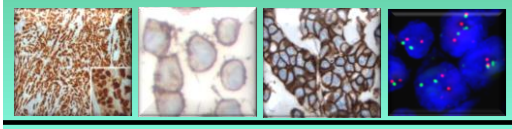

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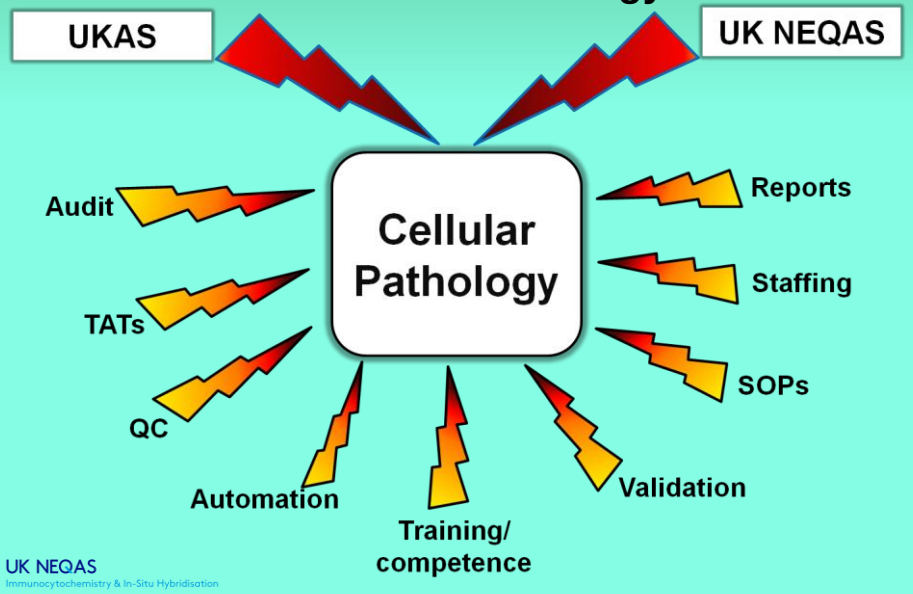
Uncertainty in Cellular Pathology: What's Yours?

Merdol Ibrahim
UK NEQAS ICC & ISH, London
merdol.ibrahim@ucl.ac.uk

Outline

- Measurement of Uncertainty – ISO
  (International Organization for Standardization)
- How To Test For Uncertainty In Cellular Pathology: Workshop Data
- UK NEQAS ICC & ISH Examples
- References

Increasing workloads & Considerations in Cellular Pathology



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Accreditation Providers (in UK overseen by UKAS)

ISO 15189 → ISO 17043

Medical laboratories
Requirements for quality and competence

Laboratories have to take part in external EQA

Proficiency testing providers (UK NEQAS)
e.g. UK NEQAS ICC & ISH

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Testing for Uncertainty

Workshop

Pinpoint where you may have uncertainty

Tools you will need

- Consideration guide
- Post-it notes
  - GREEN
  - AMBER
  - RED

Preanalytic
e.g Fixation transport

Analytic
e.g Staining Controls, Reporting

Post-Analytic
e.g TATs / IT / Audit

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Consideration Matrix Board

Breakdown of cellular pathology testing phases

Pre-analytic			Analytic				Post-analytic		
Fixation fixative? ischemia time? time in fixative? grossing (scientist / pathologist)?	**Transport** Local/remote pathology? Audit trail? Friday surgery?	**Processing / Embedding** Microwave/xylene free? Macro/standard blocks?	**HER2 IHC** Kit or lab-derived? Time from cutting to staining?	**Validation & QC** Validated procedures?	**Controls** Controls used? On-slide controls? Batch to batch variation?	**Reporting** Pathologist, scientist, both? Competence? Telepathology? Digital Reporting?	**Turn around Times (TATs)** What is your TAT? How do you calculate your TAT?	**Audit** Regular audit of HER2 rates? Weekly Monthly ?	**IT** LIMS, Barcode system / Tracking, Electronic Reporting

Post-analytic

Turn around Times (TATs)	**Audit**	**IT**
What is your TAT? How do you calculate your TAT?	Regular audit of HER2 rates? Weekly Monthly ?	LIMS, Barcode system / Tracking, Electronic Reporting

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Post-it Notes

Make comments on coloured ‘post-it’ notes

✓	Some data on referred sample fixation	No idea of sample fixation time!!
No uncertainty	Some uncertainty	A lot of uncertainty

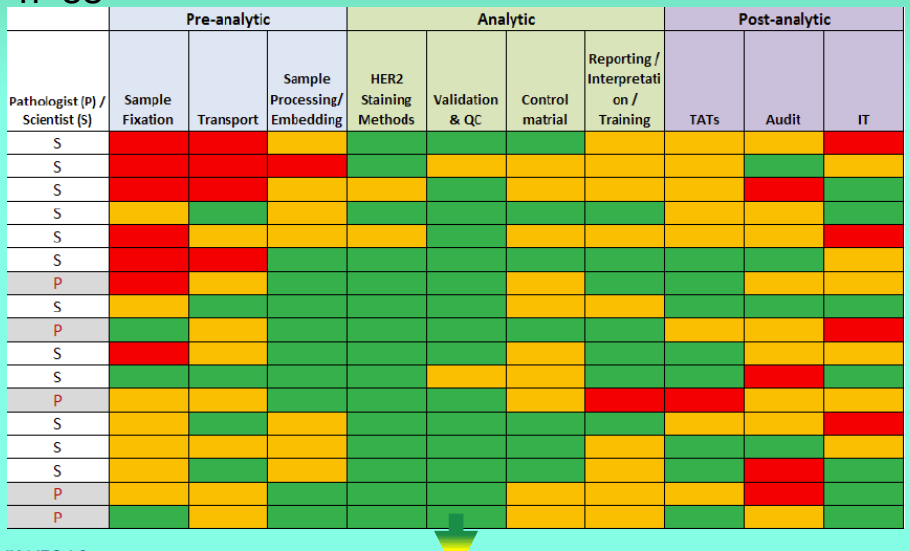
Completed Matrix Board

Place in Staff cafeteria for anonymous collection!

Build up your evidence

Heat Map of Uncertainty

n=53



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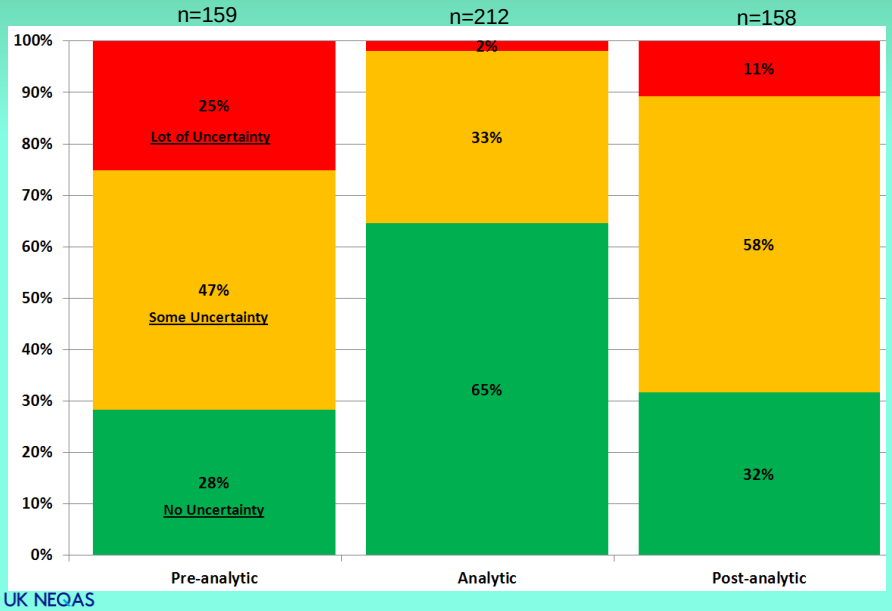
Stages of Uncertainty



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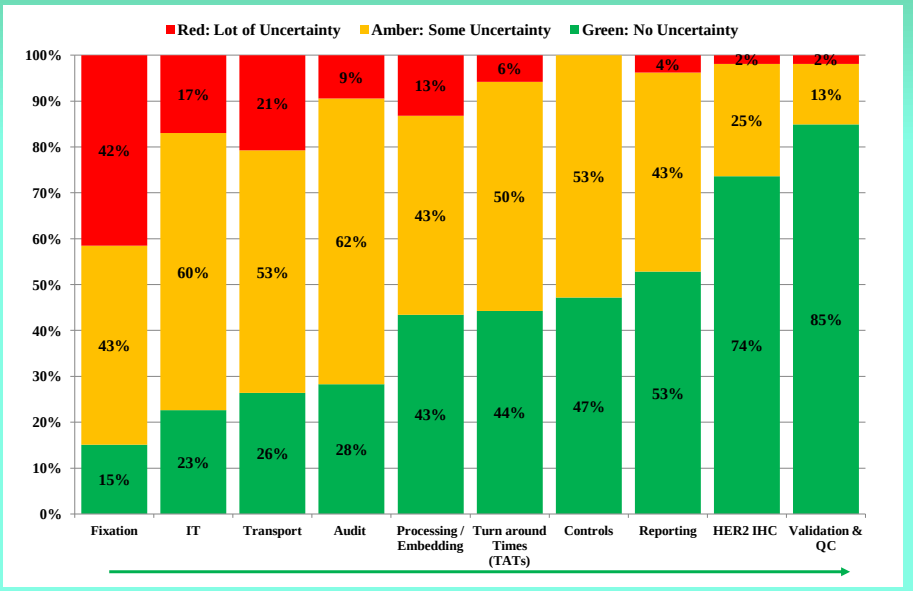
Order of Greatest Uncertainty



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Fixation

85% some/lot of uncertainty

- Sample ischemia time unknown
- Time in fixative not known / No control over fixation
- Referring site so no idea of fixation times or protocols
- Lack of forms indicating fixation times
- Weekends - samples can be over-fixed
- pH of formalin not taken
- Surgical samples not always ‘opened’: “depends on pathologist”

- Cold ischemia time audit is carried out
- Have good fixation times including min/max times
- pH taken of fixative
- Use Datix (www.datix.com) (patient safety software for healthcare risk management,) for samples fixed for >72 hours
- Commercial fixative supplied to surgery clinics

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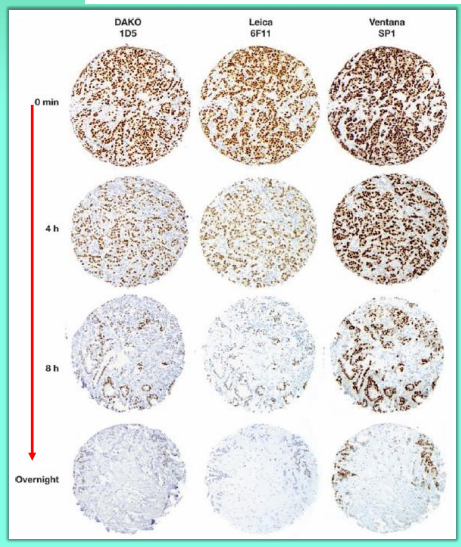
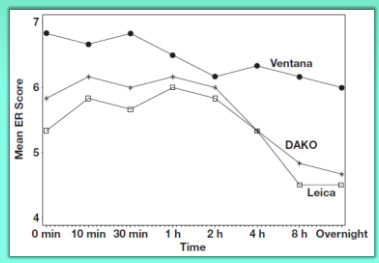
Effect of Delayed Formalin Fixation on Estrogen and Progesterone Receptors in Breast Cancer

A Study of Three Different Clones

Jingxin Qiu, MD, PhD,[1] Swati Kulkarni, MD,[2] Rameela Chandrasekhar,[3] Mark Rees, PhD,[4,6] Kathryn Hyde,[5] Gregory Wilding, PhD,[3] Dongfeng Tan, MD,[6] and Thaer Khoury, MD[1]

Am J Clin Pathol 2010;134:813-819





The effect of cold ischemic time on the immunohistochemical evaluation of estrogen receptor, progesterone receptor, and HER2 expression in invasive breast carcinoma

Isil Z Yildiz-Aktas, David J Dabbs and Rohit Bhargava

MODERN PATHOLOGY (2012) 25, 1098–1105

We have the evidence!

Need for better collaboration with surgical team/s

Fixation: **Q.V.T**

Quality

CE marked

Volume

15-20:1 (fix:tissue)

Image from: www.southend.nhs.uk

Non UK Laboratory

Time

- 6-8 hrs min for core biopsies
- 24-48hrs (72 hrs to cover weekends!) surgical excisions

Validation & QC

15% some/lot of uncertainty

- **Antibody Verification or Validation?**

- **Verification:**
  - IVD/CE marked antibodies, kits/assays e.g. ALK FISH, Her2 kits (Herceptest, Ventana 4B5 assay, Leica Oracle kit)
  - Less complex procedures
- **Validation:**
  - Lab devised Techniques (LDTs) / 'home brew' methods
  - Out of date antibodies
  - More complex procedures

Antibodies: Where to Start?

- **Verification or Validation?**
- **Commercial company recommendations / data sheets**
- **Lab devised methodologies: Onus on yourselves**
- **Best Methods database: www.ukneqasiccish.org**
- **Data analysis / statistics**
- **References**

Antibody validation

BioTechniques 48:197-209 (March 2010)

Jennifer Bordeaux, Allison W. Welsh, Seema Agarwal, Elizabeth Killiam, Maria T. Baquero, Jason A. Hanna, Valsamo K. Anagnostou, and David L. Rimm

- **Sensitivity & specificity**
- **Common pitfalls**
- **More vendor based validation procedures**

Antibody Validation: References

Updated UK Recommendations for HER2 assessment in breast cancer

Emad A Rakha,[1] Sarah E Pinder,[2] John M S Bartlett,[3] Merdol Ibrahim,[4] et al.,

J Clin Pathol. 2015 68(2):93-99

- **100 IHC and ISH cases**
- **95% concordance**

Principles of Analytic Validation of Immunohistochemical Assays

Guideline From the College of American Pathologists Pathology and Laboratory Quality Center

Patrick L. Fitzgibbons, MD; Linda A. Bradley, PhD; Lisa A. Fatheree, BS, SCT(ASCP); Randa Alsabeh, MD; et al.

Arch Pathol Lab Med 2014 138: 1432-1443

- **90% Concordance : Too low for UK?**
- **Biomarkers: 40 Cases; 20 +ve & 20 -ve (full clinical range)**
- **Non predicative assays: 10 +ve & 10 -ve**
- **Change in protocol: dilution, vendor, incubation, retrieval**
  - **2 known +ve's and 2 known -ve's**
- **Change in antibody clone: full re-validation**

Document all validation and verification procedure: SOPs

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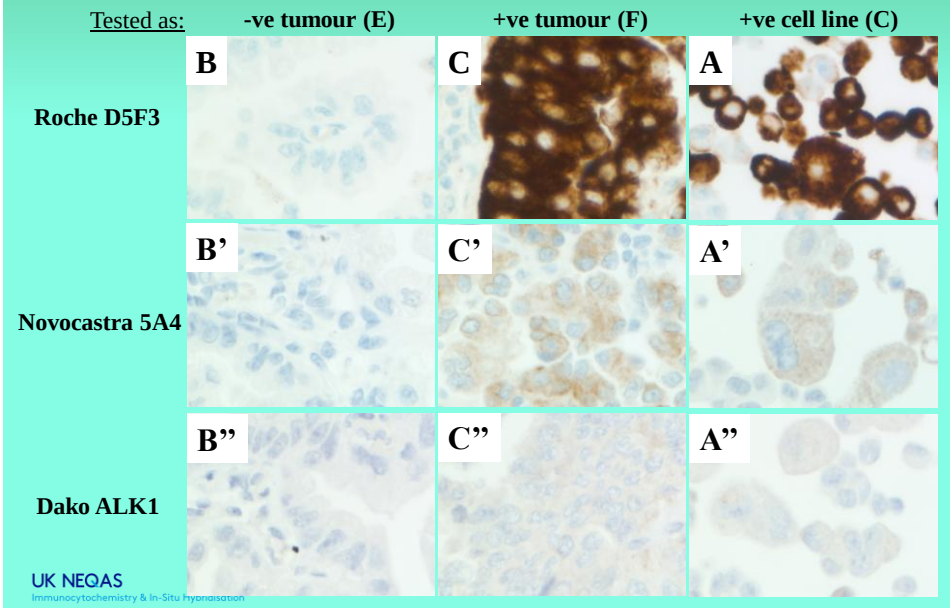
Method Matters: ALK IHC:



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ALK IHC: 38 Staining Methods

				Assessment Results on NEQAS Samples			
Primary Antibody	**Dilution**	**automation**	**Detection**	**Excellent**	**Acceptable**	**Borderline**	**Unacceptable**
Cell Signalling Tech. (D5F3)	np	Dako Autostainer Link 48	Dako EnVision FLEX+	1 (100%)	-	-	-
		Leica Bond-III	Leica Bond Polymer Refine	1 (50%)	1 (50%)	-	-
	1:100	LabVision Autostainer	Dako Envision HRP/DAB	2 (67%)	1 (33%)	-	-
		Ventana Benchmark ULTRA	Ventana OptiView Kit	1 (100%)	-	-	-
		Ventana Benchmark XT	Ventana OptiView Kit	1 (100%)	-	-	-
			Ventana UltraView Kit	0 (0%)	-	1 (100%)	-
	1:250	Leica Bond-III	Leica Bond Polymer Refine	-	1 (100%)	-	-
Dako (ALK1)	np	Dako Autostainer Link 48	DAKO ENVISION FLEX+	-	1 (100%)	-	-
		Ventana Benchmark ULTRA	Ventana UltraView Kit	1 (50%)	-	-	1 (50%)
	1:10	Ventana Benchmark XT	Ventana OptiView Kit	-	-	-	1 (100%)
	1:20	Dako Autostainer Link 48	DAKO Envision FLEX+	-	1 (50%)	1 (50%)	-
	1:25	Leica Bond-III	Leica Bond Polymer Refine	-	-	-	1 (100%)
Diag. Bio (5A4)	np	Ventana Benchmark XT	Ventana UltraView Kit	-	1 (100%)	-	-
Genemed (D5F3)	Prediluted	Ventana Benchmark XT	Ventana OptiView Kit	1 (100%)	-	-	-
Novocastra (5A4)	np	LabVision Autostainer	Dako Envision HRP/DAB	-	-	1 (100%)	-
		Leica Bond-III	Leica Bond Polymer Refine	1 (50%)	-	1 (50%)	-
		Ventana Benchmark XT	Ventana OptiView Kit	1 (100%)	-	-	-
		Ventana Benchmark ULTRA	Ventana OptiView Kit	-	-	1 (100%)	-
	1:10	Leica Bond Max	Leica Bond Polymer Refine	-	1 (100%)	-	-
		Ventana Benchmark XT	Ventana OptiView Kit	1 (100%)	-	-	-
	1:20	Ventana Benchmark XT	Ventana OptiView Kit	2 (100%)	-	-	-
	1:25	Leica Bond Max	Bond Polymer Refine Red	1 (100%)	-	-	-
	1:50	Dako Autostainer Link 48	Dako EnVision FLEX+	2 (67%)	1 (33%)	-	-
		Leica Bond-III	Leica Bond Polymer Refine	-	-	-	1 (100%)
		Ventana Benchmark ULTRA	Ventana OptiView Kit	(0%)	1 (50%)	1 (50%)	-
	1:100	Dako Autostainer Link 48	Dako EnVision FLEX+	2 (67%)	1 (33%)	-	-
		Leica Bond-III	Leica Bond Polymer Refine	1 (100%)	-	-	-
		Ventana Benchmark ULTRA	Ventana OptiView Kit	-	-	1 (100%)	-
Novocastra RTU (5A4)	Prediluted	Leica Bond Max	Leica Bond Polymer Refine	1 (25%)	3 (75%)	-	-
		Leica Bond-III	Leica Bond Polymer Refine	-	-	1 (50%)	1 (50%)
Thermo/Neomarkers (5A4)	1:10	Ventana Benchmark XT	Ventana OptiView Kit	-	-	-	1 (100%)
Ventana (D5F3)	Prediluted	Ventana Benchmark	Ventana OptiView Kit	6 (75%)	-	2 (25%)	-
		Ventana Benchmark ULTRA	Ventana OptiView Kit	9 (90%)	1 (10%)	-	-
		Ventana Benchmark XT	Ventana OptiView Kit	60 (74%)	13 (16%)	5 (6%)	3 (4%)
			Ventana UltraView Kit	1 (50%)	1 (50%)	-	-
		Ventana Benchmark XT	Ventana UltraView Kit	1 (50%)	-	-	-
Ventana Confirm (ALK01)	prediluted	Ventana Benchmark XT	Ventana OptiView Kit	1 (50%)	-	1 (50%)	-
Zytomed (p80)	1:15	None (Manual)	Zytomed ZytoChem Plus	--	-	(0%)	1 (50%)

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More Antibody Uncertainty! : PD-L1

Method	Checkpoint inhibitors	Checkpoint	All 2nd line treatment	Kit assay	Automated Platform	Cut-offs
Assay / kit	**Pembrolizumab** (Keytruda) (MSD)	PD-1	All NSCLCs (FDA and UK/EU)	**Dako 22C3** pharmDX - companion -	**Dako** Autostainer Link 48	Tumour cells 1%, 50% ?
	Nivolumab (Opdivo) (BMS)	PD-1	Squamous NSCLC (FDA and UK/EU) All NSCLC (FDA)	**Dako PD-L1 28-8** pharmDX - complementary -	**Dako** Autostainer Link 48	Tumour cells 1% ?
	Atezolizumab (Roche)	PD-L1	Not yet licensed	SP142: Kit form TBC - companion -	**Ventana**: TBC	Tumour cells + TILS
	Durvalumab (AZ/Medimmune)	PD-L1	Not yet licensed	SP263: Kit form TBC - companion -	**Ventana** : TBC	Tumour cells %?
	Avelumab (Merck KGaA & Pfiezer)	PD-1	?	?	?	?

LDTs / Home Brews

- 28-8 (RabMab): BMS clone available from Abcam
- E1L3N (RabMab): Cell Signaling
- SP142 (RabMab): Spring Bioscience

Blueprint Proposal for Companion Diagnostic Comparability (www.fda.gov/MedicalDevices/NewsEvents/

Goal: Characterize PD-L1 assay systems from **Dako and Ventana** to assess the level of analytical similarity.

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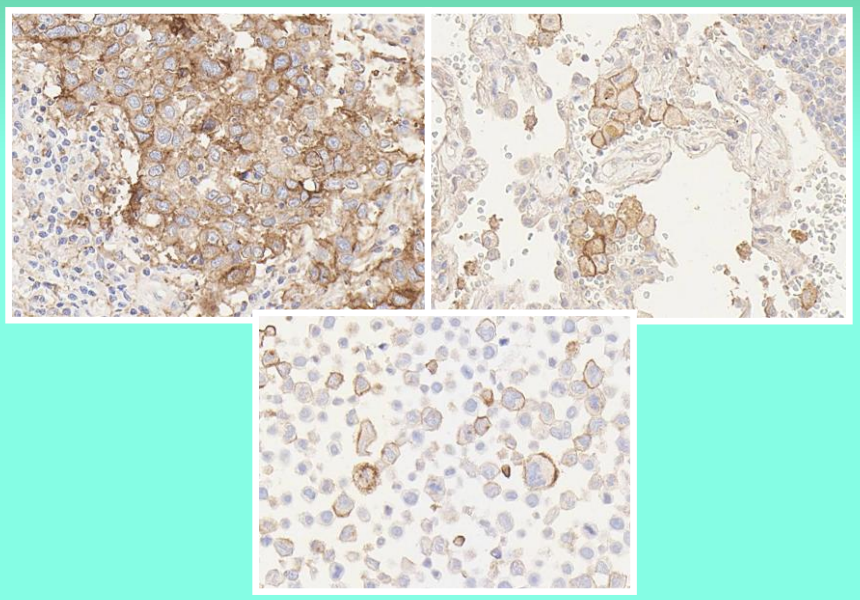
NEQAS is Preparing for PD-LI



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Out of Date Antibodies

- ISO accreditation: Appears to accept out of date antibodies
- **BUT Confusion as to what validation is exactly required**

"We are starting to feel that this standard is unachievable in Cellular Pathology"

ISO feedback

- Lab could take on the role of the 'manufacturer', & give an expiry date
- Validate across all tissue/tumour types & all possible usage situations

ISO 17025:2005

- Based on **knowledge of the performance** of the method and on the measurement scope and shall make use of, for example, **previous experience** and **validation data**

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Out of Date Antibodies

Extension of Useful Reagent Shelf Life Beyond Manufacturers' Recommendations

Raymond R. Tubbs, DO; Raymond Nagle, MD, PhD; Kevin Leslie, MD; Norman M. Pettigrew, MD; Jonathan W. Said, MD;

Arch Pathol Lab Med
1998 122 (12) 4 1051-1052

MSA, HMB45, CLA & S100

***Results.*—Only minor differences were identified for the 221 reporting laboratories in 1998 as compared with those in 1997.**

***Conclusions.*—The data suggest review of the Health Care Financing Administration's ruling on extending the useful reagent shelf life beyond manufacturers recommendations. Similar studies using more inherently quantitative methodology are suggested.**

Satisfactory Performance of Primary Antibodies Beyond Manufacturers' Recommended Expiration Dates

Balaton, André J. M.D.; Drachenberg, Cinthia B. M.D.; Rucker, Cheryl H.T.; Vaury, Philippe M.D.; Papadimitriou, John C. M.D., Ph.D.

App. Immuohistochem. Mol. Morphol. 1999 7 (3) 221-225

Antibody Expiration in the Context of Resource Limitation

What Is the Evidence Basis?

Erica C. Savage, MD, and Barry R. DeYoung, MD

Am J Clin Pathol 2010;134:60-64

CAP (College of American Pathologists) do not accept out of date antibodies!

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Out of Date Antibodies

Antibodies are forever: a study using 12–26-year-old expired antibodies

Maria C Argentieri,[1] Daniela Pilla,[1] Alice Vanzati,[1,2] Silvia Lonardi,[3] Fabio Facchetti,[3] Claudio Doglioni,[4] Carlo Parravicini[5] & Giorgio Cattoretti[1,2]

Histopathology 2013, **63**, 869–876

"Vintage" antibodies

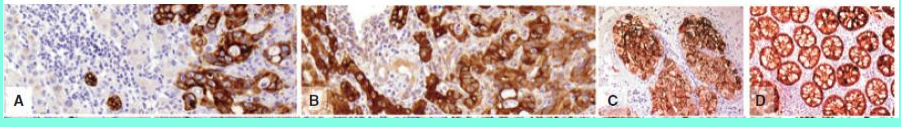

Figure 1. Immunoreactivity of vintage antibodies. **A**, Colon carcinoma (right) metastatic to the liver (left), stained for AE1. Note the unstained liver and the positive bile ductules. **B**, Colon carcinoma (right) metastatic to the liver (left), stained for AE3. **C**, EMA staining of a salivary gland. **D**, Cytokeratin staining of colon mucosa.

All Referenceable BUT…
…Is time up for out of date antibodies!

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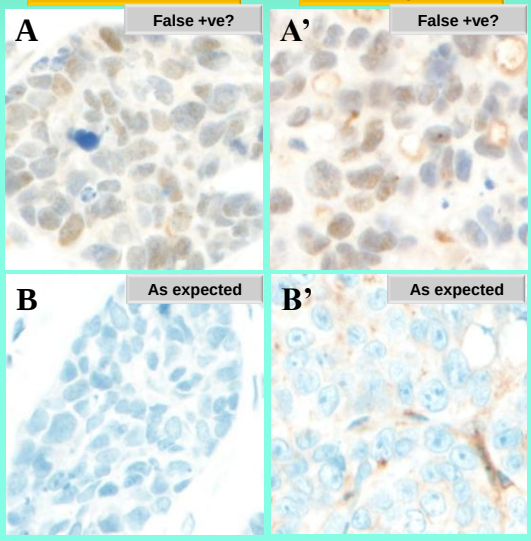
Uncertainty in Estrogen (ER) Immunohistochmistry

Same Antibody = Two Results: Whose right?

6F11

	Distributed ER -ve Breast tumour	Colon adenocarcinoma Xenograft
On Bond III + High pH retrieval + Bond Refine Detection	A — False +ve?	A' — False +ve?
Ventana Benchmark + High pH retrieval + Ultraview Detection	B — As expected	B' — As expected



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ER – Multiple Methodologies

UK Data : Assessment 96 (2012)

6F11 (Concentrate) Clone on The Leica Bond Max

Retrieval Methods: 32% Low pH (recommended protocol) & 68% High pH

11 Antibody dilutions	+	7 Retrieval Times (mins)	+	4 Incubation Times (mins)
1:20		10		15
1:50		15		20
1:60		20		20
1:75		25		30
1:80		30		60
1:100		35		
1:150		40		
1:200				
1:250				
1:300				
1:400				

22/29 (76%) * = Protocol Variations
***Labs who submitted complete Methods**

Too Many Protocols! Labs have their preferences!

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6F11: Correct Result Can be Achieved

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

Primary Antibody:	Novocastra NCL -L-ER- 6F11, 15 Mins, RT ºC, Dilution 1: 50
Automation:	Leica Bond-III
Method:	Leica BondMAx Refine KIT
Main Buffer:	Bond Wash Buffer (AR9590)
HMAR:	Leica Bond III ER 1, 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

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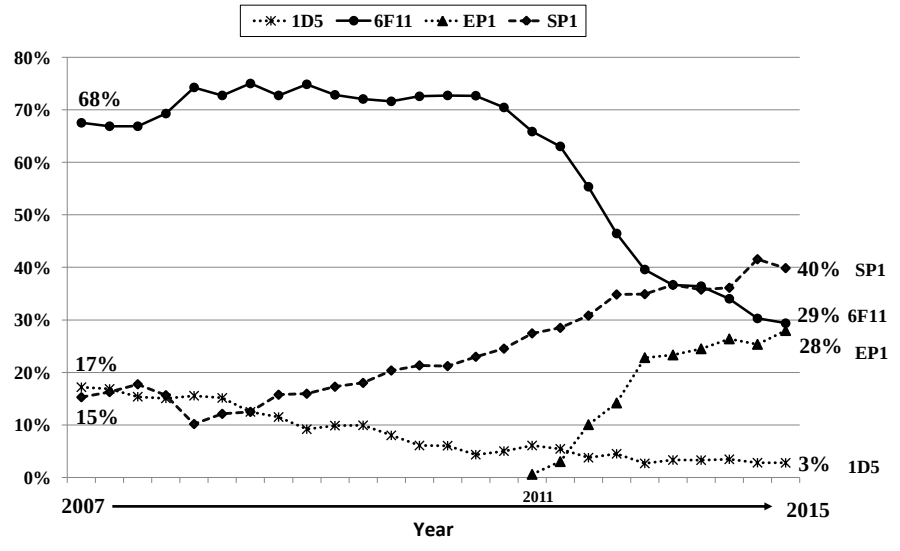
UK: Change in ER Antibody Usage



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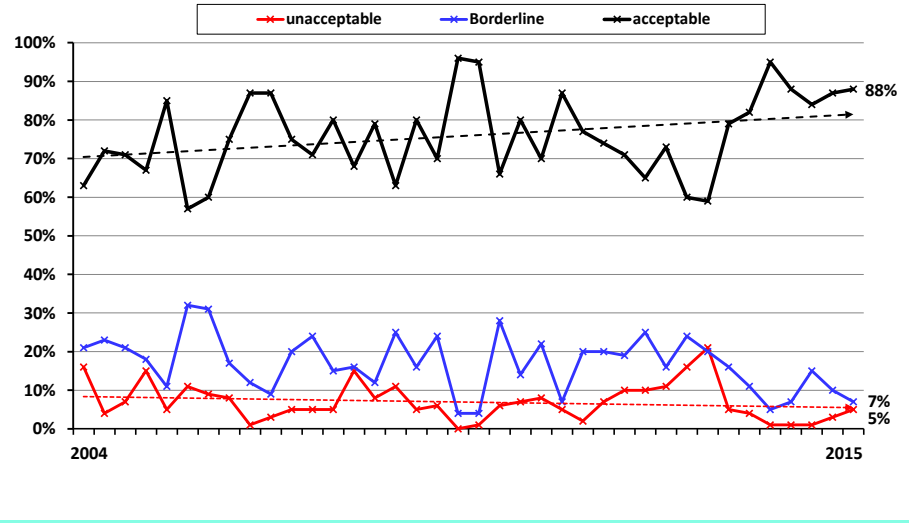
UK Breast ER
EQA Pass Rates 2004-2015



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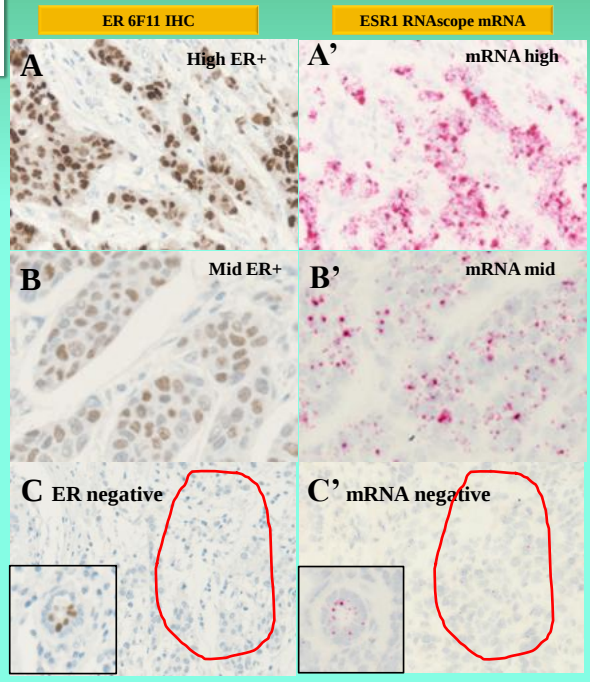
UK NEQAS R&D
IHC Vs mRNA

ER IHC & mRNA
=
Good Comparative Correlation



Reference: Advanced Cell Diagnostics
- ESR1: transcript variant 4, mRNA
- Number of double Z probe pairs: 40
- Gene region probes designed against: 677-3065 nucleotides

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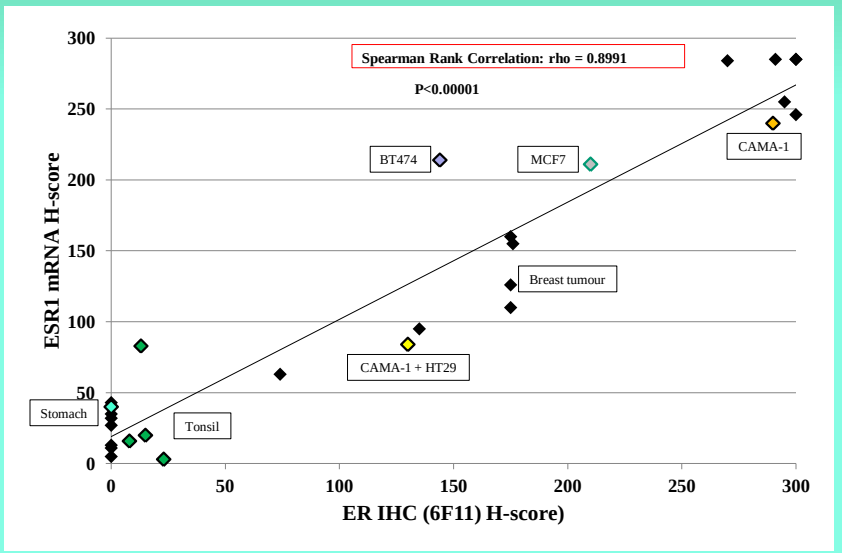
Correlation of ER (6F11) IHC & ESR1 mRNA

n= 28



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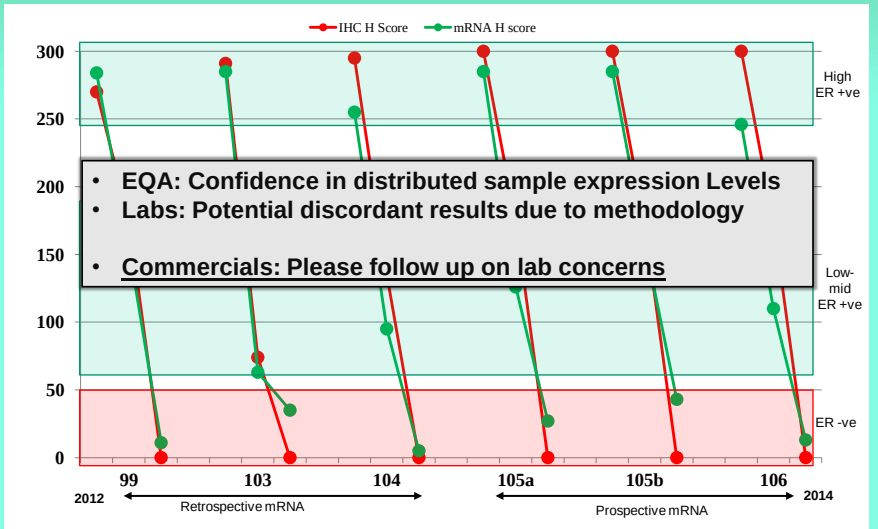
ER IHC & ESR1 mRNA H-Scores in

Distributed EQA Samples



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Breast HER2 IHC

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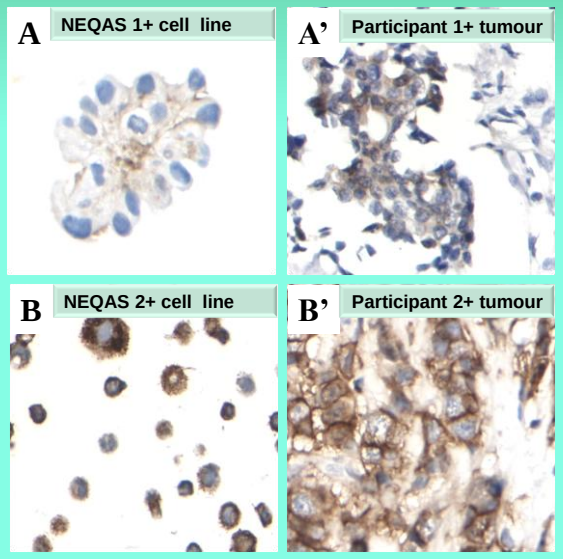
HER2 IHC EQA Sample Results Parallels that of In-house Controls



Poor analytic staining techniques

Poor preservation of in-house samples

Pre-analytic / Fixation needs addressing

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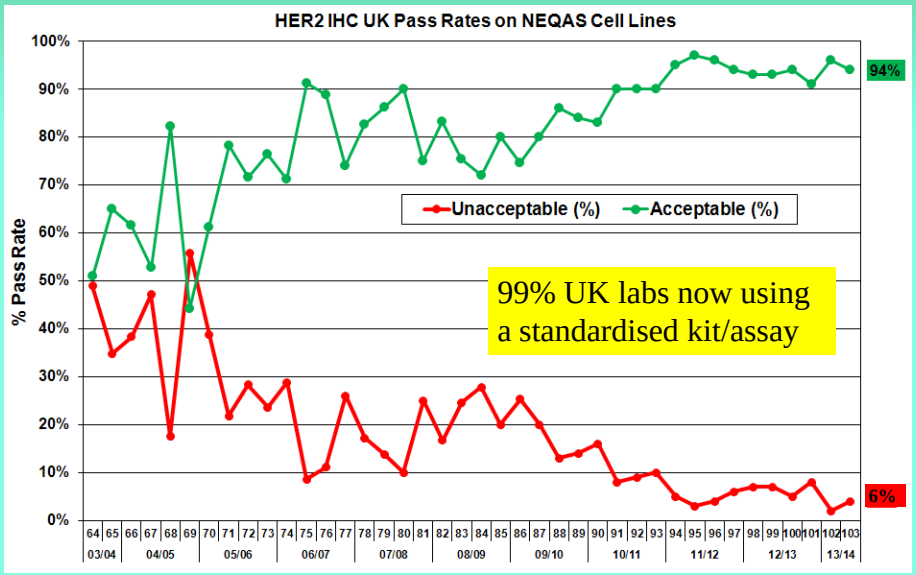
EQA Feedback does help to improve quality & confidence in HER2 testing: UK Breast HER2 IHC Rates 2003–2013



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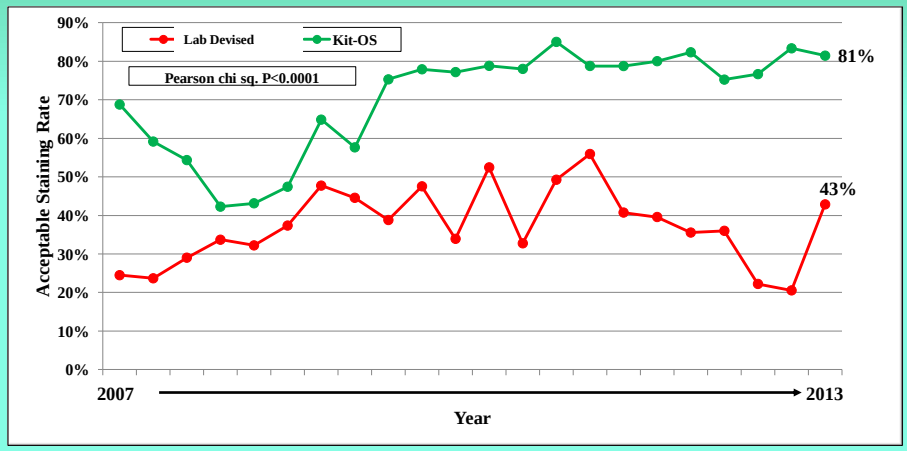
Breast HER2 IHC: Kit vs Home Brew Method
Overseas Participants



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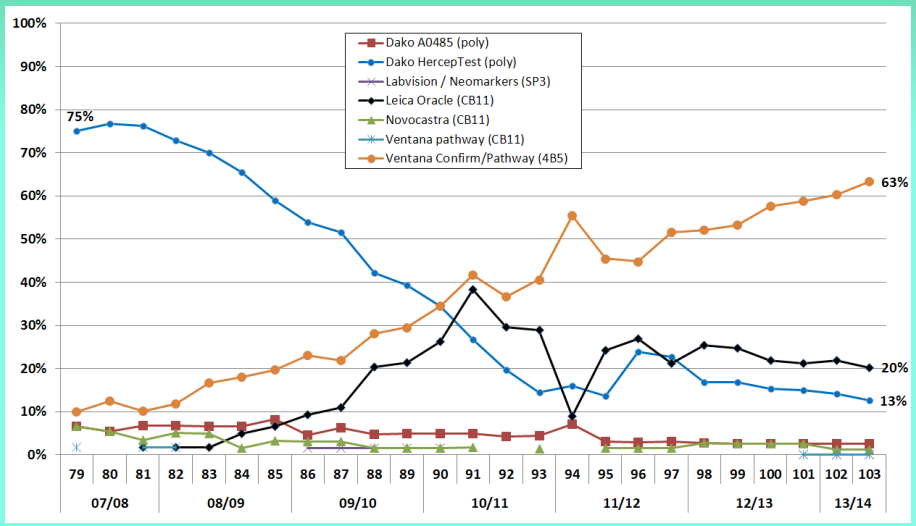
UK labs: Choice of Her-2 IHC (2007-2013)



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Transport

74% some/lot of uncertainty

- Referral centre: no idea of transport time
- No special arrangement for Fridays/weekends
- Causes bottleneck…batching of work
- Samples received later than expected
- Inter-hospital transport problems
- Delays TATs as no control over when sample arrives

- Transport not a problem
- Good transport schedule

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Audits & TATs

Audits: 71% some/lot of uncertainty

- Done only yearly / bi-yearly / Episodic
- **No time / too busy / understaffed**
- What should we audit?
- No audit of TATs
- **Only if there is a problem**
- Not regular because of IT problems

TATs: 56% some/lot of uncertainty

- Not measured: Too busy to audit
- **Presume ok if no complaint**
- Not done: staff shortage
- Often delayed due to poor tissue quality
- Variable depends on pathologist
- Behind due to workload issues
- Poor TATs due to batching

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UK HER2 Rates

Updated UK Recommendations for HER2 assessment in breast cancer

Emad A Rakha,[1] Sarah E Pinder,[2] John M S Bartlett,[3] Merdol Ibrahim,[4] Jane Starczynski,[5] Pauline J Carder,[6] Elena Provenzano,[7] Andrew Hanby,[8] Sally Hales,[9] Andrew H S Lee,[1] Ian O Ellis,[1] On behalf of the National Coordinating Committee for Breast Pathology

J Clin Pathol. 2015 68(2):93-99

Table 1 Proportion of HER2-positive primary and metastatic breast cancers*

	0	1+	2+	3+	ISH +	Overall HER2-positive
Overall (%)	32.8	33.1	21.8	11.6	14.7	14.5
Primary carcinoma (%)	32.6	33.7	21.8	11.5	14.6	14.3
Metastatic lesion (%)	36.7	27.2	21.1	14.9	15.8	18.0*

*UK NEQAS ICC & ISH combined 5 year national audit data (unpublished data). ISH, *in situ hybridisation*; ISH+, proportion of 2+ carcinomas that are amplified; UK NEQAS ICC & ISH, UK National External Quality Assessment Scheme for Immunocytochemistry and *In Situ* Hybridisation.

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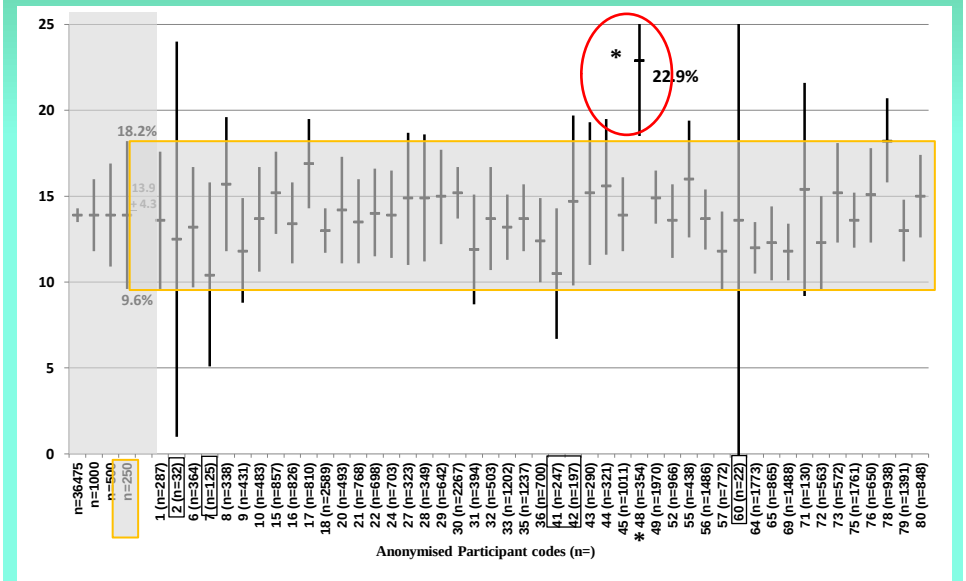
HER2 Positivity (2009): Individual Participant Data + 95% Conf. intervals



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Control Material

53% some/lot of uncertainty

- No on-slide controls
- Kit controls, only one per run
- Problems sourcing control material
- Variability in quality of control material
- Yes, but no on-slide
- **In-house control a pain!**
- Commercial controls too expensive

- Use on slide control
- Use controls to monitor batch to batch variability

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Control Material

Standardization of Negative Controls in Diagnostic Immunohistochemistry: Recommendations From the International Ad Hoc Expert Panel

Emina E. Torlakovic, MD, PhD, †‡ Glenn Francis, MBBS, FRCPA, MBA, FFSc (RCPA)* et al.,

Appl Immunohistochem Mol Morphol 22, Number 4, 2014

Standardization of Positive Controls in Diagnostic Immunohistochemistry: Recommendations From the International Ad Hoc Expert Committee

Emina E. Torlakovic, MD, PhD, † Søren Nielsen, HT, CT,‡§ Glenn Francis, MBBS, FRCPA,* et al.,

Appl Immunohistochem Mol Morphol 23, Number 1, 2015

Summary 1

- **Lack of tissue homogeneity makes it impossible to determine the 'true value' of measurement in cellular pathology.**
- **Laboratory should consider where they have 'Uncertainty'**
  - Create a simple **matrix board of uncertainty:** post-its!
  - Consider which methods are best to achieve clinically reliable measurements…. **Verify or Validate methods**
  - Ensure lab equipment is calibrated regularly to a **traceable standard**
  - Make sure all **staff are competent** in the area they are working
  - Assess equipment achieves the desired objectives… **Audits**
  - Well defined **Protocols & Methods**

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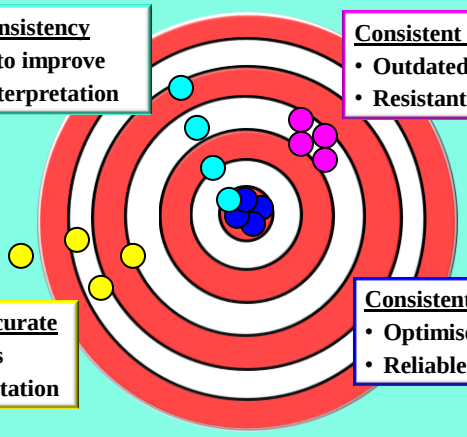
Summary 2

External quality Assessments Provide Laboratories with a Means to Gauge Staining Quality Over Time

Improving Consistency
- Conscious effort to improve
- More Reliable Interpretation

Consistent but NOT Accurate
- Outdated Methods
- Resistant to Change!



Inconsistent & Inaccurate
- Unreliable Methods
- Unreliable Interpretation

Consistent & Accurate
- Optimised Methods
- Reliable Interpretation

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Thank you

Acknowledgments

- Advanced Cell Diagnostics: RNAScope: mRNA staining
- Dako: Providing ER/PR Cell lines
- Roche products, Leica and Dako: Staining of NEQAS samples using recommended protocols

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