UK NEQAS
Education | Quality | Global

Quality Immunocytochemistry Staining: Every Step Counts

Suzanne Parry
Scheme Manager
UK NEQAS ICC & ISH

ilac-MRA
UKAS PROFICIENCY TESTING
7833
ISO 17043:2023 Accredited

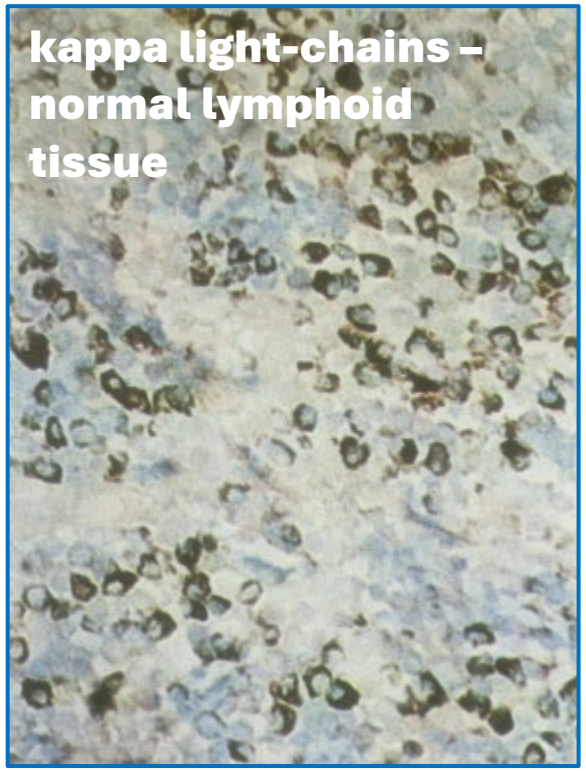
Introduction

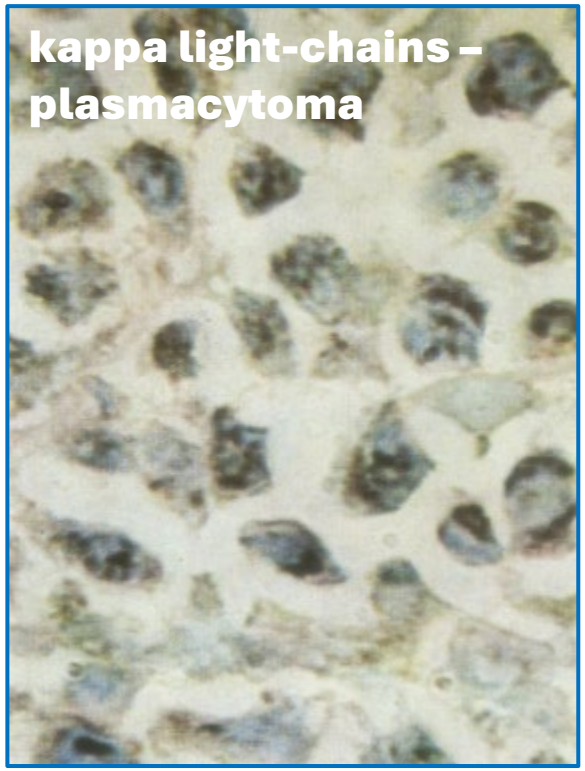
- Historical context – the early days of immunohistochemistry
- The concept of the Total Test – every step counts
- Pre-analytics
- Analytical phase
- Post-analytical considerations

Early days

1974. Widely acknowledged as the paper that started immunohistochemistry on its journey…

- In 1974 this was the ‘state of the art’.





J. clin. Path., 1974, 27, 14-20

The demonstration of plasma cells and other immunoglobulin-containing cells in formalin-fixed, paraffin-embedded tissues using peroxidase-labelled antibody

C. R. TAYLOR AND J. BURNS

From the Department of Pathology, Gibson Laboratories, Radcliffe Infirmary, Oxford

SYNOPSIS A method is described for the demonstration of specific immunoglobulin in plasma cells and other lymphoid cells in sections taken from routine surgical histology specimens which have been formalin fixed and paraffin embedded.

An indirect sandwich technique was employed using specific rabbit antihuman immunoglobulin antisera (anti-K, L, G, A, and M) and a swine antirabbit serum Ig G, conjugated with horseradish peroxidase. The presence of plasma cells was revealed by staining the tissue-bound peroxidase-labelled antibody, having previously stained the endogenous peroxidase a contrasting colour.

It was possible to demonstrate clearly immunoglobulin in the plasma cells of tissues processed and embedded several years previously.

Some of the potential uses of the method are discussed.

Plasma cells have been recognized by histologists since the classical description of Marschalkó (1895a and b). The relationship of these cells to antibody production became widely accepted following the work of Amano *et al* in 1944 (see 'The morphology of plasma cells', Feldman, 1972) and Fagreus (1948), and the development of fluorescein labelling methods (Coons, Creech, and Jones, 1941; Coons, 1956). Fluorescent antibody techniques have since been used extensively to demonstrate the presence of immunoglobulins within cells or bound to tissue components.

The routine use of immunofluorescence is inhibited by the requirement for frozen unfixed tissue or cold alcohol-fixed tissue (Sainte-Marie, 1962). There is also the problem of standardization (Holborow 1970), while rapid fading of positive fluorescence makes it difficult to refer back directly to previous work. Formalin-fixed tissue does not lend itself to immunofluorescence methods, because the enhancement of intrinsic tissue autofluorescence masks any specific fluorescent staining.

The use of antibody labelled with horseradish peroxidase (Nakane and Pierce, 1966; Nakane, 1968; Avrameas, 1969; Avrameas and Ternynck, 1969) has resolved some of these difficulties as it affords a permanent preparation. Tissue-bound immunoglobulin has been successfully demonstrated, eg, on renal basement membranes (Davey and Busch, 1970) using peroxidase-conjugated antibody and the specificity and sensitivity of the method have been favourably compared with established fluorescence methods (Nakane and Pierce, 1966; Davey and Busch, 1970; Petts and Roitt, 1971).

This paper presents a method for demonstrating the presence of immunoglobulin in lymphoid cells in routine formalin-fixed, paraffin-embedded tissues using a peroxidase-conjugated antibody and an indirect sandwich technique.

Materials and Methods

Paraffin-embedded tissue blocks were selected from surgical histology specimens received in the Department of Morbid Anatomy at the Radcliffe Infirmary. Tissues had been routinely processed on the laboratory Histokinettes by the recommended schedule using industrial methylated spirit and chloroform

Received for publication 17 September 1973

Taylor CR, Burns J. J Clin Pathol. 1974 Jan;27(1):14-20.

And then everything changed

1998. Dako's HercepTest: the first IHC-based companion diagnostic…

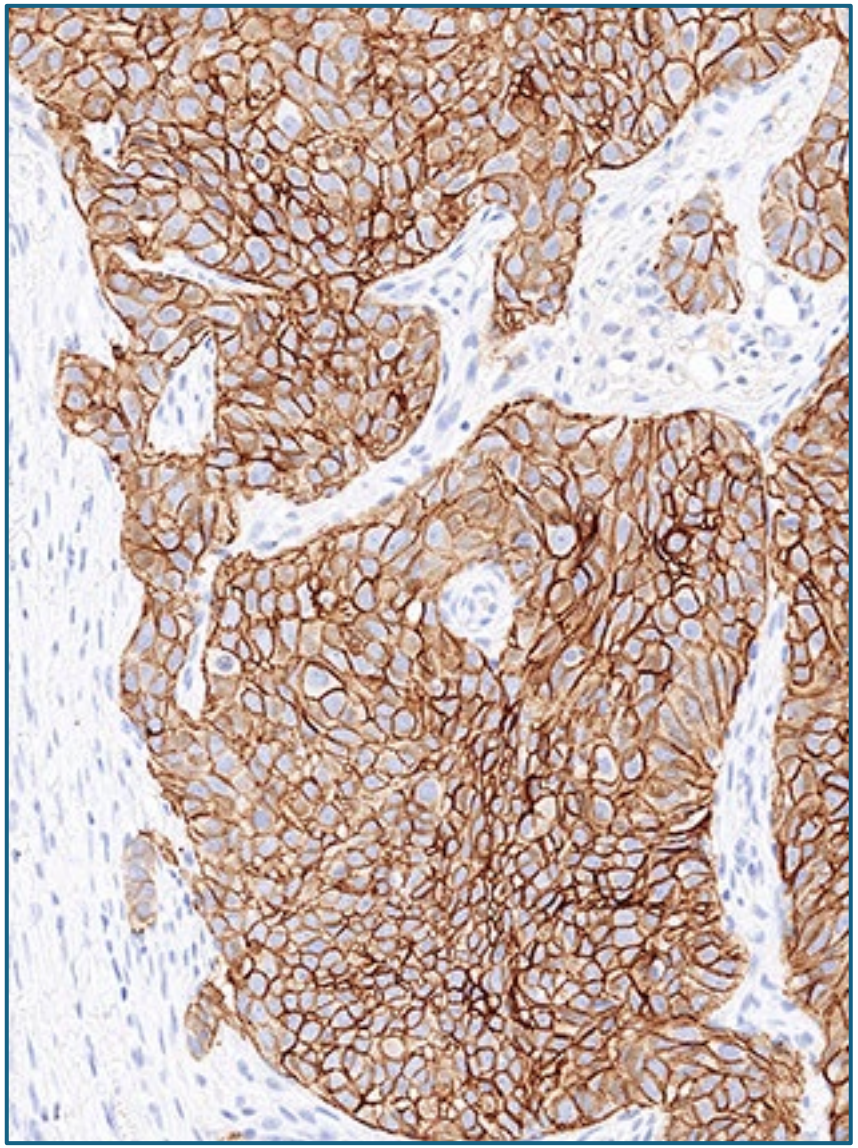
DAKO Corporation
6392 Via Real
Carpinteria, California 93013

SEP 25 1998

Re: P980018
DAKO HercepTest
Filed: May 18, 1998
Amended: June 2, June 4, August 4, August 10, August 18, August 24, August 31 and September 25, 1998.

Dear Dr. Murray:

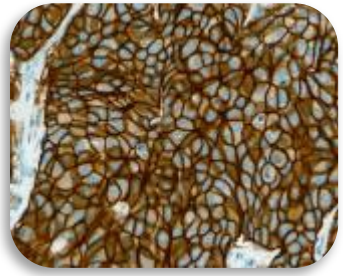
The Center for Devices and Radiological Health (CDRH) of the Food and Drug Administration (FDA) has completed its review of your premarket approval application (PMA) for the DAKO Herceptest. This device is a semi-quantitative immunohistochemical assay to determine HER2 overexpression in breast



HER2 protein overexpression (3+) in breast cancer

Multidisciplinary approach for quality

From Operating Theatre to the Laboratory



In the Lab

Diagnostics

Therapeutics

Technical Test
Pre-analytical:
Test selection: indication for the test
Specimen handling, from operating room to histology laboratory
Fixation: total fixation time and type of fixative
Paraffin embedding, storage, and sectioning
De-paraffinization
Analytical:
Antigen retrieval (exact method)
Assay (staining) method and protocol
Reagent validation
Controls (reference standards)
Technologist and laboratory certification
Proficiency testing and quality assurance
Post-analytical:
Reading of result(s)/scoring/quantification
Diagnostic, prognostic, or predictive significance
Report
Turn-around time
Outcomes analysis/economics/reimbursement

Taylor CR. *The total test approach to standardization of immunohistochemistry. Arch Pathol Lab Med 2000; 124: 945-951.*

The Technical Test

The Pre-Analytical Phase

Step	Source of error	Examples/consequence of error
Tissue arrival	Wrongly labelled	
	Not into fixative soon enough	
	Not in the correct fixative	
	Not arriving to the lab soon enough	
	Error with booking in	
Cut-up/grossing	Which part of the sample is required ?	
	Which orinetation?	
Fixative	Type	Under or over fixation
	Volume	Affects of poor fixation
	Time	
Decal	Type	Under or over decalcification
	Time	
Processing	Time	Inappropriate processing
	Change of solutions	Affect of poor processing
	Lost sample	Not using a biopsy pad when necessary
	Cassette not sealed	Lost or floating sample(s)
Embedding	Inappropriate cassette size	Inappropriate cassette size
	Incorrect orientation	Cushing and not embedded correctly
	Carry over	Forceps or in wax bath or floated through the cassette
	Incorrect labelling of cassette	
Cutting sections/microtomy	Not full face	Miss detail if not full face
	Too thick/thin	Too thick or too thin important particularly for membrane stains e.g. HER2 and HER2-low
	Serials in order	
Drying/baking	Traped water	Trapped water leads to artefact if not drained effectively
	Over baked	Potential distruction of material
	Under baked	Potential to lift
	Oven temperature too high	Potential destruction of material: Some samples delecate

Technical Test

Pre-analytical:

- Test selection: indication for the test
- Specimen handling, from operating room to histology laboratory
- Fixation: total fixation time and type of fixative
- Paraffin embedding, storage, and sectioning
- De-paraffinization

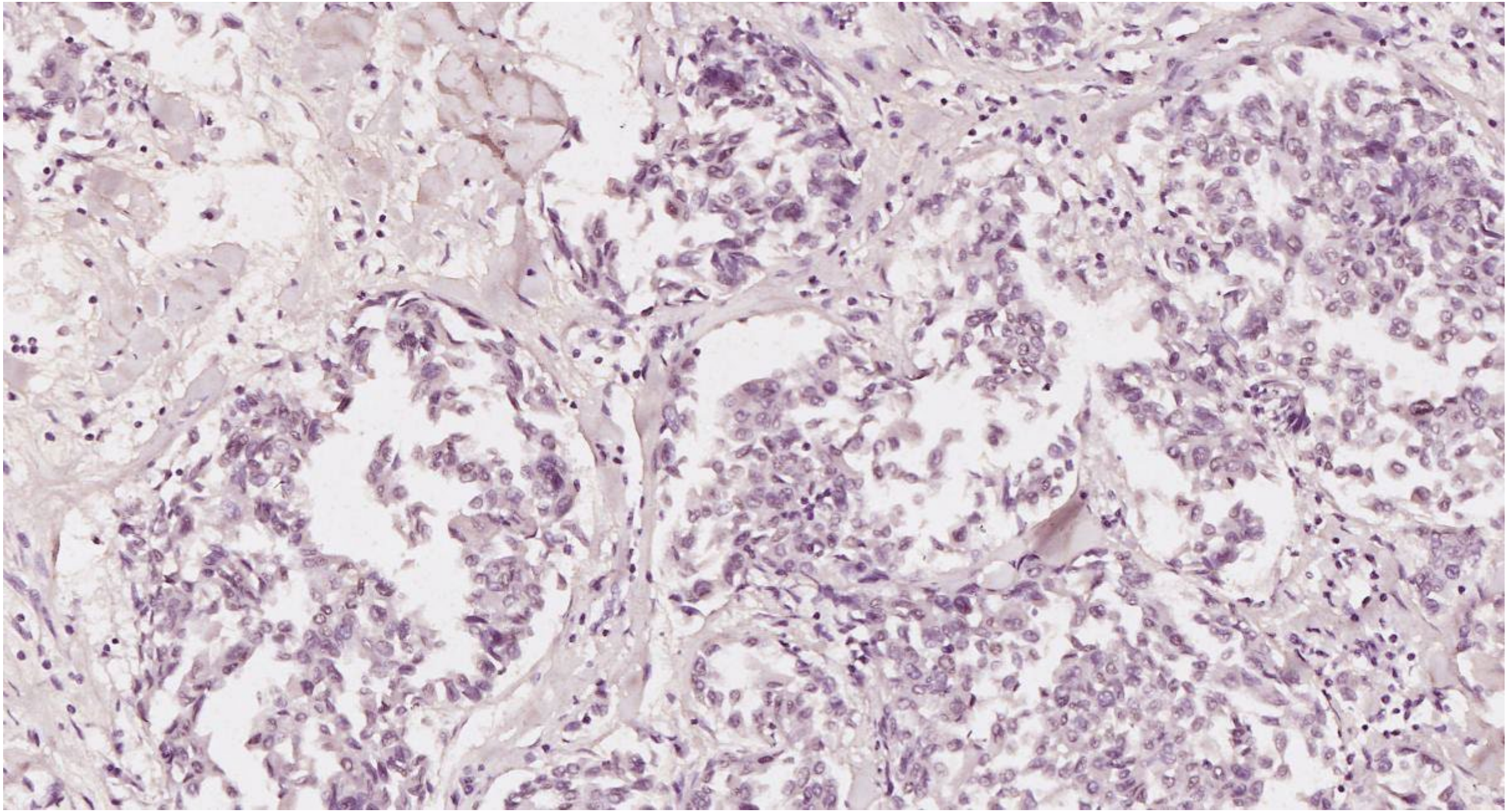
Analytical:

- Antigen retrieval (exact method)
- Assay (staining) method and protocol
- Reagent validation
- Controls (reference standards)
- Technologist and laboratory certification
- Proficiency testing and quality assurance

Post-analytical:

- Reading of result(s)/scoring/quantification
- Diagnostic, prognostic, or predictive significance
- Report
- Turn-around time
- Outcomes analysis/economics/reimbursement

Taylor CR. The total test approach to standardization of immunohistochemistry. Arch Pathol Lab Med 2000; 124: 945-951.



The Technical

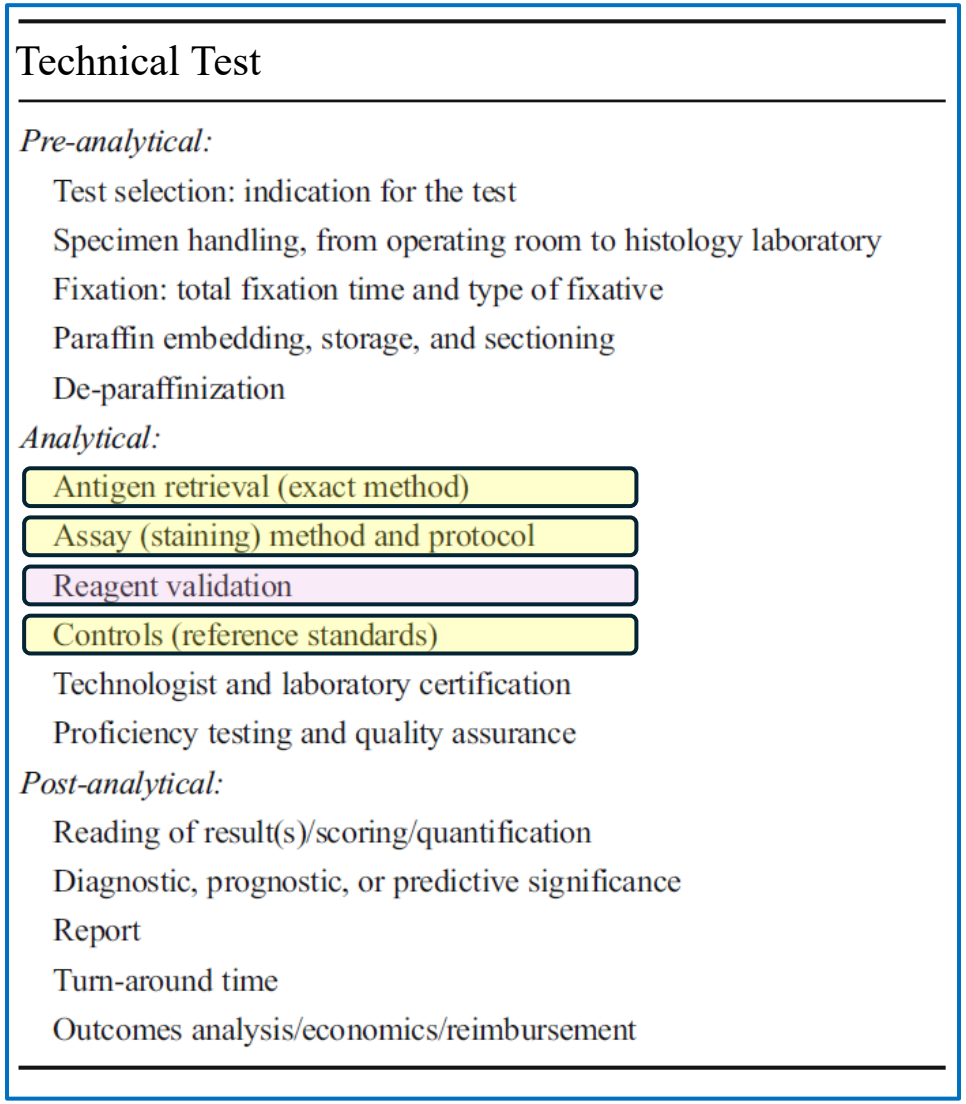
The Analytical Phase *(or when is an assay not an assay).*

Antigen retrieval (exact method)

Assay (staining) method and protocol

[Reagent verification]

Controls (reference standards)



Taylor CR. The total test approach to standardization of immunohistochemistry. Arch Pathol Lab Med 2000; 124: 945-951.

The Technical Test

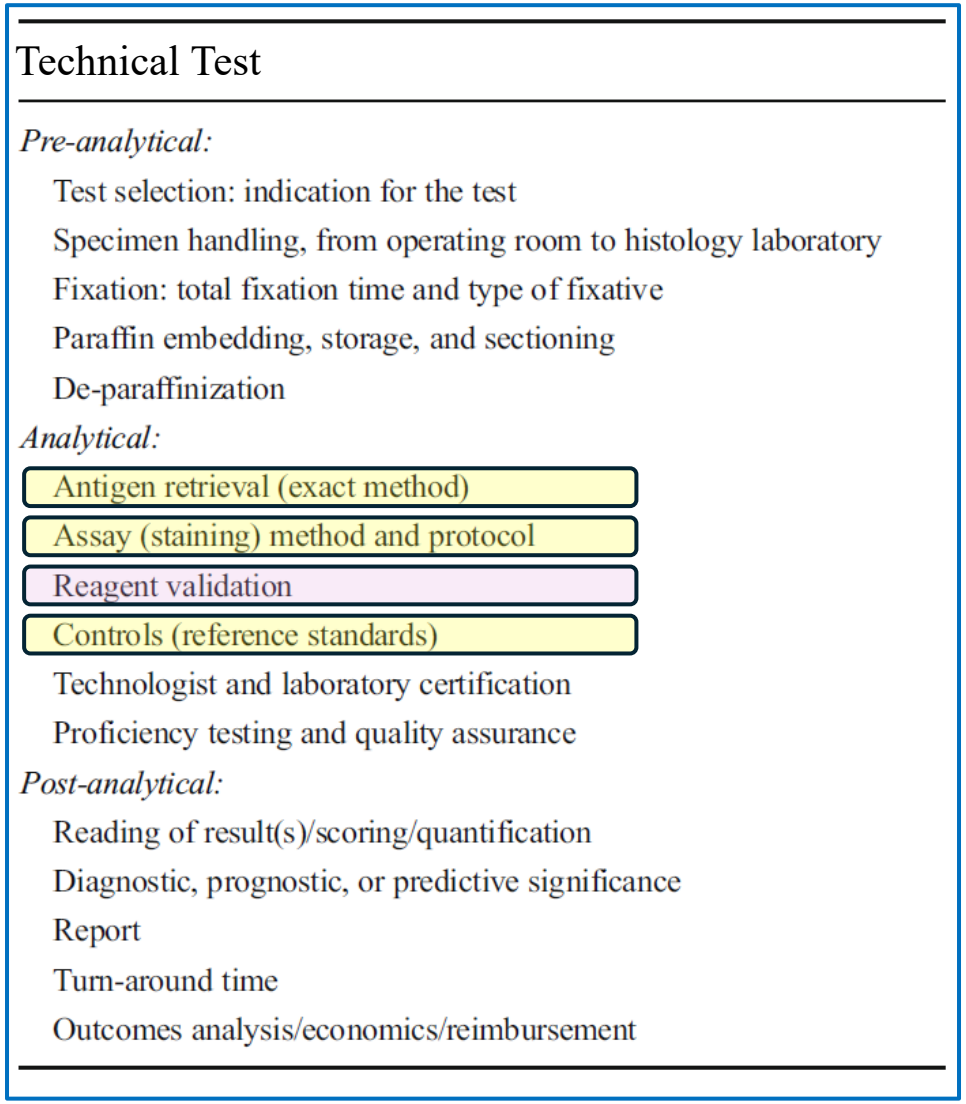
The Analytical Phase *(when is an assay not an assay).*

Antigen retrieval (exact method)

Assay (staining) method and protocol

[Reagent verification]

Controls (reference standards)



Taylor CR. The total test approach to standardization of immunohistochemistry. Arch Pathol Lab Med 2000; 124: 945-951.

Antigen retrieval: PD-L1 testing in non-small cell lung cancer (NSCLC)

SP263 (CE-IVD, Ventana). Data from the UK NEQAS ICC & ISH EQA programme.

IHC method	Antigen retrieval	Detection system	Ancillary reagents	Automation	Count of submissions	Proportion of submissions	Mean quality score (95% CIs)	P value (1)	Significance (2)
All	Various	Various	Various	Various	789	100.0%	14.9 (14.7 - 15.0)	0.057	ns
CDx	CC1 (64 min)	OptiView	None	BenchMark	402	51.0%	15.1 (14.9 - 15.4)	n/a	n/a
All LDTs	Various	Various	Various	Various	387	49.0%	14.6 (2.9 - 20.4)	0.001	***
LDT-1	CC1 (64 min)	OptiView	Amplifier	BenchMark	34	4.3%	14.9 (14.1 - 15.8)	0.828	ns
LDT-2	CC1 (not 64 min)	OptiView	None	BenchMark	286	36.2%	14.8 (14.5 - 15.1)	0.026	*
LDT-3	CC2	OptiView	Amplifier	BenchMark	12	1.5%	14.4 (13.0 - 15.8)	0.269	ns
LDT-4	CC1 (not 64 min)	OptiView	Amplifier	BenchMark	25	3.2%	14.3 (13.1 - 15.5)	0.163	ns
LDT-5	CC1 (64 min)	Ultraview	None	BenchMark	5	0.6%	13.0 (8.4 - 17.7)	0.101	ns
LDT-6	ER2	Bond Refine	None	Bond-III	16	2.0%	12.8 (11.2 – 14.3)	0.0003	****
LDT-7	ER1	Bond Refine	None	Bond-III	6	0.8%	11.0 (8.4 - 13.6)	0.0001	****

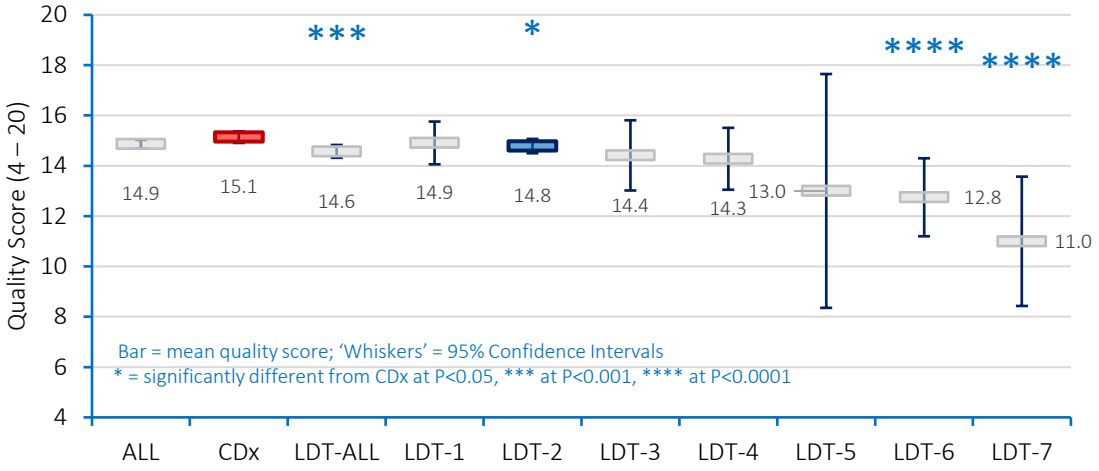
CDx = companion diagnostic; LDT = laboratory developed test; CIs = confidence intervals; n/a = not applicable; ns = not significant; Note 1: t-test for difference from CDx; Note 2: significant at P<0.05

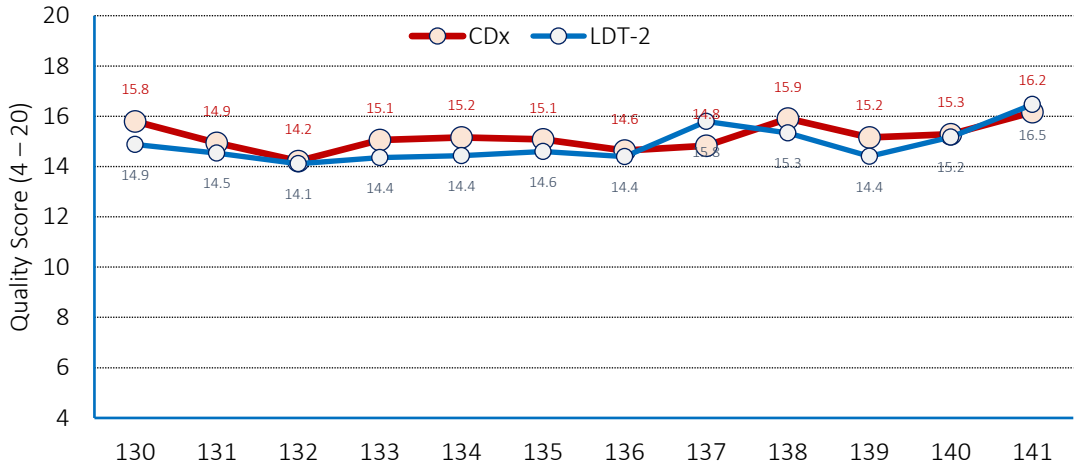
Antigen retrieval: PD-L1 testing in non-small cell lung cancer (NSCLC)

SP263 (CE-IVD, Ventana). Data from the UK NEQAS ICC & ISH EQA programme.

IHC method	Antigen retrieval	Detection system	Ancillary reagents	Automation	Count of submissions	Proportion of submissions	Mean quality score (95% CIs)	P value (1)	Significance (2)
All methods	Various	Various	Various	Various	789	100.0%	14.9 (14.7 - 15.0)	0.057	ns
CDx	**CC1 (64 min)**	**OptiView**	**None**	**BenchMark**	**402**	**51.0%**	**15.1 (14.9 - 15.4)**	**n/a**	**n/a**
All LDTs	Various	Various	Various	Various	387	49.0%	14.6 (14.3 - 15.0)	0.001	***
LDT-1	CC1 (64 min)	OptiView	Amplifier	BenchMark	34	4.3%	14.9 (14.1 - 15.8)	0.828	ns
LDT-2	**CC1 (not 64 min)**	**OptiView**	**None**	**BenchMark**	**286**	**36.2%**	**14.8 (14.5 - 15.1)**	**0.026**	*
LDT-3	CC2	OptiView	Amplifier	BenchMark	12	1.5%	14.4 (13.0 - 15.8)	0.269	ns
LDT-4	CC1 (not 64 min)	OptiView	Amplifier	BenchMark	25	3.2%	14.3 (13.1 - 15.5)	0.163	ns
LDT-5	CC1 (64 min)	Ultraview	None	BenchMark	5	0.6%	13.0 (8.4 - 17.7)	0.101	ns
LDT-6	ER2	Bond Refine	None	Bond-III	16	2.0%	12.8 (11.2 – 14.3)	0.0003	****
LDT-7	ER1	Bond Refine	None	Bond-III	6	0.8%	11.0 (8.4 - 13.6)	0.0001	****

CDx = companion diagnostic; LDT = laboratory developed test; CIs = confidence intervals; n/a = not applicable; ns = not significant; Note 1: t-test for difference from CDx; Note 2: significant at P<0.05





The Technical Test

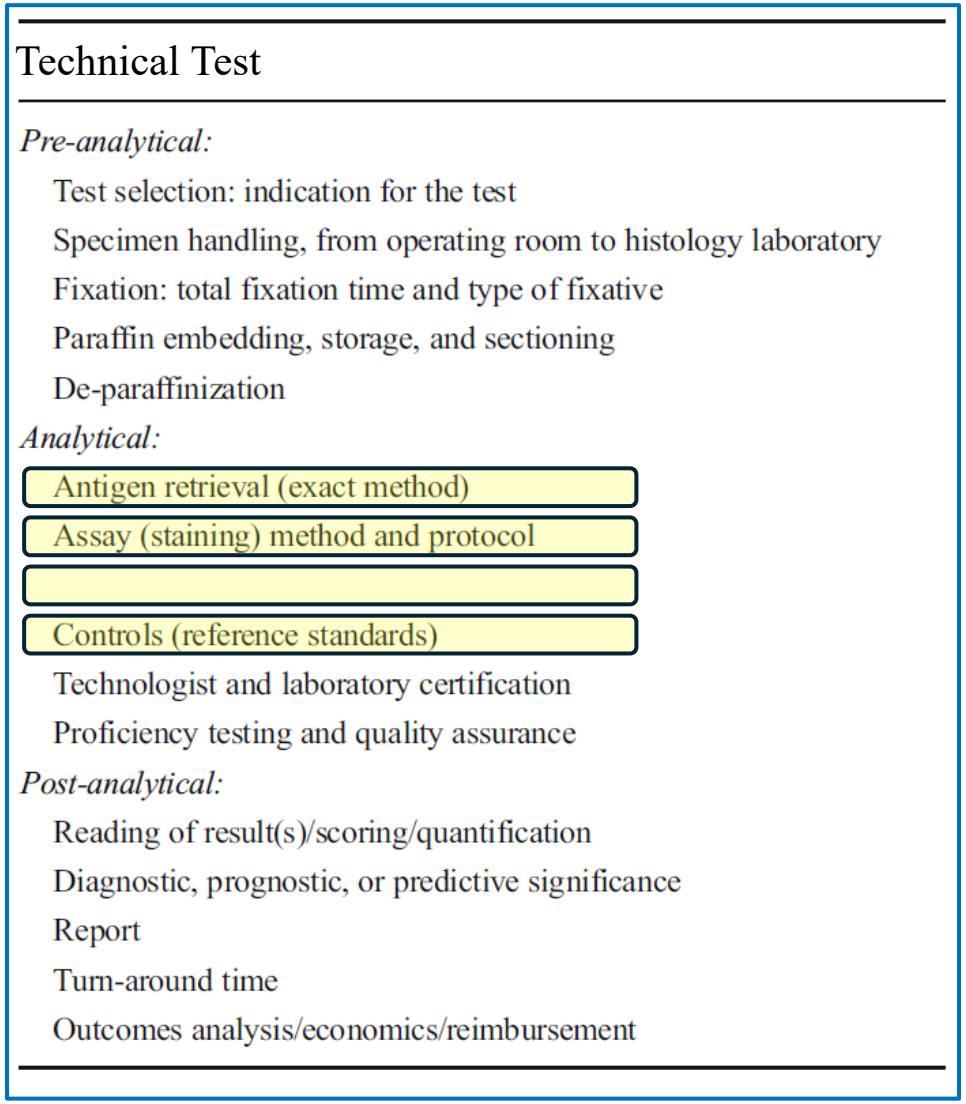
The Analytical Phase *(when is an assay not an assay).*

Antigen retrieval (exact method)

Assay (staining) method and protocol

[Reagent verification]

Controls (reference standards)

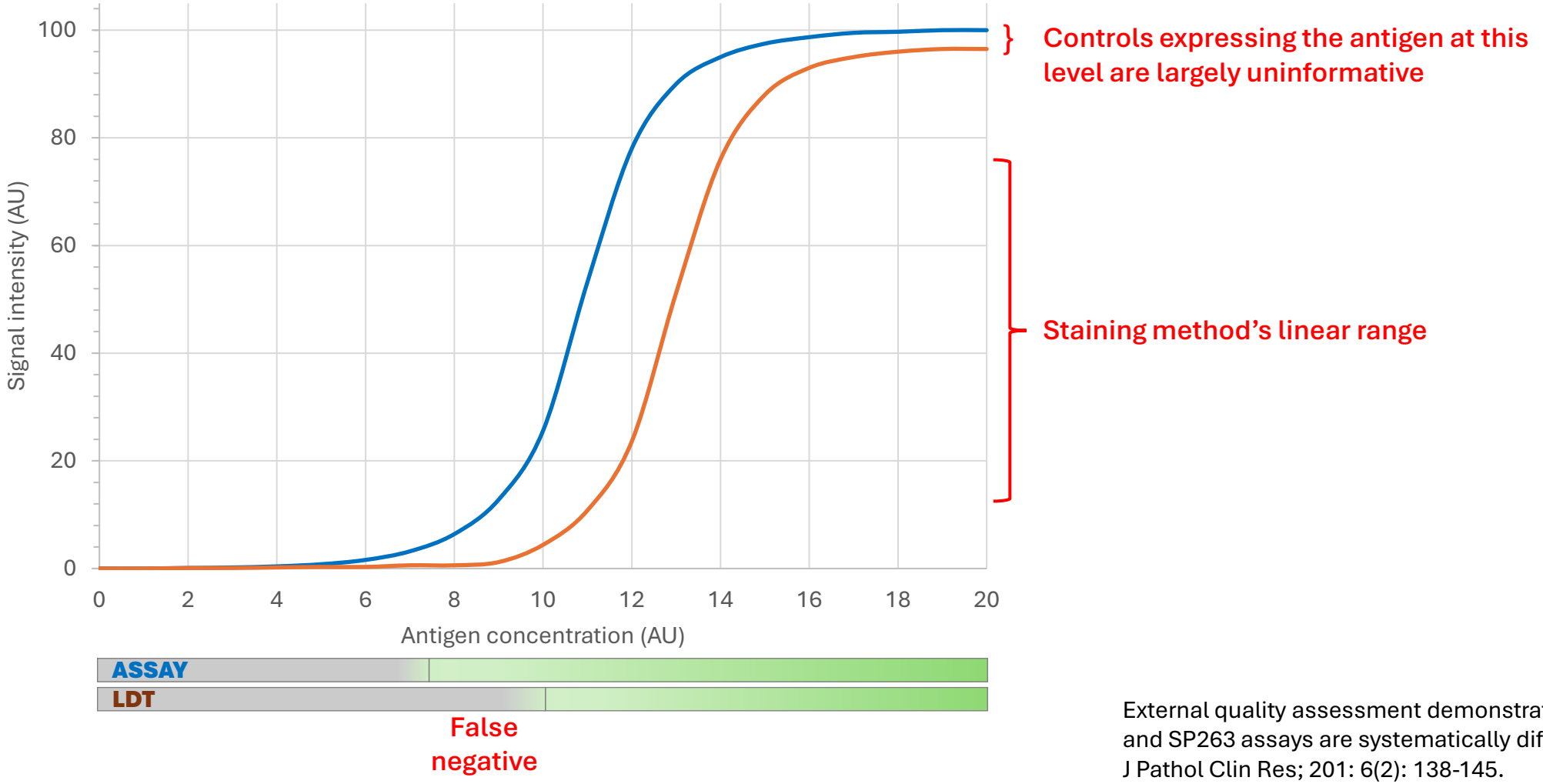


Taylor CR. The total test approach to standardization of immunohistochemistry. Arch Pathol Lab Med 2000; 124: 945-951.

Controls: PD-L1 testing in non-small cell lung cancer (NSCLC)

UK NEQAS
Immunocytochemistry & In-Situ Hybridisation

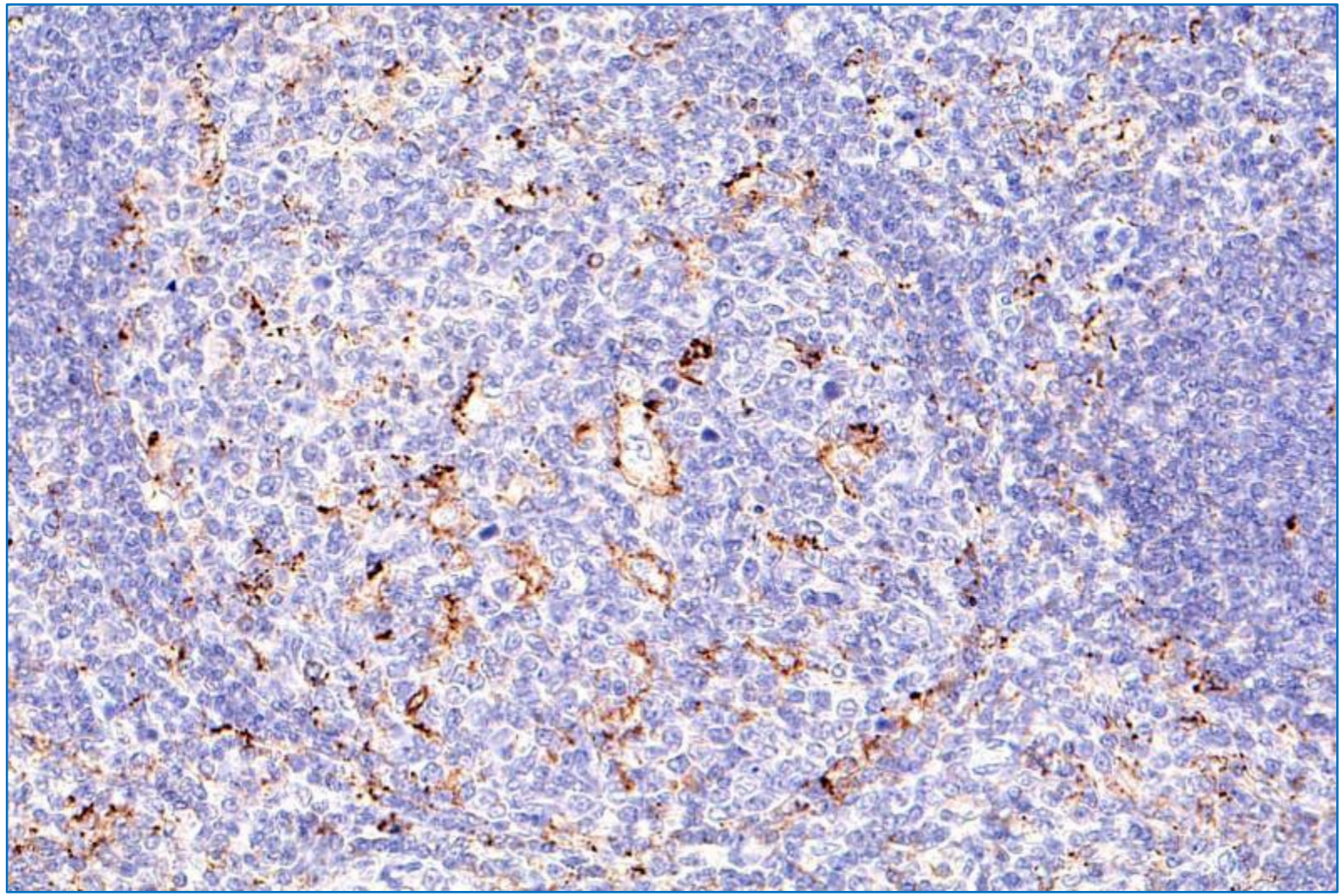
SP263 (CE-IVD, Ventana). Data from the UK NEQAS ICC & ISH EQA programme.



External quality assessment demonstrates that PD-L1 22C3 and SP263 assays are systematically different. Dodson A, *et al*. J Pathol Clin Res; 201: 6(2): 138-145.

Controls: PD-L1 testing in non-small cell lung cancer (NSCLC)

UK NEQAS
Immunocytochemistry & In-Situ Hybridisation

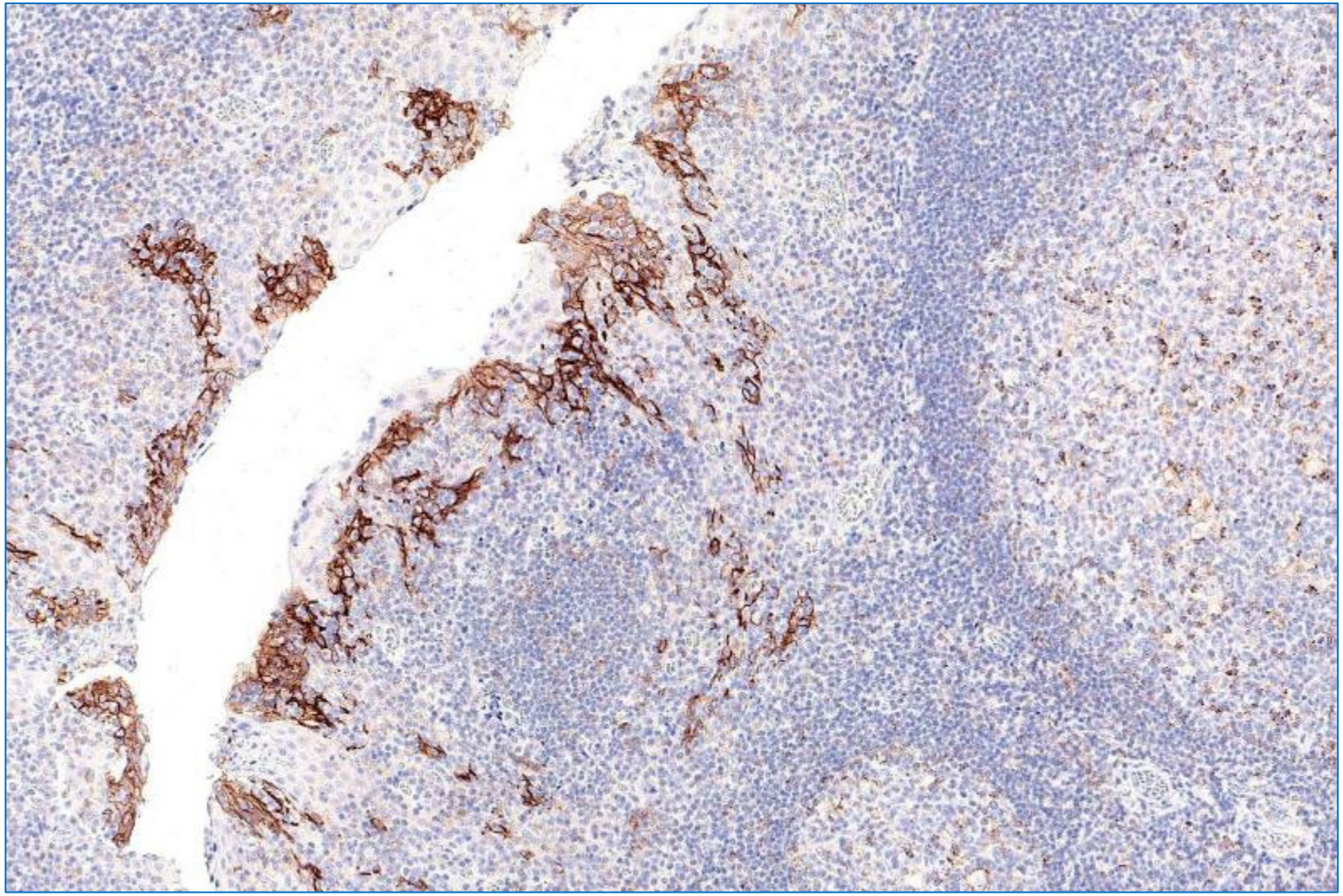


- PD-L1 demonstrated using SP263 (Ventana)

- Normal tissues showing a range of expression, including low-level expression near to the LOD are ideal.
- In the follicles, follicular macrophages showing moderate to strong membrane staining are prominently demonstrated
- Small antigen presenting cells located with the follicles show strong expression of PD-L1.
- PD-L1 is also present in a sub-set of lymphocytes in the intra-follicular areas.
- PD-L1 staining is well-localised to cellular membranes.
- Cellular morphology and nuclear detail has been retained; indicative of adequate pre-analytical treatment.
- The level of nuclear counterstain (haematoxylin) is well-balanced so as to show the nuclear detail but not obscure weak PD-L1 staining.

Controls: PD-L1 testing in non-small cell lung cancer (NSCLC)

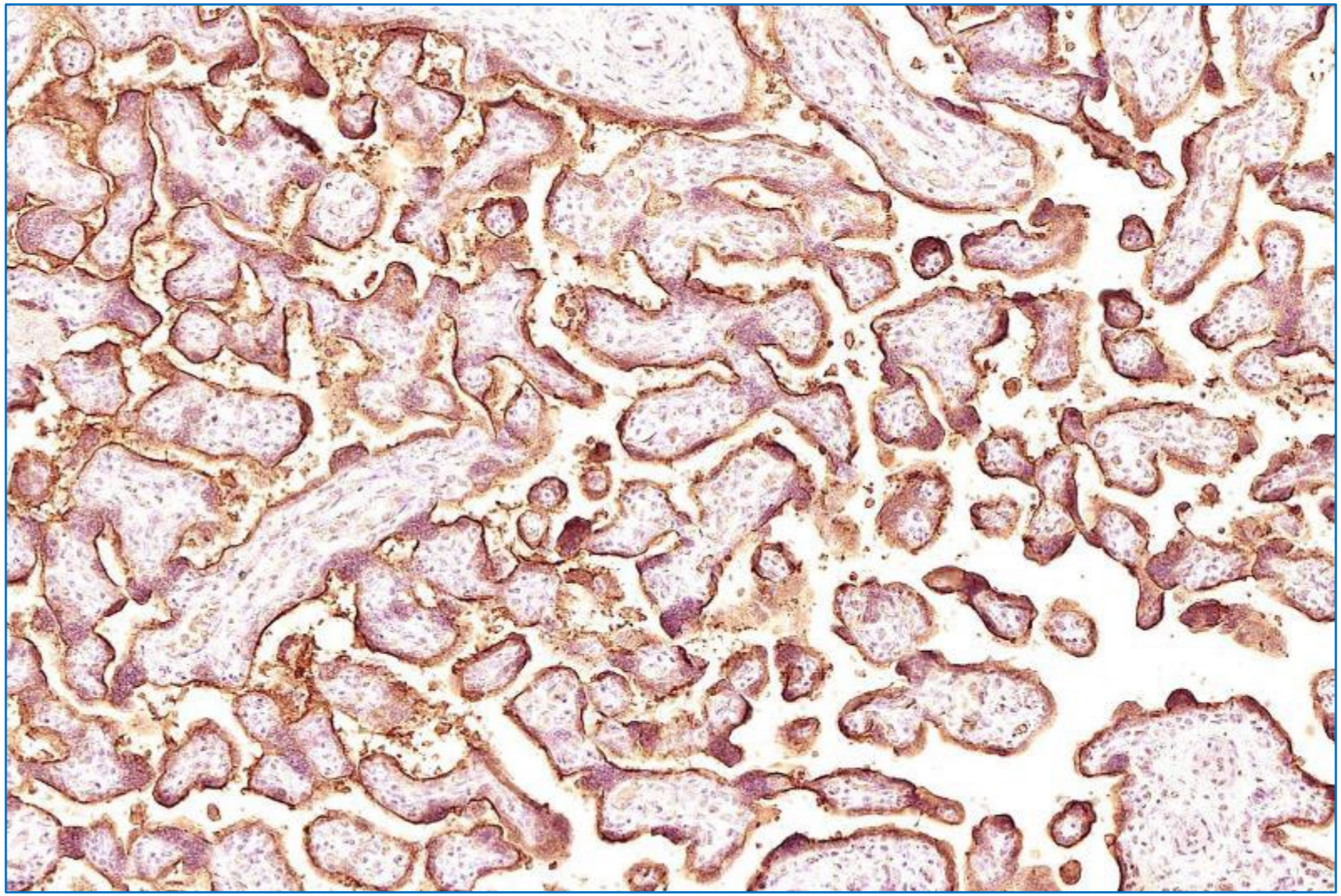
UK NEQAS
Immunocytochemistry & In-Situ Hybridisation



- PD-L1 demonstrated using SP263 (Ventana)
- The reticulated crypt epithelium is universally strongly positive.
- The method sensitivity required to produce staining in crypt epithelium is much less than that required in testing NSCLC.
- **You cannot rely on the presence of staining in the crypt epithelium alone to quality control the sensitivity of your method.**

Controls: PD-L1 testing in non-small cell lung cancer (NSCLC)

UK NEQAS
Immunocytochemistry & In-Situ Hybridisation



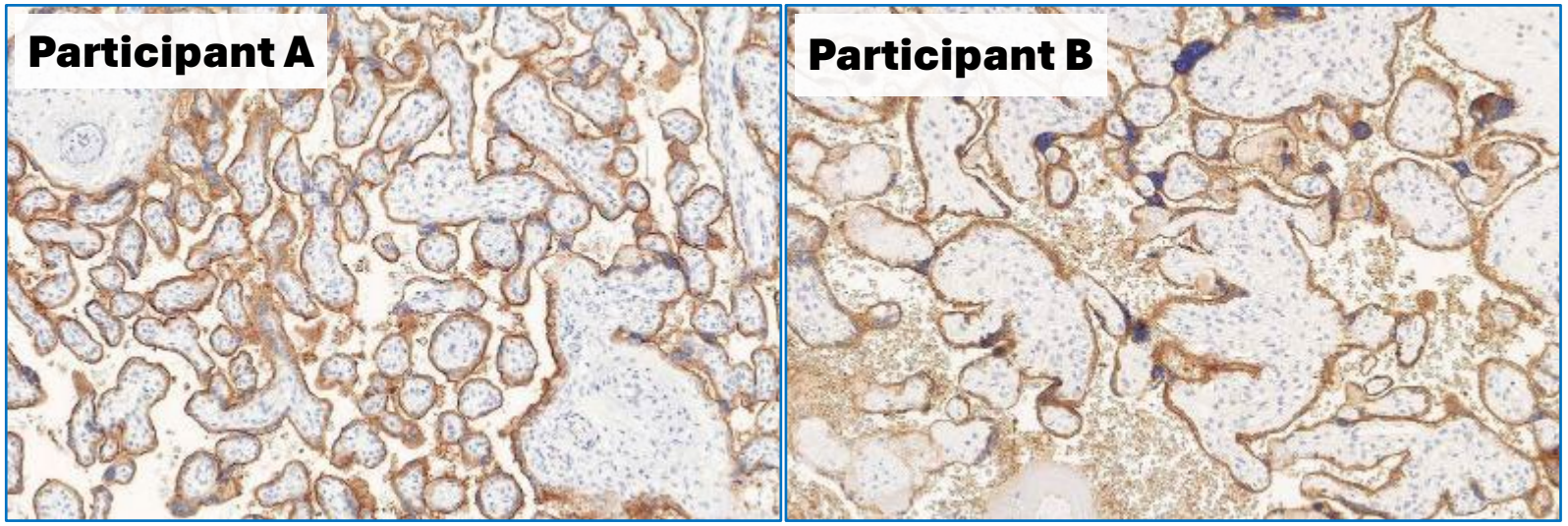
- PD-L1 demonstrated using SP263 (Ventana)

- Placenta is recommended by some primary antibody manufacturers as a suitable control.
- Trophoblasts show strong membrane and moderate to weak cytoplasmic staining.
- '*...stromal tissue and vasculature can be used for assessment of any background staining...*'

- UK NEQAS does not recommend the use of placenta as a control for PD-L1 staining in the companion diagnostic setting.
- Here's why...

Controls: PD-L1 testing in non-small cell lung cancer (NSCLC)

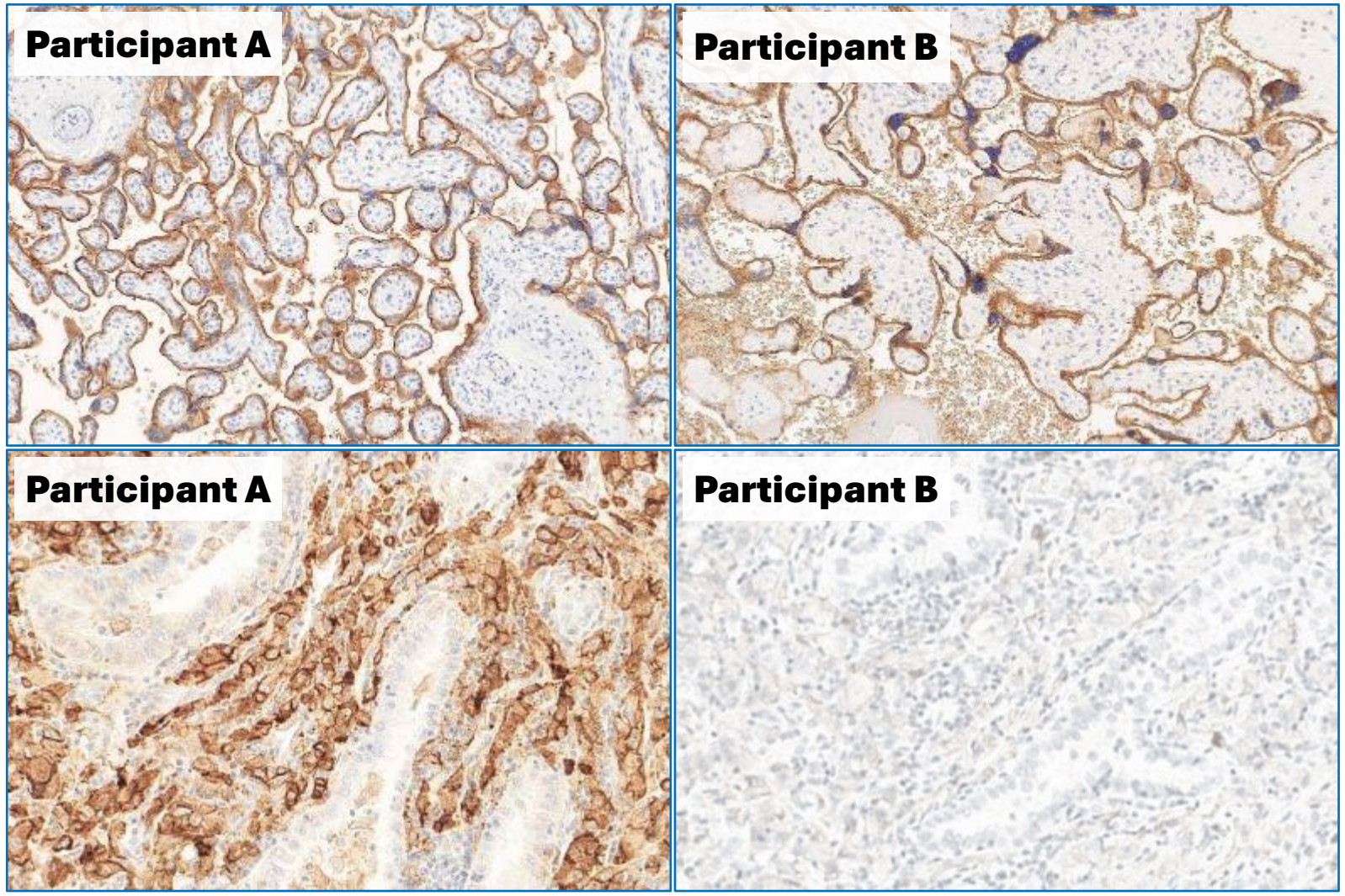
UK NEQAS
Immunocytochemistry & In-Situ Hybridisation



- PD-L1 demonstrated using SP263 assay (Ventana).
- In-house control submissions from two Participants, both used placenta as their in-house control.

Controls: PD-L1 testing in non-small cell lung cancer (NSCLC)

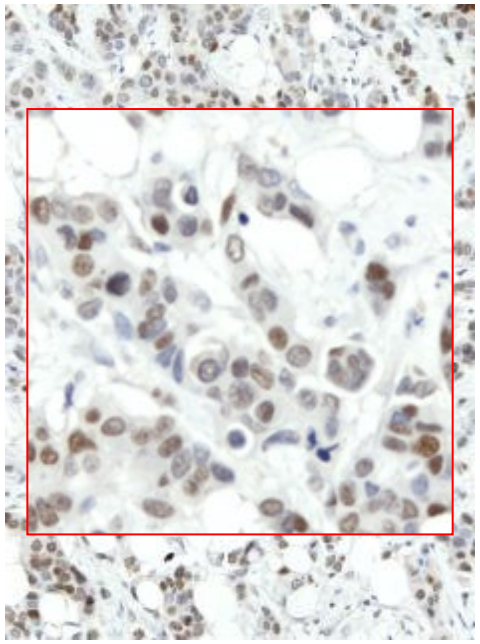
UK NEQAS
Immunocytochemistry & In-Situ Hybridisation



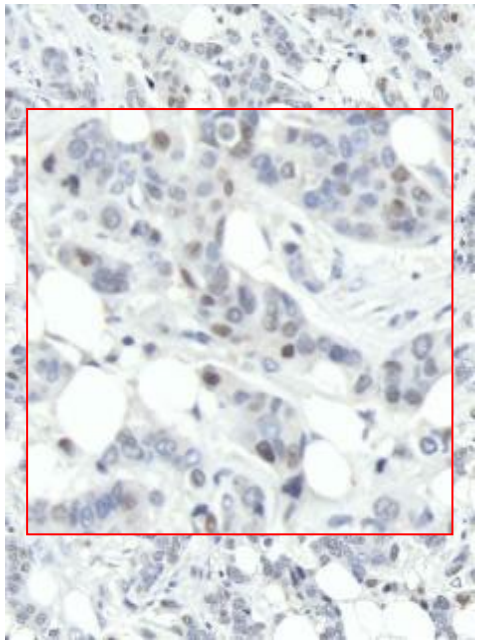
- PD-L1 demonstrated using SP263 assay (Ventana).
- In-house control submissions from two Participants, both used placenta as their only in-house control.
- Their respective staining results on the UK NEQAS NSCLC sample provided to them which was expected to stain at a level of approximately 70%.

Antigen loss on storage

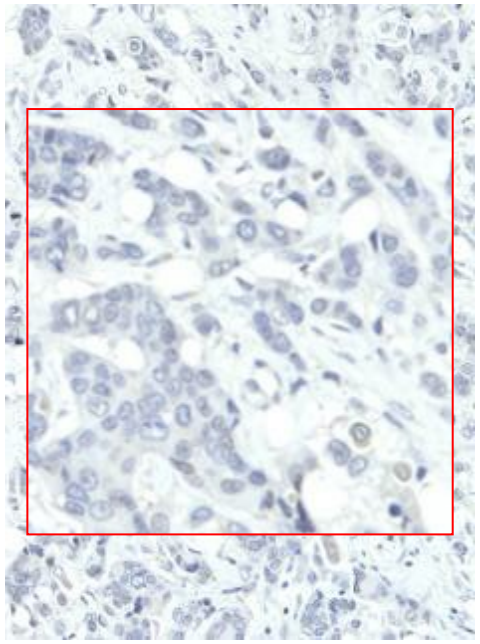
Stained 1 day after cutting



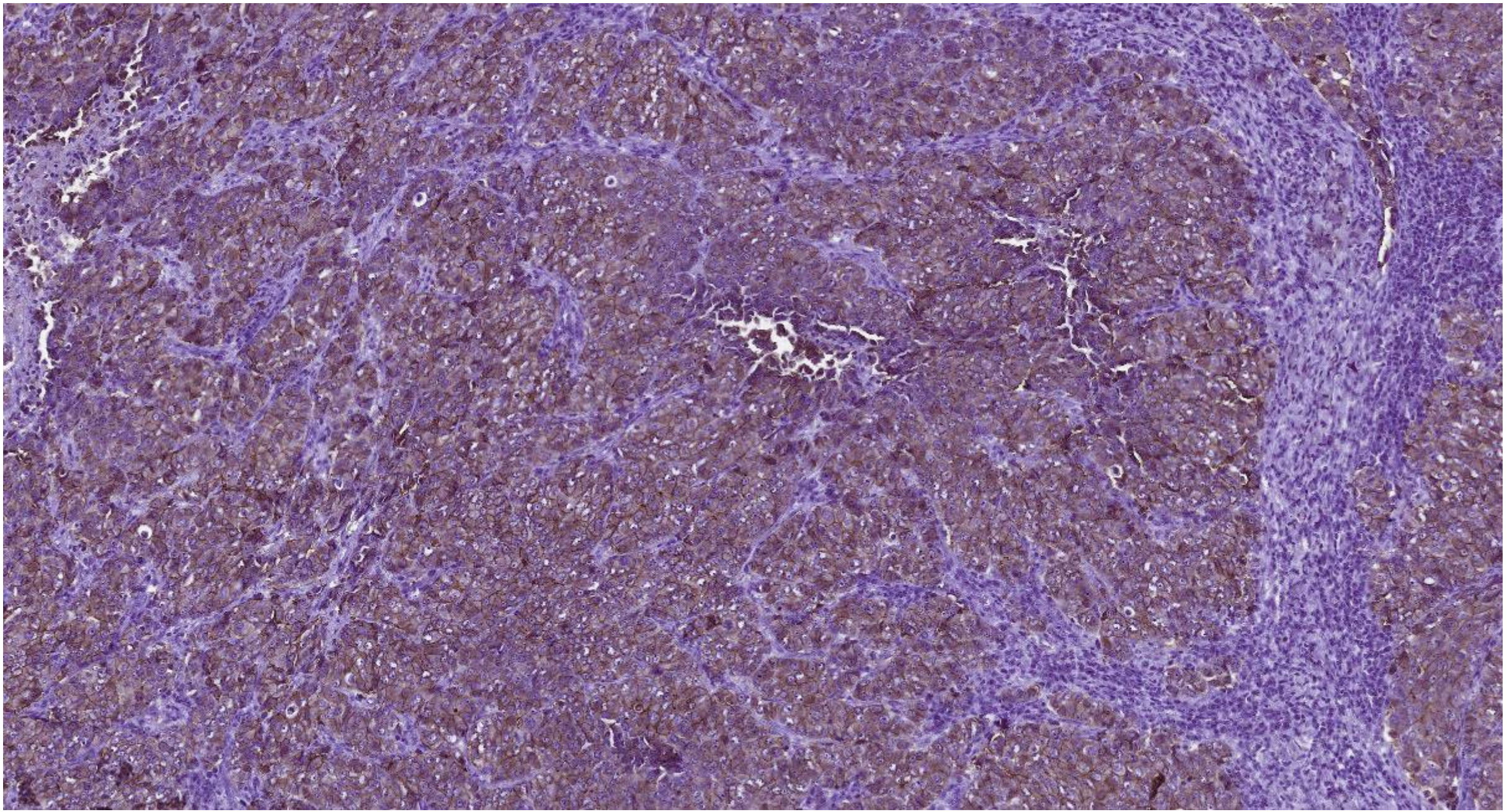
Stained 3 weeks after cutting



Stained 6 weeks after cutting



Parry S *et al*. *Immunocytochemistry UKNEQAS ICC & ISH e-journal* (2012) 96, 4-50



The Technical Test

The Post-analytical Phase.

- The way in which the interpretation of an IHC test is done is very much dependent the purpose of the test.
  - **Diagnosis** of disease in a symptomatic patient e.g., diagnosis of pulmonary carcinoma in a case of unknown primary using a panel of primary antibodies.
  - **Diagnostic screening** in a patient who already has a primary diagnosis e.g., mismatch repair protein deficiency in colorectal carcinoma.
  - **Prognosis** of the diagnosed disease e.g., Ki-67 labelling
  - **Predictive** testing for response to a targeted therapy e.g., HER2 in breast or gastric tumours; PD-L1 in immuno-oncology.

Technical Test
Pre-analytical:
Test selection: indication for the test
Specimen handling, from operating room to histology laboratory
Fixation: total fixation time and type of fixative
Paraffin embedding, storage, and sectioning
De-paraffinization
Analytical:
Antigen retrieval (exact method)
Assay (staining) method and protocol
Reagent validation
Controls (reference standards)
Technologist and laboratory certification
Proficiency testing and quality assurance
Post-analytical:
Reading of result(s)/scoring/quantification
Diagnostic, prognostic, or predictive significance
Report
Turn-around time
Outcomes analysis/economics/reimbursement

Taylor CR. The total test approach to standardization of immunohistochemistry. Arch Pathol Lab Med 2000; 124: 945-951.

Diagnostic Screening Tests

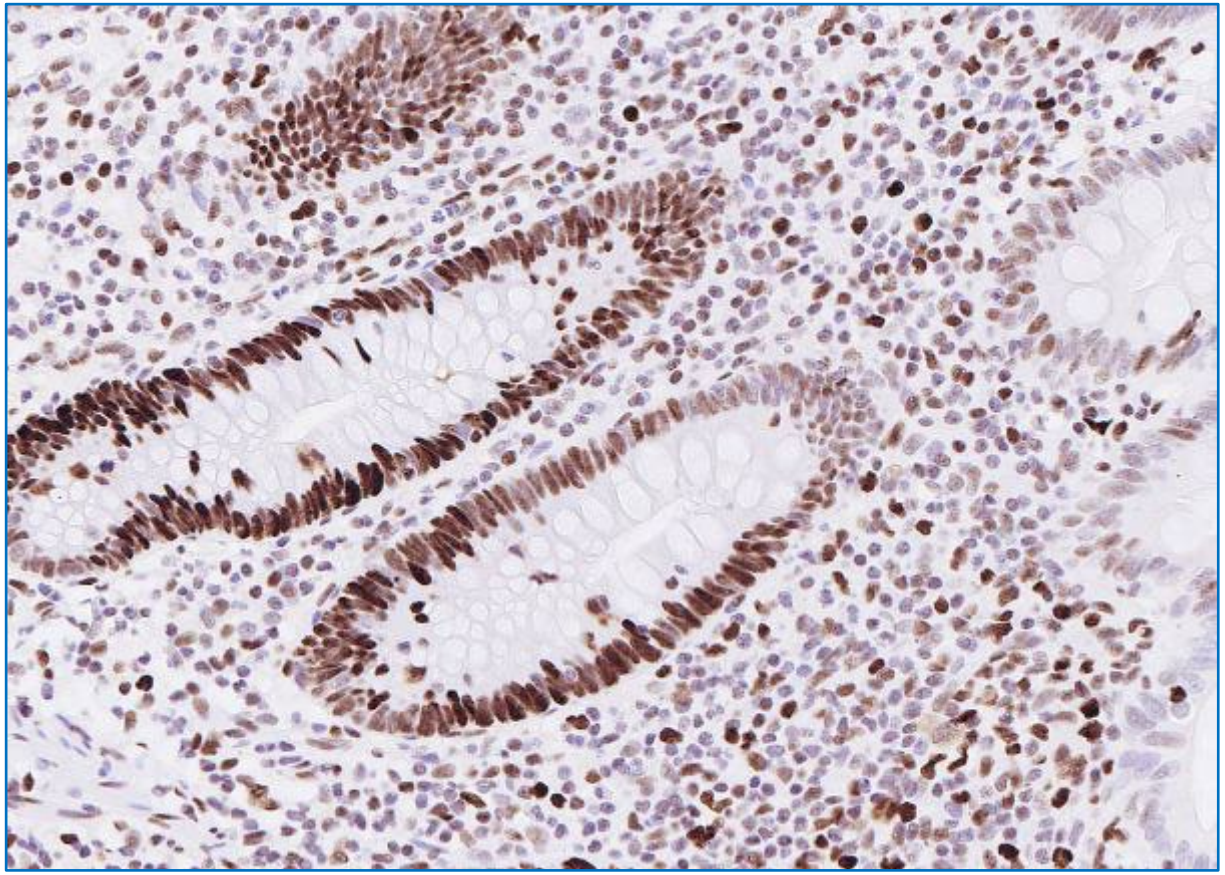
Diagnostic screening in a patient who already has a primary diagnosis e.g., mismatch repair protein deficiency in colorectal carcinoma.

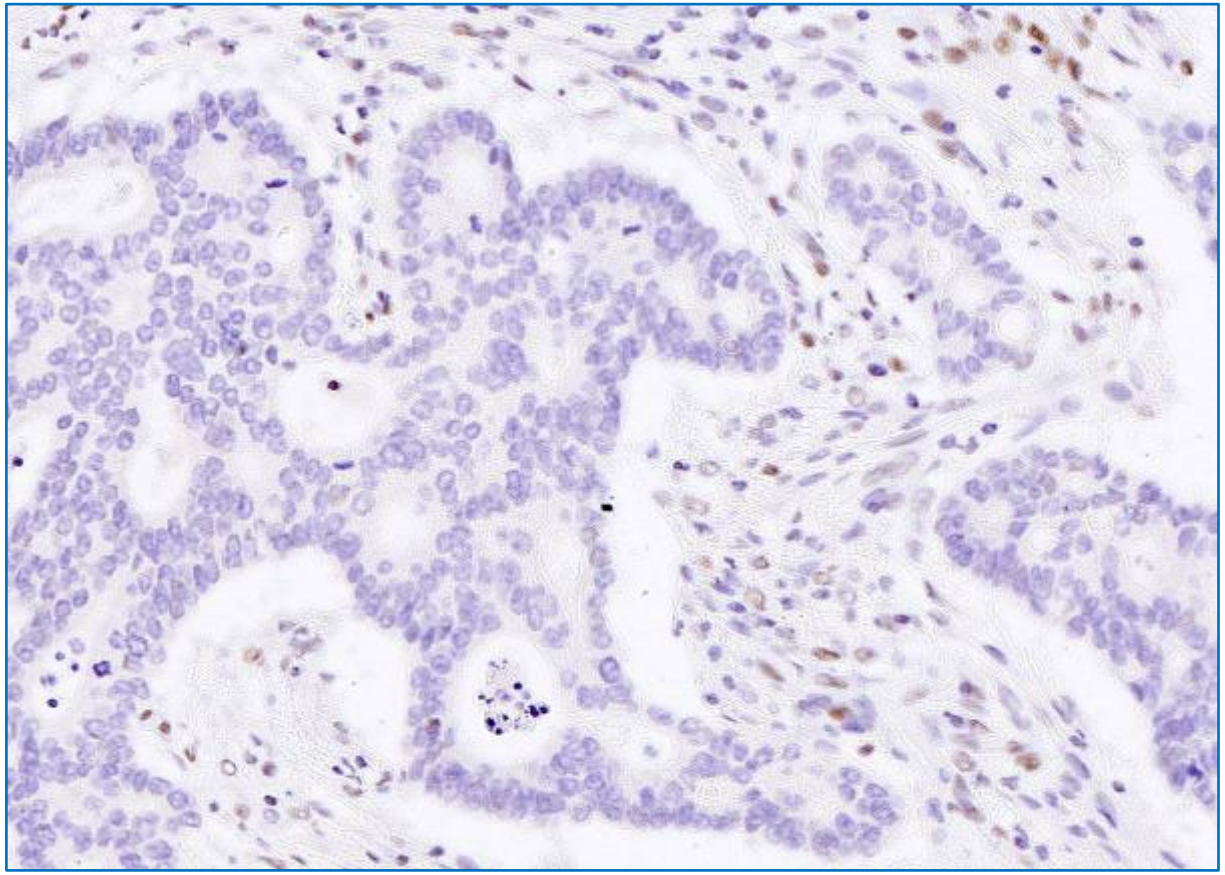
Three different cases for us to diagnose…

Diagnostic Screening Tests

UK NEQAS
Immunocytochemistry & In-Situ Hybridisation

Diagnostic screening in a patient who already has a primary diagnosis e.g., mismatch repair protein deficiency in colorectal carcinoma.





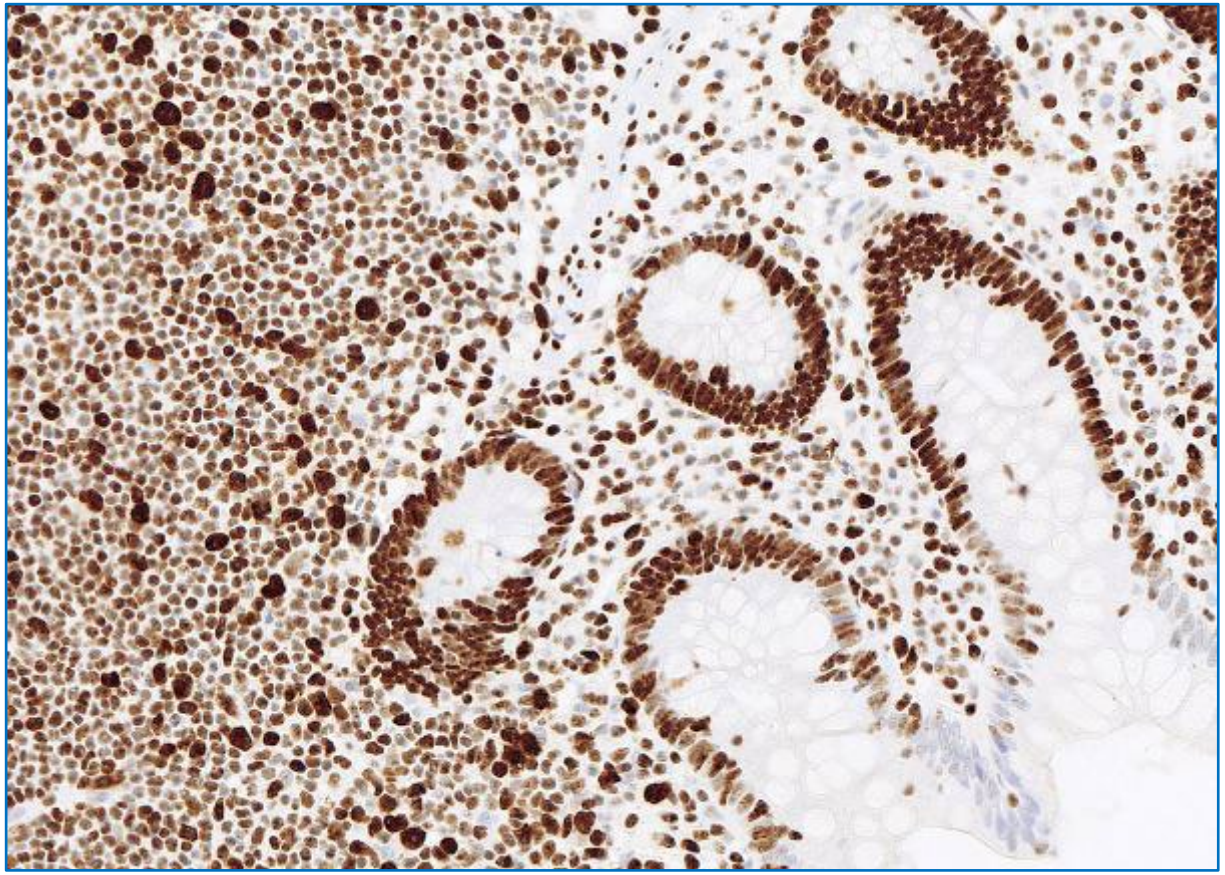
Case 1 (MSH6, x30 obj. mag.)

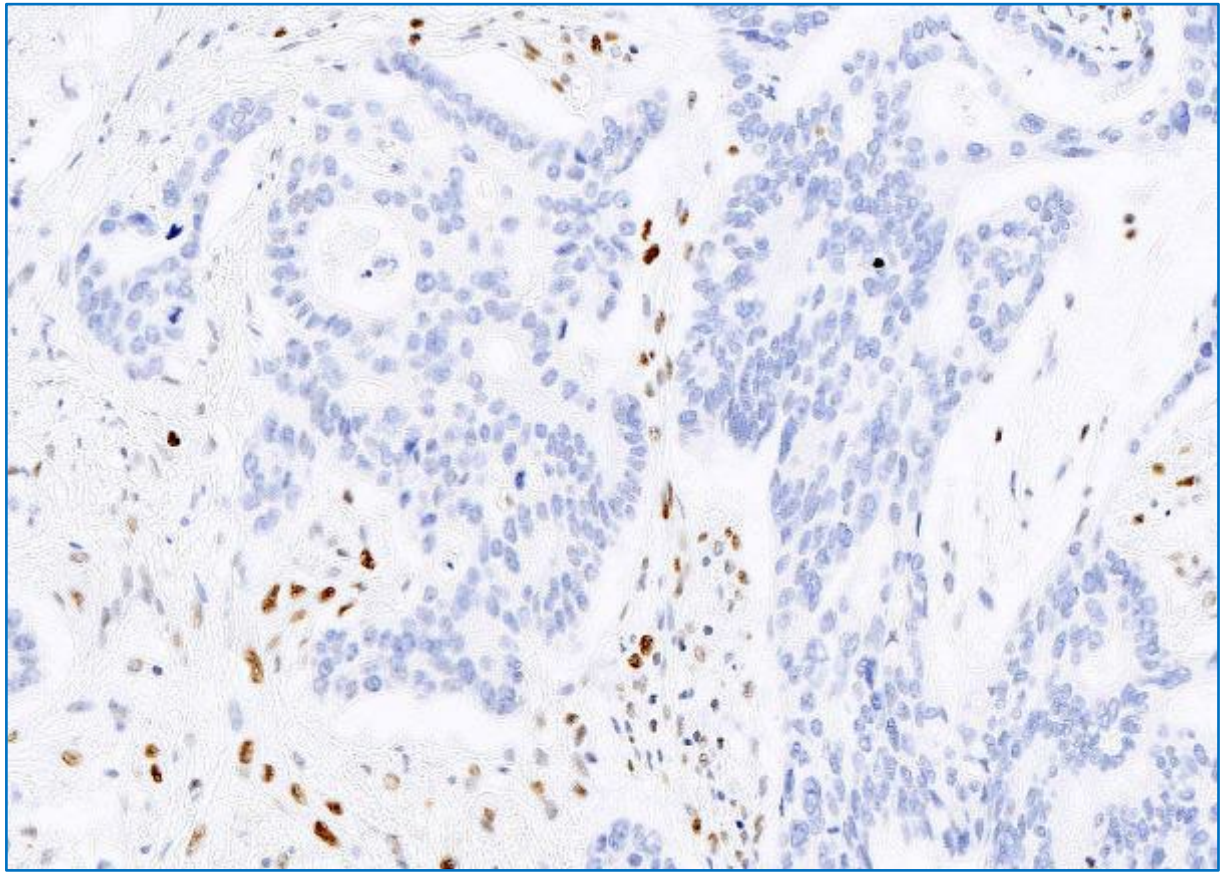
MSH6 proficient, MSH6 deficient, Equivocal, Uninterpretable.

Diagnostic Screening Tests

UK NEQAS
Immunocytochemistry & In-Situ Hybridisation

Diagnostic screening in a patient who already has a primary diagnosis e.g., mismatch repair protein deficiency in colorectal carcinoma.





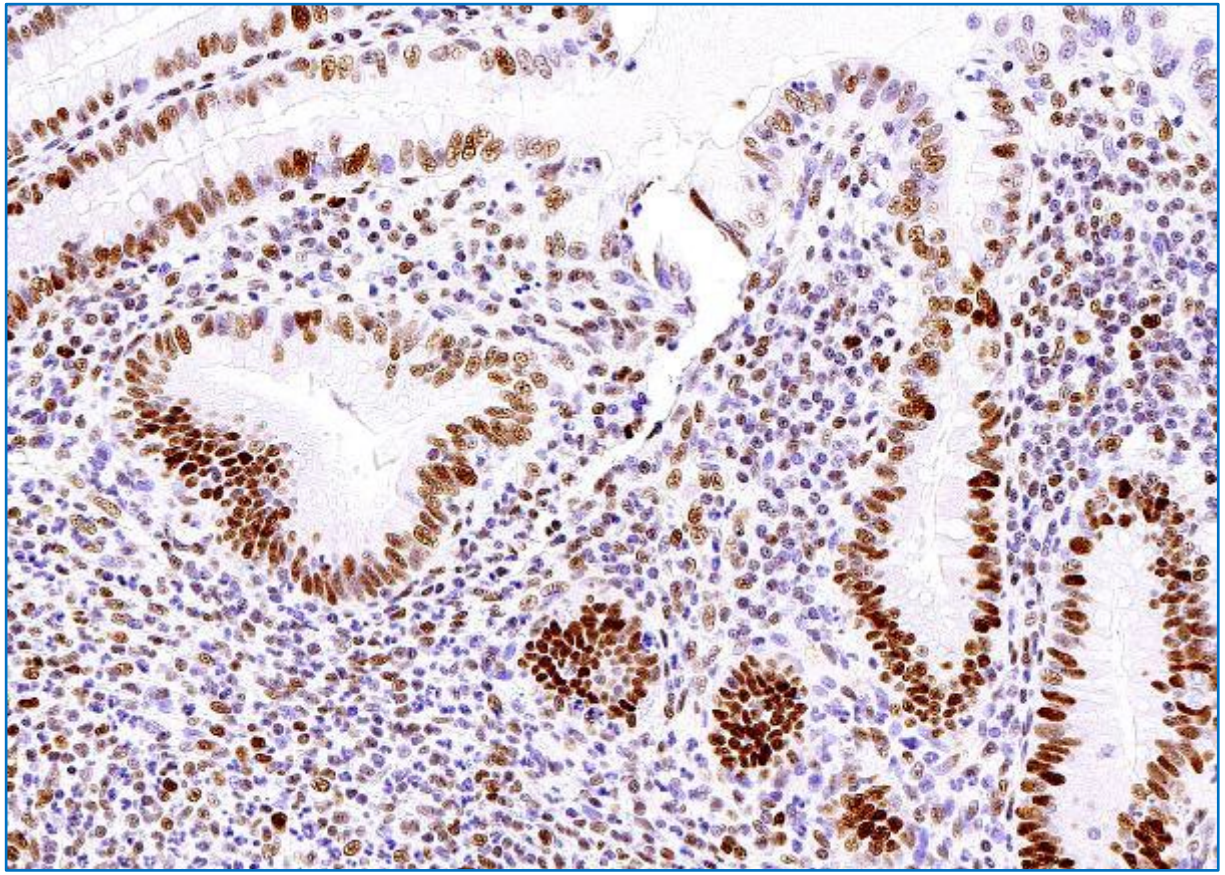
Case 2 (MSH6, x30 obj. mag.)

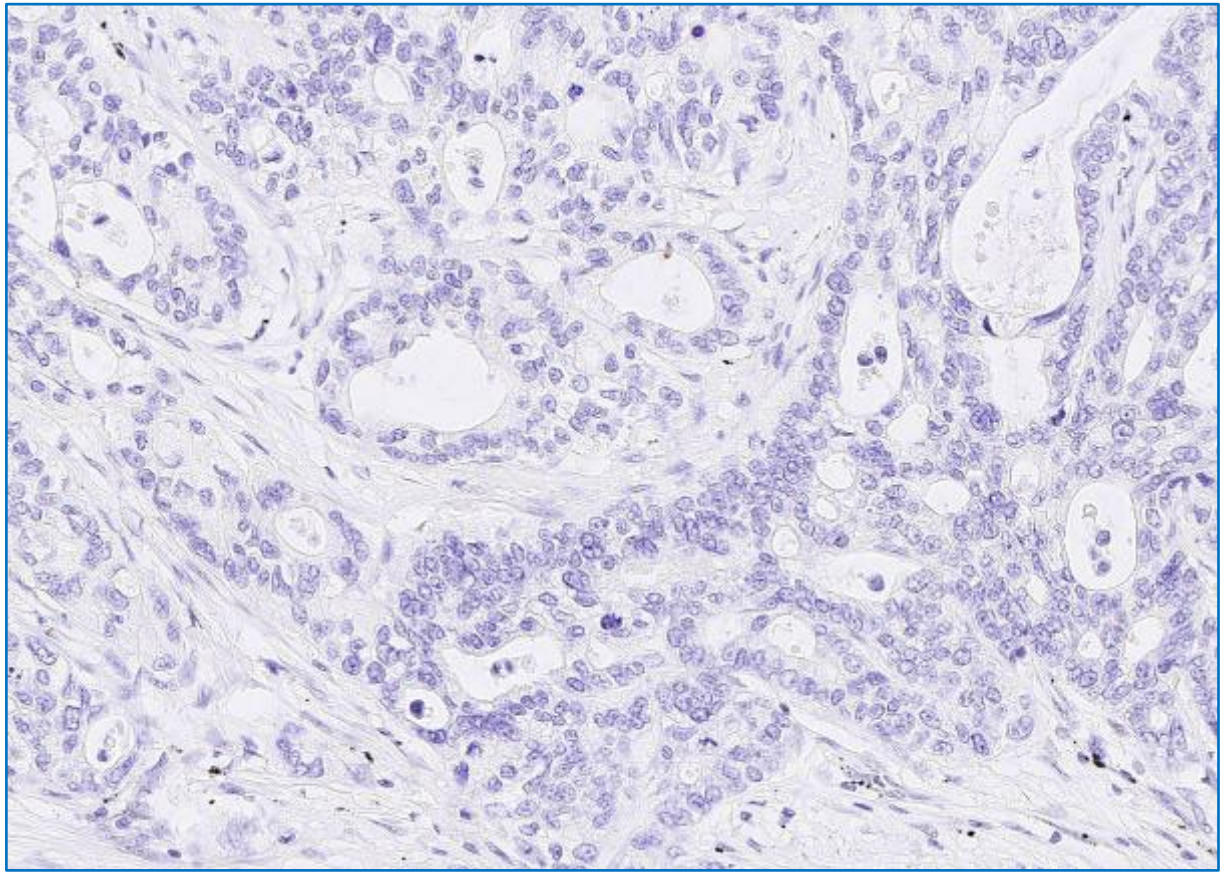
MSH6 proficient, MSH6 deficient, Equivocal, Uninterpretable.

Diagnostic Screening Tests

UK NEQAS
Immunocytochemistry & In-Situ Hybridisation

Diagnostic screening in a patient who already has a primary diagnosis e.g., mismatch repair protein deficiency in colorectal carcinoma.





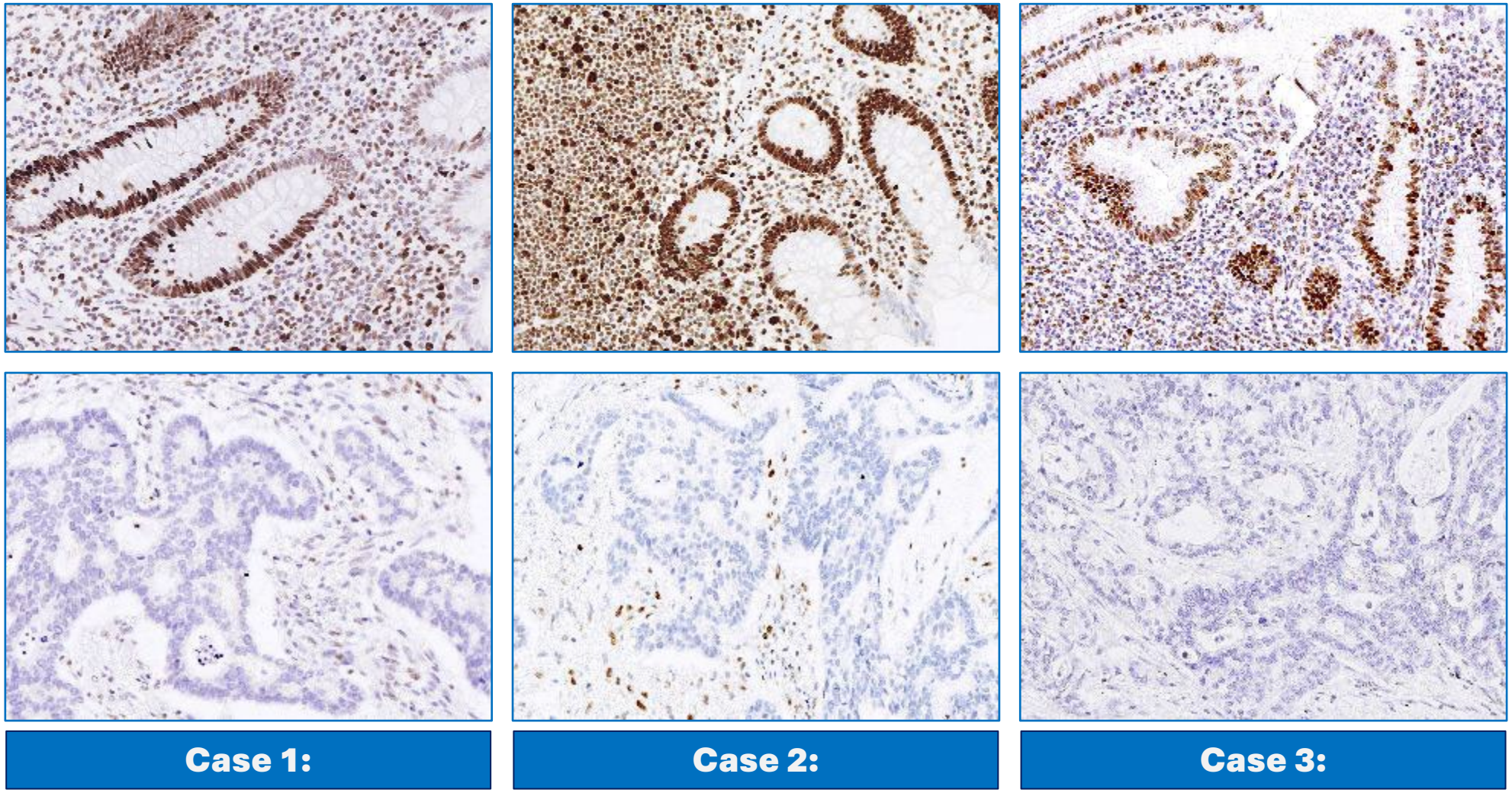
Case 3 (MSH6, x30 obj. mag.)

MSH6 proficient, MSH6 deficient, Equivocal, Uninterpretable.

Diagnostic Screening Tests

UK NEQAS
Immunocytochemistry & In-Situ Hybridisation

Diagnostic screening in a patient who already has a primary diagnosis e.g., mismatch repair protein deficiency in colorectal carcinoma.

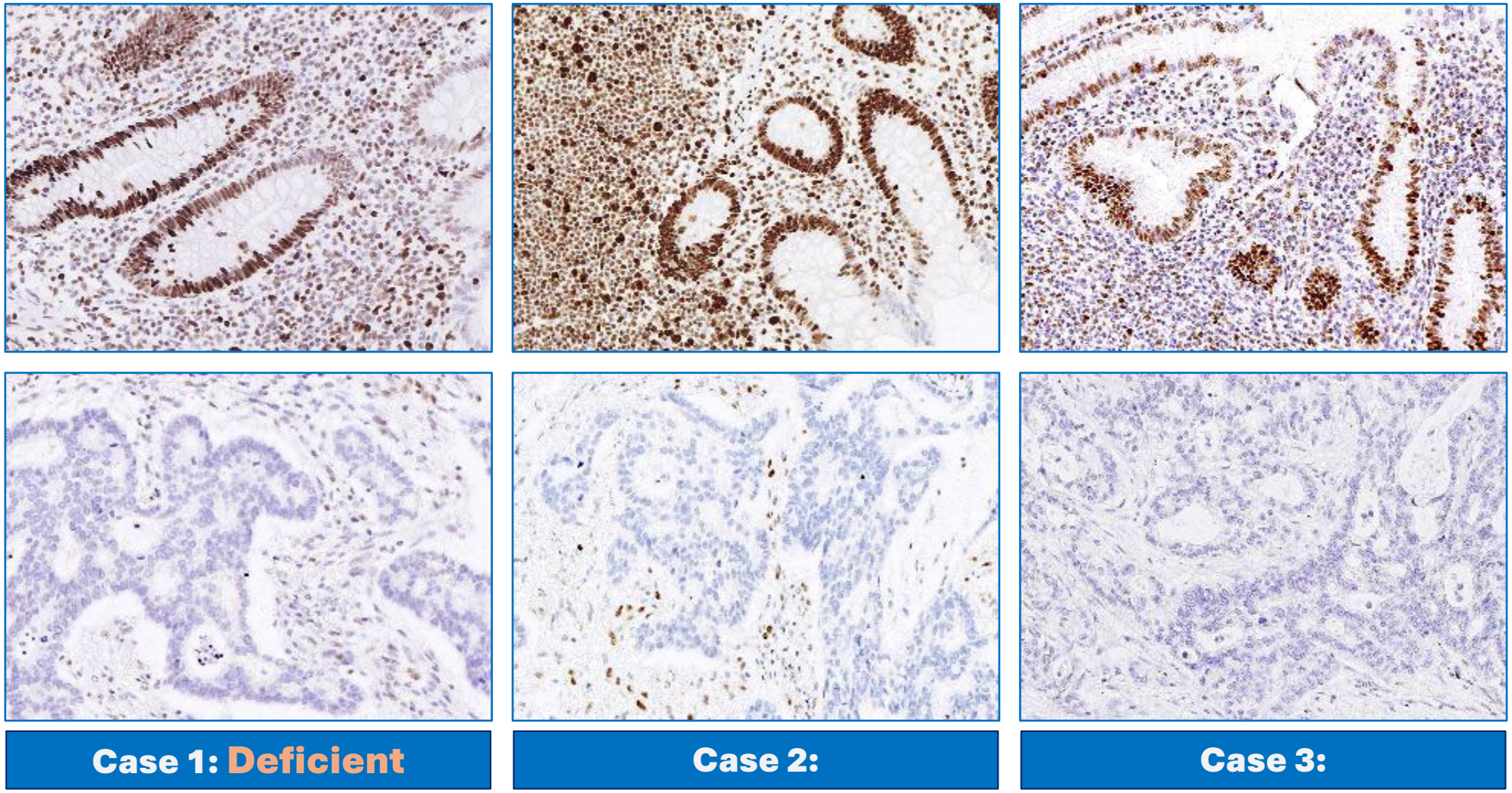


Case 1:	Case 2:	Case 3:

Diagnostic Screening Tests

UK NEQAS
Immunocytochemistry & In-Situ Hybridisation

Diagnostic screening in a patient who already has a primary diagnosis e.g., mismatch repair protein deficiency in colorectal carcinoma.

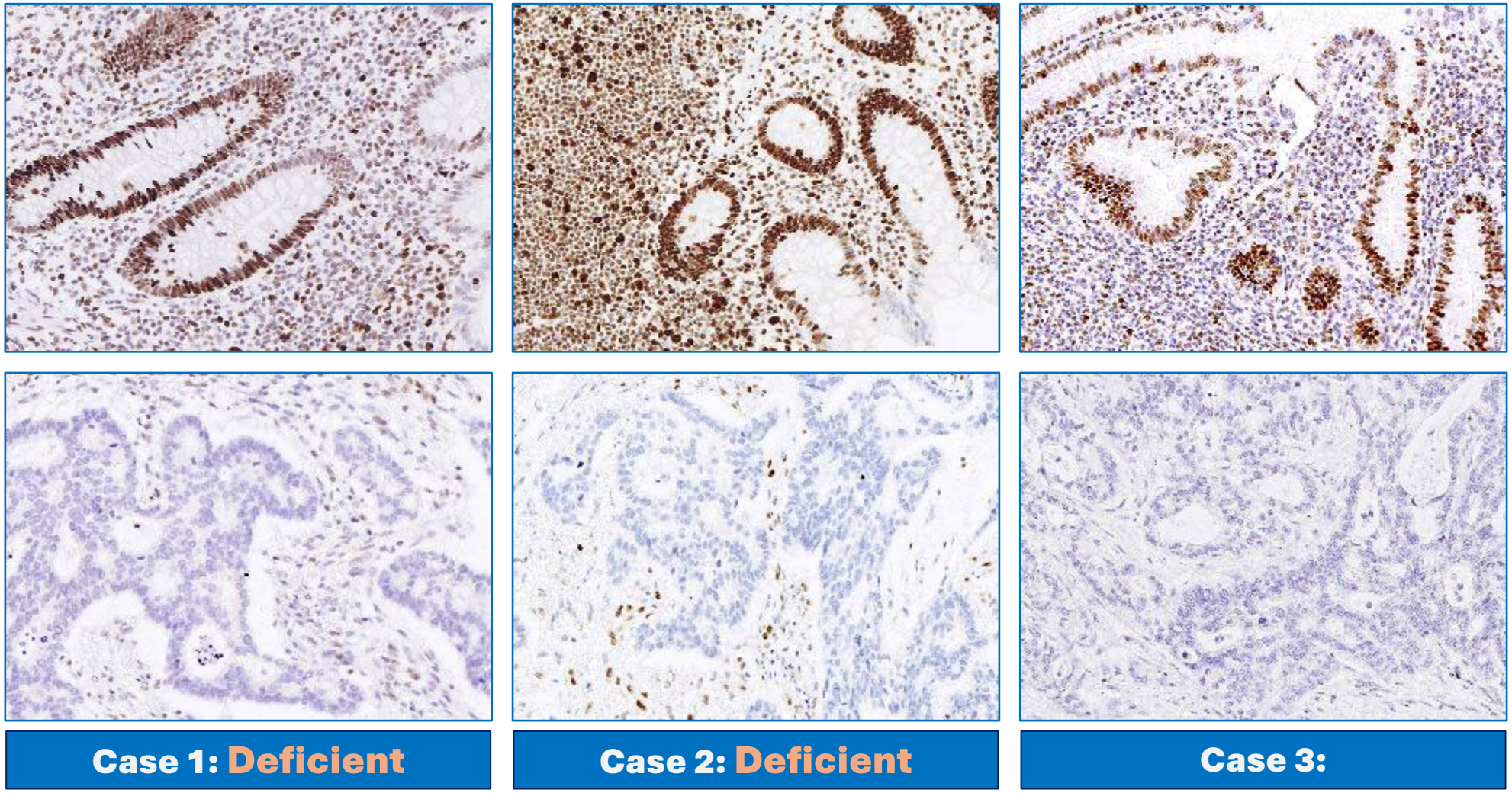


Case 1: Deficient	Case 2:	Case 3:

Diagnostic Screening Tests

UK NEQAS
Immunocytochemistry & In-Situ Hybridisation

Diagnostic screening in a patient who already has a primary diagnosis e.g., mismatch repair protein deficiency in colorectal carcinoma.

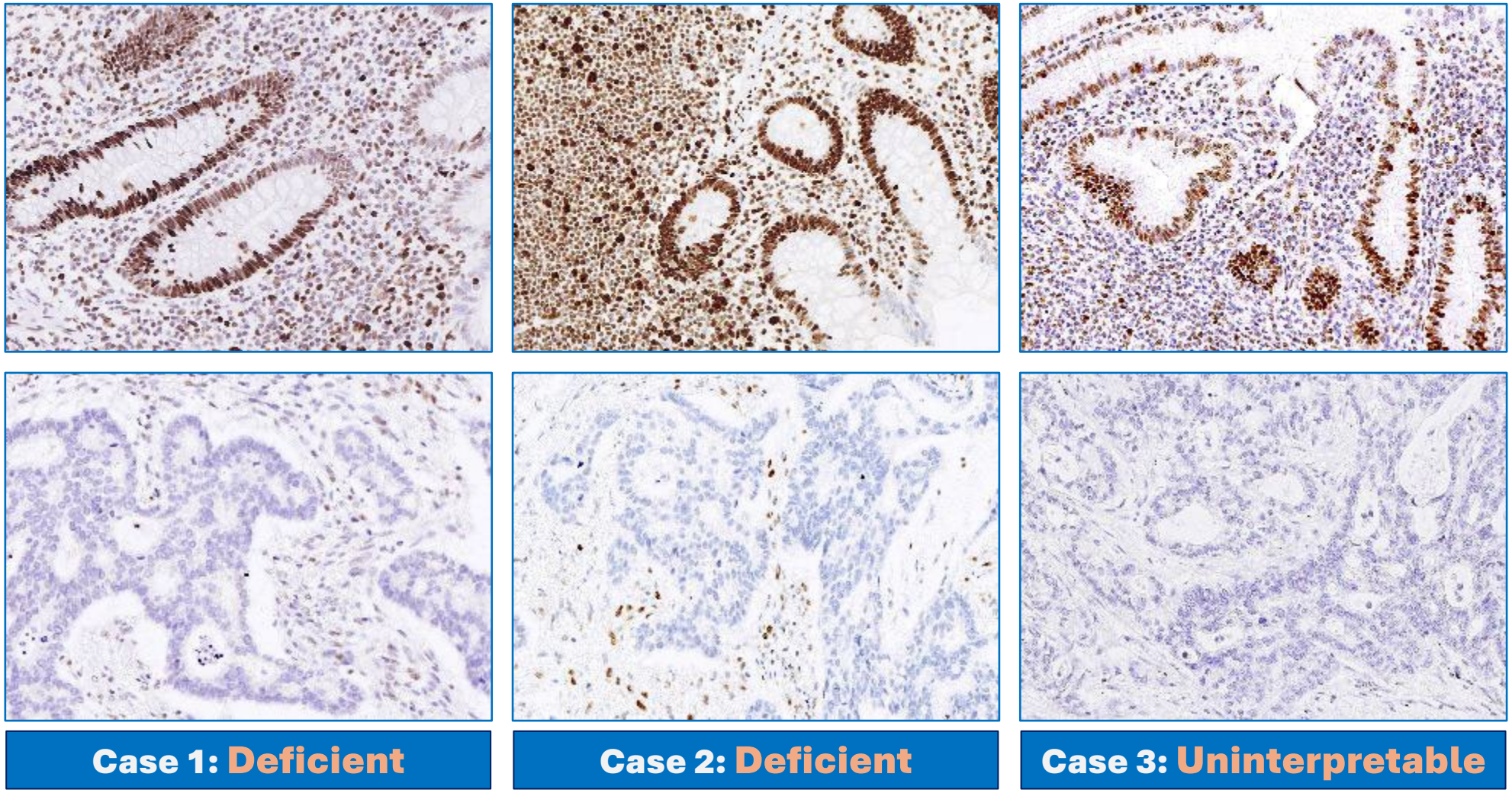


Case 1: Deficient	Case 2: Deficient	Case 3:

Diagnostic Screening Tests

UK NEQAS
Immunocytochemistry & In-Situ Hybridisation

Diagnostic screening in a patient who already has a primary diagnosis e.g., mismatch repair protein deficiency in colorectal carcinoma.



Case 1: Deficient	Case 2: Deficient	Case 3: Uninterpretable

Thank You

Acknowledgements

- All the UK NEQAS ICC & ISH staff
- Sponsors
- Participants