)	3.08 (0.65)	2.44 (0.54)	1.26 (0.24)
Ratio: Ventana DDISH (780/800-4422)	52	18.35 (4.63)	2.10 (0.56)	9.02 (2.46)	2.06 (0.62)	1.74 (0.3)	1.22 (0.36)	2.93 (0.95)	2.13 (0.54)	1.35 (0.38)	3.24 (1.17)	1.94 (0.5)	1.74 (0.57)
Ratio: China Medical Technologies Kit	15	21.01 (4.99)	2.28 (0.33)	9.28 (2.33)	2.01 (0.6)	1.81 (0.56)	1.15 (0.32)	2.76 (0.74)	2.28 (0.75)	1.23 (0.36)	3.33 (0.62)	2.53 (0.78)	1.32 (0.38)
Ratio: Other - FISH	14	17.48 (6.02)	2.11 (0.23)	7.92 (2.32)	2.00 (0.2)	1.78 (0.19)	1.12 (0.11)	2.8 (0.59)	2.14 (0.32)	1.30 (0.17)	2.80 (0.35)	2.13 (0.26)	1.33 (0.24)
Ratio: Leica HER2 FISH TA9217	8	20.19 (4.67)	2.46 (0.33)	8.25 (1.63)	1.98 (0.18)	1.82 (0.18)	1.10 (0.08)	2.92 (0.82)	2.23 (0.46)	1.31 (0.25)	3.22 (0.45)	2.37 (0.32)	1.37 (0.14)
Ratio: Ventana BDISH 800-098/505	8	18.03 (8.65)	2.1 (0.99)	8.12 (1.09)	2.21 (1.17)	1.73 (0.92)	1.17 (0.42)	2.93 (1.56)	2.28 (1.24)	1.31 (0.24)	3.53 (1.67)	2.13 (1.08)	1.78 (0.91)
Ratio: Zytovision ZytoLight	8	19.17 (5.22)	2.05 (0.46)	9.31 (1.25)	1.95 (0.93)	1.71 (0.3)	1.14 (0.11)	2.61 (0.71)	2.18 (0.47)	1.19 (0.12)	2.87 (0.68)	2.08 (0.45)	1.38 (0.1)
Ratio: Dako IQFISH pharmDX	6	20.27 (9.9)	2.53 (0.24)	7.85 (1.90)	1.94 (0.82)	1.75 (0.73)	1.12 (0.09)	3.22 (1.4)	2.51 (1.06)	1.31 (0.14)	3.00 (1.27)	2.3 (1.02)	1.42 (0.4)
Ratio: Dako Pharm Dx	5	18.15 (6.7)	2.13 (0.29)	8.52 (2.92)	2.06 (0.22)	1.61 (0.27)	1.29 (0.15)	3.61 (1.19)	2.34 (0.59)	1.41 (0.67)	2.64 (0.71)	1.89 (0.32)	1.40 (0.33)
Ratio: Ventana Inform Silver ISH	5	20.3 (11.25)	2.46 (1.15)	8.74 (5.08)	2.23 (0.24)	1.58 (0.37)	1.32 (0.6)	2.21 (0.63)	1.63 (0.45)	1.42 (0.23)	2.99 (0.92)	1.62 (0.5)	1.70 (0.80)
Ratio: Kreatech Probes	4	24.01 (2.97)	2.35 (0.12)	10.27 (1.49)	1.81 (0.21)	1.56 (0.16)	1.17 (0.10)	2.92 (1.02)	2.23 (0.46)	1.28 (0.22)	3.14 (0.18)	2.39 (0.29)	1.33 (0.13)
Ratio: In house FISH	4	16.5 (5.81)	2.38 (0.29)	6.81 (1.80)	1.95 (1.01)	1.81 (0.34)	1.23 (0.15)	3.04 (1.11)	2.58 (0.56)	1.41 (0.29)	3.00 (1.52)	2.38 (0.39)	1.54 (0.42)
Ratio: Dako DuoCISH	2	14.28 (1.03)	1.85 (0.07)	7.71 (0.25)	1.95 (0.21)	1.68 (0.39)	1.18 (0.16)	4.09 (0.3)	3.15 (0.35)	1.31 (0.24)	2.48 (0.03)	1.78 (0.57)	1.47 (0.48)
Ratio: Zytovision ZytoDot 2C	1	nd (n/a)	nd (n/a)	9.86 (n/a)	nd (n/a)	nd (n/a)	1.52 (n/a)	nd (n/a)	nd (n/a)	1.13 (n/a)	nd (n/a)	nd (n/a)	2.3 (n/a)

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

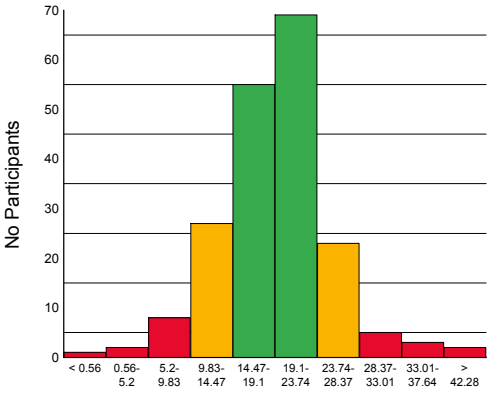
Ratio Method	N	% Pass (score ≥ 30/36)
Ratio: China Medical Technologies Kit	15	73%
Ratio: Dako DuoCISH	2	50%
Ratio: Dako IQFISH pharmDX	6	83%
Ratio: Dako Pharm Dx	5	40%
Ratio: In house FISH	4	50%
Ratio: Kreatech Probes	4	100%
Ratio: Leica HER2 FISH TA9217	8	100%
Ratio: Other - FISH	14	100%
Ratio: Pathvysion Vysis Kit	64	73%
Ratio: Ventana BDISH 800-098/505	8	50%
Ratio: Ventana DDISH (780/800-4422)	52	54%
Ratio: Ventana Inform Silver ISH	5	40%
Ratio: Zytovision ZytoDot 2C	1	0%
Ratio: Zytovision ZytoLight	8	75%

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

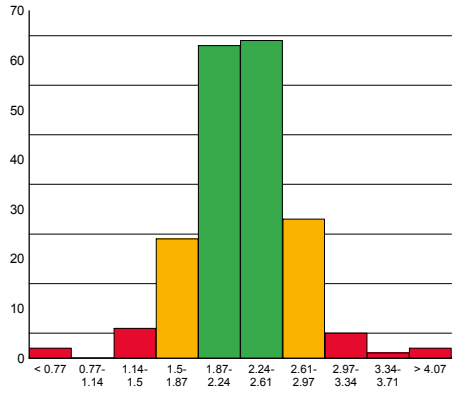
FREQUENCY HISTOGRAMS

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

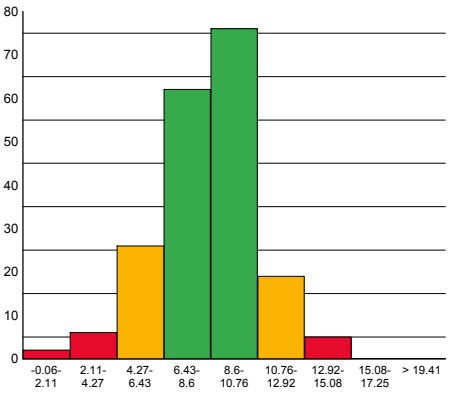
Green = mean +/- 1 Stdev Amber = mean +/- 2 Stdev Red = mean ≥ +/- 3 stdev



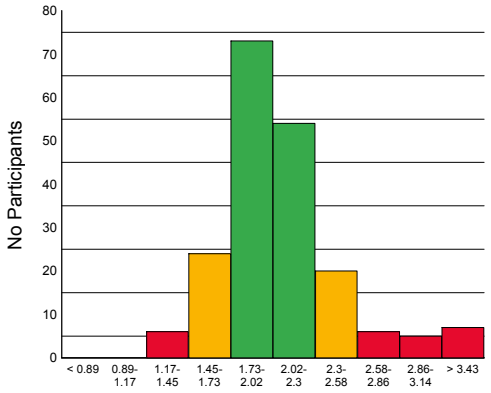
Sample A: Av. HER2 copy



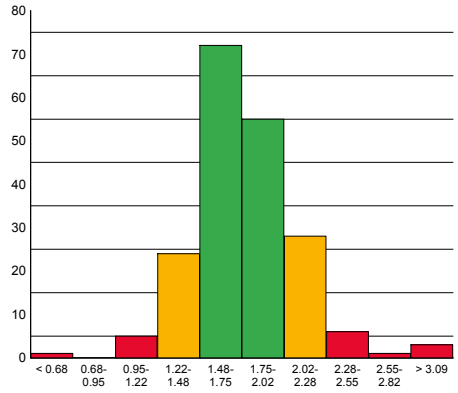
Sample A: Av. Chr17



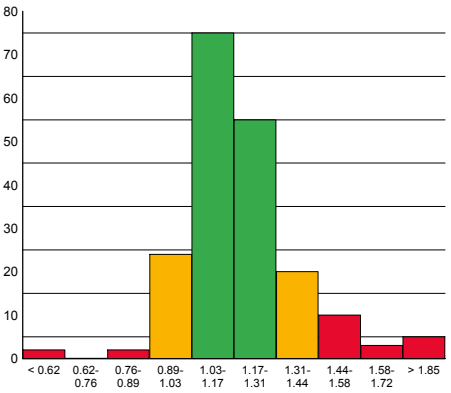
Sample A: Ratio



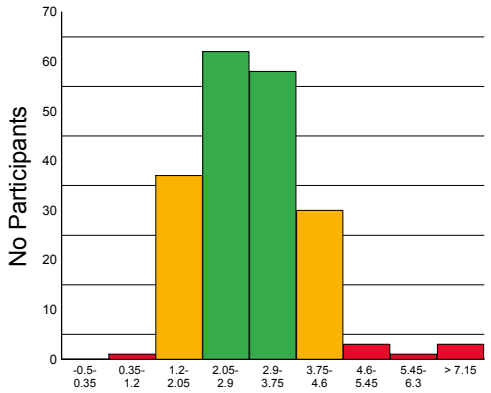
Sample B: Av. HER2 copy



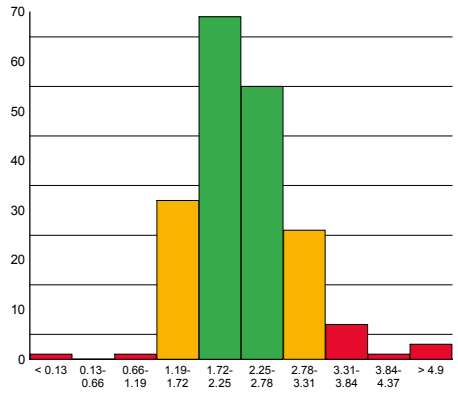
Sample B: Av. Chr17



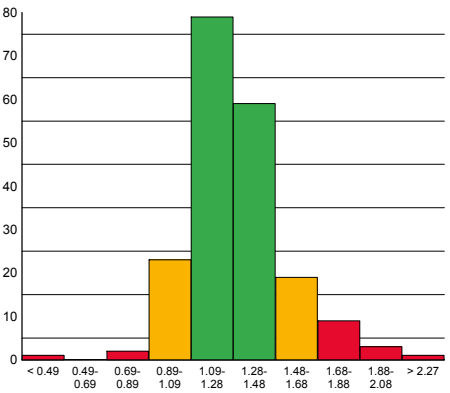
Sample B: Ratio



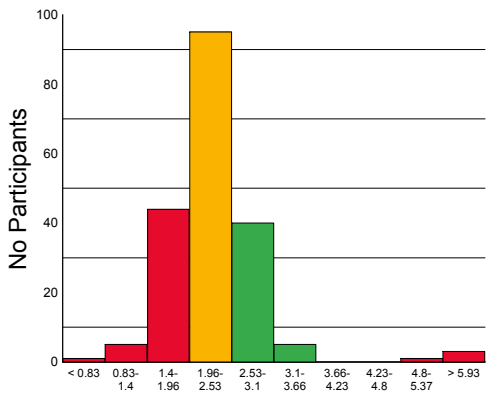
Sample C: Av. HER2 copy



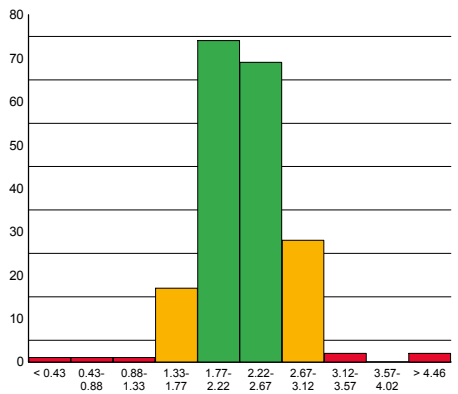
Sample C: Av. Chr17



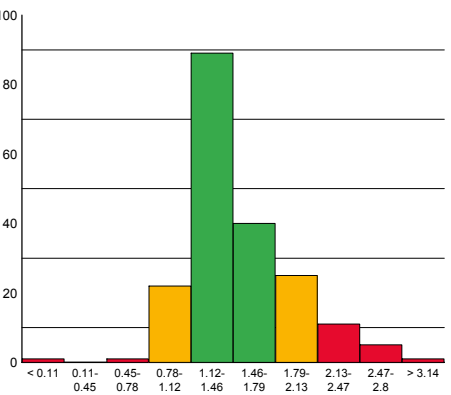
Sample C: Ratio



Sample D: Av. HER2 copy



Sample D: Av. Chr17



Sample D: Ratio

The HER2 ISH Module - Technical (Pilot) Run 37

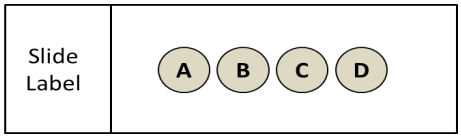
Merdol Ibrahim and Suzanne Parry

Gene Assessed:	HER2
Sections circulated:	Composite slide consisting of 4 breast carcinoma sections (see table below)
Number of Registered Participants:	226
Number of Participants Taking Part This Run	182 (81%) (118 Fluorescent & 64 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

Tissue Section	HER2 status by IHC	HER2 status by FISH
A	**3+**	Amplified
B	**2+**	Non-Amplified
C	**2+**	Non-Amplified
D	**2+**	Non-Amplified

Tissue Section Positioning:
Tissue sections were positioned on microscope slides as illustrated below (Same slide as in the interpretive



Assessment Procedure

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

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

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

Each of the UK NEQAS tissue sections (A-D) (as well as in-house samples where submitted) are individually assessed for the quality of ISH staining. Assessors **do not count** the HER2/ CEN17 signals. The accuracy of signal enumeration is assessed in the 'interpretive' section of the HER2 ISH module.

Important: If two separate slides are submitted with separate CEN17 and HER 2 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.

Note that 'Kit control' slides are not requested for submission for this module and will not be scored.

Results Summary

CISH Results

Selected images showing the acceptable and unacceptable levels of staining for the different methods are illustrated in figures 1-6.

The overall results from the CISH technical assessment were very much the same as the previous Run (36), with only 36% receiving an acceptable pass, a further 30% achieving a borderline level of staining, and 35% failing on the UK NEQAS distributed material. Similarly to the last Run, the main reason for unacceptable results was due to very weak or no CEN17 signals, which was mostly seen on slides stained with the Ventana BDISH or DDISH methods. It was also noted that many of the slides stained with these kits showed little or no staining of the internal normal cells, which obviously has an impact on the sensitivity of the ISH assays. The DDISH and BDISH kits were used by the vast majority of labs carrying out brightfield ISH staining (54%).

Important: Any laboratory experiencing staining problems with their CISH method should contact the relevant company for further support.

FISH Results

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

Overall the FISH results showed much better pass rates to the CISH methods, with 70% of labs achieving an acceptable pass, and a further 10% receiving a borderline. However, there was still a fail rate of 19%. The main reason for unacceptable results was due to weak or no signals. The Pathvysion Vysis kit was the most popular FISH method, used by 60% of labs for this assessment, and this method showed an acceptable pass rate

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

of 62%. A further 15% of Pathvysion Vysis kit users received a borderline pass.

Validating ISH

It is important to emphasise that when introducing ISH into the laboratory or when changing methodology, e.g. from FISH to CISH, the new technique must be fully validated and staff must undergo extra training to gain expertise with the new method employed.

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

- Staff perform a minimum of 100 ISH evaluations in parallel with an experienced member of staff to ensure scoring consistency before working independently.
- A minimum concordance of 95% is recommended for both diagnostic results (amplified and non-amplified status) and
- numerical results (for both HER2 and CEN17).
- Clinical and UK NEQAS samples should be assessed for quality of hybridisation prior to interpretation. You should make sure that the probe signals are easily discernible, normal cells show the expected HER2 and/or CEN17 signal, and there is minimal background and leaching of the signal.
- Further validation guidelines can be found in: Bartlett et al., (2011) HER2 testing in the UK: Recommendations for Breast and Gastric In-situ Hybridisation Methods. J Clin Pathol.64(8):649-653.

Recommendations for Returning FISH Slides for NEQAS Assessments

a. Sections should be mounted using a fluorescence antifade mounting media, which prevents loss/quenching of fluorescence e.g. Vectashield (Vector labs), Dako Fluorescence Mounting Medium (Dako), Fluoromount (SIGMA/Aldrich, VWR). Note: The Abbott Vysis kit already contains DAPI in phenylenediamine dihydrochloride, which is an anti fading reagent, but we have found that some laboratories also used 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

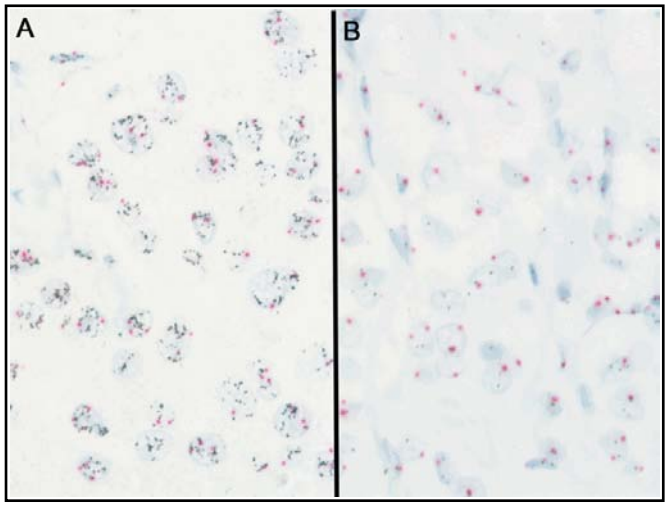


Fig 1. Acceptable Ventana DDISH in the UK NEQAS samples. (A) Amplified tissue 'sample A' and (B) non-amplified tissue ' sample B'. In both cases distinct HER2 signals (black) and Ch17 signals (red) are clearly demonstrated with the expected level of copies per cell.

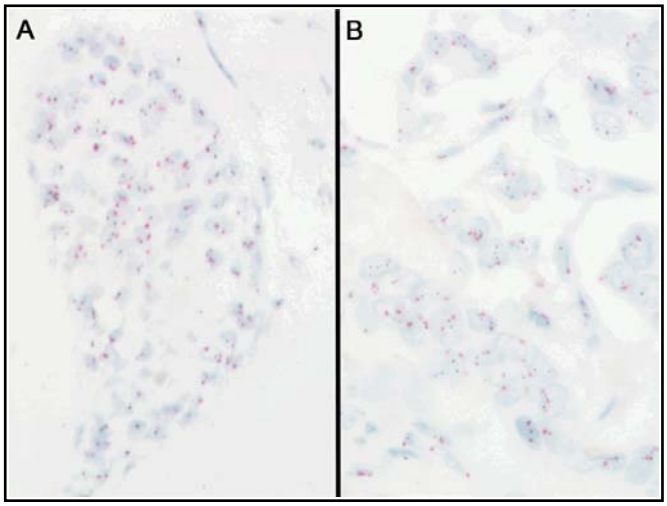


Fig 2. Acceptable Ventana DDISH in the UK NEQAS samples: (A) Non-amplified tissue 'sample C' and (B) non-amplified tissue 'sample D'. In both examples the HER2 (black) and Ch17 (red) signals are strong and clear and show the expected level of copies per nuclei.

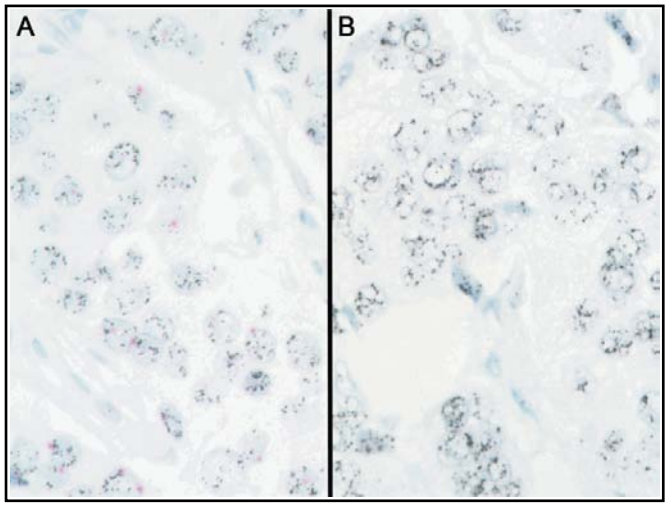


Fig 3. Two examples of unacceptable DDISH staining from UK NEQAS amplified tissue 'sample A'. (A) Ch17 signals are very weak with normal stromal cells also lacking signal. (B) Ch17 signals are absent and morphology of the nuclei are damaged, most likely due to over-digestion. Both slides stained with Ventana DDISH.

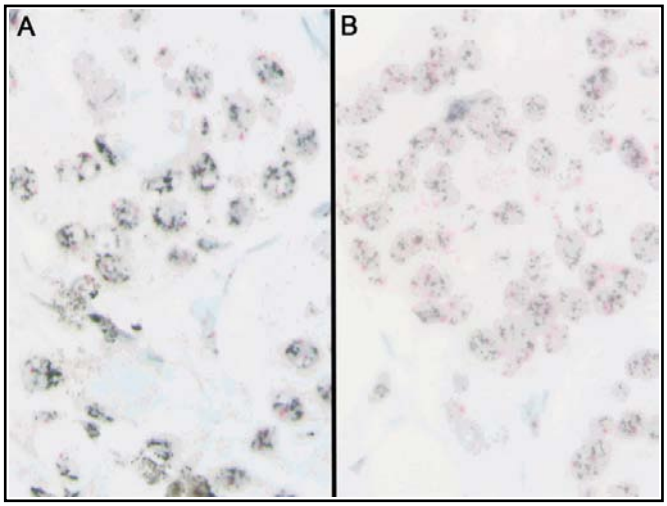


Fig 4. Two further examples of slides with unacceptable level of staining from UK NEQAS distributed 'sample A' (A) Very weak Ch17 signal and background HER2 signal (B) Leaching of Ch17 signal and a red hazy background noise making interpretation difficult.

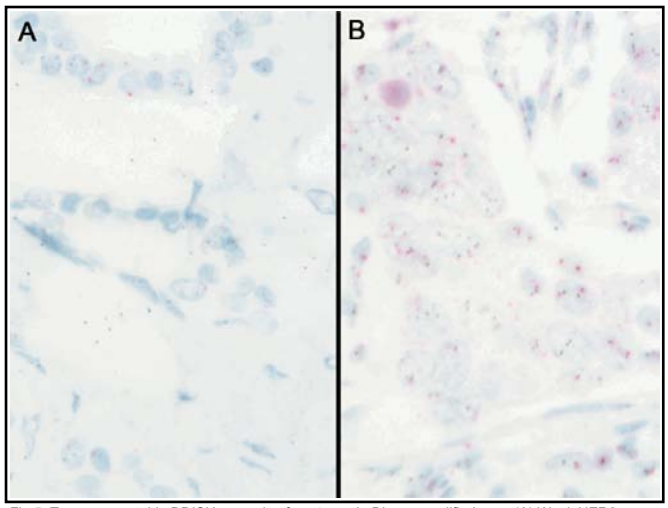


Fig 5. Two unacceptable DDISH examples from 'sample D' non-amplified case (A) Weak HER2 signal with background pepering of non-specific HER2 signal and very weak Chr17 signal. (B) Leeching of Chr17 signal.

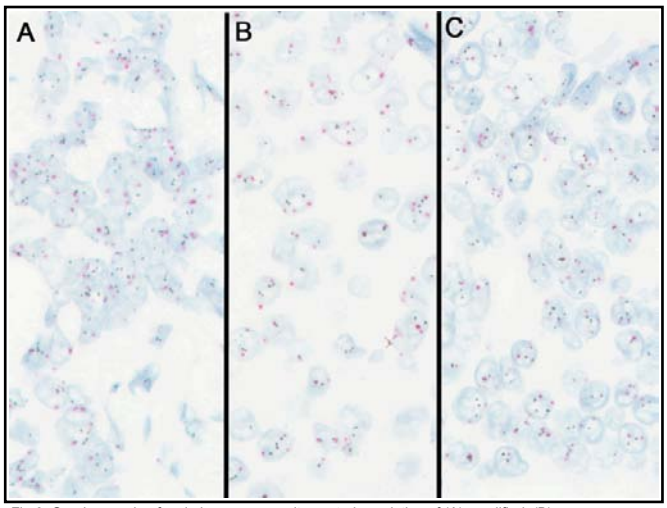


Fig 6. Good example of an in house composite control consisting of (A) amplified, (B) non-amplified and (C)non=amlplified cases. Each case shows strong, clear and distinct HER2 (black) and Ch17 signals (red).

Selected Images showing Optimal and Sub-optimal Immunostaining

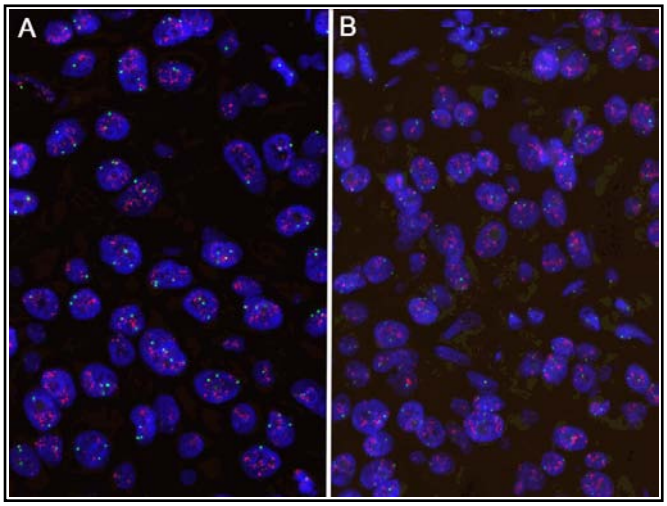


Fig 7. Acceptable FISH examples from UK NEQAS distributed amplified case (Sample 'A') showing crisp multiple HER2 copies (red) and Ch17 signals (green). (A) Stained using Abbott Pathvysion Vysis and (B) Dako pharm DX.

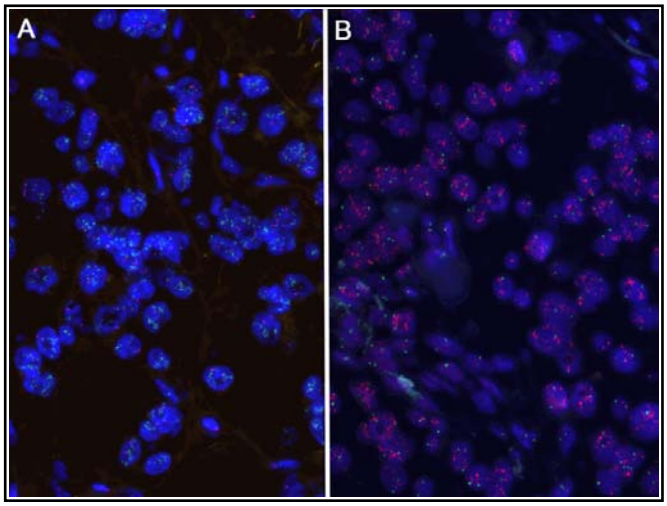


Fig 8. Acceptable FISH examples from UK NEQAS distributed amplified case (Sample 'A') showing crisp multiple HER2 copies (red) and Ch17 signals (green). (A) Stained using Zytovision Zytolight and (B) China Medical Technologies kit.

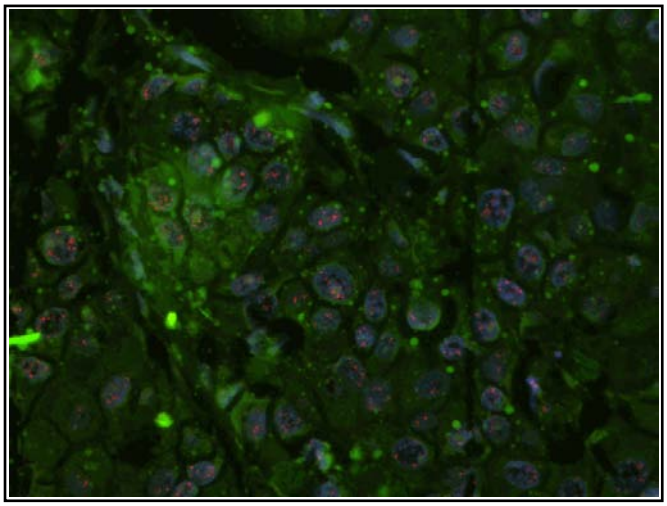


Fig 9. Unacceptable FISH from the UK NEQAS distributed 'sample A' amplified case. Although red HER2 signals are present, the Chr17 signal is not distinguishable due to green non-specific 'globules'. This may be due to microscope objective oil getting under the cover slip. Stained using Abbott Pathvysion Vysis.

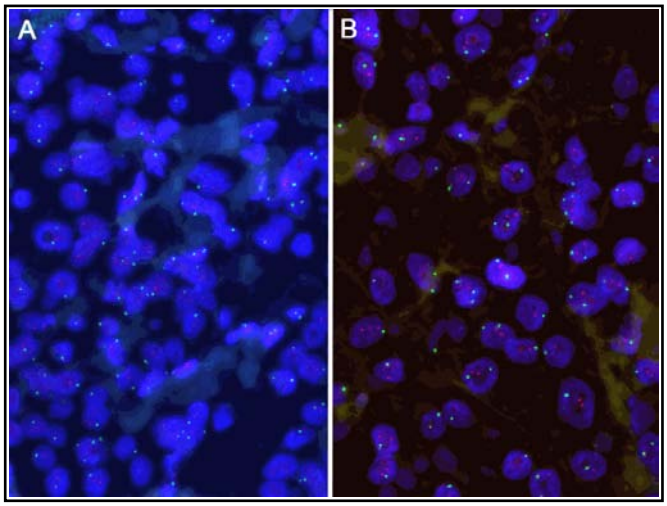


Fig 10. Acceptable FISH examples from UK NEQAS distributed non-amplified case (Sample 'B') showing 1-2 HER2 copies (green) and 1-2 Chr17 signals (red) per nuclei.

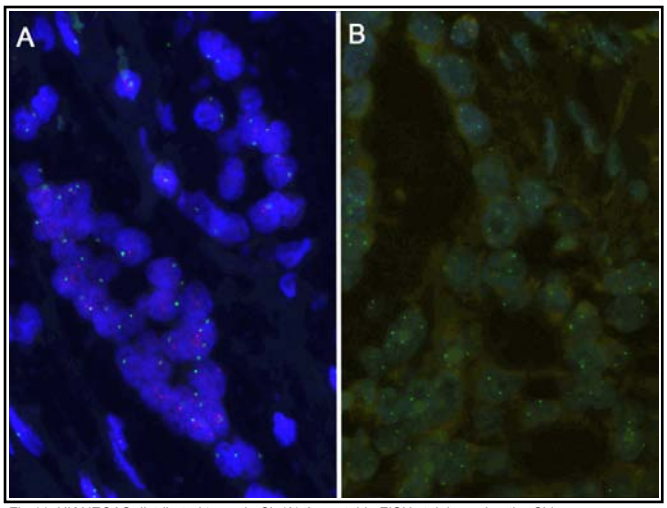


Fig 11. UK NEQAS distributed 'sample C'. (A) Acceptable FISH staining using the China Medical Technologies kit. (B) Chr17 signals are present but there are no HER2 signals rendering this sample uniterpretable. Stained using the Dako IQFISH.

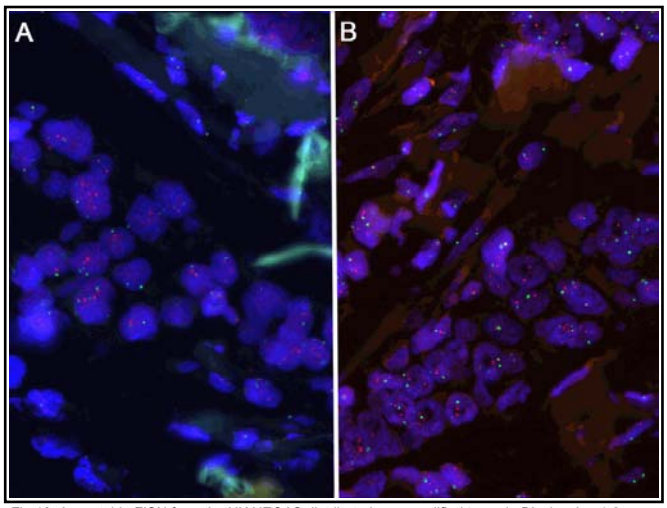
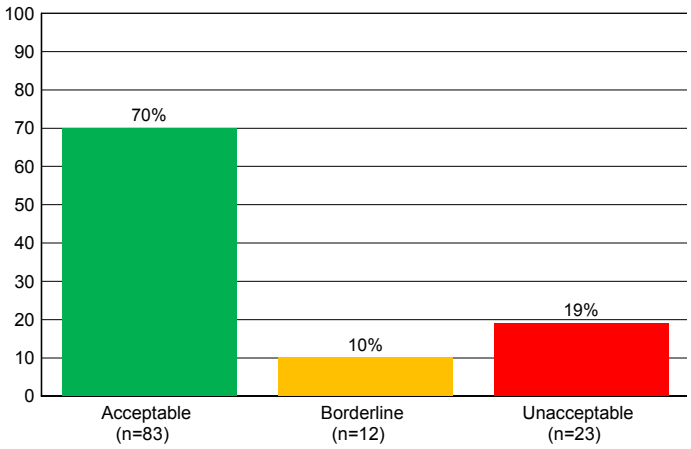


Fig 12. Acceptable FISH from the UK NEQAS distributed non-amplified 'sample D', showing 1-2 HER2 (red) and Chr17 (green) signals. Stained using (A) China Medical Technologies kit and (B) Abbott Pathvysion Vysis.

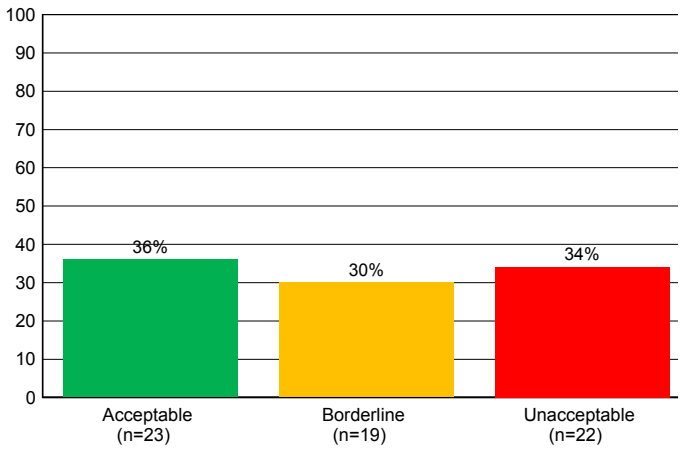
Technical ISH: Pass Rates and Methods

Overall Pass Rates

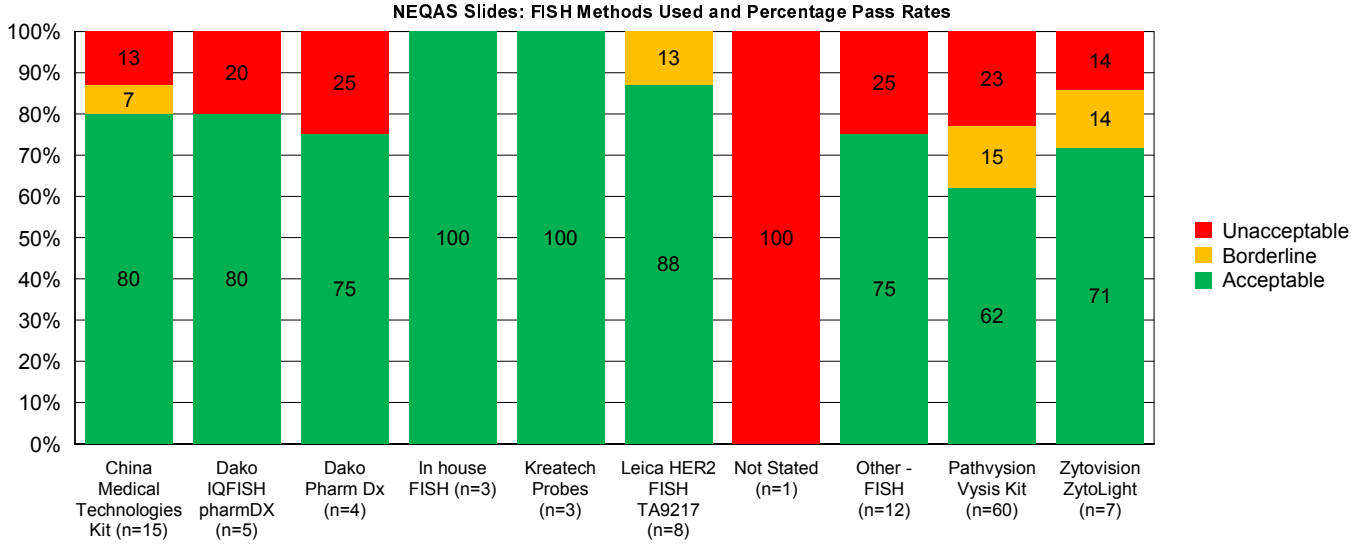
FISH NEQAS slide (n=118)



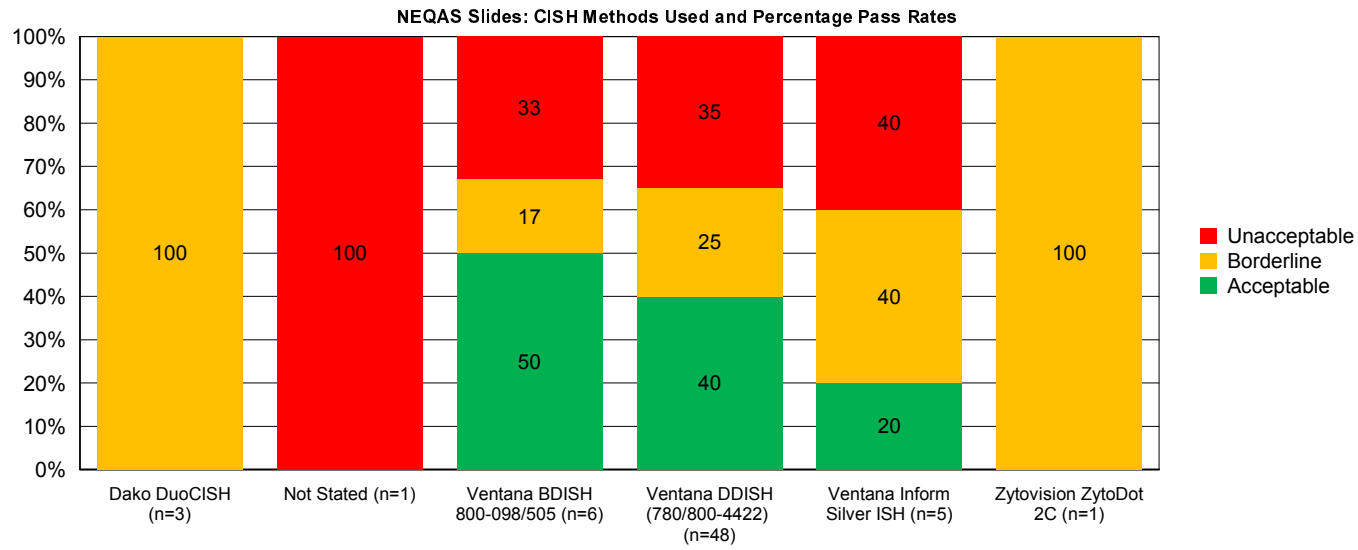
CISH NEQAS Slide (n=64)



NEQAS Slides: FISH Methods Used and Percentage Pass Rates



NEQAS Slides: CISH Methods Used and Percentage Pass Rates

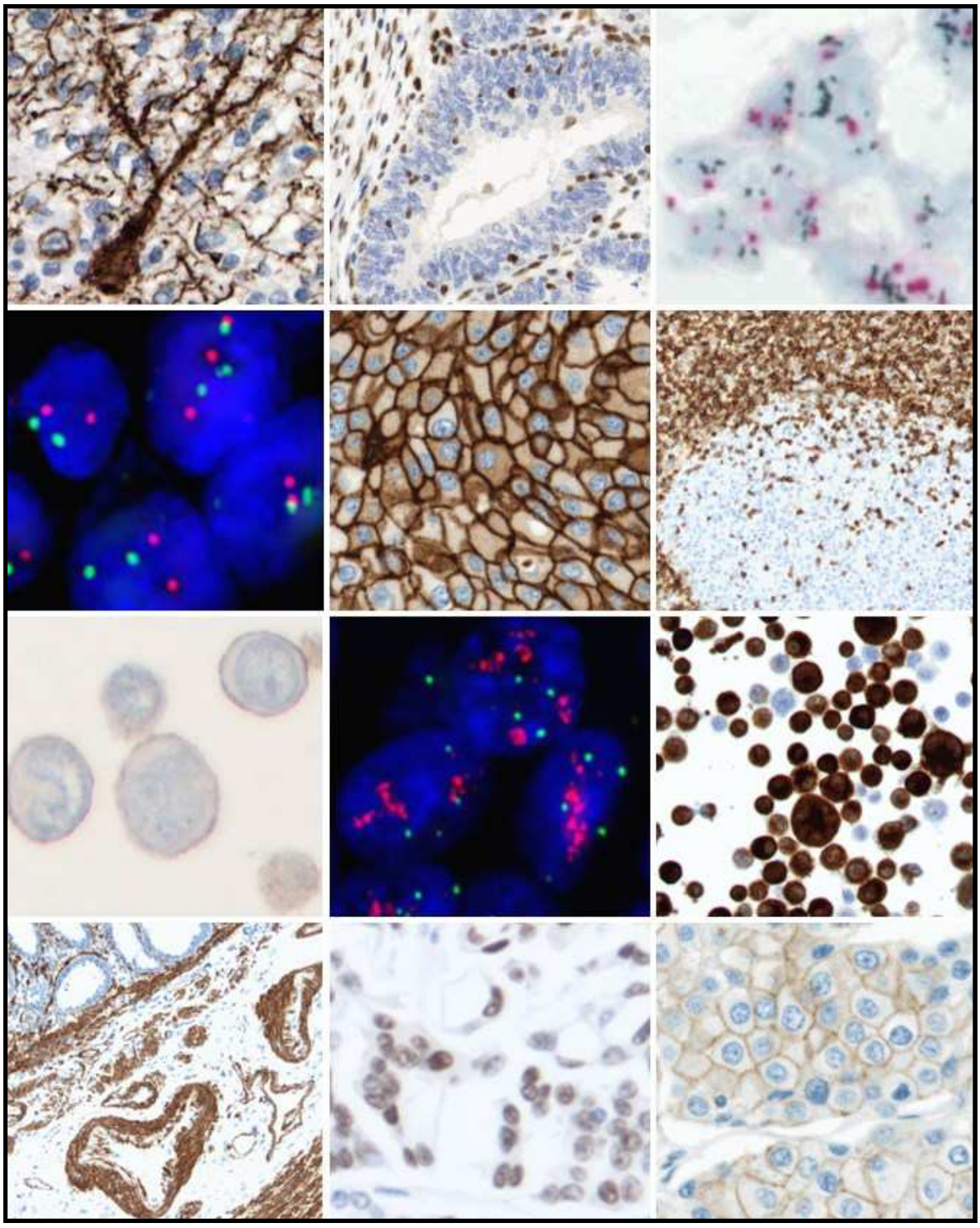


UK NEQAS
International Quality Expertise

CPA
Accredited EQA Scheme
Reference No: 044

UK NEQAS ICC & ISH

Improving Immunocytochemistry for over 25 Years



For advertising opportunities contact:
Neil Bilbe: n.bilbe@ucl.ac.uk