Nordic immunohistochemical Quality Control

NordiQC

Workshop in Diagnostic Immunohistochemistry
Aalborg University Hospital, October 2023

Technical aspects of immunohistochemistry & pitfalls
Pre-Analytical - Analytical (I & II) - Post Analytical phase

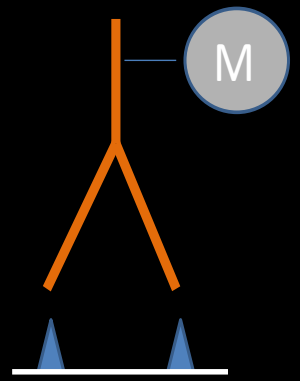
Michael Bzorek

Histotechnologist

Department of Surgical Pathology

University Hospital, Region Zealand, Denmark

Technical aspects of immunohistochemistry & pitfalls - Analytical phase



Immunohistochemistry – A simple technique ?

NordiQC

Info Modules Assessments Protocols Controls Events Login

26-Apr-2024

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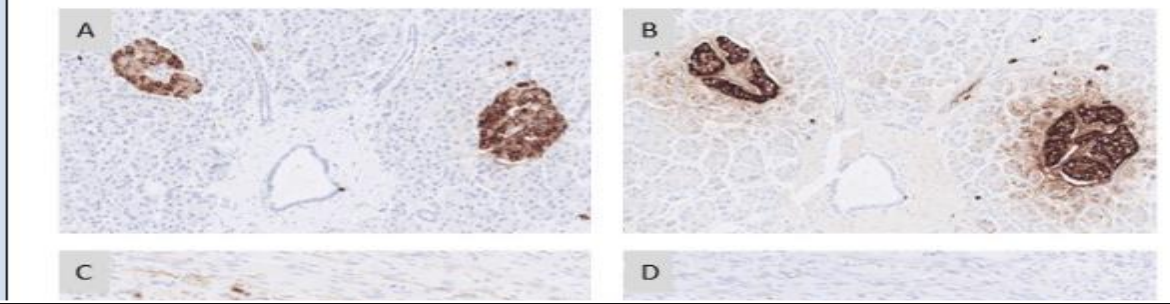
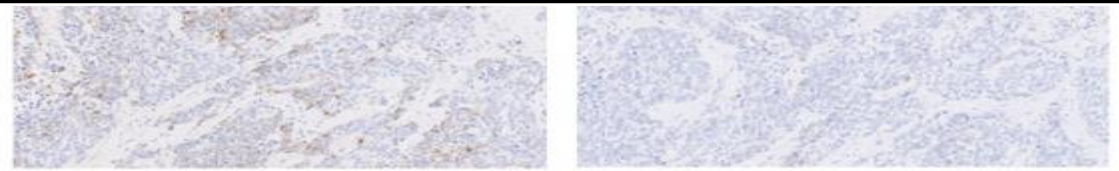
Results - Run 70, B37 and H25



External Quality Assurance programs

Staining quality varies significantly between different laboratories depending on the individual selection of methods



IHC for CGA in two laboratories:
Lab 1 (A+C+E): Optimal results in pancreas (A), appendix (C) and SCLC (E)
In the pancreas vast majority of endocrine islet cells show a strong and distinct cytoplasmic staining reaction. Normal ganglion cells and axons in the nerve plexus give a moderate and distinct, granular cytoplasmic staining reaction. The majority of neoplastic cells in the SCLC show a weak to moderate dot-like accentuation.
Lab 2 (B+D+F): Insufficient result due to inefficient HIER. In the pancreas the expected intense staining reaction is obtained, whereas both a reduced proportion, too weak staining and false negative staining reaction is seen in appendix and the SCLC.

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

The total test paradigm:
Key elements in the IHC procedure

The analytic phase :

Begins with dewax of the cut slides and is completed with the coverslipping of the stained slides.

Unlike the pre-analytic factors, analytic parameters can easily be modified and controlled within the immunohistological laboratory.

Pre-analytic phase

Pre-fixation
Fixation
Post-Fixation/Decalcification
Processing
Dehydration & clearing
Paraffin embedding
Sectioning
Drying/Storage

Analytic phase

Platform (manual/ Automated)
Epitope retrieval
Blocking
Primary Antibody
Detection system
Chromogen
Counterstain
Mounting

Post-analytic phase

Design of controls
Critical stain indicators
Internal/External control
Interpretation
Positive/Negative
Localization
Quantification
Cutt-of levels
Reporting

Technical aspects of IHC and pitfalls – Analytical phase

Optimization of the IHC assay – issues to be addressed

- Purpose and/or “fit-for-purpose” (assay validated for intended use)
- How to establish “best practice protocol” of the IHC test (Calibration of the IHC assay with focus clone, antigen retrieval, titer & detection system)
  - Is the IHC test reproducible/robust (pre-analytic conditions)
  - Evaluation of the analytical sensitivity and specificity
- Identification of most robust controls providing information that the established level of detection is obtained in each test performed in daily practice.

Tissue materials are essential for all these processes (calibration, validation and controls)

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Immunohistochemistry: Calibration of a biomarker/antibody depend on the type of marker and purpose of the test

IHC-type 1 markers (Diagnostic)

Often calibrated to produced appropriate level of sensitivity and specificity (positive versus negative)

IHC-type 2 markers (Disease screening, predictive treatment & prognosis)

Predictive markers: The assays are calibrated to provide information of which patients will or will not benefit from a specific treatment (HER-2, PD-L1 …….)

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Purpose and Intended use

Melan A (MLA)/MART-1
IHC-Type 1 marker

Malignant melanoma → **Optimal results (NQC)**

mAb A103
mAb BS52
mAb M2-7C10
rmAb EP43
……

Steroid producing tumors (e.g., Granulosa cell tumor of the ovary) → **Optimal results (NQC)**

mAb A103

mAb clone A103, cross-react with an unknown protein in steroid hormone producing cells and tumours.

Table 1. **Antibodies and assessment marks for MLA, Run 56**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **A103**	69 19 5 1 1 1 1	Dako/Agilent Novocastra/Leica Cell Marque Diagnostic BioSystems Immunologic Monosan Thermo Scientific	22	43	24	8	67%	73%
mAb clone **BS52**	1	Nordic Biosite	1	0	0	0	-	-
mAb clone cocktail **HMB45+MC-7310 +M2-9E3+T311**	3	Biocare	1	2	0	0	-	-
mAb clone cocktail **M2-7C10+M2-9E3**	1	NeoMarkers	0	1	0	0	-	-
mAb clone **M2-7C10**	1	Zytomed	1	0	0	0	-	-
rmAb **EP43**	5 3 3	Nordic Biotite Cell Marque Epitomics	9	1	1	0	91%	100%
Ready-To-Use antibodies								
mAb clone **A103 790-2990**	87	Ventana/Roche	8	31	45	3	45%	66%
mAb clone **A103, IR633/IS633**	26	Dako/Agilent	10	14	2	0	92%	95%
mAb clone **A103, IR633/IS633** [3]	29	Dako/Agilent	9	12	8	0	72%	-
mAb clone **A103, IR633/IS633** [4]	9	Dako/Agilent	1	2	5	1	-	-
mAb clone **A103, PA0233**	9	Novocastra/Leica	1	8	0	0	-	-
mAb clone **A103, PA0233** [5]	1	Novocastra/Leica	0	0	1	0	-	-
mAb clone **A103, 281M-87/281M-88**	3	Cell Marque	1	0	2	0	-	-
mAb clone **A103, API3114**	1	Biocare	0	1	0	0	-	-
mAb clone **A103, MAB-0275**	1	Maixin	1	0	0	0	-	-
mAb clone cocktail **HMB45+A103+T311, 904H-08**	1	CellMarque	1	0	0	0	-	-
mAb clone cocktail **HMB45+A103+T311, 790-4677**	1	Ventana/Roche	0	1	0	0	-	-
rmAb clone **EP43, MAD-000695QD-7/N**	2	Master Diagnostica	0	1	1	0	-	-
rmAb clone **EP43, 8319-C010**	2	Sakura Finetek	1	1	0	0	-	-
Total	286		67	118	89	12	-	
Proportion			24%	41%	31%	4%	65%	

1) Proportion of sufficient stains (optimal or good)
2) Proportion of sufficient stains with optimal protocol settings only, see below.
3) RTU system developed for the Dako/Agilent semi-automatic system (Dako Autostainer), but used by laboratories on the Dako fully-automatic platform (Dako Omins)
4) RTU system developed for the Dako/Agilent semi-automatic system (Dako Autostainer), but used by laboratories on different platforms (e.g. Ventana BenchMark)
5) RTU system developed for the Leica Bond system, but used on the Ventana BenchMark system.

Purpose and Intended use

Material
The slide to be stained for MLA comprised:

1. Skin, 2. Kidney, 3. Adrenal gland, 4-5. Malignant melanoma, 6. Granulosa cell tumour.

All tissues were fixed in 10% neutral buffered formalin.

Melan A clone A103 (Run 56)

Melanoma/melanocyte assessment: 62% Sufficient/ 20% Optimal

Optimal results: Efficient HIER (alkaline buffer), high conc. of the primary Ab (app. 1:10-50) and a sensitive detection system (3-step)

Melanoma/melanocyte + steroid hormone assessment): Suff. 29%

Sufficient steroid hormone related A103 (cross)reaction can be very difficult to obtain on e.g., the Ventana BenchMark and Dako Omnis platforms.

Melan A (MLA) / MART-1:

263 participants ~ 92% used clone A103 as single reagent (no cocktails)

Table 1. **Antibodies and assessment marks for MLA, Run 60**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **A103**	57 19 6 1 1 1 1 1	Dako/Agilent Novocastra/Leica Cell Marque Abcam Biocare Monosan Biogenex Zeta Corporation	21	57	9	0	90%	24%
mAb clone **BS52**	3	Nordic Biosite	3	0	0	0	-	-
mAb clone **M2-7C10**	1	Zytomed	1	0	0	0	-	-
mAb clone cocktail **HMB45+MC-7310 +M2-9E3+T311**	3	Biocare	2	1	0	0	-	-
mAb clone cocktail **HMB45+A103+T311**	1	Diagnostic Biosystems	0	0	0	1	-	-
mAb clone cocktail **M2-7C10+M2-9E3**	1	Thermo F. Scientific	1	0	0	0	-	-
rmAb clone **EP1442Y**	1	Abcam	1	0	0	0	-	-
rmAb clone **EP43**	9 9 1	Nordic Biotite Epitomics Cell Marque	18	1	0	0	100%	95%
Ready-To-Use antibodies								
mAb clone **A103 790-2990**[3]	3	Ventana/Roche	0	0	3	0	-	-
mAb clone **A103 790-2990**[4]	94	Ventana/Roche	6	74	11	3	85%	6%
mAb clone **A103, IR633/IS633**[3]	14	Dako/Agilent	1	13	0	0	100%	7%
mAb clone **A103, IR633/IS633**[4]	56	Dako/Agilent	12	36	7	1	85%	21%
mAb clone **A103, PA0233/PA0044**[3]	7	Leica Biosystems	2	5	0	0	100%	29%
mAb clone **A103, PA0233/PA0044**[4]	10	Leica Biosystems	6	4	0	0	100%	60%
mAb clone **A103, 281M-87/281M-88**	1	Cell Marque	1	0	0	0	-	-
mAb clone **A103, API3114**	1	Biocare	0	0	1	0	-	-
mAb clone **M2-7C10, 281M-97/281M-98**	2	Cell Marque	1	1	0	0	-	-
mAb clone cocktail **HMB45+A103+T311, 904H-08**	1	Cell Marque	0	0	1	0	-	-
mAb clone cocktail **HMB45+A103+T311, 790-4677**	1	Ventana/Roche	0	1	0	0	-	-
mAb clone cocktail **HMB45+MC-7310 +M2-9E3+T311, PM165**	1	Biocare	0	1	0	0	-	-
rmAb clone **BP6086, I1064**	1	Tuling Biotechnology	0	0	1	0		
rmAb clone **EP43, MAD-000695QD-7/N**	1	Master Diagnostica	1	0	0	0	-	-
rmAb clone **EP43, 8319-C010**	3	Sakura Finetek	3	0	0	0	-	-
Total	312		80	194	33	5	-	
Proportion			26%	62%	11%	1%	88%	

1) Proportion of sufficient results (optimal or good). (≥5 asessed protocols).
2) Proportion of Optimal Results (OR).
3) Vendor Recommended Protocol Settings (VRPS) to a specific RTU product applied on the vendor recommended platform(s) (
asessed protocols).
4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product applied either on the vendor recommended platform(
validated semi/fully automatic systems or used manually (≥5 asessed protocols).

Purpose and Intended use

Material
The slide to be stained for MLA comprised:

1. Kidney, 2. Skin, 3-4. Malignant melanoma, 5. Colon Adenocarcinoma.

All tissues were fixed in 10% neutral buffered formalin.

Melan A/MART-1 (Run 60)

MLA, A103 (melanoma/melanocyte assessment): 87% Sufficient / 18% Optimal.

Optimal results: Efficient HIER (alkaline buffer), high conc. of the primary Ab (app. 1:10-50) and a sensitive detection system (3-step).

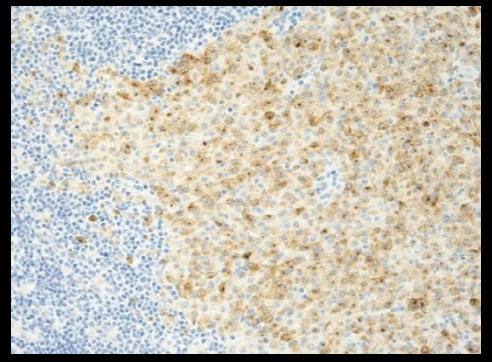
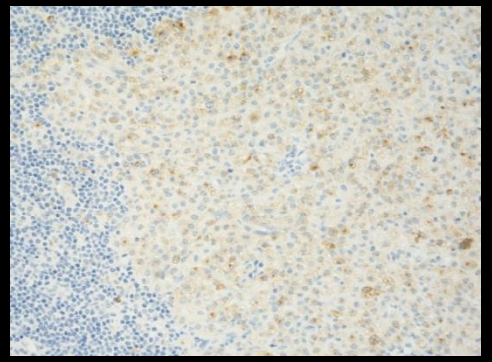
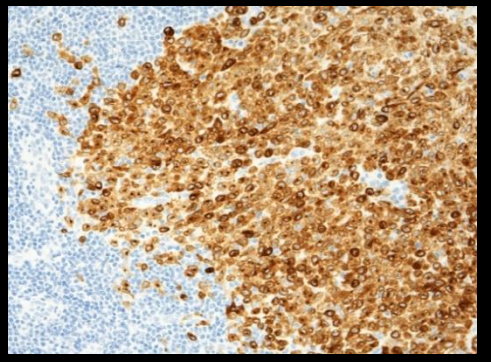
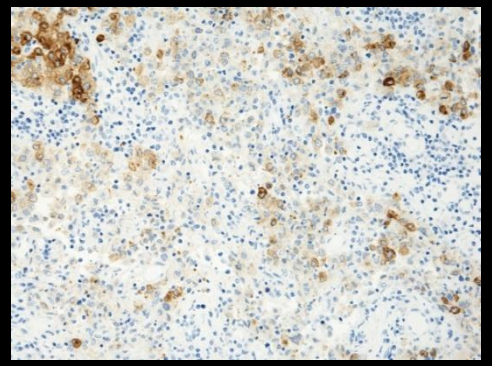
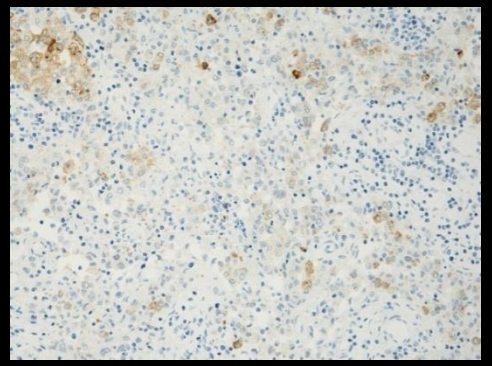
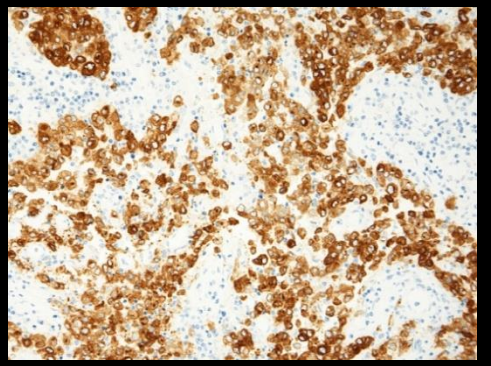
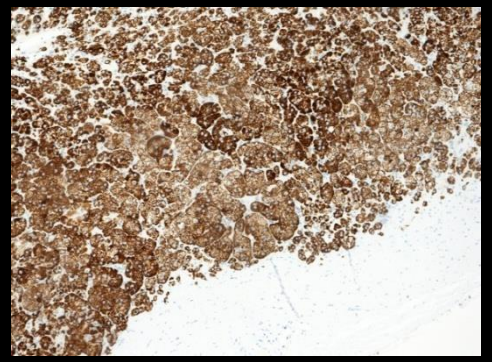
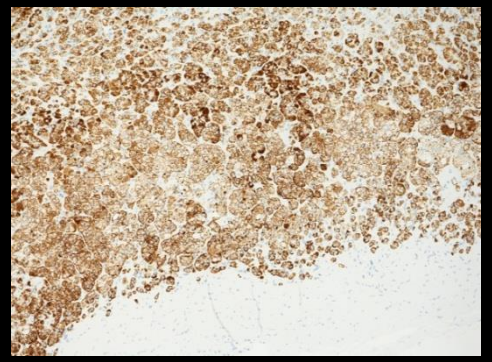
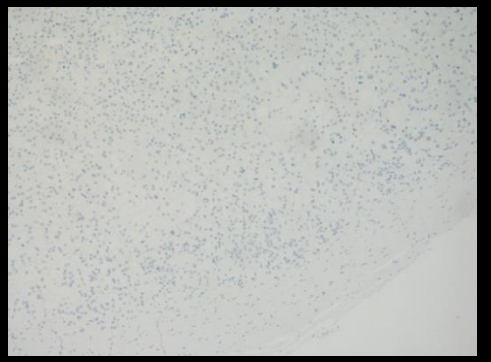
Performance of MLA clone A103 is sensitive to the chosen platform e.g., Omnis or Benchmark.

Melan A (MLA) / MART-1:
273 participants ~ 88% used clone A103 as single reagent (no cocktails)

Is MLA , A103 the best primary Ab for detection of melanomas ?

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Melan A /MART1

	MLA, A103 1:25 AutoStainer	MLA, A103 1:25 Omnis	MART1, EP43 1:30 Omnis
Melanoma (Sentinel node)			
Melanoma (Lymph node)			
Adrenal gland			

TRS High pH/Flex+ protocols

PRAME clone EPR20330 (Biocare Medical)

Purpose ?

Fit-for-purpose (validated for intended use)

PRAME: Purpose and "fit-for-purpose"

PReferentially expressed Antigen in MElanoma

Antibody: PRAME
Clone: EPR20330
Company/Supplier: Biocare M.
Date: 22-03-2023

Controls (High. Low and None expression):

HIGH: Malignant melanoma. The majortity of the neoplastic cells should display an at least moderate but distinct nuclear reaction.

LOW: Tonsil. Dispersed intragerminal centre lymphatic cells should show a weak to moderate and distinct nuclear staining reaction.

NONE: Appendix. Epithelial cells should be negative.

Remark: Subsets of lymphocytes/macrophages are positive in most of the tissue cores. In addition, smooth muscle cells might be positive in some cores. Only expression in normal epithelial or tumour cells were assessed.

Normal Tissue Study (TMA Normal)				
	Tissue	Stained (+)	Total #	Notes
1a	Liver	0	1	
1b	Spleen	0	1	
1c	Placenta	NA	NA	Tissue core
2a	Tonsil (5h)	0	1	Dispersed
2b	Tonsil (29h)	0	1	Dispersed
2c	Tonsil (173h)	0	1	Follicles ar
2d	Tonsil (27h)/EDTA (26h)	0	1	Dispersed
3a	Uterine ecto-cervix (120h)	0	1	
3b	Uterine cervix (120h)	0	1	
3c	Uterine endometrium	1	1	
3d	Breast	0	1	
4a	Colon	0	1	
4b	Pancreas	0	1	
4c	Salivary Gland	0	1	
4d	Placenta	0	1	
5a	Kidney	1	1	Few po
5b	Adrenal gland	0	1	
5c	Prostate	1	1	
5d	Testis	0	1	
6a	Thyroid	0	1	
6b	Striated muscle	0	1	
6c	Cerebellum (240h)	0	1	Norr
6d	Skin			

General tumour Study (TMA Tumour)				
	Tissue	Stained (+)	Total #	Notes
1f	Liver	0	1	
2a	Lung SCC	1	1	Weak reactions.
2b	Lung Adenocarcinoma (Skin)	1	1	Faint to weak reactions.
2c	Lung Adenocarcinoma (Small intestine)	1	1	Faint to weak reactions.
2d	Breast carcinoma (Lobular)	0	1	
2e-f	Breast carcinomas (Ductal)	1	2	Faint reaction in dispersed cells
3a	Medullar carcinoma (Thyroid)	0	1	
3b	Follicular carcinoma (Thyroid)	0	1	
3c	Renal cell carcinoma	0	1	
3d	Clear cell carcinoma (Kidney)	0	1	
3e-f	Colon adenocarcinoma	0	2	
4a	Carcino-Sarcoma (Uterus)	1	1	Moderate to intense nuclear staining reaction of neoplastic cells.
4b	Endometroid Adc. (Uterus)			Not assessed due to lack of tissue core.
4c	Endometroid Ad...	NA		Faint and weak reaction in few dispersed neoplastic cells.
				Intense nuclear staining reaction of neoplastic cells.
				Weak to moderate intensity.
				Weak staining intensity.
				Weak to strong intensity.
				Faint staining reactions.
				0
				Core 6d (weak intensity) and core 6f (strong intensity). Core 6e not assessed (lacking).
				not assessed due to lack of tumour cells. Dispersed germinal centre lymphatic cells are positive of normal follicles.

Purpose: Discriminate malignant from benign melanocytic lesions

Specific Disease Tissue Study				
Tissue	# Stained (+)	# Stained (+) >76%[1]	Total #	Notes
Nevus	3	0	10	Focal and/or dispersed cells
Nevus atypical/dysplastic nevus	2	1	10	Both positive cases with focal staining pattern.
Melanoma In Situ	10	9	10	In two difficult cases, PRAME was positive in dispersed melanocytic cells in one case and in the second case, displayed focal but diffuse reaction pattern, and thus regarded as positive using the criteria of >76% positive neoplastic cells.
Melanomas	16	16	17	4/16 (weak reaction of >76% of the neoplastic cells). The cores 2c and 4c are without neoplastic cells, and thus, not assessed (NA).

Rare isolated junctional melanocytes with immunoreactivity for PRAME were also seen in benign non-lesional skin.

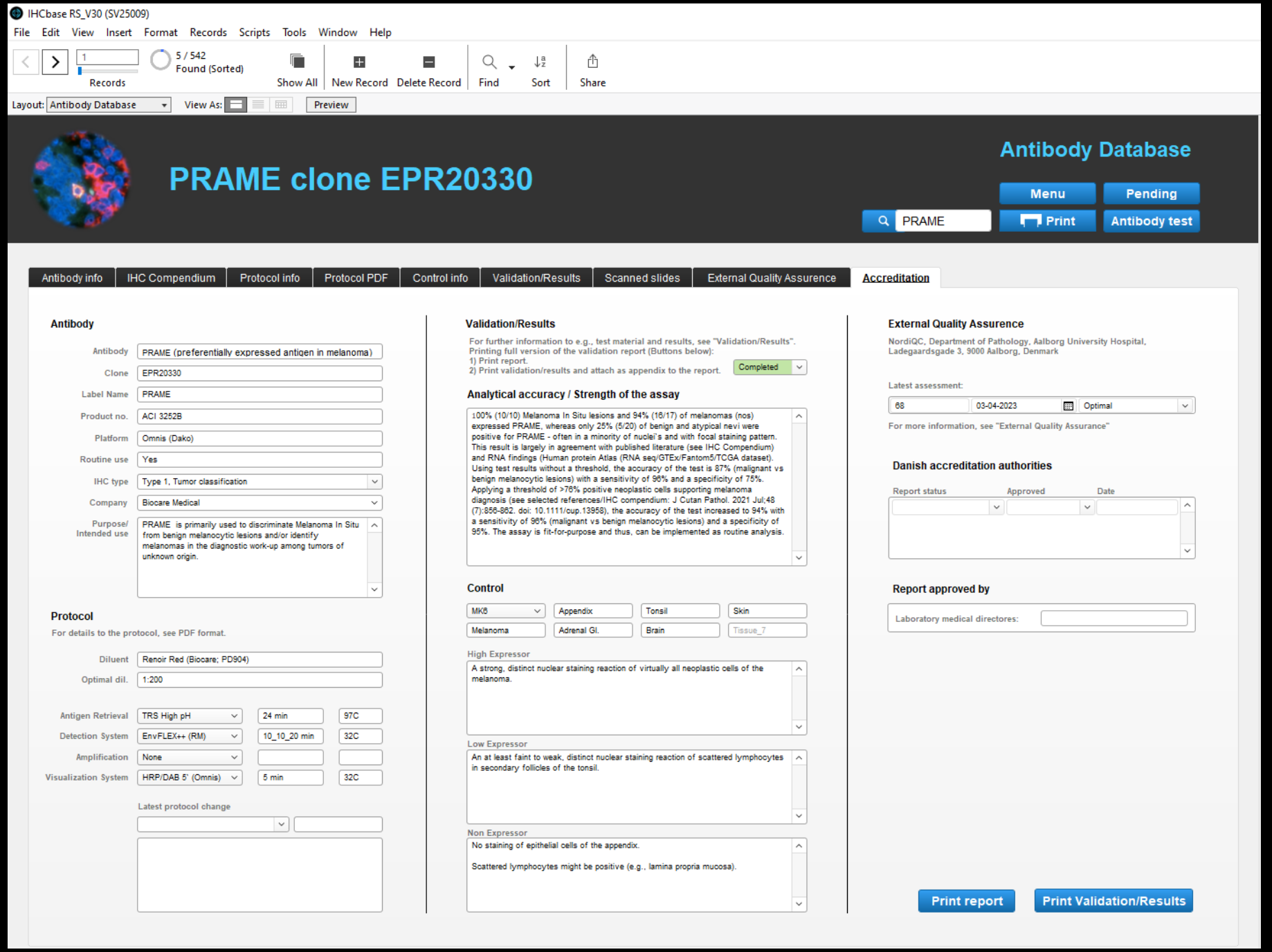
[1]Threshold according to literature supporting melanoma diagnosis (see selected references/IHC compendium: J Cutan Pathol. 2021 Jul;48(7):856-862. doi: 10.1111/cup.13958.)

Summery/Conclusion: 100% (10/10) Melanoma In Situ lesions and 94% (16/17) of melanomas (nos) expressed PRAME, whereas only 25% (5/20) of benign and atypical nevi were positive for PRAME - often in a minority of nuclei`s and with focal staining pattern. This result is largely in agreement with published literature (see IHC Compendium) and RNA findings (Human protein Atlas (RNA seq/GTEx/Fantom5/TCGA dataset). Using test results without a threshold, the accuracy of the test is 87% (malignant vs benign melanocytic lesions) with a sensitivity of 96% and a specificity of 75%. Applying a threshold of >76% positive neoplastic cells supporting melanoma diagnosis (see selected references/IHC compendium), the accuracy of the test increased to 94% with a sensitivity of 96% (malignant vs benign melanocytic lesions) and a specificity of 95%. The assay is fit-for-purpose and thus, can be implemented as routine analysis.

J Cutan Pathol. 2021 Jul;48(7):856-862

Best practice protocol

PRAME: Ready for accreditation



Technical aspects of IHC and pitfalls – Analytical phase

Optimization of the IHC assay – issues to be addressed

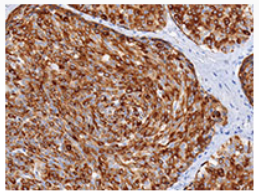
- Purpose and/or “fit-for-purpose” (assay validated for intended use)
- How to establish “best practice protocol” of the IHC test (Calibration of the IHC assay with focus clone, antigen retrieval, titer & detection system)
  - Is the IHC test reproducible/robust (pre-analytic conditions)
  - Evaluation of the analytical sensitivity and specificity
- Identification of most robust controls providing information that the established level of detection is obtained in each test performed in daily practice.

Tissue materials are essential for all these processes (calibration, validation and controls)

Calcitonin optimization (data sheets ?)

anti-Calcitonin (SP17), Rabbit Monoclonal Primary Antibody

Roche/ Ventana/ Cell Marque



Catalog Number:	760-4705
Ordering Code:	06586554001
Quantity:	50 tests
Controls:	Medullary Carcinoma of Thyroid
Isotypes:	IgG
Clone Name:	SP17
Species:	Rabbit
Localization:	Cytoplasmic
Regulatory Status:	IVD

This antibody is intended for in vitro diagnostic (IVD) use. Calcitonin (SP17) antibody is intended for qualified laboratories to qualitatively identify by light microscopy the presence of associated antigens in sections of formalin-fixed, para test methods. This antibody is used as an aid in the id diagnosis of thyroid medullary carcinoma within the c clinical history, and other diagnostic tests determined

Recommended staining protocol with *ultra*View	
Procedure Type	Method
Deparaffinization	Selected
Cell Conditioning (Antigen Unmasking)	Cell Conditioning 1, Mild
Enzyme (Protease)	Not required
Antibody (Primary)	BenchMark ULTRA instrument: 16 minutes, 36°C BenchMark XT instrument: 16 minutes, 37°C BenchMark GX instrument: 16 minutes, 37°C
	inutes

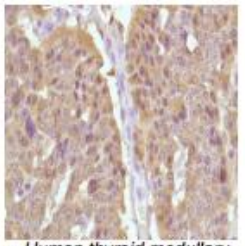
Optimizing an assay can be confusing

(Vendor recommendations)

SPRING BIOSCIENCE

Rabbit Anti-Human Calcitonin Monoclonal Antibody (Clone SP17)

Spring Cat#	Roche P/N	Product Description
M3170	05492769001	0.1 ml rabbit monoclonal antibody supplied as tissue culture supernatant in TBS/1% BSA buffer pH 7.5 with less than 0.1% sodium azide.
M3172	06970419001	0.5 ml rabbit monoclonal antibody supplied as tissue culture supernatant in TBS/1% BSA buffer pH 7.5 with less than 0.1% sodium azide.
M3174	05298725001	1.0 ml rabbit monoclonal antibody supplied as tissue culture supernatant in TBS/1% BSA buffer pH 7.5 with less than 0.1% sodium azide.
M3171	05298717001	7.0 ml pre-diluted rabbit monoclonal antibody supplied as tissue culture supernatant in TBS/1% BSA buffer pH 7.6 with less than 0.1% sodium azide. (For manual IHC only)



Human thyroid medullary carcinoma stained with anti-calcitonin antibody

INTENDED USE: For Research Use Only. Not for use in diagnostic procedures.

CLONE: SP17

IMMUNOGEN: Synthetic human calcitonin 1-32 amino acid peptide.

IG ISOTYPE: Rabbbit IgG

EPITOPE: Not determined

MOLECULAR WEIGHT 15kDa

SPECIES REACTIVITY: Human (tested). (See www.springbio.com for information on species reactivity predicted by sequence homology.)

DESCRIPTION: Calcitonin is a 32 amino acid peptide which can be demonstrated in C cells of the normal and hyperplastic thyroid. Staining for calcitonin may be used for the identification of a spectrum of C cell proliferative abnormalities ranging from C cell hyperplasia to invasive tumors. Staining for calcitonin in medullary carcinoma of the thyroid produces a fine granular pattern in the cytoplasm. Amyloid deposits within the tumor may also exhibit varying degrees of calcitonin activity.

APPLICATIONS: Immunohistochemistry (IHC)

IHC PROCEDURE:
Specimen Preparation: Formalin-fixed, paraffin-embedded tissues are suitable for use with this primary antibody.
Deparaffinization: Deparaffinize slides using xylene or xylene alternative and graded alcohols.
Antibody Dilution: If using the concentrate format of this product, dilute the antibody 1:100 in Antibody Diluent (Cat# ADS-125). The dilutions are estimates; actual results may differ because of variability in methods and protocols.
Antigen Retrieval: None
Primary Antibody Incubation: Incubate for 30 minutes at room temperature.
Slide Washing: Slides must be washed in between steps. Rinse slides with PBS/0.05% Tween.
Detection: Detect the antibody as instructed by the instructions provided with the detection system.

POSITIVE CONTROL: Thyroid medullary carcinoma

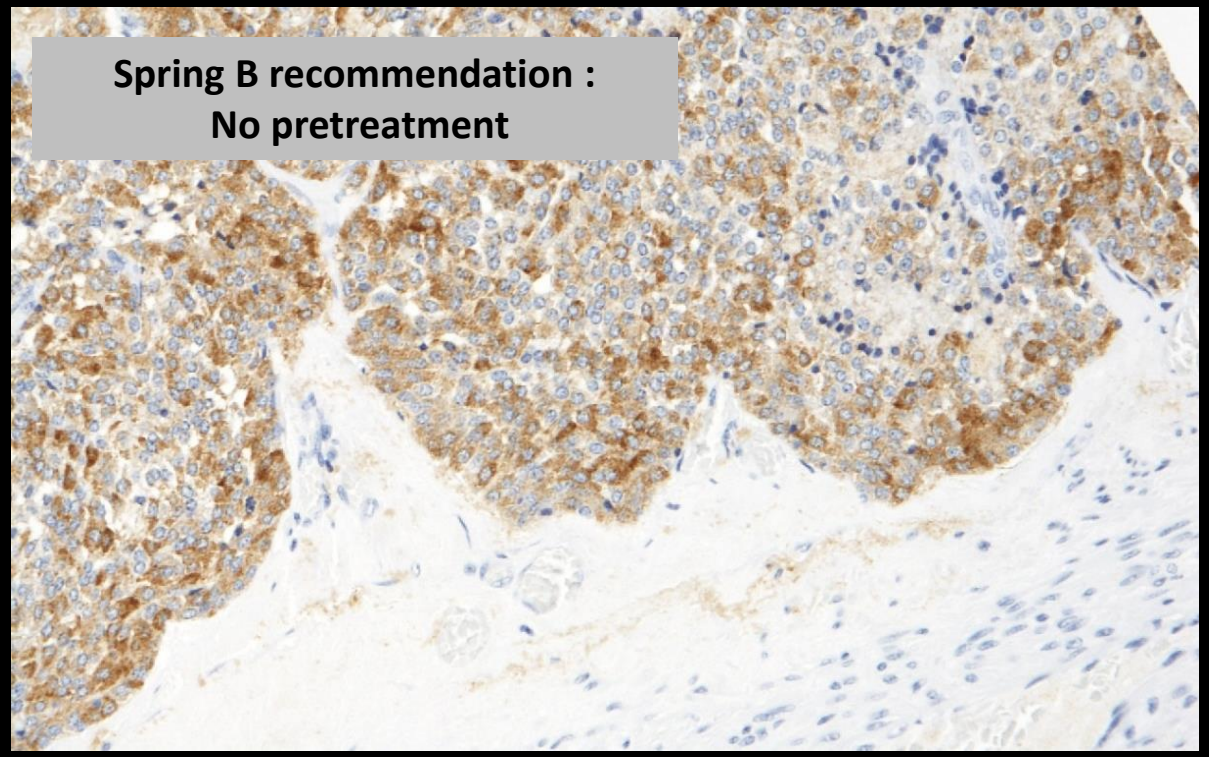
CELLULAR LOCALIZATION: Cytoplasm

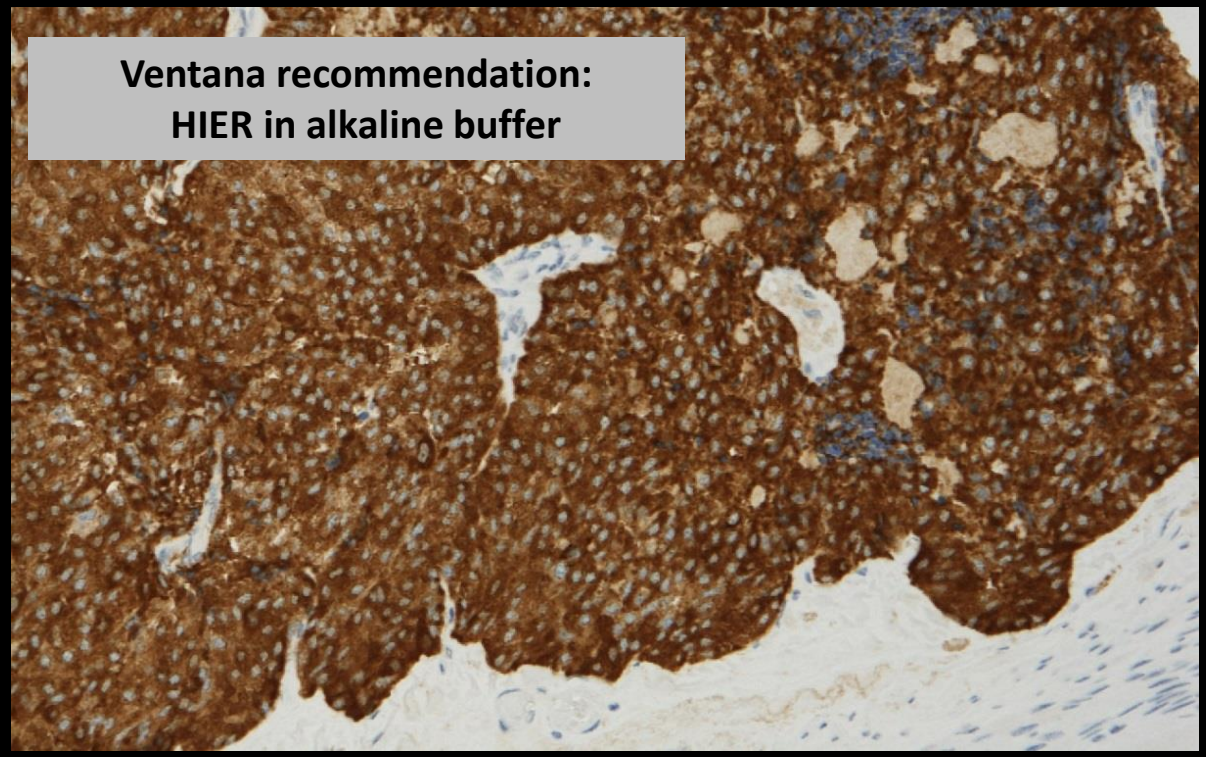
Can we use the recommendations provided by the manufactures spec sheets?

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

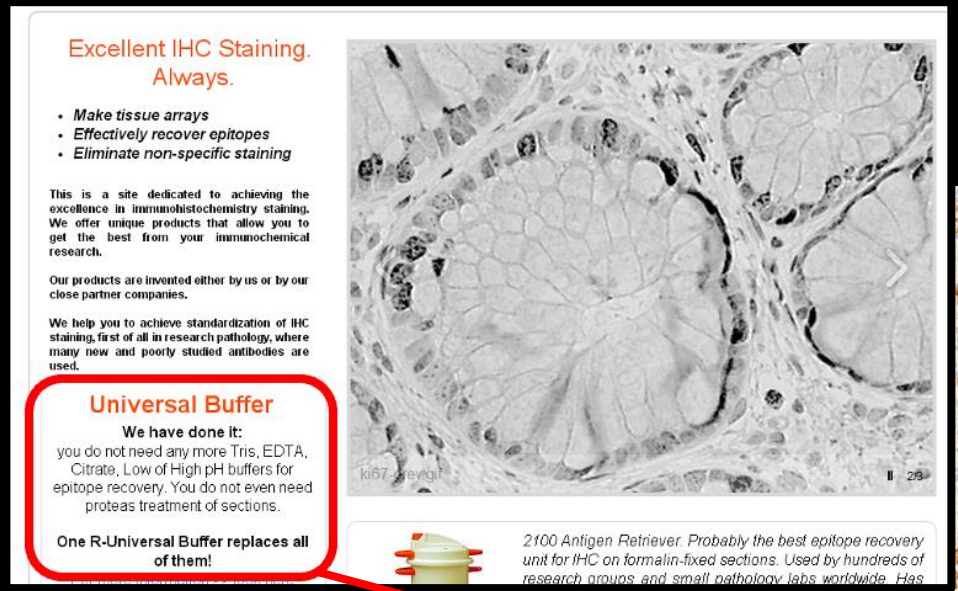
Calcitonin: Vendor recommendations - spec sheets ?

Thyroid medullary carcinoma



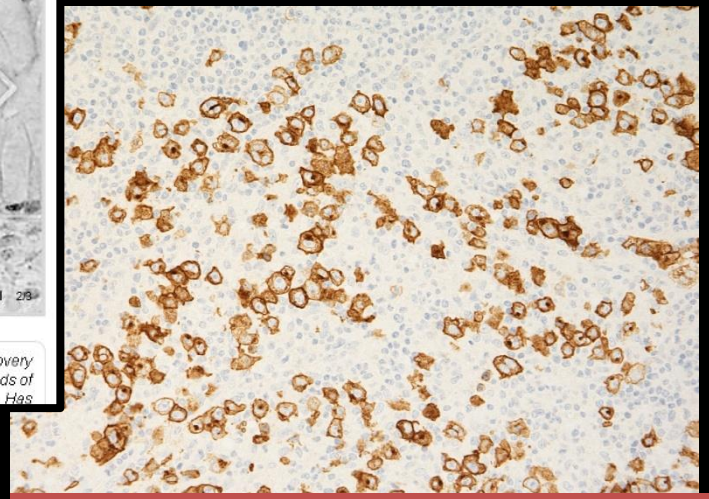


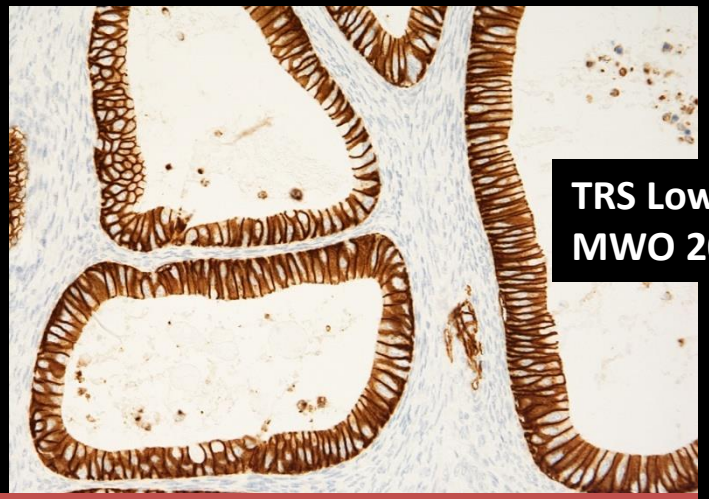
Calcitonin, SP17 (1:800), Omnis, Flex+



CD30, ConD6/D5 (1:50)

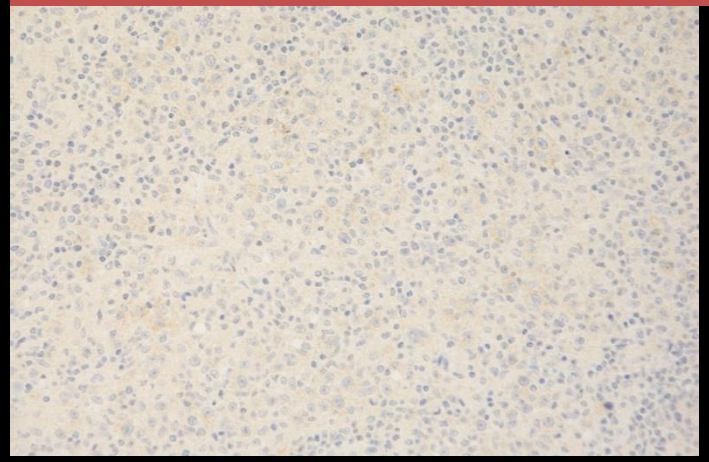
EP-CAM, MOC-31 (1:50)

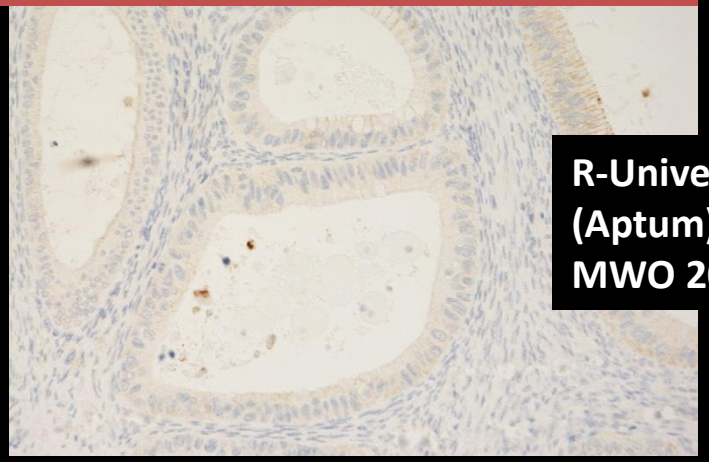




TRS Low (Dako)
MWO 20`

Be critical – Do your own tests





R-Universal buffer
(Aptum)
MWO 20`

Hodgkin Lymphoma

Uterus / Endometrium

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Optimizing IHC test (IHC-type 1 markers /conc. formats) - parameters to consider

Use a “Test Battery Approach” (TBA) (involves different pre-treatment regimes and dilution ranges)

Test more than one antibody clone against an antigen of interest before implementation for routine purpose

Test with robust, specific & sensitive detection system

Test/validate on normal and tumor tissue material with broad spectrum of antigen densities (specificity/sensitivity)

Compare results with external quality assurance programs, literature or colleagues

No antibody should be acquired without the basic knowledge of its performance characteristics and expected expression pattern

Hadi Yaziji and Todd Barry – Adv Anat Pathol ● Vol. 13, Number 5, September 2006

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

The “test battery approach” should be tailored to the individual IHC instruments or manual set-ups

Omnis + Autostainer platforms

Antibody Performance Testing (“Test Battery approach”)

	Dil. 1	Dil.2	Dil.3
A	None	None	None
B	Enzyme (1) 5 min.	Enzyme (1) 5 min.	Enzyme (1) 5 min.
C	HIER TRS Low pH 6.1 (30`)	HIER TRS Low pH 6.1 (30`)	HIER TRS Low pH 6.1 (30`)
D	HIER TRS High pH 9.0 (24`)	HIER TRS High pH 9.0 (24`)	HIER TRS High pH 9.0 (24`)
E	TRS Low (20`) + Pep (12`) ✷	TRS Low (20`) + Pep (12`)	TRS Low (20`) + Pep (12`)
F	HIER TRS High pH 9.0 (30`)	HIER TRS High pH 9.0 (30`)	HIER TRS High pH 9.0 (30`)

✷ Combined pre-treatment using Cytology Pepsin solution (Zytovision, #ES-0002-50)

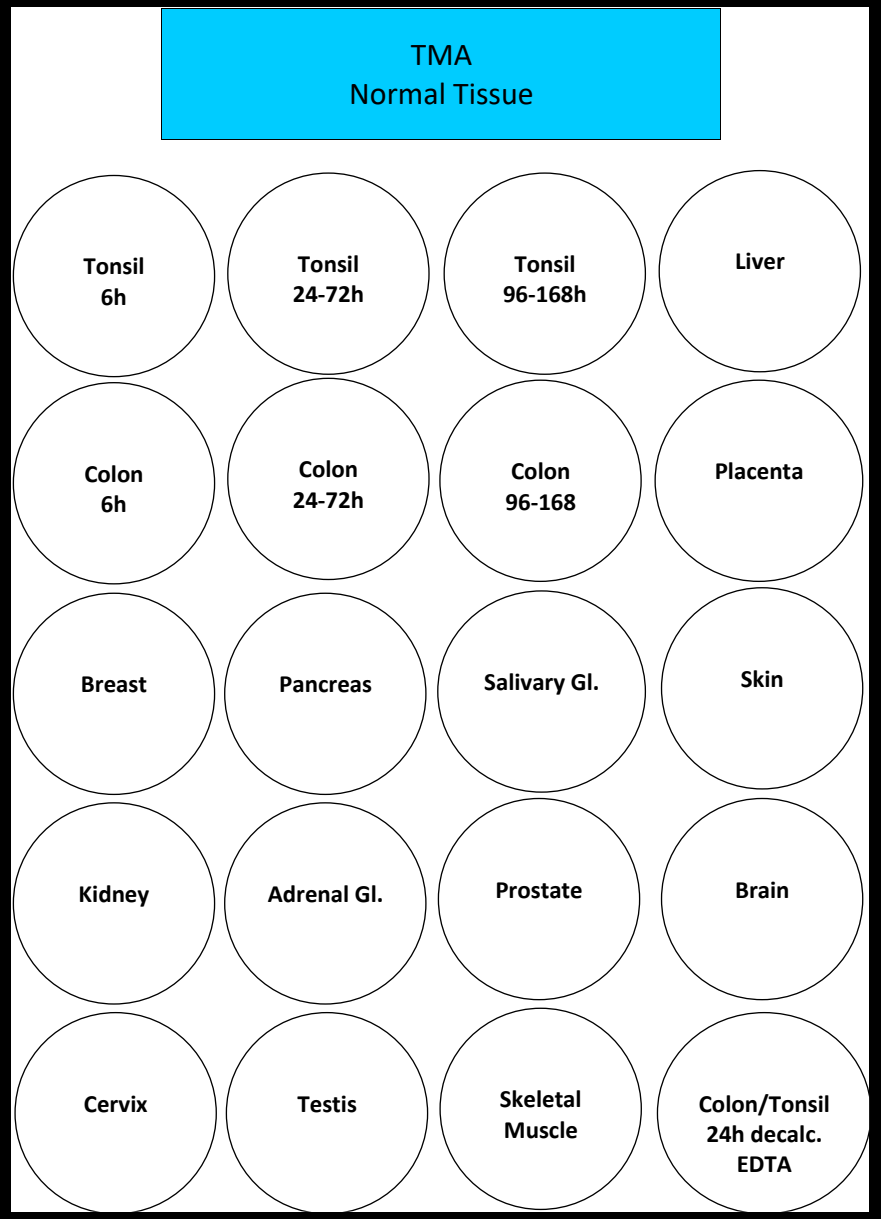
Omnis

Protocol A:	0.0 %
Protocol B:	2.0 %
Protocol C:	10.0 %
Protocol D:	84.0 %
Protocol E:	1.0 %
Protocol F:	3.0 %

Routine purpose : App 220 markers

Identify the protocol that discriminate between the desired (specific) positive staining and any unwanted (non-specific) background staining

Technical aspects of immunohistochemistry & pitfalls - Analytical phase



Protocol set-up: Evaluate analytic sensitivity and specificity

Normal tissue including fixation and decalcification controls

Identification of the best practice protocol: Test Battery Approach (clone, titer, retrieval etc.)

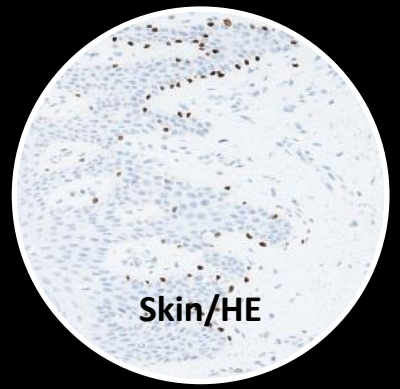
SOX10, BS7 (dil. range 1:50-800): HIER High pH 24`/ 1:350 (after re-test) / EnvFlex+

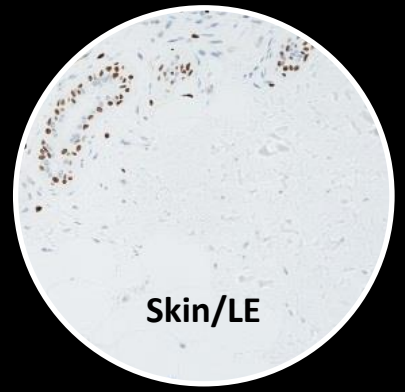
Establishing robustness of the IHC assay / pre-analytic parameter`s ?

SOX10, BS7; Robust to both fixation time in NBF and decalcification

Identification of robust controls

SOX10, BS7; IHC Critical Assay performance Controls (high, low and non exp.)





Diagnostic potential
Analytical validation

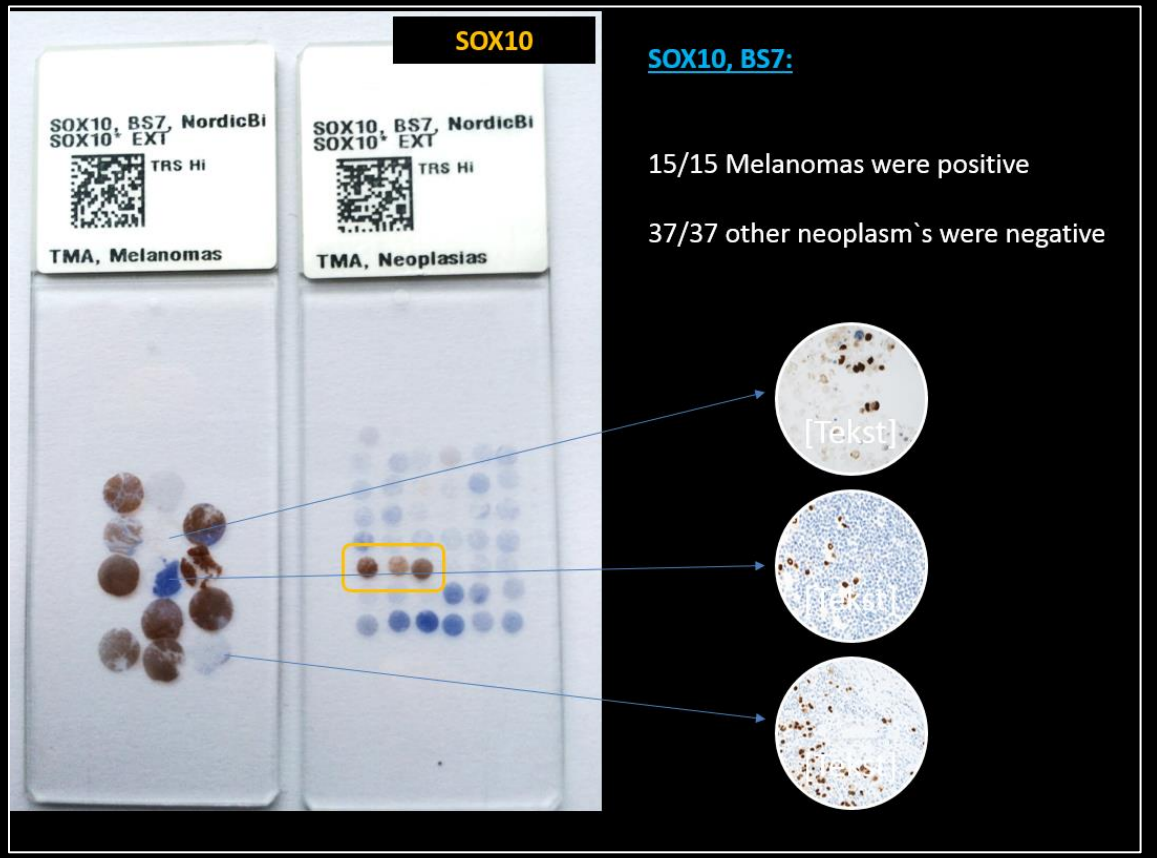
TMA
Malignant Melanomas

Melanoma 1 | Kidney
Melanoma 2 | Melanoma 3 | Melanoma 4
Melanoma 5 | Melanoma 6 | Melanoma 7
Melanoma 8 | Melanoma 9
Melanoma 10 | Melanoma 11 | Melanoma 12

TMA
Mixed tumors

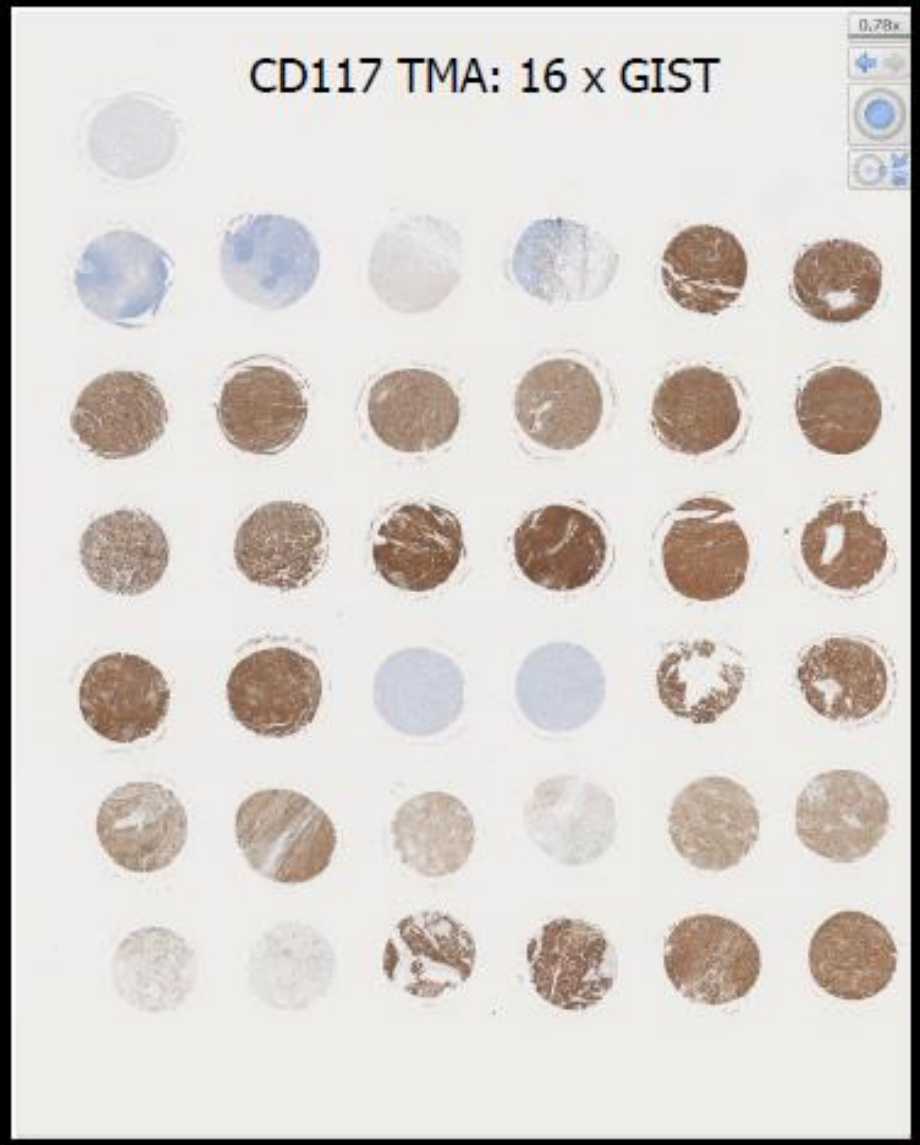
SOX10

Liver					
Mamma Ductal carcinoma	Mamma Ductal carcinoma	Mamma Lobular carcinoma	Lung Adeno carcinoma	Lung Adeno carcinoma	Lung Squam. carcinoma
Colon Adeno carcinoma	Colon Adeno carcinoma	Kidney Clear cell carcinoma	Kidney Clear cell carcinoma	Thyroid Follicular carcinoma	Thyroid Medullar carcinoma
Ovary Serous carcinoma	Ovary Serous carcinoma	Ovary Clear cell carcinoma	Ovary Endom. carcinoma	Ovary Endom. carcinoma	Uterus Carc. Sarcoma
Tonsil	Testis Seminoma	Testis Seminoma	Prostate Adeno carcinoma	Prostate Adeno carcinoma	Colon Carcinoid
Lymph n. Melanoma	Skin Melanoma	Lymph n. Melanoma	Pancreas Adeno carcinoma	Bladder Uroth. cell carcinoma	Bladder Uroth. cell carcinoma
Colon GIST	Uterus Leiomyo sacrcoma	Testis Lipo Sarcoma	Hodgkin L. Classic	Hodgkin L. Mixed	Diffuse L. B Cell lymphoma
Diffuse L. B Cell lymphoma	CLL	Follicular B Cell lymph.	Mantle Cell Lymph.	Pheriph. T Cell lymph.	Large C. Anaplastic T Cell lymph.



Other SOX10+ tumours: Schwannoma´s, neurofibroma`s, myoepithelial carcinomas, triple negative breast cancer……..?

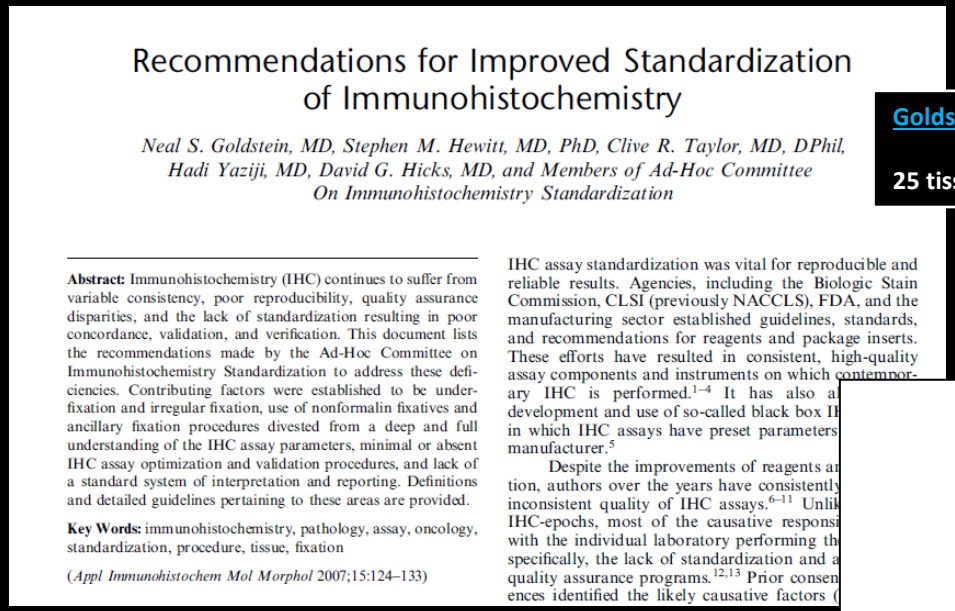
IHC – The Technical Test Approach



Courtesy by Søren Nielsen, Aalborg, DK (slide modified)

The technical test approach – Analytical phase

Analytical Validation - Evaluation of sensitivity and specificity



Recommendations for Improved Standardization of Immunohistochemistry

Neal S. Goldstein, MD, Stephen M. Hewitt, MD, PhD, Clive R. Taylor, MD, DPhil, Hadi Yaziji, MD, David G. Hicks, MD, and Members of Ad-Hoc Committee On Immunohistochemistry Standardization

Abstract: Immunohistochemistry (IHC) continues to suffer from variable consistency, poor reproducibility, quality assurance disparities, and the lack of standardization resulting in poor concordance, validation, and verification. This document lists the recommendations made by the Ad-Hoc Committee on Immunohistochemistry Standardization to address these deficiencies. Contributing factors were established to be underfixation and irregular fixation, use of nonformalin fixatives and ancillary fixation procedures divested from a deep and full understanding of the IHC assay parameters, minimal or absent IHC assay optimization and validation procedures, and lack of a standard system of interpretation and reporting. Definitions and detailed guidelines pertaining to these areas are provided.

Key Words: immunohistochemistry, pathology, assay, oncology, standardization, procedure, tissue, fixation

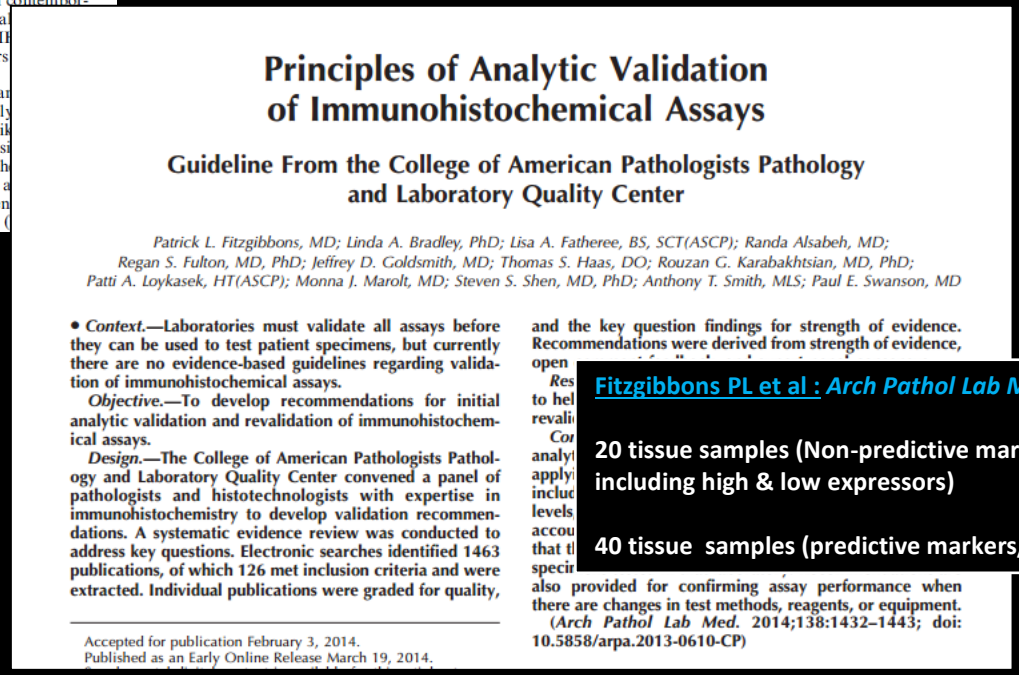
(*Appl Immunohistochem Mol Morphol* 2007;15:124–133)

IHC assay standardization was vital for reproducible and reliable results. Agencies, including the Biologic Stain Commission, CLSI (previously NACCLS), FDA, and the manufacturing sector established guidelines, standards, and recommendations for reagents and package inserts. These efforts have resulted in consistent, high-quality assay components and instruments on which contemporary IHC is performed.[1–4] It has also a
development and use of so-called black box I
in which IHC assays have preset parameters
manufacturer.[5]

Despite the improvements of reagents a
tion, authors over the years have consistently
inconsistent quality of IHC assays.[6–11] Unlik
IHC-epochs, most of the causative responsi
with the individual laboratory performing th
specifically, the lack of standardization and a
quality assurance programs.[12,13] Prior consen
ences identified the likely causative factors (

[Goldstein NS et al :](#) *Appl Immunohistochem Mol Morphol 2007 Mar; 15 : 124-133*

25 tissue samples (Non-predictive markers/ IHC-type I: 10 high, 10 low and 5 non-expressors)



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; Regan S. Fulton, MD, PhD; Jeffrey D. Goldsmith, MD; Thomas S. Haas, DO; Rouzan G. Karabakhtsian, MD, PhD; Patti A. Loykasek, HT(ASCP); Monna J. Marolt, MD; Steven S. Shen, MD, PhD; Anthony T. Smith, MLS; Paul E. Swanson, MD

• *Context.*—Laboratories must validate all assays before they can be used to test patient specimens, but currently there are no evidence-based guidelines regarding validation of immunohistochemical assays.

Objective.—To develop recommendations for initial analytic validation and revalidation of immunohistochemical assays.

Design.—The College of American Pathologists Pathology and Laboratory Quality Center convened a panel of pathologists and histotechnologists with expertise in immunohistochemistry to develop validation recommendations. A systematic evidence review was conducted to address key questions. Electronic searches identified 1463 publications, of which 126 met inclusion criteria and were extracted. Individual publications were graded for quality,

Accepted for publication February 3, 2014.
Published as an Early Online Release March 19, 2014.

and the key question findings for strength of evidence. Recommendations were derived from strength of evidence,
open
Res
to hel
revali
Cor
analyt
applyi
includ
levels
accou
that t
specir
also provided for confirming assay performance when there are changes in test methods, reagents, or equipment.
(*Arch Pathol Lab Med.* 2014;138:1432–1443; doi: 10.5858/arpa.2013-0610-CP)

[Fitzgibbons PL et al :](#) *Arch Pathol Lab Med 2014;138:1432-1443*

20 tissue samples (Non-predictive markers/IHC-type I: 10 positive and 10 negative cases including high & low expressors)

40 tissue samples (predictive markers/IHC-type 2: 20 positive and 20 negative cases

How many tissue samples are needed for the analytical validation process ?

Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine: Part 1: Fit-for-Purpose Approach to Classification of Clinical Immunohistochemistry Biomarkers

Carol C. Cheung, MD, PhD, JD,† Corrado D'Arrigo, MB, ChB, PhD, FRCPath,‡§‖ Manfred Dietel, MD, PhD,¶ Glenn D. Francis, MBBS, FRCPA, MBA, FFSc (RCPA),#**†† C. Blake Gilks, MD,‡‡ Jacqueline A. Hall, PhD,§§‖‖ Jason L. Hornick, MD, PhD,¶¶ Merdol Ibrahim, PhD,## Antonio Marchetti, MD, PhD,*** Keith Miller, FIBMS,## J. Han van Krieken, MD, PhD,††† Soren Nielsen, BMS,‡‡‡§§§ Paul E. Swanson, MD,‖‖‖ Clive R. Taylor, MD,¶¶¶ Mogens Vyberg, MD,‡‡‡§§§ Xiaoge Zhou, MD,###**** and Emina E. Torlakovic, MD, PhD,*†††‡‡‡‡*

From the International Society for Immunohistochemistry and Molecular Morphology (ISIMM) and International Quality Network for Pathology (IQN Path)

original purpose for which an IHC test is developed and its

Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine – Part 2: Immunohistochemistry Test Performance Characteristics

Emina E. Torlakovic, MD, PhD,†‡ Carol C. Cheung, MD, PhD, JD,*§ Corrado D'Arrigo, MB, ChB, PhD, FRCPath,‖¶# Manfred Dietel, MD, PhD,** Glenn D. Francis, MBBS, FRCPA, MBA, FFSc (RCPA),††‡‡§§ C. Blake Gilks, MD,‖‖ Jacqueline A. Hall, PhD,¶¶ Jason L. Hornick, MD, PhD,## Merdol Ibrahim, PhD,*** Antonio Marchetti, MD, PhD,††† Keith Miller, FIBMS,*** J. Han van Krieken, MD, PhD,‡‡‡ Soren Nielsen, BMS,§§§‖‖‖ Paul E. Swanson, MD,¶¶¶ Mogens Vyberg, MD,§§§‖‖‖ Xiaoge Zhou, MD,###**** Clive R. Taylor, MD,†††† and*

From the International Society for Immunohistochemistry and Molecular Morphology (ISIMM) and International Quality Network for Pathology (IQN Path)

Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine. Part 3: Technical Validation of Immunohistochemistry (IHC) Assays in Clinical IHC Laboratories

Emina E. Torlakovic, MD, PhD,†‡ Carol C. Cheung, MD, PhD, JD,*§ Corrado D'Arrigo, MB, ChB, PhD, FRCPath,‖¶# Manfred Dietel, MD, PhD,** Glenn D. Francis, MBBS, FRCPA, MBA, FFSc (RCPA),††‡‡§§ C. Blake Gilks, MD,‖‖ Jacqueline A. Hall, PhD,¶¶ Jason L. Hornick, MD, PhD,## Merdol Ibrahim, PhD,*** Antonio Marchetti, MD, PhD,††† Keith Miller, FIBMS,*** J. Han van Krieken, MD, PhD,‡‡‡ Soren Nielsen, BMS,§§§‖‖‖ Paul E. Swanson, MD,¶¶¶ Mogens Vyberg, MD,§§§‖‖‖ Xiaoge Zhou, MD,###**** and Clive R. Taylor, MD,††††*

From the International Society for Immunohistochemistry and Molecular Morphology (ISIMM) and International Quality Network for Pathology (IQN Path)

Evolution of Quality Assurance for Clinical Immunohistochemistry in the Era of Precision Medicine: Part 4: Tissue Tools for Quality Assurance in Immunohistochemistry

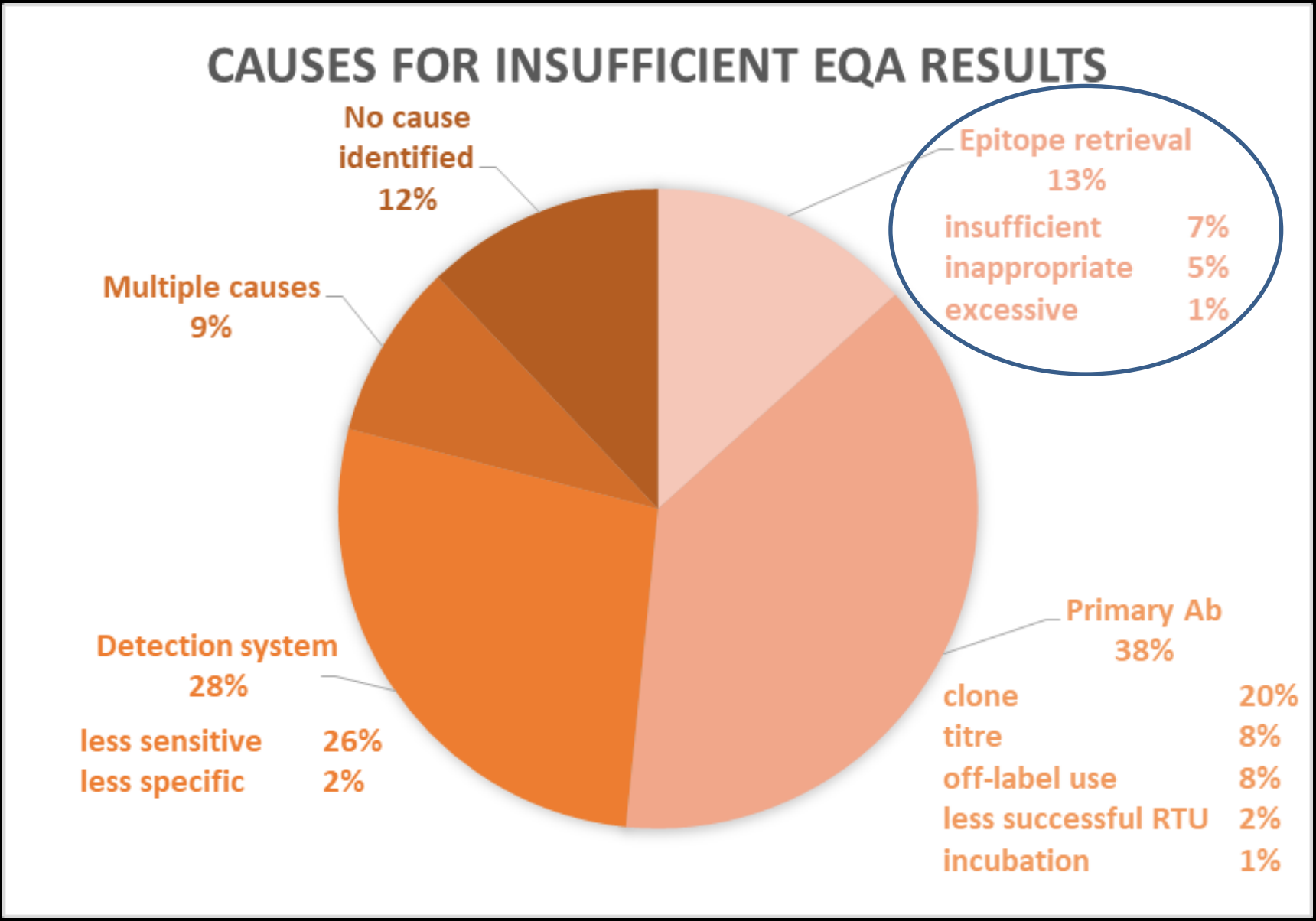
Carol C. Cheung, MD, PhD, JD,† Corrado D'Arrigo, MB, ChB, PhD, FRCPath,‡§‖ Manfred Dietel, MD, PhD,¶ Glenn D. Francis, MBBS, FRCPA, MBA, FFSc (RCPA),#**†† Regan Fulton, MD, PhD,‡‡ C. Blake Gilks, MD,§§ Jacqueline A. Hall, PhD,‖‖¶¶ Jason L. Hornick, MD, PhD,## Merdol Ibrahim, PhD,*** Antonio Marchetti, MD, PhD,†††‡‡‡ Keith Miller, FIBMS,*** J. Han van Krieken, MD, PhD,§§§ Soren Nielsen, BMS,‖‖‖¶¶¶ Paul E. Swanson, MD,### Clive R. Taylor, MD,**** Mogens Vyberg, MD,‖‖‖¶¶¶ Xiaoge Zhou, MD,†††‡‡‡‡ and Emina E. Torlakovic, MD, PhD,*§§§§‖‖‖‖*

From the International Society for Immunohistochemistry and Molecular Morphology (ISIMM) and International Quality Network for Pathology (IQN Path)

Article sequence (part 1-4) published in Appl Immunohistochem Mod Morphol (2017) systematically describing/defining all aspects of the IHC test - what is required for a full technical validation and implementation of a new test.

Full technical validation

NordiQC External Quality Assurance program



App. 20-30% of all results are evaluated as insufficient

79 % of insufficient results are related to the choice/use of:

- **Epitope retrieval procedure**
- **Primary Ab (including stainer platform)**
- **Detection system**

False positive and/or negative results

Courtesy of Birgit Truumees et al (NordiQC): Poster presented at the annual USCAP meeting 2024

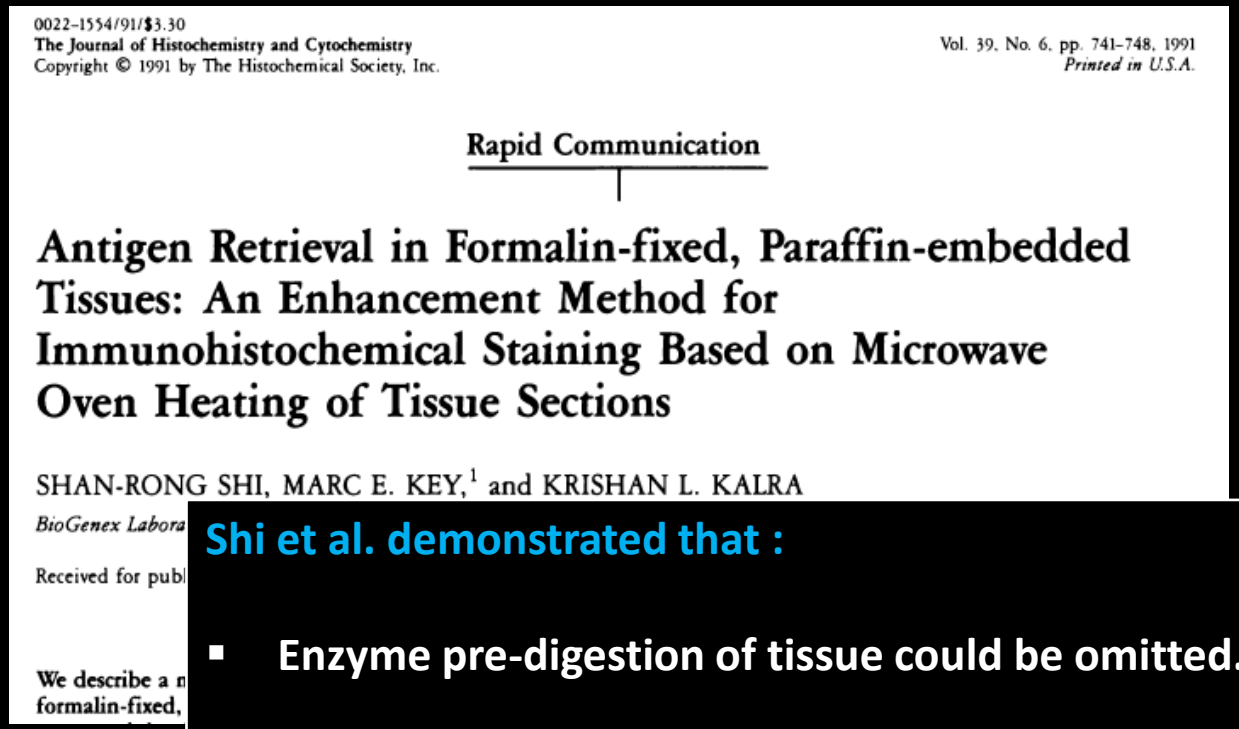
Technical aspects of immunohistochemistry & pitfalls - Analytical phase

The purpose of antigen retrieval is to unmask antigenic determinants (epitopes) and recover immuno-reactivity

Antigen retrieval procedures for formalin fixed tissue:

- ❑ **Heat Induced Epitope Retrieval (HIER)**
- ❑ **Tissue digestion using proteolytic enzymes**
- ❑ **Combined pre-treatment (HIER with proteolytic digestion)**

Technical aspects of immunohistochemistry & pitfalls - Analytical phase



0022-1554/91/$3.30
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Printed in U.S.A.

Rapid Communication

Antigen Retrieval in Formalin-fixed, Paraffin-embedded Tissues: An Enhancement Method for Immunohistochemical Staining Based on Microwave Oven Heating of Tissue Sections

SHAN-RONG SHI, MARC E. KEY,[1] and KRISHAN L. KALRA

BioGenex Labora

Received for publ

We describe a n
formalin-fixed,

HIER

Shi et al. demonstrated that :

- **Enzyme pre-digestion of tissue could be omitted.**
- **Incubation time with primary antibodies could be reduced, or dilutions of primary antibodies could be increased.**
- **Staining could be achieved on long-term formalin fixed tissue that failed to stain with conventional methods.**
- **Antibodies which where typically unreactive with formalin-fixed tissue gave excellent staining.**

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

The mechanism of HIER is not completely understood, and several hypothesizes has been proposed:

Heating tissue sections in an appropriate buffer may unmask epitopes by :

- ❑ Hydrolysis/disruption of methylene cross-links formed by formalin fixation
- ❑ Extraction of diffusible blocking proteins
- ❑ Precipitation/denaturation of proteins
- ❑ Rehydration of the tissue section allowing better penetration of the antibody
- ❑ Removal of tissue-bound calcium ions by chelating substances
- ❑ Other mechanism´s ?

Reviewed by Ramos-Vara JA: Vet Pathol 42:405-426, 2005

J Mol Hist (2008) 39:389–399
DOI 10.1007/s10735-008-9177-y

ORIGINAL PAPER

Hypothesis for the mechanism for heat-induced antigen retrieval occurring on fresh frozen sections without formalin-fixation in immunohistochemistry

Kochi Kakimoto · Susumu Takekoshi · Katsuhiro Miyajima · R. Yoshiyuki Osamura

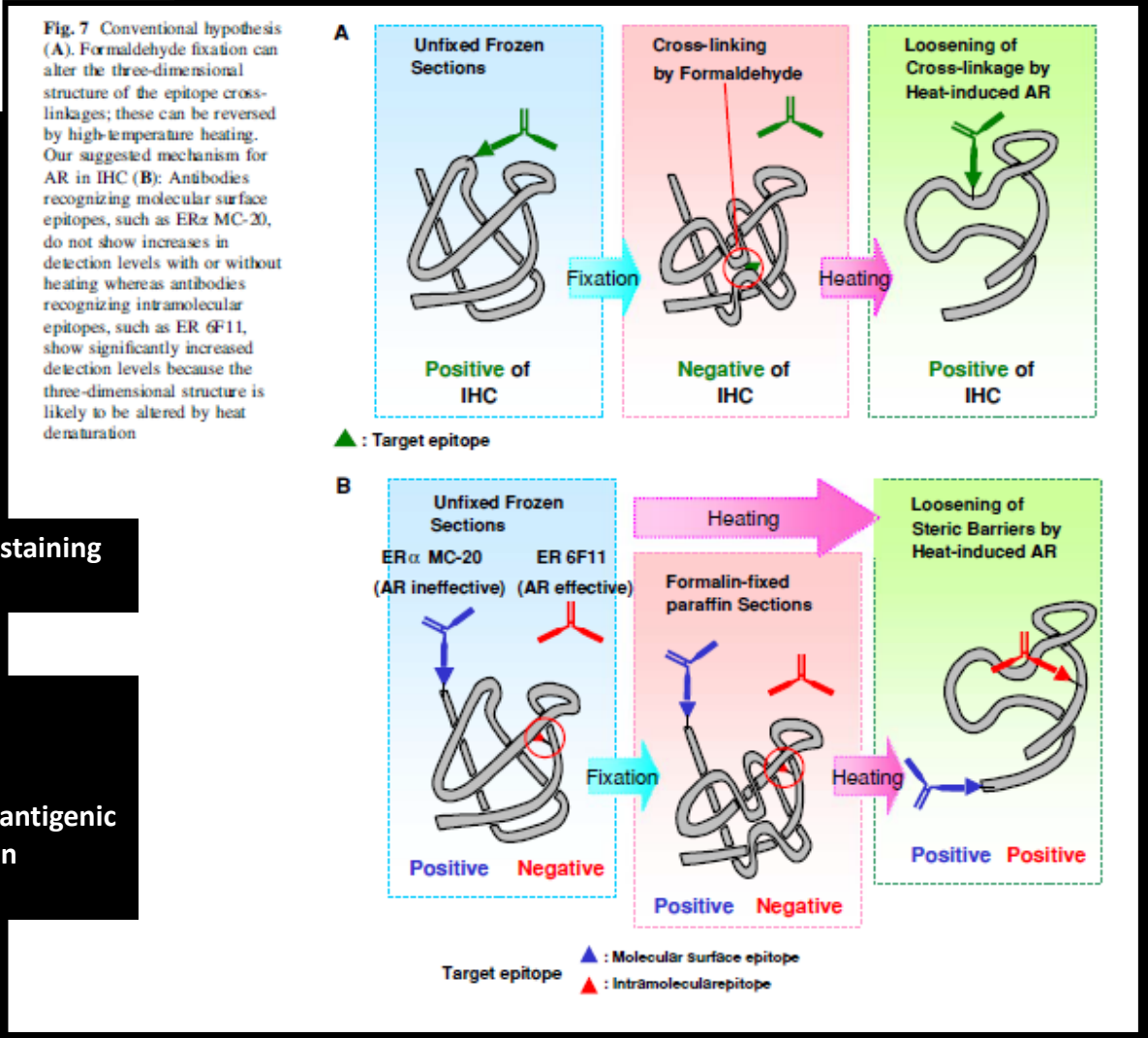


Fig. 7 Conventional hypothesis (A). Formaldehyde fixation can alter the three-dimensional structure of the epitope cross-linkages; these can be reversed by high-temperature heating. Our suggested mechanism for AR in IHC (B): Antibodies recognizing molecular surface epitopes, such as ERα MC-20, do not show increases in detection levels with or without heating whereas antibodies recognizing intramolecular epitopes, such as ER 6F11, show significantly increased detection levels because the three-dimensional structure is likely to be altered by heat denaturation

Demonstrated that: unfixed frozen sections, which did not show immunostaining with nine antibodies, were clearly stained after heating the sections

These results indicate that other mechanisms than breaking formalin-induced cross-linkages may be present.

The authors proposed that : the accessibility to some target epitopes of antigenic proteins is limited by natural steric barriers even in the fresh state (frozen sections) caused by the antigenic protein itself

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

HIER buffers used by the vast majority of NordiQC participants

App. 95 % of all pretreatment protocols

In house	Agilent Dako AS/Omnis	Roche Ventana Benchmark	Leica Biosystems Bond	Biocare	Thermo S LAB Vision
Low pH buffers					
Citric based buffers pH 6-6.7	TRS Low (3-in-1) pH 6.1	CC2 pH 6	BERS-1 pH 6	Diva Decloaker pH 6.2	
High pH buffer					
EDTA/EGTA based buffers pH 8-9	TRS High (3-in-1) pH 9	CC1 pH 8.5	BERS-2 pH 9	Borg Decloaker pH 9.5	HIER buffer H pH 9
	TRS High pH 9				

Challenges:

The platform often dictates the choice of HIER buffers

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Efficient HIER depends on several parameters:

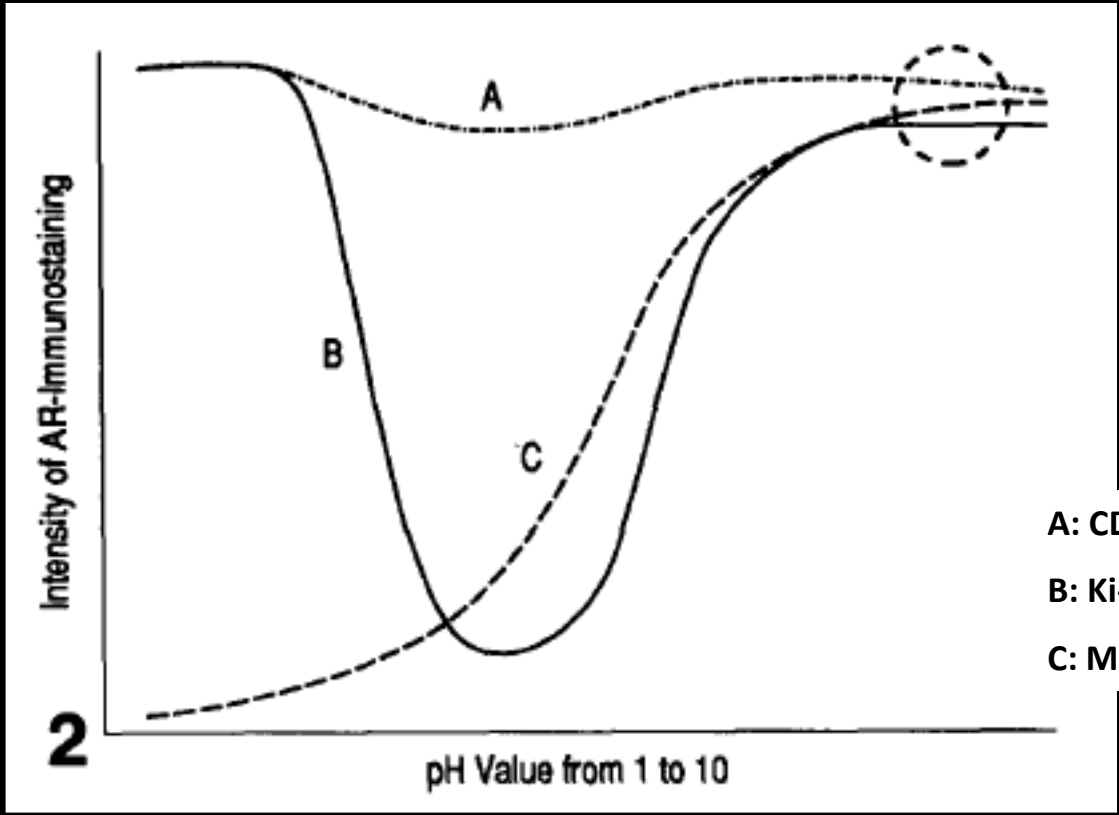
- ❑ pH of the HIER buffer
- ❑ Temperature
- ❑ Time
- ❑ Chemicals/components of the HIER buffer (e.g., Citrate; TRIS; EDTA; TRIS-EDTA)

Less sensitive to routinely fixed tissue (formalin) compared to enzymatic pre-treatment

> 95% of all commonly used antibodies require HIER

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Shi SR et al. *J Histochem Cytochem 1995 43:193-201*



Efficient HIER - Influence of pH value

A: CD20 (clone L26)

B: Ki-67 (clone MIB1)

C: MSA (clone HMB45)

Demonstrated that the performance of monoclonal antibodies were highly influenced by pH of the Antigen Retrieval buffer (AR).

Also, the results indicate the advantage of using an AR solution of higher pH value (8-9).

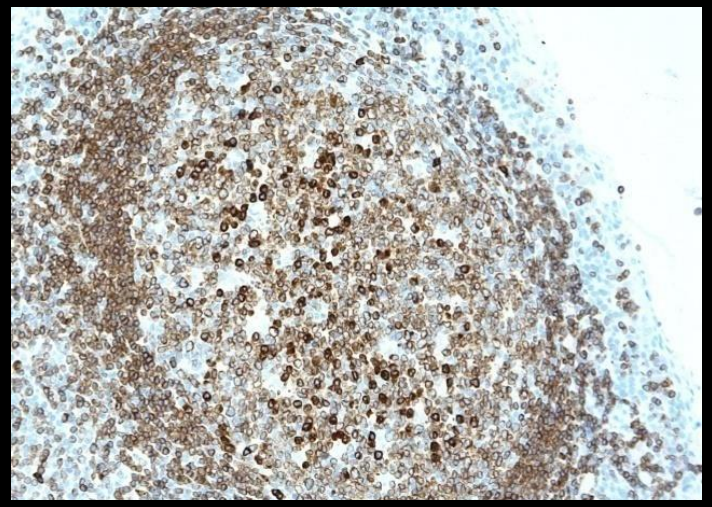
Technical aspects of immunohistochemistry & pitfalls - Analytical phase

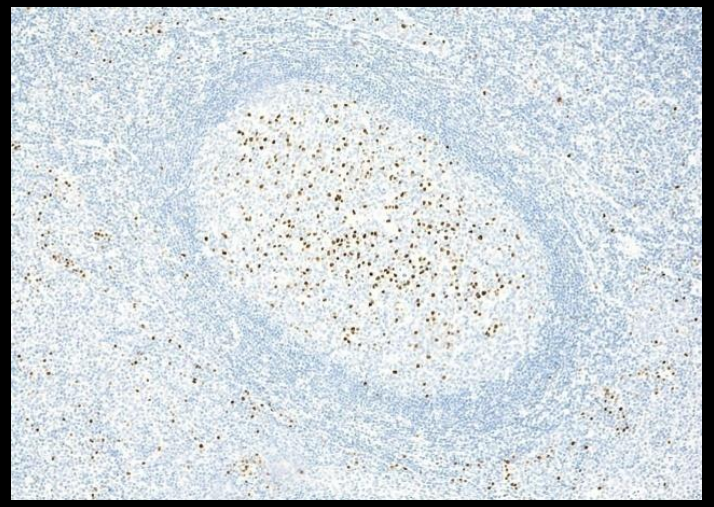
Efficient HIER - Influence of pH

CD79, JCB117 (1:300)

MUM-1, MUM1p (1:400)

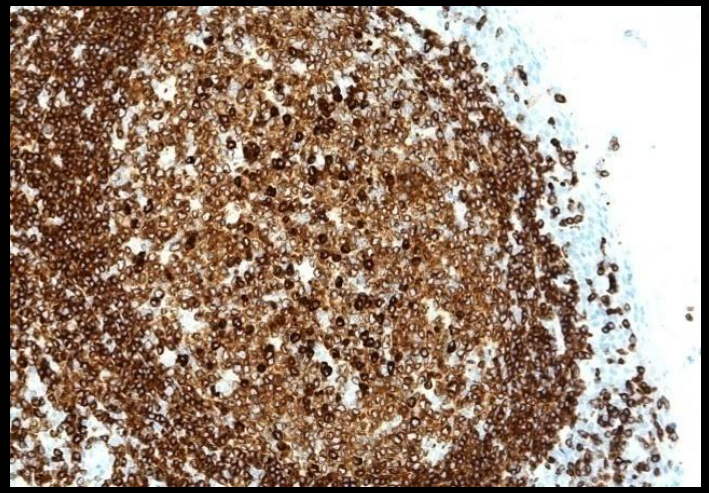
HIER in TRS pH 6.1
(20 min at 97°C)

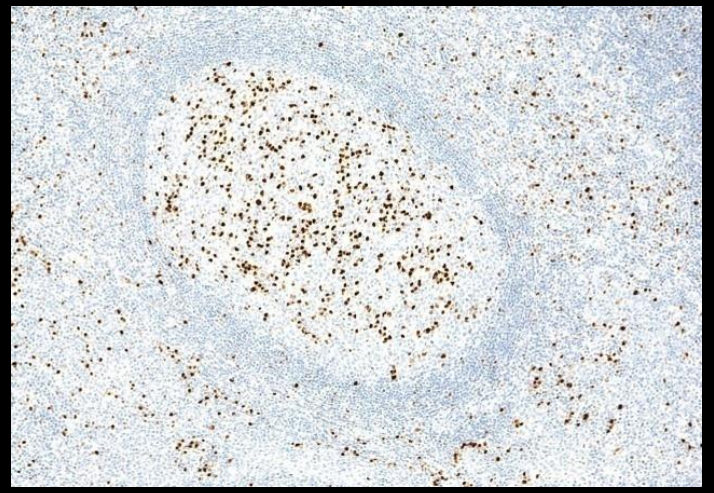




Autostainer: Flex+ as the detection system

HIER in TRS pH 9
(20 min at 97°C)





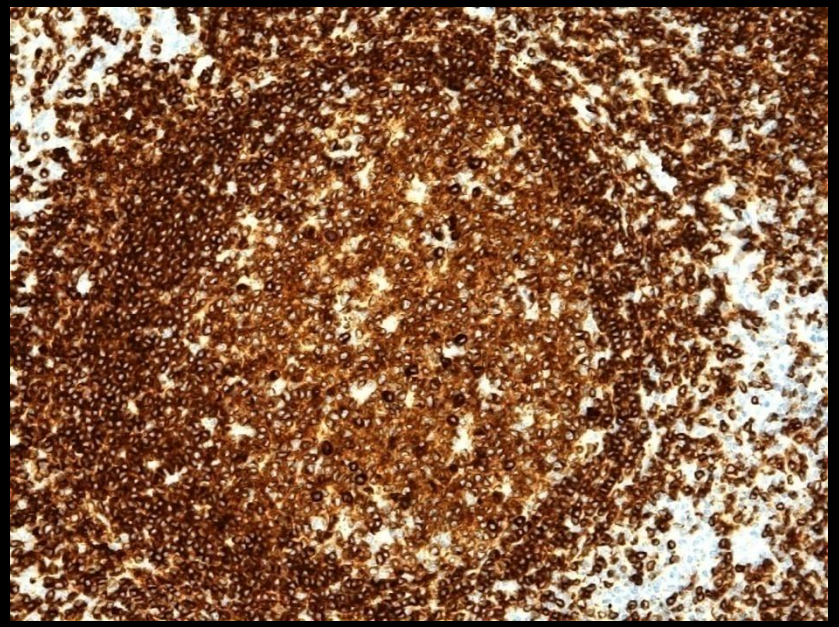
Tonsillar tissue fixed in 10% NBF (48h).

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Efficient HIER - Influence of pH

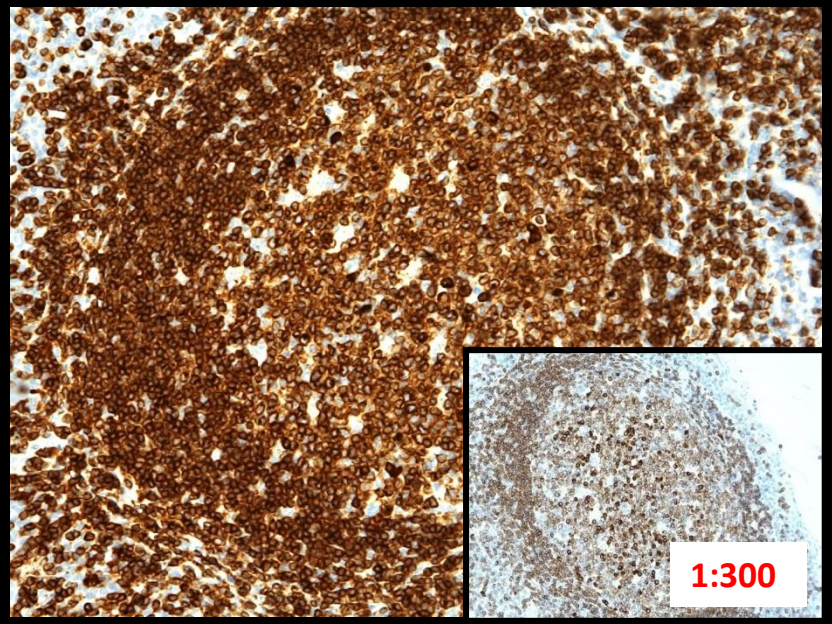
HIER in TRS pH 9

CD79, JCB117 (1:300)



HIER in TRS pH 6.1

CD79, JCB117 (1:50)



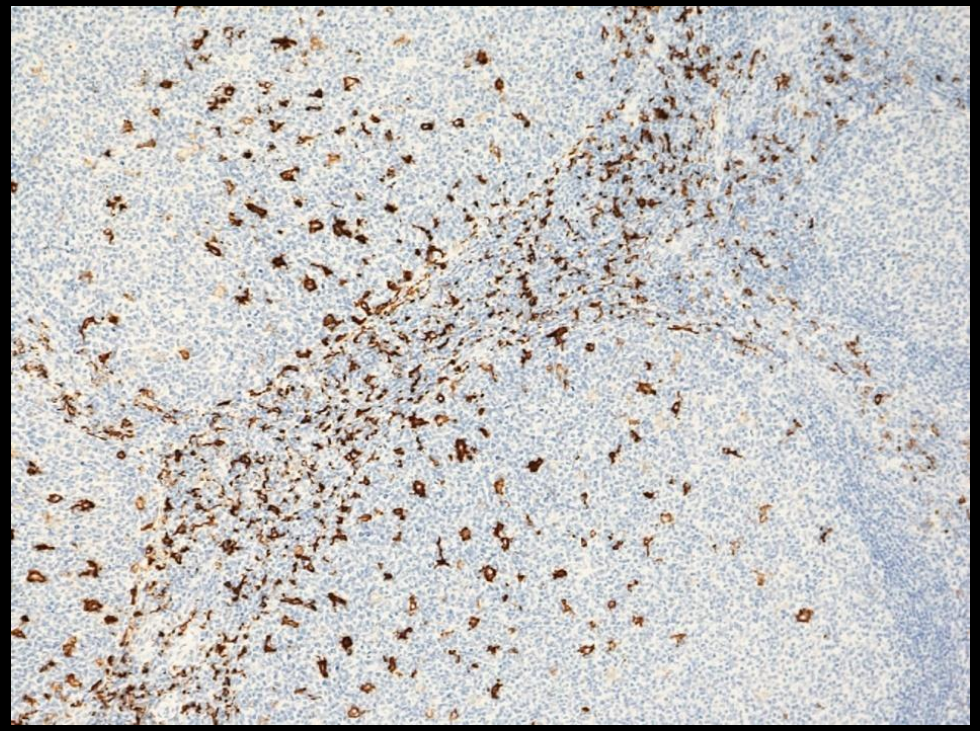
Tonsillar tissue fixed in 10% NBF (48h).
Flex+ as detection system

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Efficient HIER - Influence of pH

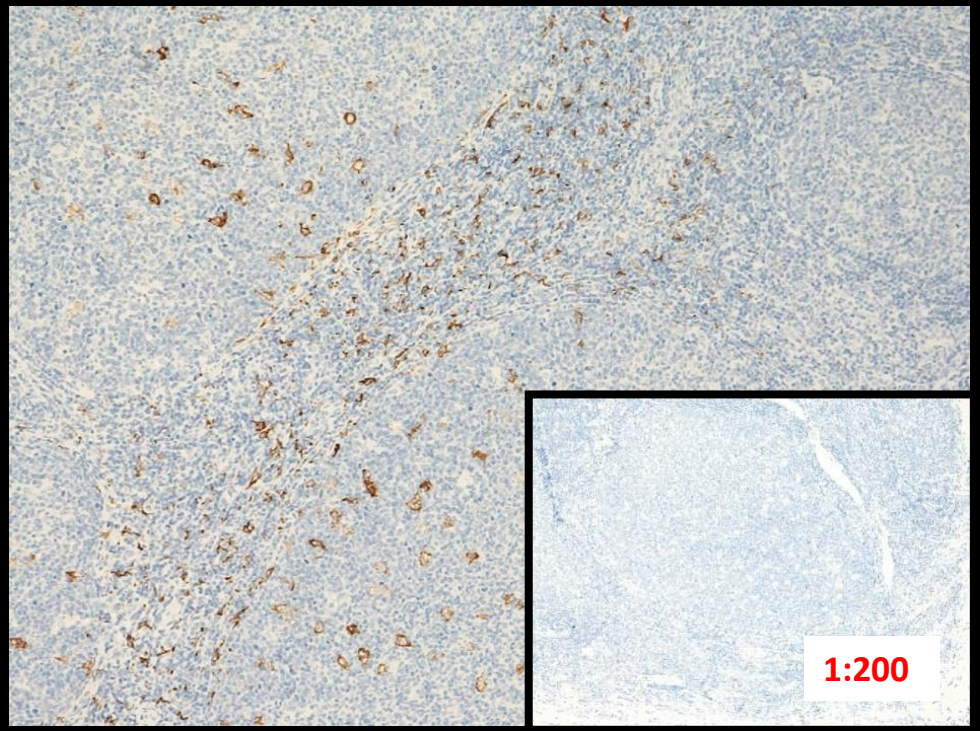
HIER in TRS pH 9

CD163, MRQ-26 (1:200)/Flex+



HIER in TRS pH 6.1

CD163, MRQ-26 (1:25)/Flex+



For app. 85-90% of the epitopes, HIER in buffers at pH 8-9 is preferable to pH6

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Ready To Use products: Recommendations to antigen retrieval provided by the manufactures ?

CK7 Run 62

Table 3. **Proportion of sufficient and optimal results for CK7 for the most commonly used RTU IHC systems**

RTU systems	Recommended protocol settings*		Laboratory modified protocol settings**	
	Sufficient	Optimal	Sufficient	Optimal
Dako AS mAb OV-TL 12/30 **IR619**	100% (12/12)	92% (11/12)	100% (11/11)	91% (10/11)
Dako Omnis mAb OV-TL 12/30 **GA619**	100% (32/32)	97% (31/32)	100% (26/26)	92% (24/26)
Leica Bond III/MAX mAb RN7 **PA0942/PA0138**	100% (6/6)	17% (1/6)	100% (11/11)	45% (5/11)
VMS Ultra/XT rmAb SP52 **790-4462**	100% (16/16)	69% (11/16)	97% (98/101)	85% (86/101)

* Protocol settings recommended by vendor – Retrieval method and duration, Ab incubation times, detection kit, IHC stainer/equipment.
** Significant modifications: retrieval method, retrieval duration and Ab incubation time altered, detection kit – only protocols performed on the specified vendor IHC stainer integrated.

17% (1/6)
BERS1 (citrate-based HIER buffer)

83% (5/6)
BERS2 (alkaline based HIER buffer)

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Efficient HIER - Influence of time and temperature

Taylor CR et al : *Applied Immunohistochemistry 1996; 4(3) : 144-166* - ***Temperature and time are inversely related*** :

Similar strong intensity of staining could be generated by the following heating conditions:

100°C for 20 min = 90°C for 30 min = 80°C for 50 min = 70°C for 10 h

Balaton AJ et al : *Applied Immunohistochemistry 1996; 4(4) : 259 - 263*

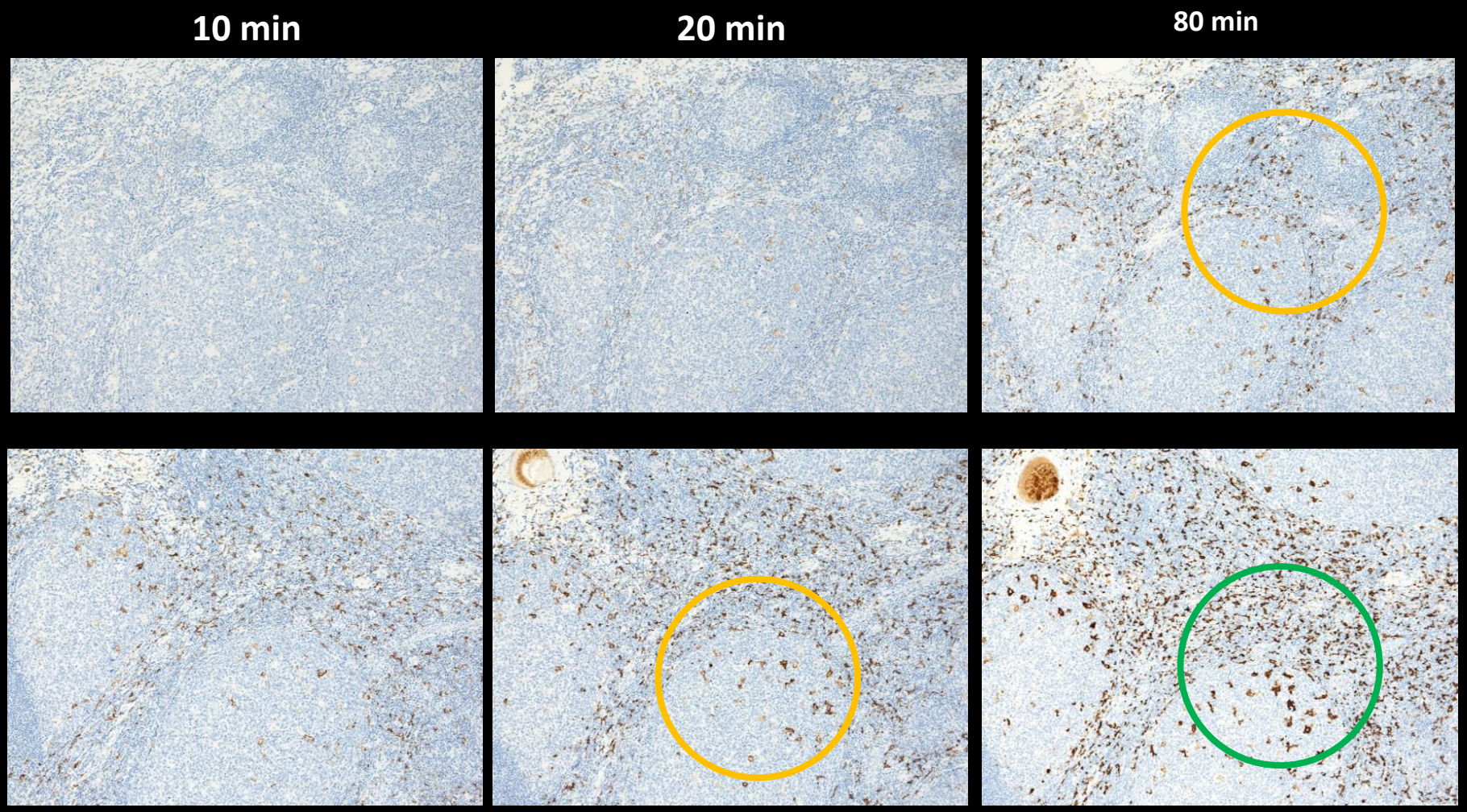
Optimal staining intensity could be generated by the following heating conditions:

Pressure cooker at 120°C for 3 min = MWO at 100°C for 20 min

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

HIER buffer - Influence of time and temperature

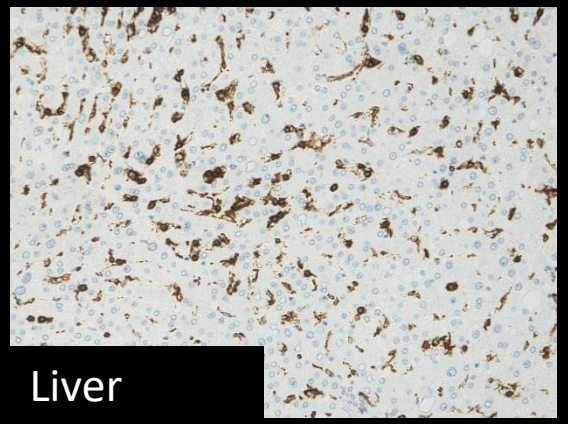
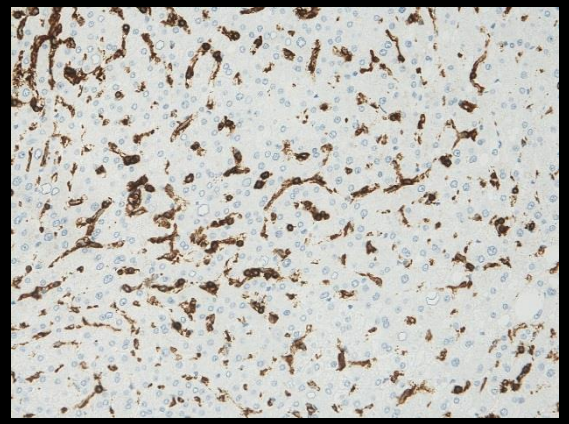
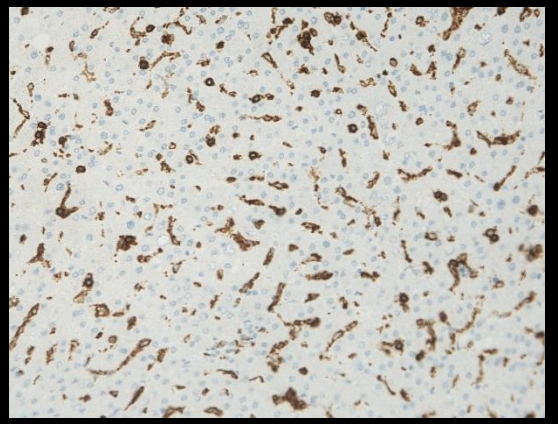
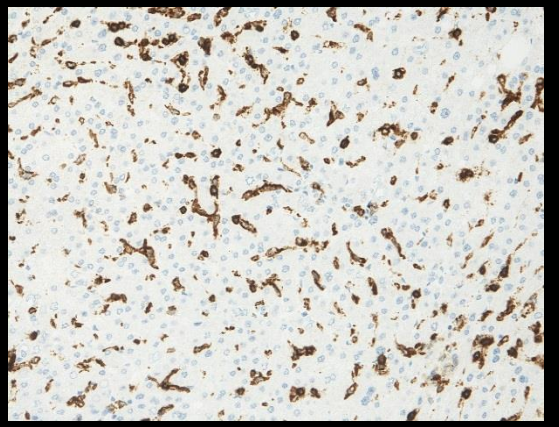
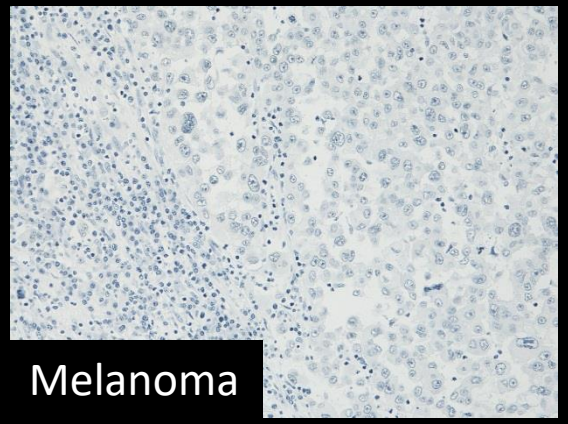
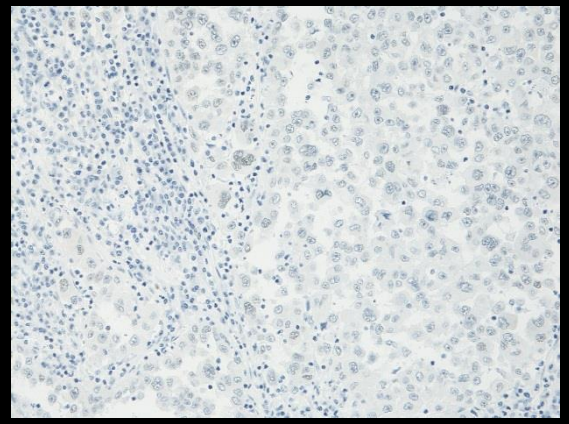
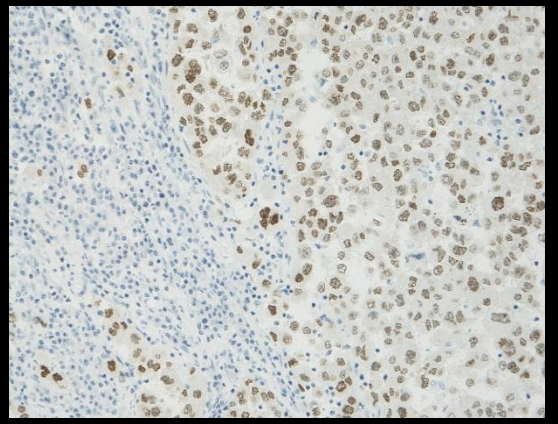
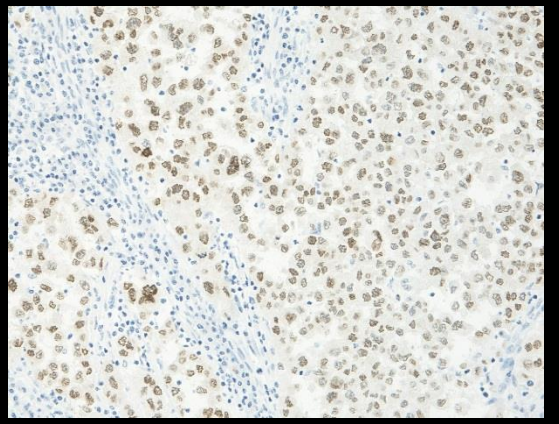
CD163, MRQ-26 (1:200)



Tonsillar tissue fixed in 10% formalin (48h).

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Efficient HIER - Influence of time and temperature

	TRS pH 9 (80°C/60`)	TRS pH 9 (90°C/30`)	TRS pH9 (90°C/60`)	TRS pH9 (97°C/24`)
CD45/LCA (2B11+PD7/26)	 Liver			
SOX10 (BS7)	 Melanoma			

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ANTIGEN RETRIEVAL TECHNIQUES IN IMMUNOHISTOCHEMISTRY: COMPARISON OF DIFFERENT METHODS

STEFANO A. PILERI[1]*, GIOVANNA RONCADOR[1], CLAUDIO CECCARELLI[1], MILENA PICCIOLI[1], ASPASIA BRISKOMATIS[1], ELENA SABATTINI[1], STEFANO ASCANI[1], DONATELLA SANTINI[1], PIER PAOLO PICCALUGA[1], ORNELLA LEONE[1], STEFANIA DAMIANI[1], CESARINA ERCOLESSI[1], FEDERICA SANDRI[1], FEDERICA PIERI[1], LORENZO LEONCINI[2] AND BRUNANGELO FALINI[3]

[1]*Second Service of Pathologic Anatomy and Haematopathology Section, Bologna University, Italy*
[2]*Institute of Pathologic Anatomy, Siena University, Italy*
[3]*Haematopathology Laboratory, Institute of Haematology, Perugia University, Italy*

Chemical components of the HIER buffer`s

Standard low pH buffer`s (e.g., citrate based pH 6.0)

Standard high pH buffer`s (e.g., TE based pH 8-10)

Modified low pH buffers pH 6.1-6.2 : S1699/S1700 (Dako) or Diva Decloaker (Biocare)

Overall best performance:

HIER in EDTA pH 8.0 (compare with Tris-HCL pH 8.0)

Table I—Results obtained under different methodological conditions with the antibodies most commonly used in the study of the haemolymphopoietic system and related disorders

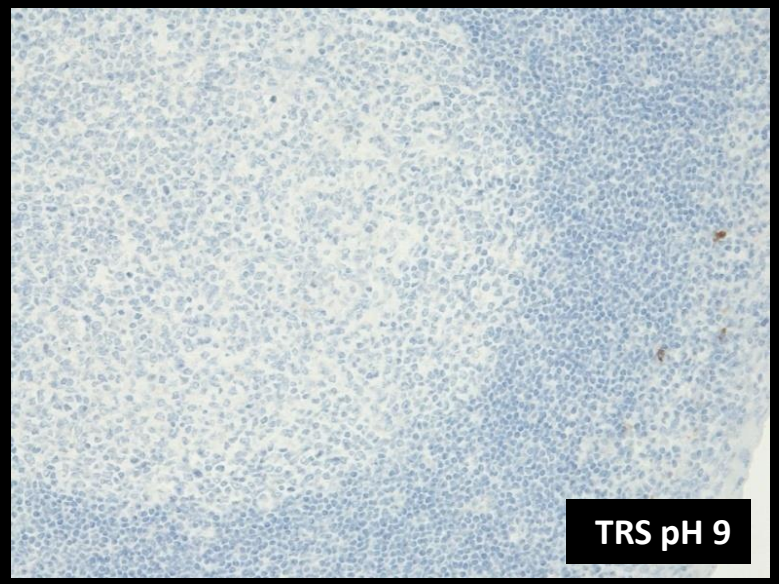
Clone	Specificity	Source	Dilution	No AgR	PT	HBAR+ citrate	HBAR+ Tris-HCl pH8	HBAR+ EDTA pH8
O10	CD1a	Immunotech	1:40	–	–	+– – –	++– –	++++
Poly	CD3	DAKO	1:300	–	++– –	+– – –	++– –	++++
C8/144B	CD8	Dr Mason	1:6	–	–	++– –	++– –	++++
			1:400	–	–	+– – –	+++–	++++
C3D-1	CD15	DAKO	1:6	+– – –	–	+– – –	++– –	++++
			1:320	++– –	–	+++–	+++–	++++
L26	CD20	DAKO	1:200	+– – –	+– – –	++++	++– –	++++
			1:3200	+– – –	+– – –	++++	++++	++++
IF8	CD21	DAKO	1:10	–	++++	–	–	–
MHM6	CD23	DAKO	1:50			+– – –	+++–	++++
Ber-H2	CD30	Professor Stein	1:10	–	–	++– –	+++–	++++
			1:320	–	–	+– – –	+++–	++++
QBEND-10	CD34	BioGenex	1:20	+– – –	–	+++–	+++–	++++
			1:400	+– – –	–	++– –	++– –	++++
BerMACDRC	CD35	DAKO	1:5	+– – –	++++	–	+– – –	+– – –
MAB89	CD40	Immunotech	1:100	–	++++	–	–	–
DF-T1	CD43	DAKO	1:200	+– – –	–	+++–	+++–	++++
			1:1600	++– –	–	+++–	++++	++++
PD7/26+2B11	CD45	DAKO	1:200	–	+– – –	+++–	++++	++++
			1:4000	–	–	+– – –	+++–	++++
UCHL-1	CD45R0	DAKO	1:120	+– – –	++– –	++– –	++++	+++–
KI-B3	CD45R	Professor Parwaresch	1:80	++– –	+– – –	+++–	++++	++++
			1:320	++– –	+– – –	+++–	++++	++++
4KB5	CD45RA	DAKO	1:20	++– –	–	++++	+++–	+++–
	CD57	Becton	1:20	++– –	++– –	+++–	+++–	++++
Y2/51	CD61	DAKO	1:5	–	+++–	+– – –	+– – –	+++–
KP1	CD68	DAKO	1:640	+– – –	++– –	++++	++– –	++++
PG-M1	CD68	Professor Falini	1:20	+– – –	++– –	++– –	++– –	++++
JCB117	CD79a	Dr Mason	1:10	+– – –	–	+++–	+++–	++++
Kim-4p	Follicular dendritic cells	Professor Parwaresch	1:5	–	++++	++– –	++– –	+– – –
DBA.44	Hairy cells	Professor Delsol	1:5	++– –	–	++++	+++–	++++
JC159	GlycophorinA	DAKO	1:320	+– – –	–	++++	+++–	+++–
NP57	Neutrophilic elastase	DAKO	1:10	++++	–	–	–	–
M616	FVIII RAg	DAKO	1:6	+– – –	++– –	++++	++– –	++++
Poly	Lysozyme	DAKO	1:800	++– –	+++–	++++	++++	++++
Poly	IgA	DAKO	1:2000	+– – –	+++–	++++	+++–	++++
Poly	IgG	DAKO	1:5000	++– –	++++	++++	++++	++++
Poly	IgM	DAKO	1:5000	–	++– –	++++	++++	++++
Poly	IgD	DAKO	1:1000	–	–	+++–	+++–	++++
Poly	κ-Ig light chain	DAKO	1:10 000	++– –	+++–	++++	+++–	+++–
Poly	λ-Ig light chain	DAKO	1:12 000	++– –	+++–	++++	+++–	+++–
Poly	Protein S-100	DAKO	1:2000	++– –	+++–	++++	+++–	++++
Poly	MPO	DAKO	1:10 000	++– –	+++–	+++–	+++–	++++

CD=cluster of differentiation; No AgR=no antigen retrieval; PT=proteolytic treatment; HBAR=heat-based antigen retrieval; Poly=polyclonal antibody; FVIII RAg=Factor VIII-related antigen; MPO=myeloperoxidase.
In bold: overnight incubation of the primary antibody+SABC technique.
– – – –=completely negative result; +– – –=weak positivity in a percentage of cells expected to be positive; ++– –=weak positivity in all cells expected to be positive; +++–=moderately strong positivity in all cells expected to be positive; ++++=very strong positivity in all cells expected to be positive.

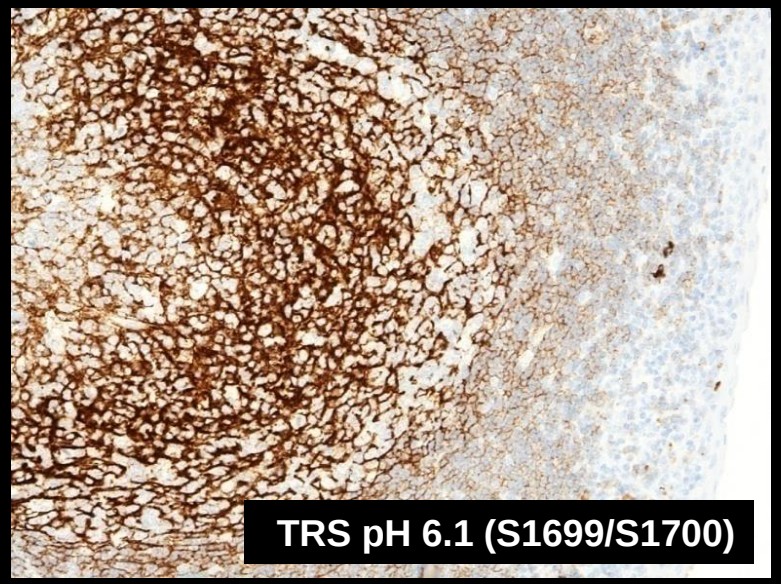
Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Modified low pH buffers

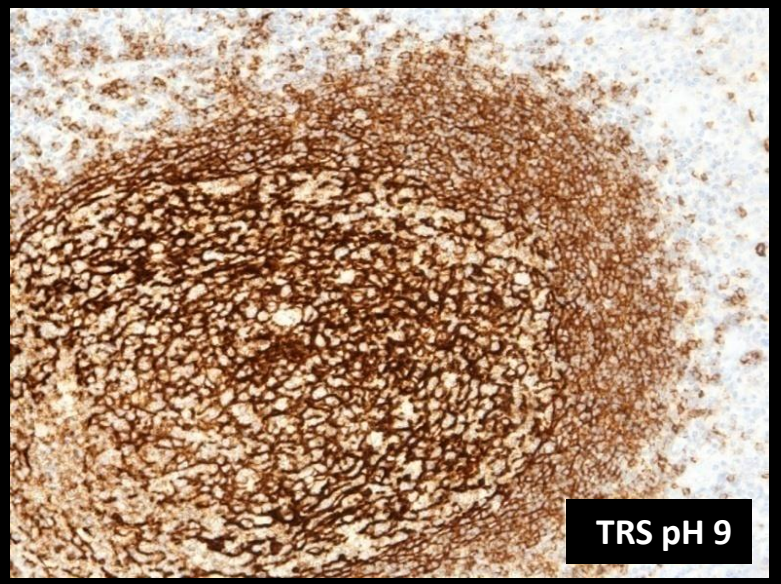
CD21, clone 1F8



CD21, clone 1F8



CD21, clone 2G9



HIER time 20`/ Flex+

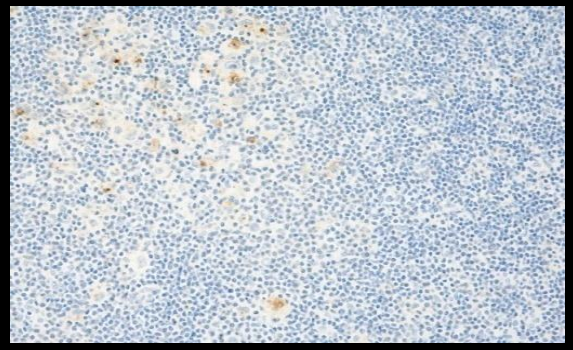
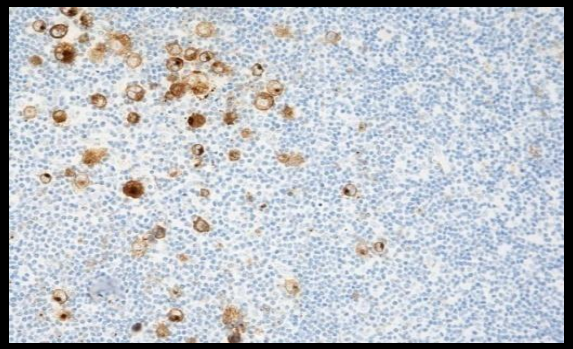
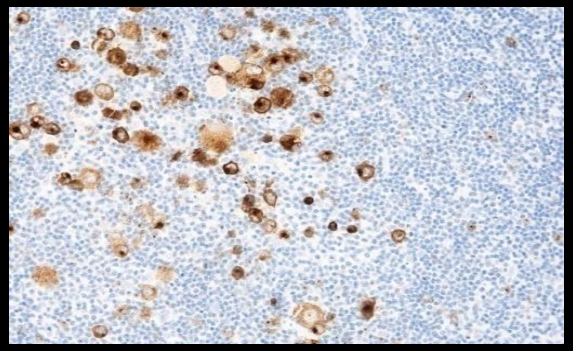
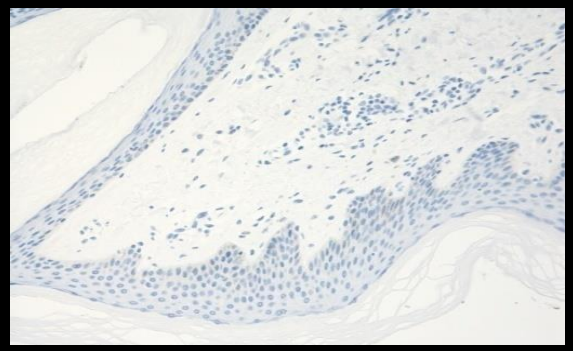
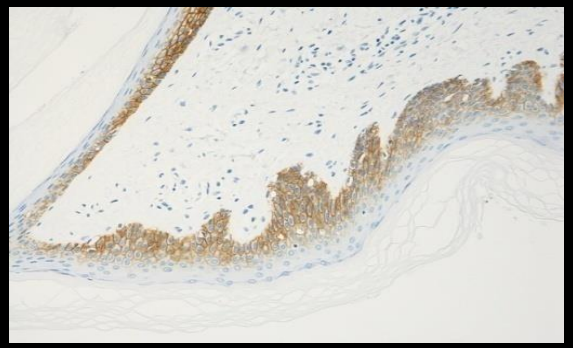
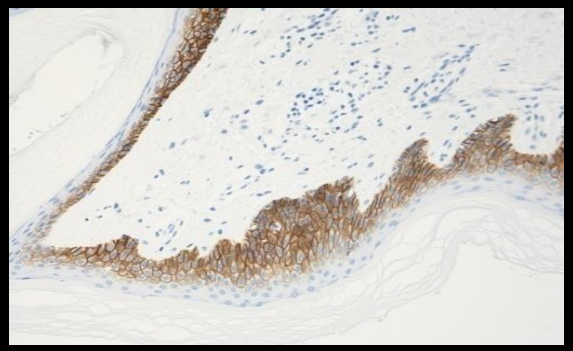
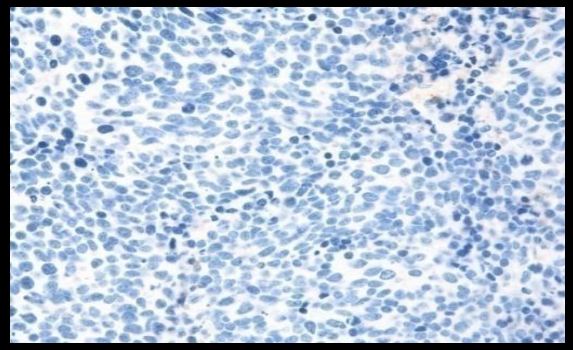
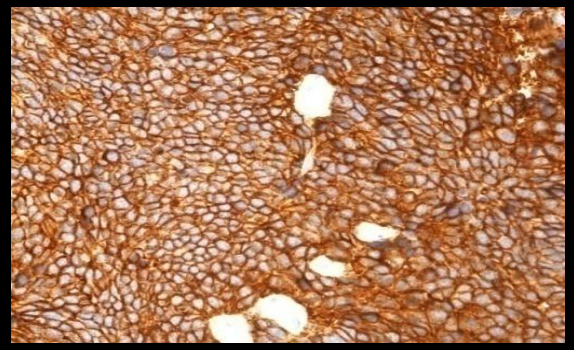
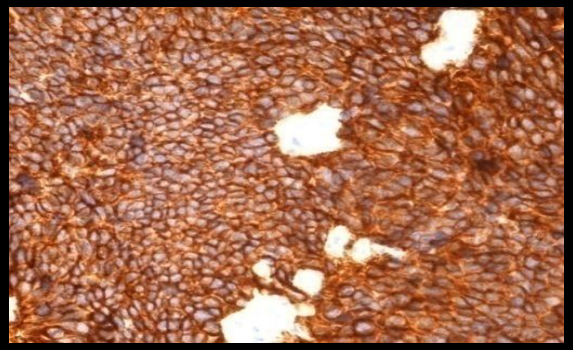
Markers requiring the TRS Low pH 6.1 (Dako, S1699/S1700) or Diva Decloaker pH 6.2 (Biocare, DV2004) :

EPCAM (clone EP-4 or MOC-31 or “VU-1D9”); GP200 (clone SPM 314 or 66.4.C2); CD21 clone 1F8; CD61 clone Y2/51; NGFR clone MRQ-21; Desmoglein-3 clone BC11, PHH3 clone BC37 and …….

Mandatory for : CD7 clone CBC 3.7; CD30 clone ConD6/B5; CD5 clone Leu1……

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

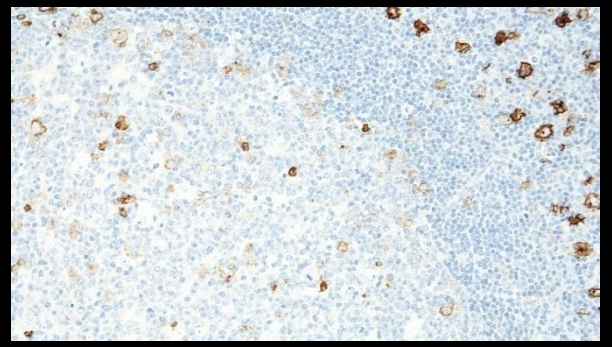
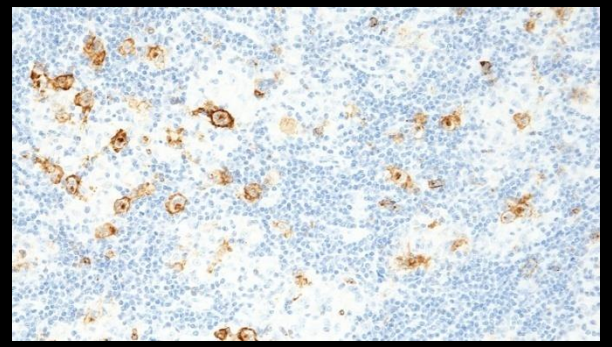
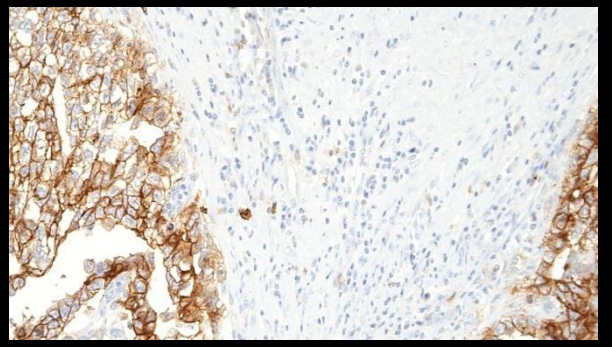
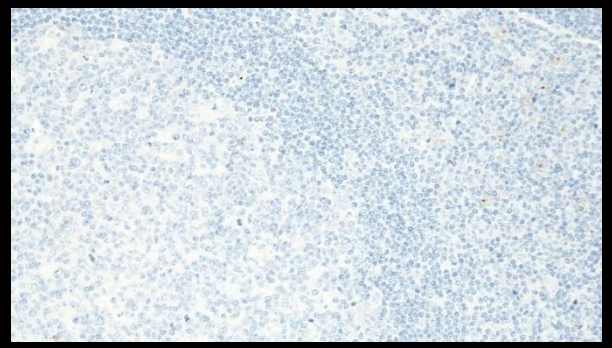
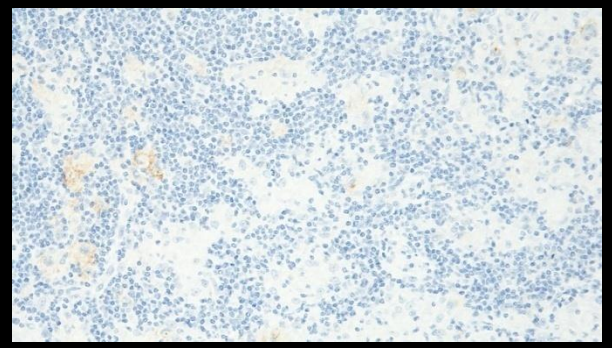
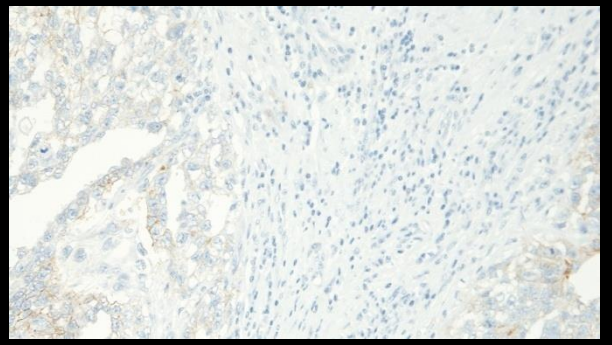
Modified low pH buffers

	TRS pH 9 (Dako) PT / 99° / 20 min	TRS pH 6.1 (Dako S1700) PT / 99° / 20 min	Diva Decloaker (Biocare) PT / 99° / 20 min
CD30, ConD6/D5 (1:50) (Hodgkin Lymphoma)			
Desmoglein-3, BC11 (1:25) (Skin)			
EP-CAM, MOC-31 (1:20)/EP4 (Small cell carcinoma)			

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Modified low pH buffers

CD30 clone ConD6/B5

	Tonsil	Hodgkin lymphoma	Embryonal carcinoma
HIER buffer, TRS pH 6.1 (Dako S 1700)			
HIER buffer, Low pH (LabVision TA-999-DHBL)			

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

The purpose of antigen retrieval is to unmask antigen epitopes /restore antigenic determinants and recover immuno-reactivity

Antigen retrieval procedures for formalin fixed tissue:

- ❑ Heat Induced Epitope Retrieval (HIER)
- ❑ **Tissue digestion using proteolytic enzymes** →
- ❑ Combined pre-treatment (HIER with proteolytic digestion)

Proteolytic enzymes cleave more or less specific amino acid sequences within peptide chains.

→ **Improves penetration of immuno-reagents into tissue structures and enhance accessibility to the epitopes of interest.**

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Enzymatic digestion - the problem?

A significant proportion of Labs still use enzymatic pre-treatment for e.g, “old” markers as cytokeratins and S100 introduced back in 1980-1990.

Only few markers require enzymatic digestion for “optimal performance”.

NQC Run 64, 2022:
Desmin

Vendor recommended protocol setting

Table 4. **Pass rates of Ventana/Roche RTU DE-R-11 antibody on the Benchmark platform for different epitope retrieval methods.**

	Pass rate							
	Total		HIER		Proteolysis		HIER + proteolysis	
	Protocols	Sufficient	Protocols	Sufficient	Protocols	Sufficient	Protocols	Sufficient
mAb DE-R-11 760-2513	139	98 (71%)	74	73 (99%)	49	11 (22%)	16	13 (81%)

None optimal

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

”Optimal” enzymatic digestion depends on:

Enzyme type

Concentration

Time

Temperature

Fixation type & time

Tissue type

Most common Enzymes

Proteinase K
Pronase XIV
Pronase XXIV
Pepsin
Trypsin

Difficult to control and to standardizes within routine LAB

Short time formalin fixation = gentle proteolysis
Long time formalin fixation = prolonged proteolysis

Markers requiring enzymatic pretreatment :

FVIII (poly), LMV CK (CAM 5.2), PAN CK (MNF116), EGFR (various), TCR-β (8A3)……..

Extracellulare matrix proteins (COLL-III (poly), Laminin (poly) and COLL-IV (CIV-22) ……..

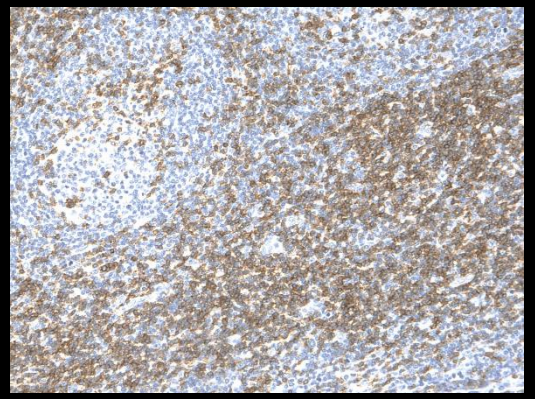
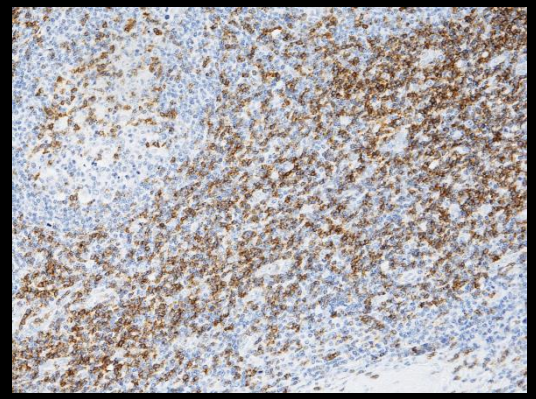
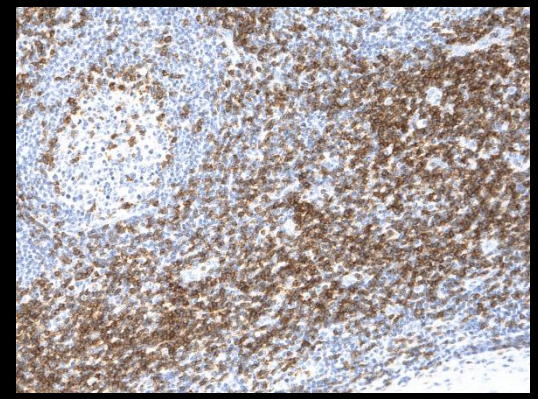
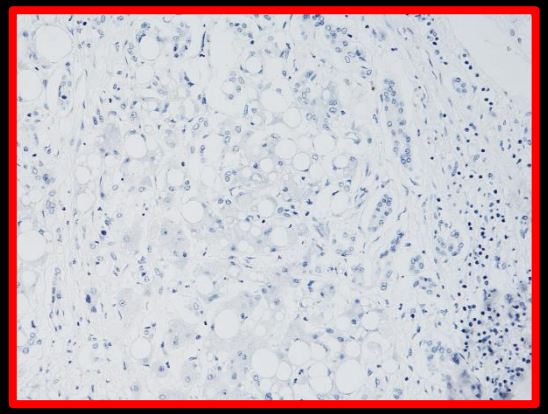
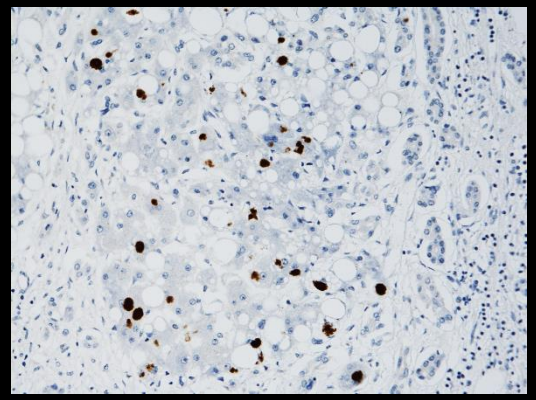
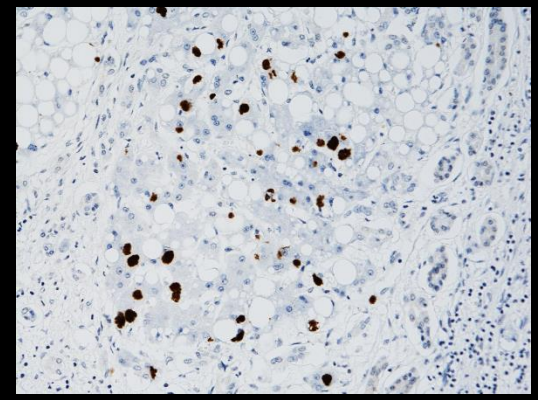
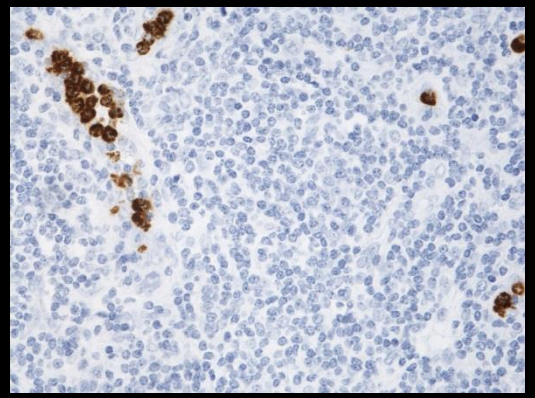
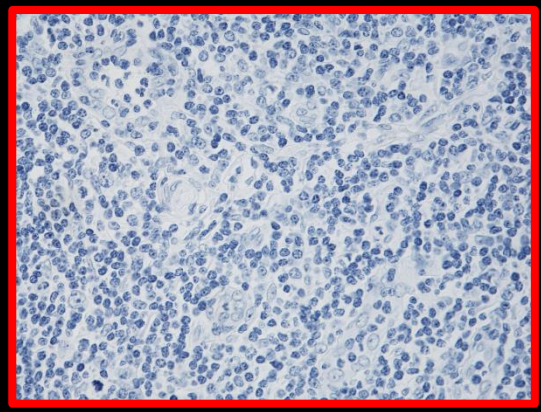
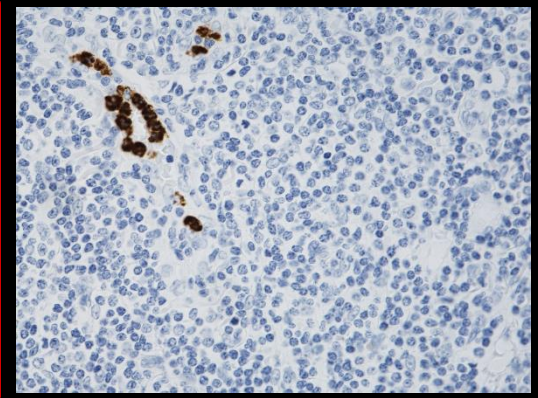
≤ 2% of all commonly used antibodies require enzymatic pre-treatment

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Most common enzymes used in IHC:

Enzyme	Typical working conc.	Activation Temperature	Typical Incubation time	Cleavage nature
Proteinase K	0.1%, pH 8.0	25-37 °C	5-10 min.	Broad - all amino acids
Trypsin	0.1-0.25%, pH 7.6	37 °C	10 min.	Arginin / Lysin
Pepsin	0.2-0.4%, pH 2.0	37 °C	5-20min.	Broad ,favor peptides with aromatic amino-groups
Protease XXIV	0.05-0.1%, pH 7.6	37 °C	5-10 min.	Broad - all amino acids
Protease XIV	0.05-0.1%,pH 7.6	25-37 °C	10-30min.	Broad, favor peptides with aromatic residues

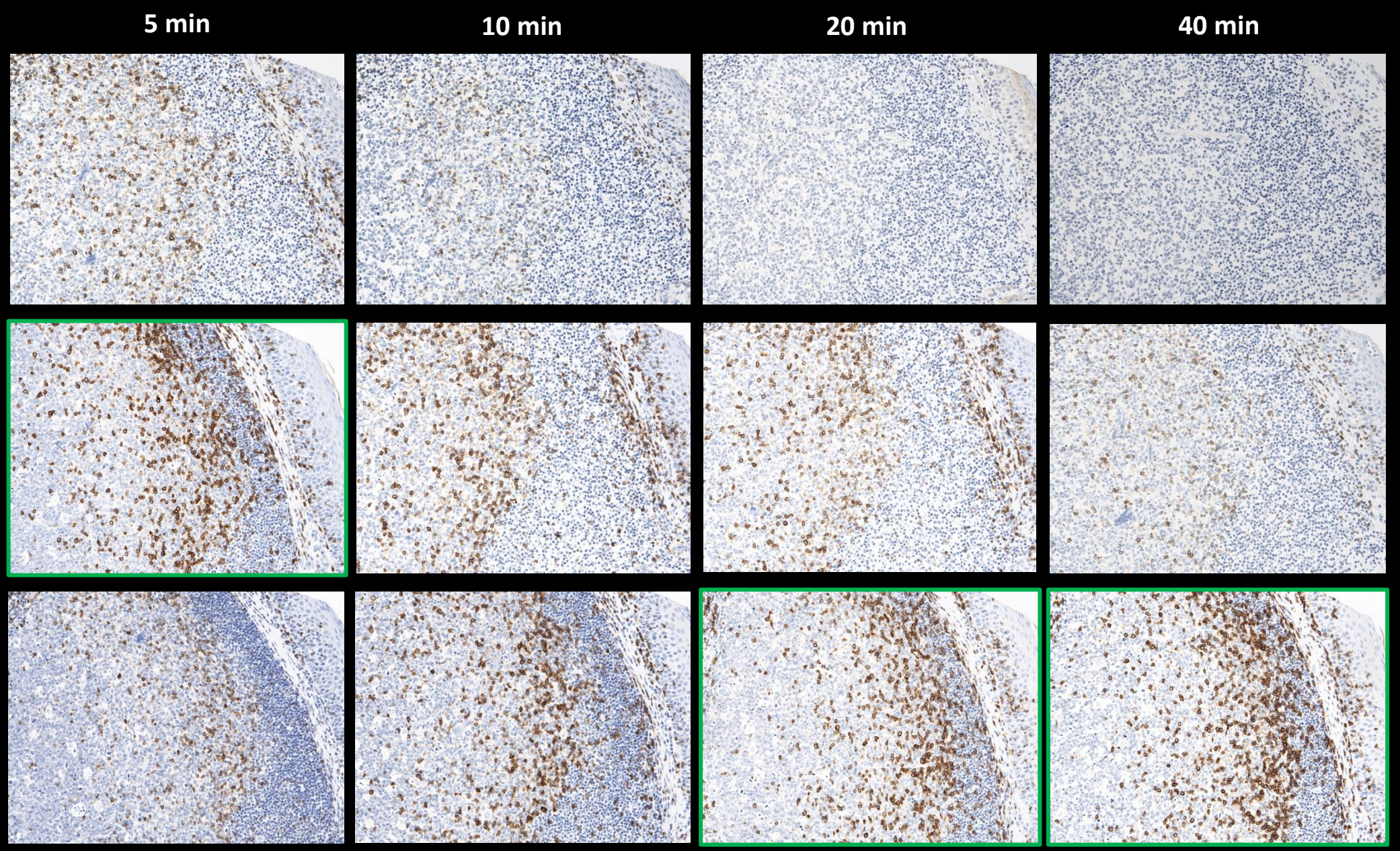
Choice of proteolytic enzyme

	Trypsin (Biocare, RTU) 40`	Pepsin (ZytoVision, RTU-H) 15`	Proteinase K (Dako, RTU) dil. 1:4 / 5`
TCR-β, 8A3, 1:200 RR Tonsil			
Ubiquitin, Ubi-1 1:750 Liver/ Mallory bodies			
Neutrophil Elastase, NP57 1:1000 Tonsil			

Proteolytic enzyme & digestion time ?

Tonsil NBF 48h

Digestion temp. 32°C



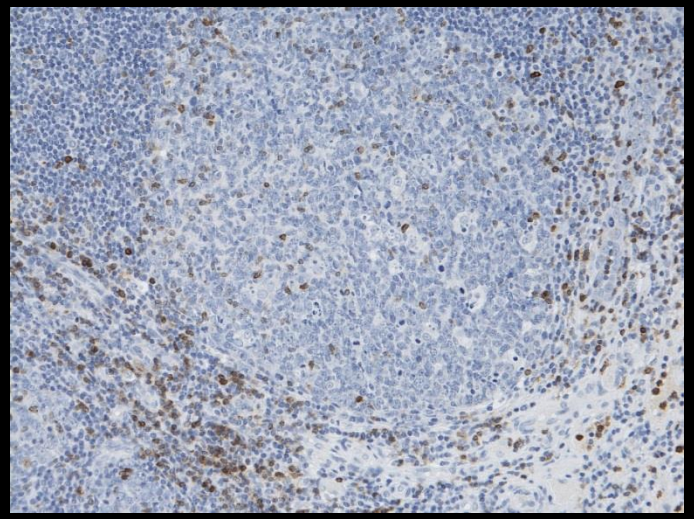
TCR-β clone 8A3 (1:200 RR) / Flex+ (Omnis)

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

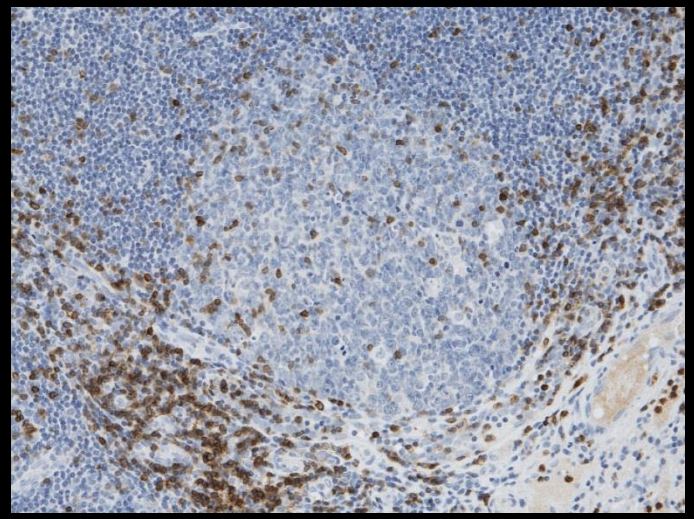
Proteolytic enzyme & digestion temperature ?

TCR β clone 8A3 (1:200 RR) / Flex+ (Tonsil NBF 48h)

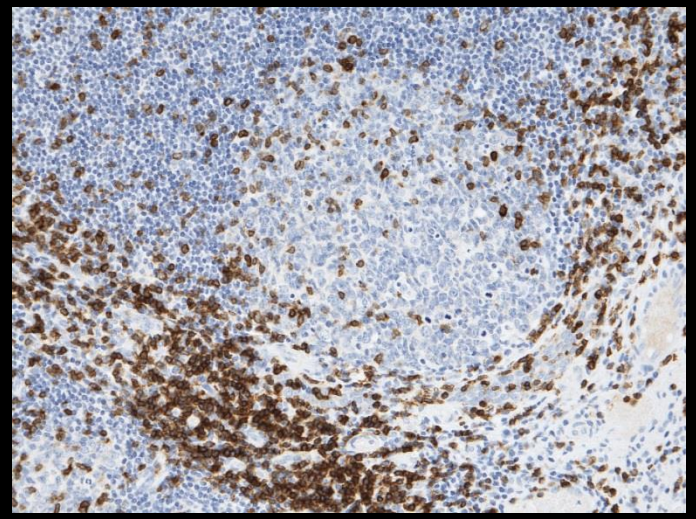
Trypsin Digestion temp. 4°C (10 `)



Trypsin Digestion temp. 24°C (10`)



Trypsin Digestion temp. 37°C (10`)



Increased intensity of TCR β positive T-cells

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Enzymatic digestion (Influence of fixation time)

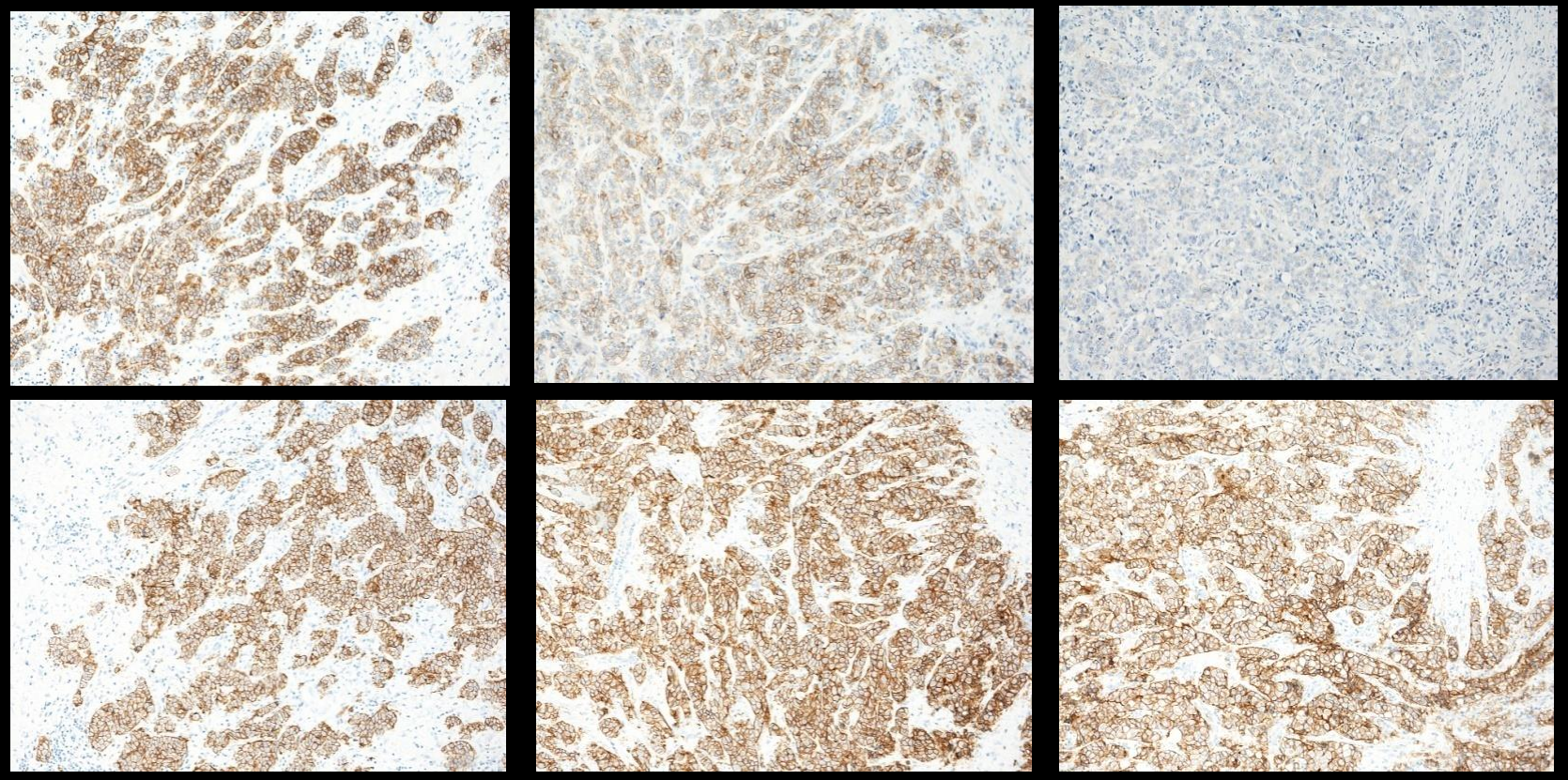
EPCAM, clone MOC-31, dilution 1:20

Pepsin / (Dako, S3002)

10 min/37°C

HIER , Low pH (S1700)

20 min / 97°C



NBF 24 h | **NBF 48 h** | **NBF 120h**

Adenocarcinoma (Breast) fixed in 10% Formalin

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

The purpose of antigen retrieval is to unmask antigen epitopes /restore antigenic determinants and recover immuno-reactivity

Antigen retrieval procedures for formalin fixed tissue:

- Heat Induced Epitope Retrieval (HIER)
- Tissue digestion using proteolytic enzymes
- **Combined pre-treatment (HIER with proteolytic digestion)**

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Only few markers require combined pre-treatment for "optimal performance".

CK-PAN

Table 4. **Pass rates for antibody cocktails combined with epitope retrieval methods in the last three NordiQC runs**

	Pass rate for compiled data from run 54, 58 and 71							
	Total		HIER		Proteolysis		HIER + proteolysis	
	Protocols	Sufficient	Protocols	Sufficient	Protocols	Sufficient	Protocols	Sufficient
mAb AE1/AE3	609	449 (74%)	579	442 (76%)	12	1 (8%)	9	1 (11%)
mAb AE1/AE3/5D3	21	12 (57%)	21	12 (57%)	-	-	-	-
mAb AE1/AE3/PCK26	323	208 (64%)	39	22 (56%)	28	3 (11%)	254	181 (71%)
mAb MNF116	27	2 (7%)	18	0 (0%)	7	2 (29%)	1	0
mAb BS5	38	36 (95%)	38	36 (95%)	-	-	-	-

RTU (Ventana)

Applying a combined pre-treatment protocol require a gentle/careful calibration of all parameters involved in the process (both HIER and proteolytic digestion steps)

WT1 clone 6H-F2 (RUN43)

HIER in CC1
(Ventana, Benchmark Ultra)

HIER in CC1 + Protease 3
(Ventana, Benchmark Ultra)

NordiQC — Nordic immunohistochemical Quality Control

NordiQC runs

Run 43 (general module), **B19** (breast cancer module) and **H7** (HER-2 ISH module) opened by 10th December, deadline for protocol submission was 7th January. Results are available by **22** April, see **Newsletter**

Run 44 (general module) opened by 26 February, deadline for protocol submission was 16 March. Results are available by 8 July

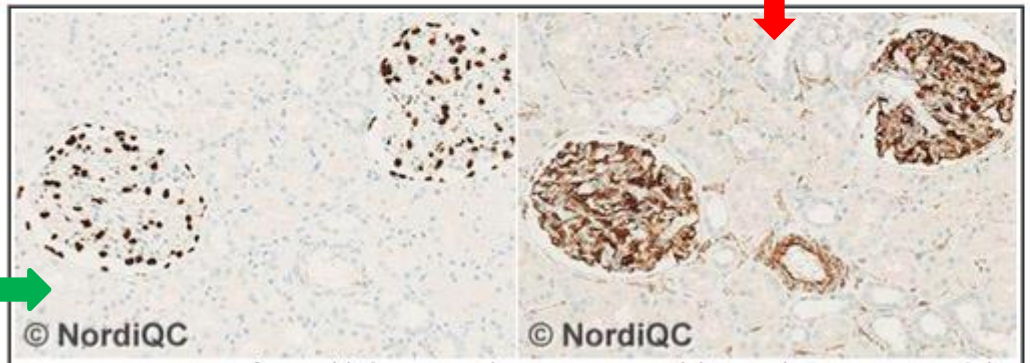
Run 45 (general module), **B20** (breast cancer module) **and H8** (HER-2 ISH module) opens by 12 August. Deadline for protocol submission is 10 September

NordiQC teaching events

The 2nd NordiQC Conference on Applied Immunohistochemistry
Aalborg, Denmark, June 9-12, 2015

NordiQC Workshop in Diagnostic Immunohistochemistry
Aalborg, Denmark, 16-18 September 2015
All seat taken. Enrolment for 2016 will soon be available

NordiQC Academy of Immunohistochemistry
Krakow, Poland, October 12-13, 2015

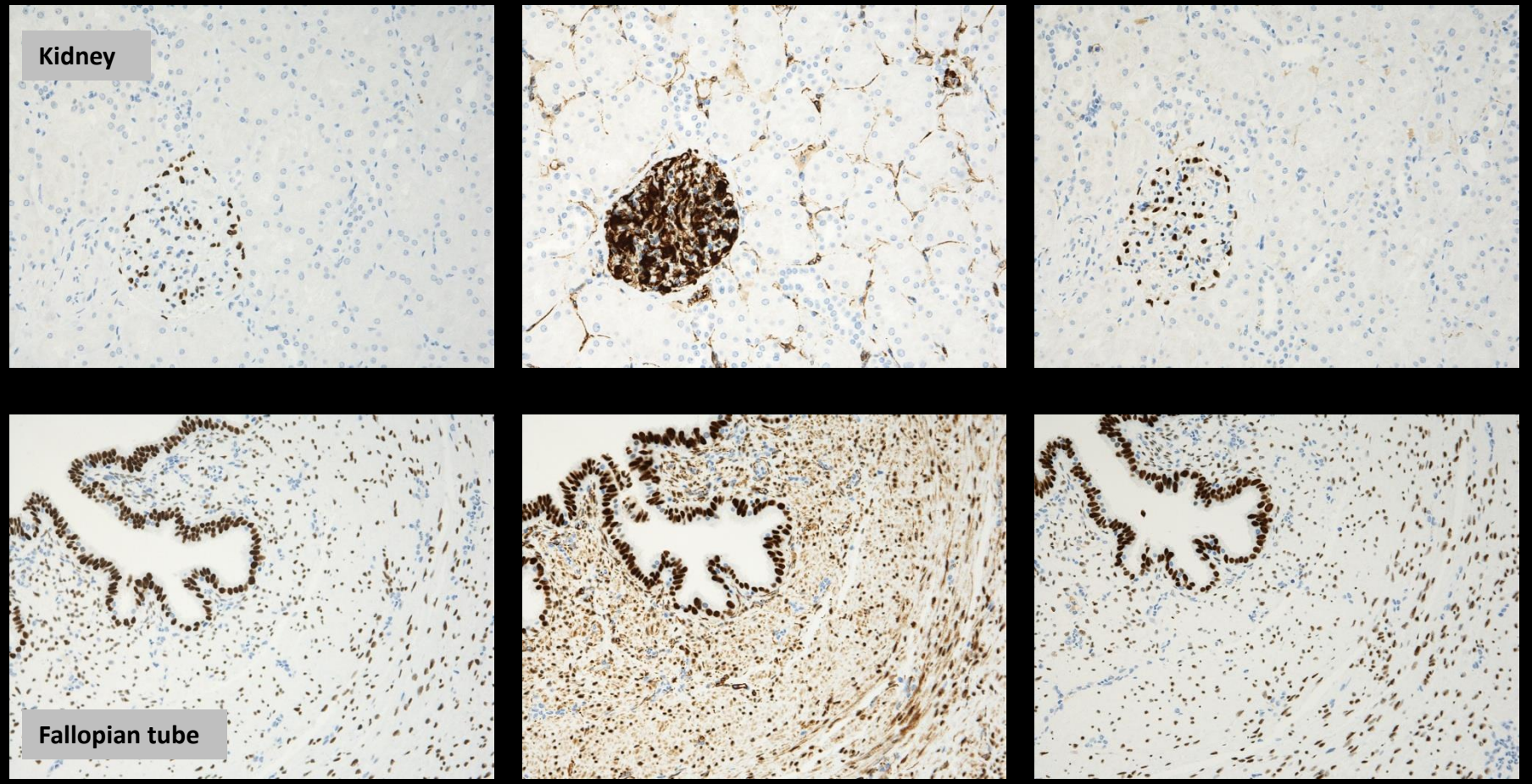


Run 43. WT1 staining of normal kidney using clone 6F-H2 in two labs. See how you can avoid the unwanted cytoplasmic reaction on WT1

WT-1 clone WT49
HIER High pH 20`/97°C

WT-1 clone 6H-F2
HIER High pH 20`/97°C

WT-1 clone 6H-F2
Pepsin 8`+ HIER High pH 20`/97°C



Proteolysis (Pepsin solution, RTU/Zytovision cat. no. ES-0001-50) followed by HIER

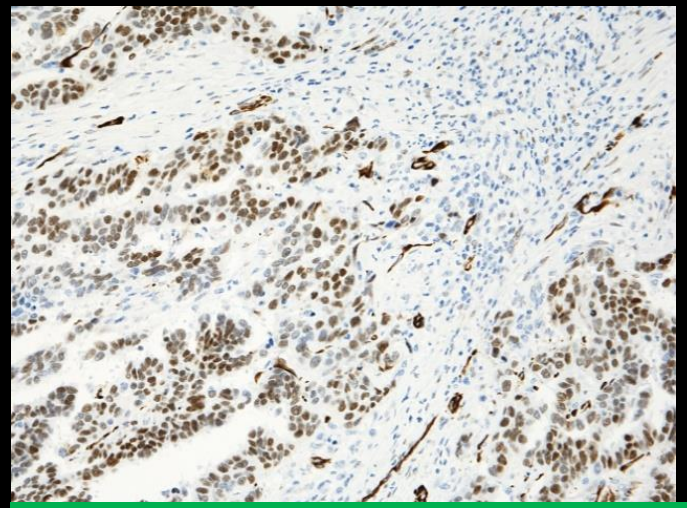
Technical aspects of immunohistochemistry & pitfalls - Analytical phase

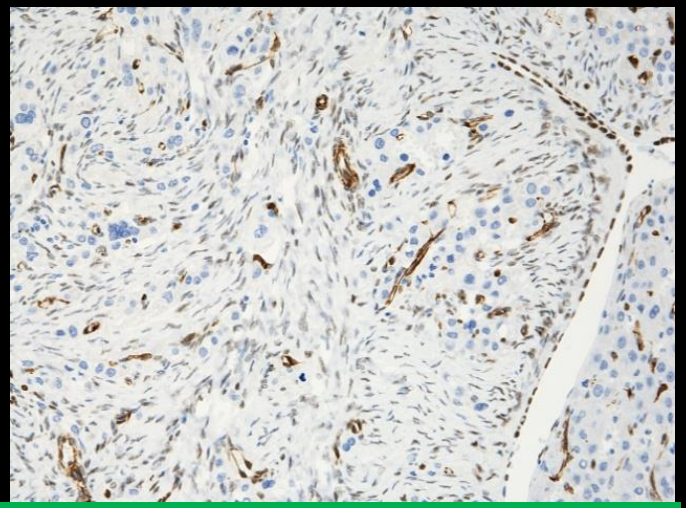
Combined pre-treatment (Enzymatic digestion followed by HIER)

Ovarian Serous Carcinoma

Ovarium/Peritoneum
Metastasis (Hepatocellular carc.)

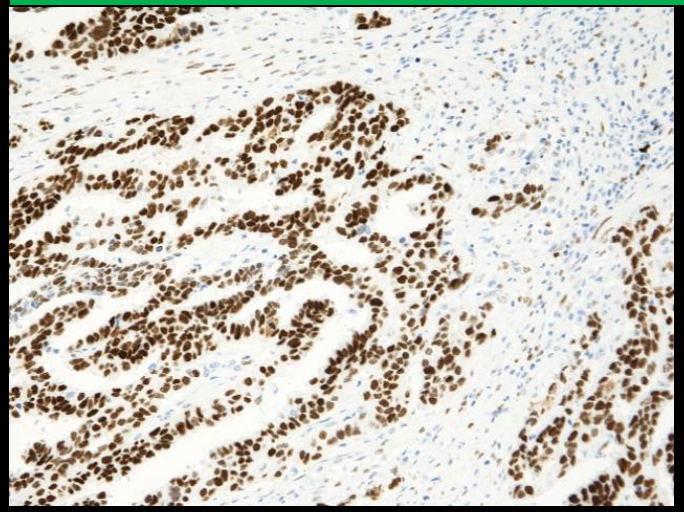
WT-1 clone 6H-F2
HIER High pH 20`/97°C

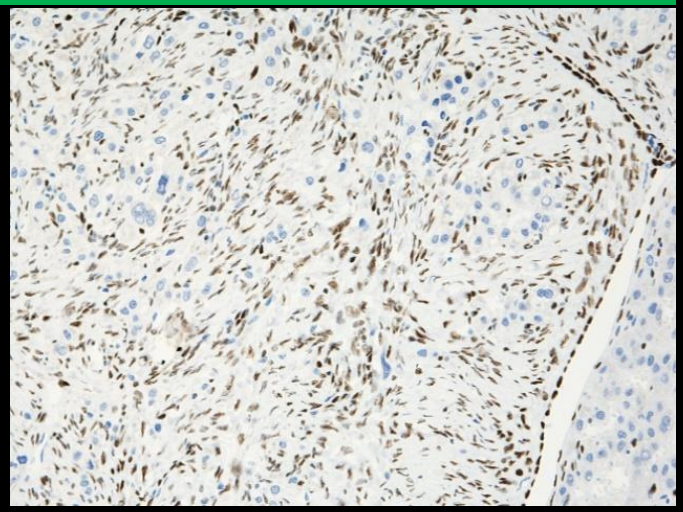




Improves sensitivity & signal to noise ratio

WT-1 clone 6H-F2
Pepsin 8`+ HIER High pH 20`/97°C





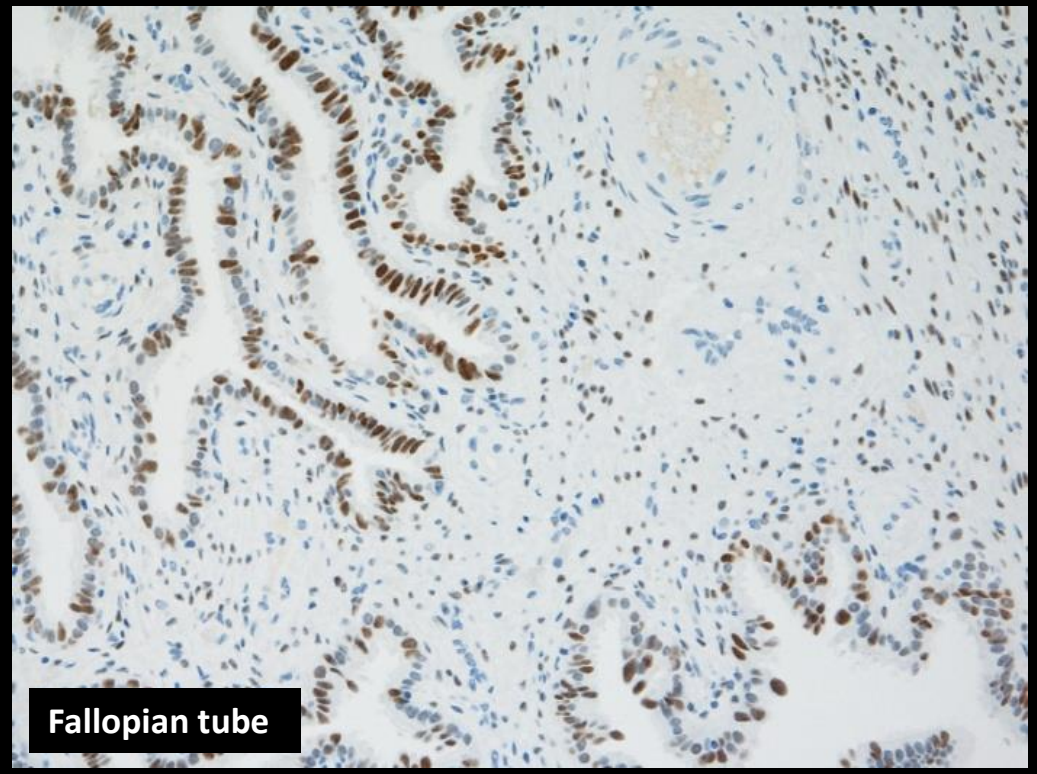
Proteolysis (Pepsin solution, RTU/Zytovision cat. no. ES-0001-50) followed by HIER

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

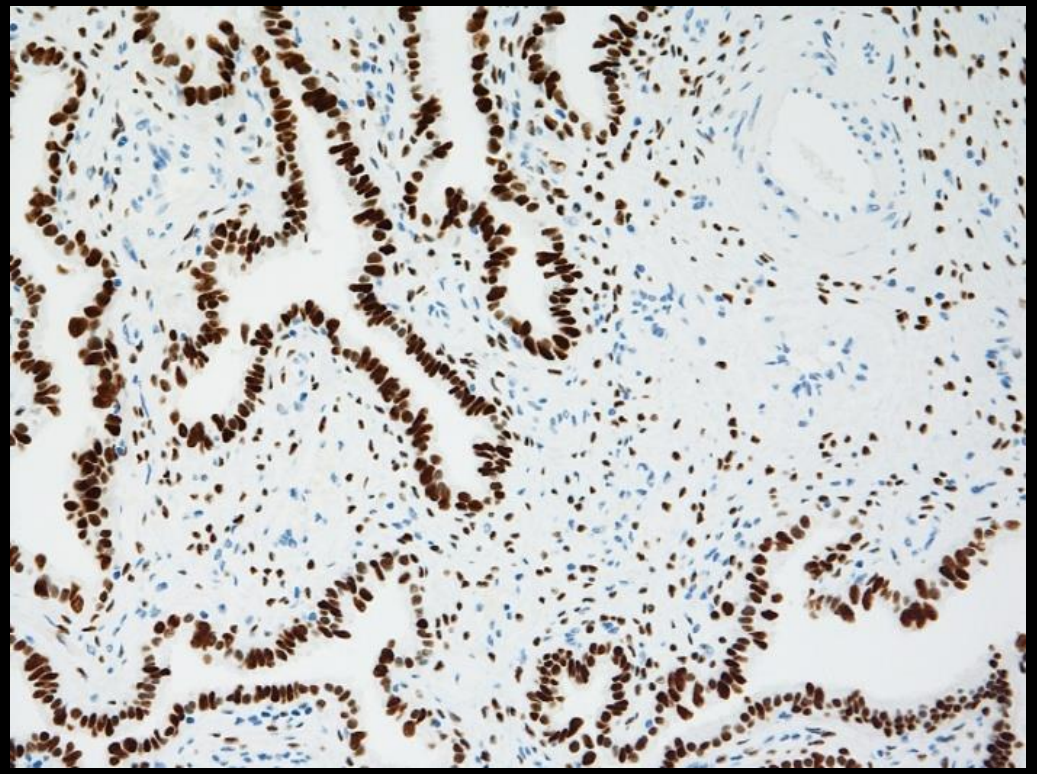
Combined pre-treatment (HIER followed by Enzymatic digestion)

WT1, EP122 1:25 (Omnis)

HIER TRS pH9 (24`/97°C)



HIER TRS pH9 (24`/97°C) + Pep © (3`)

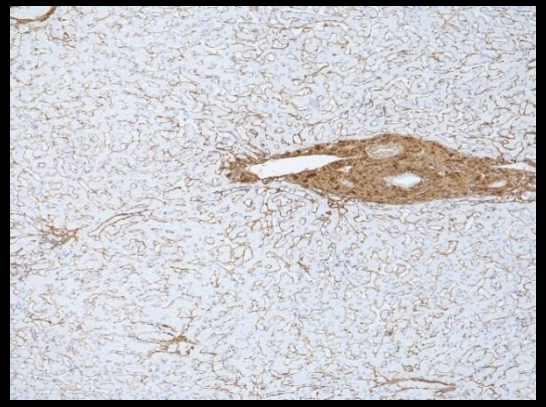
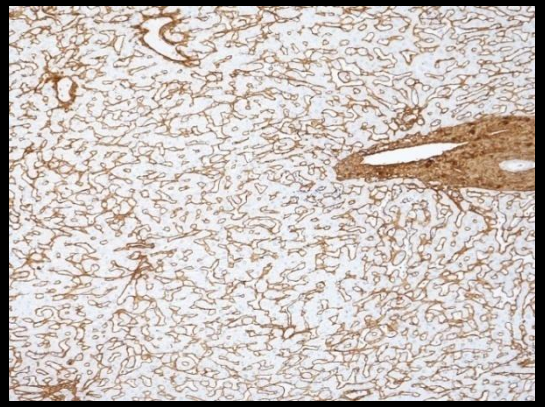
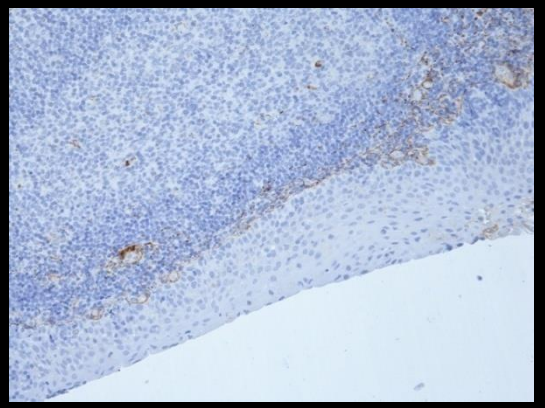
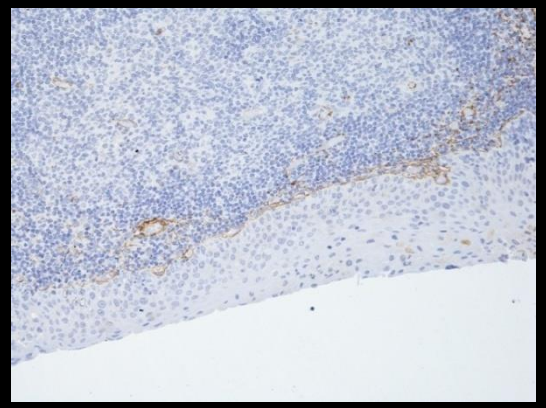
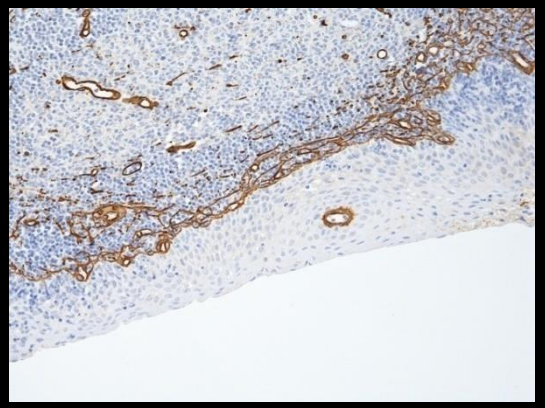


Pep © ~ Cytology Pepsin Solution (ZytoVision cat. no. ES-0002/50) / 3 min at 32°C

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Extracellulare matrix proteins: Combined pre-treatment (HIER followed by Enzymatic digestion)

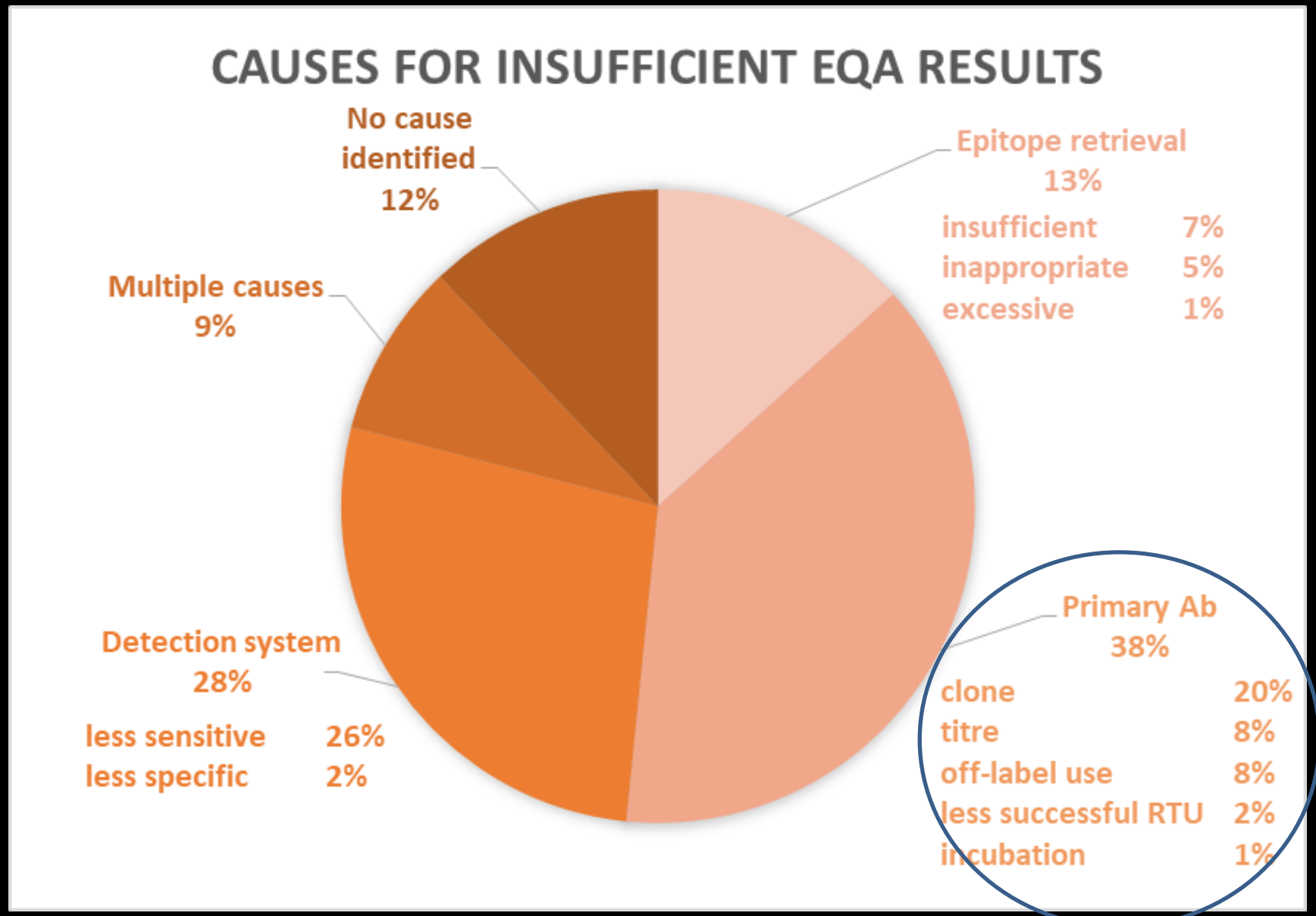
Collagen III (pAb 1:1000/ LSBio)

	HIER / Low pH TRS pH 6.1 (S1700)	HIER / High pH TRS pH 9 (Dako S2375)	HIER / Low pH + Pepsin 5` (RTU/ZytoVision)
Hepar			
Tonsil			

Pep © ~ Cytology Pepsin Solution (ZytoVision cat. no. ES-0002/50) / 3 min at 32°C

Pause

NordiQC External Quality Assurance program



App. 20-30% of all results are evaluated as insufficient

79 % of insufficient results are related to the choice/use of:

- **Epitope retrieval procedure**
- **Primary Ab (including stainer platform)**
- **Detection system**

False positive and/or negative results

Courtesy of Birgit Truumees et al (NordiQC): Poster presented at the annual USCAP meeting 2024

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Antibody company (producer) - Quote:

In the world of next generation immuno-oncology research, having confidence in your immunoassay results is vital.

Unfortunately, 75% of antibodies in today's market are non-specific or simply do not work at all.

Primary Antibodies

Volume 57(1): 7–8, 2009
Journal of Histochemistry & Cytochemistry
http://www.jhc.org

PERSPECTIVE

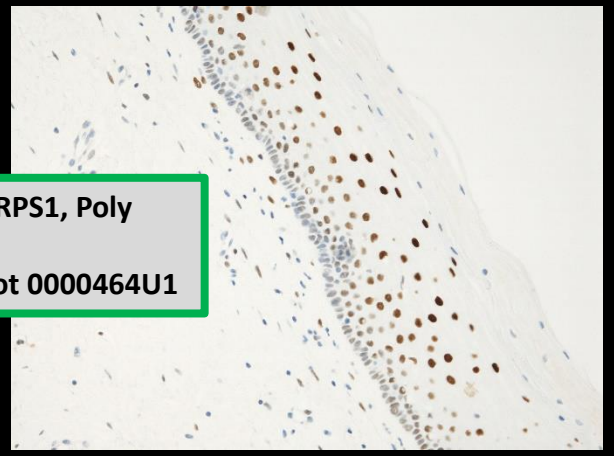
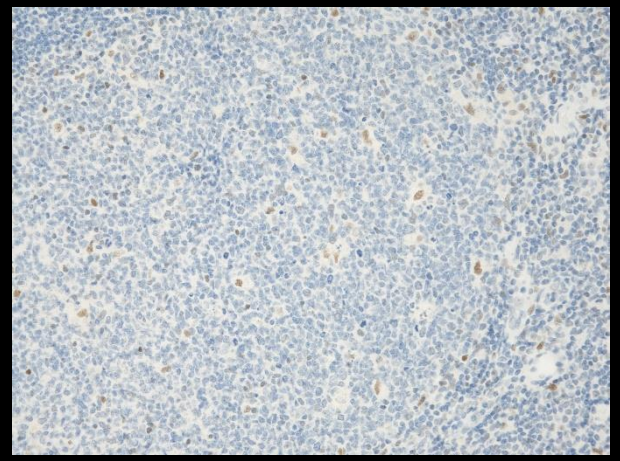
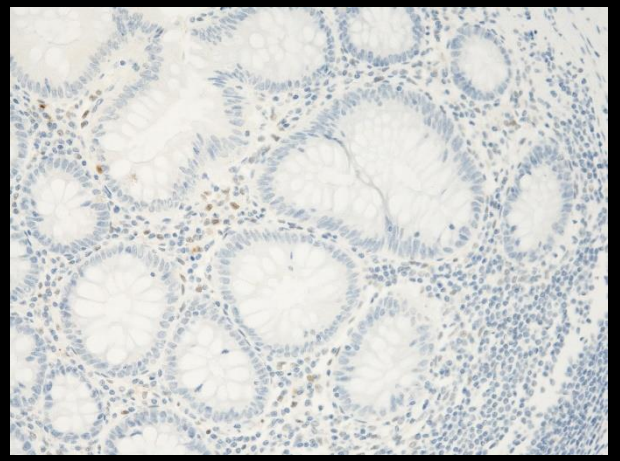
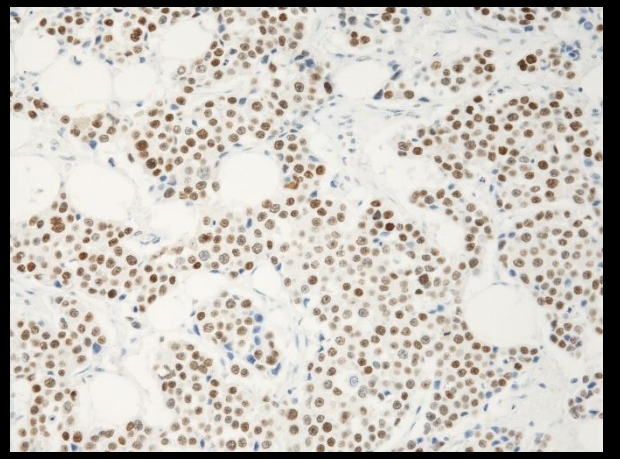
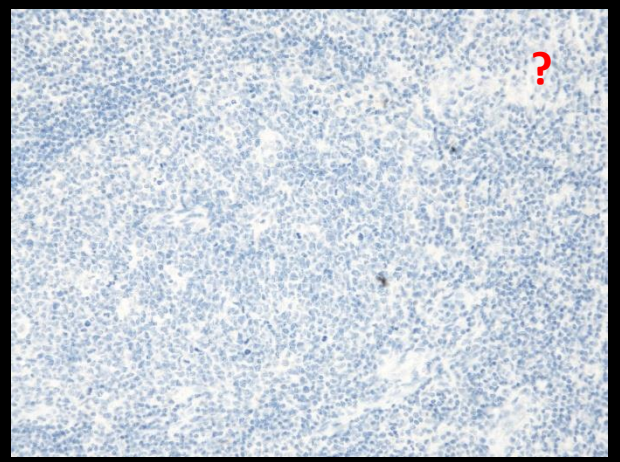
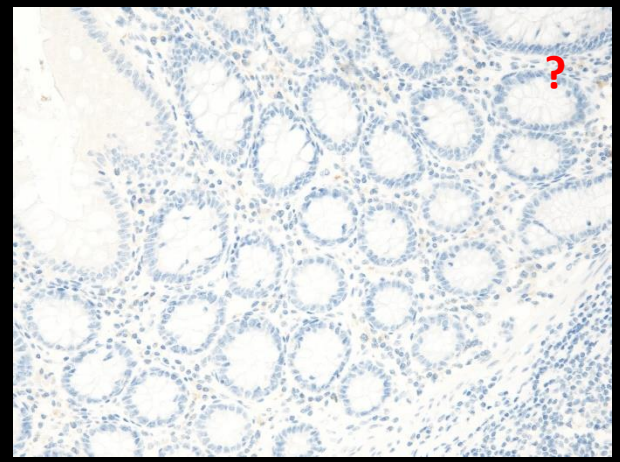
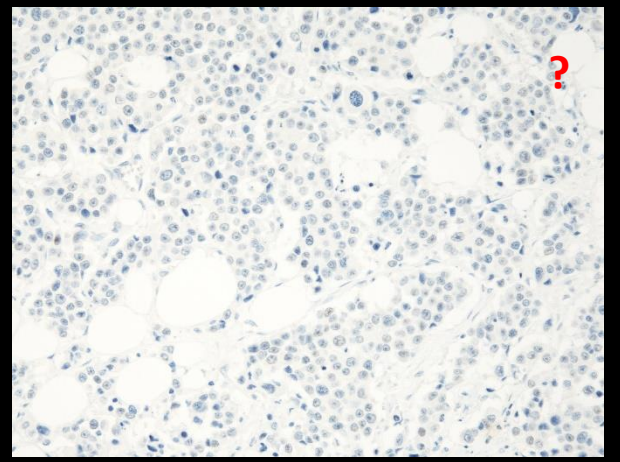
Commercial Antibodies: The Good, Bad, and Really Ugly

John R. Couchman
Biomedicine Institute, University of Copenhagen, Biocenter, Copenhagen, Denmark

SUMMARY The range of antibodies available commercially grows ever larger. Perhaps as a consequence, quality control is not always what it could and should be. Investigators must be aware of potential pitfalls and take steps to assure themselves that the specificity of each antibody is as advertised. Additionally, companies should provide the necessary information about the antigen and antibody to investigators, including references, so that the appropriate controls can be included. (J Histochem Cytochem 57:7–8, 2009)

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Antibody choice: TRPS1, Poly (Invitrogen) 1:400 - Poor lots (same vendor)

	Uterine ecto-cervix (HE)	Tonsil (LE)	Appendix (NE)	Breast Carcinoma
TRPS1, Poly Lot 0000464U1				
TRPS1, Poly Lot 000051339 Lot 000051034				

HIER High pH 24`; Flex+ Rabbit linker

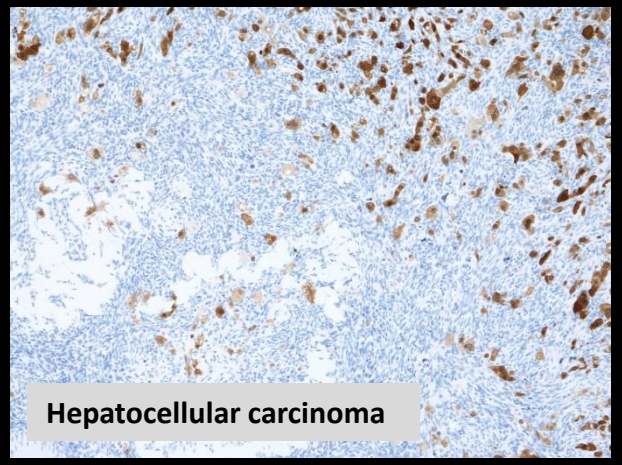
Technical aspects of immunohistochemistry & pitfalls - Analytical phase

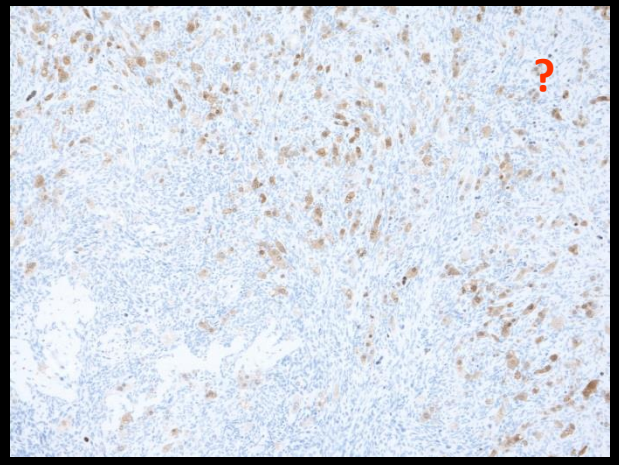
Antibody choice: Specificity (different vendors)

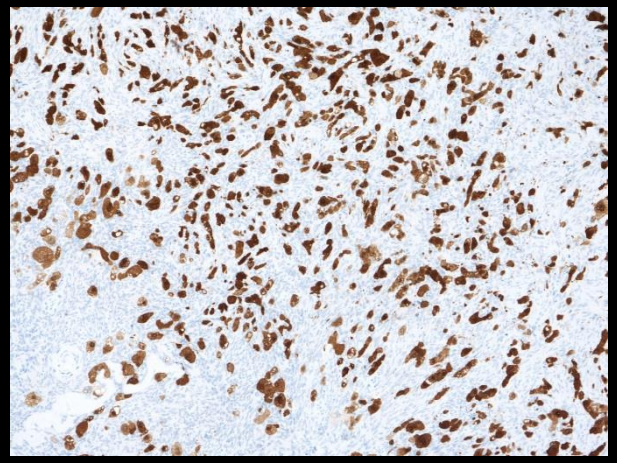
ARG1, SP156 Cell M 1:50

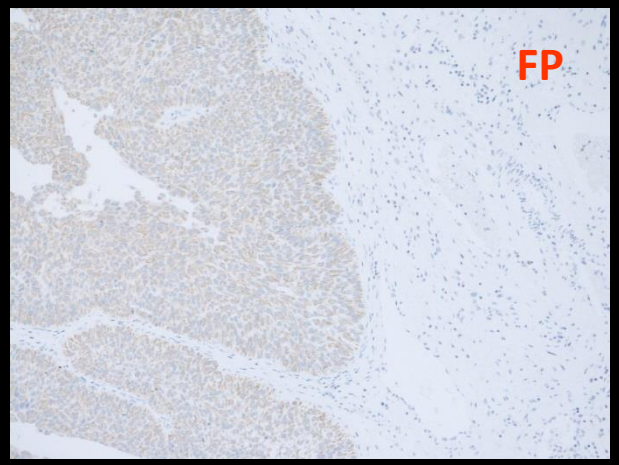
ARG1, SP156 Cell M 1:100

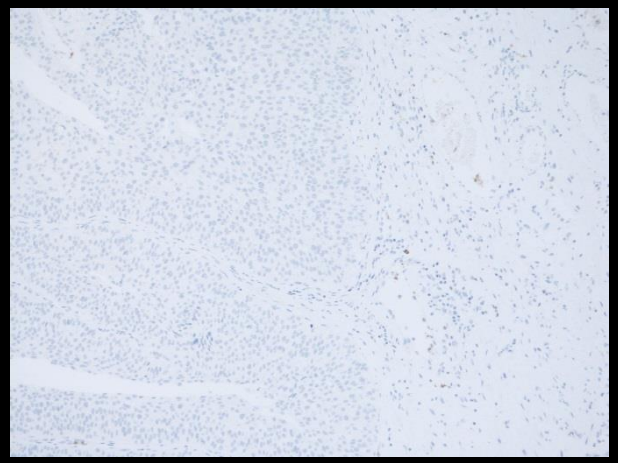
ARG1, SP156 Spring B. 1:25







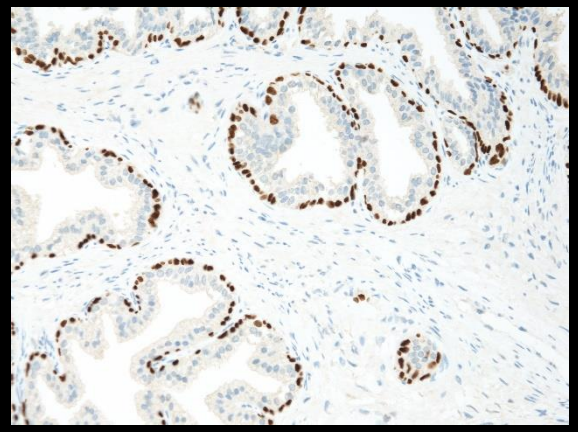


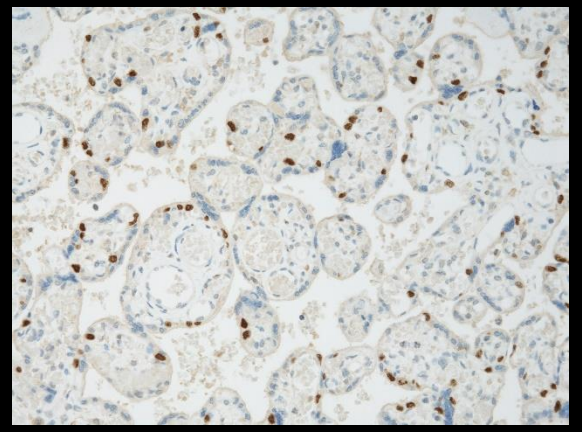


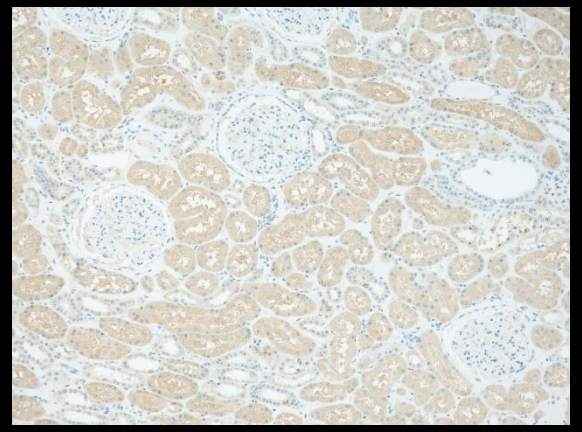
HIER High pH 24`; Flex+ Rabbit linker

Dako Envision Flex+ - Poor Polymer-HRP batch(es) on the Omnis

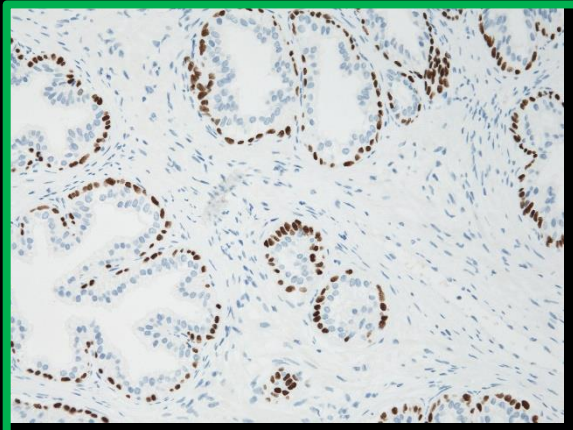
P40 - Standard Envision Flex+ system
(Poor HRP lot)

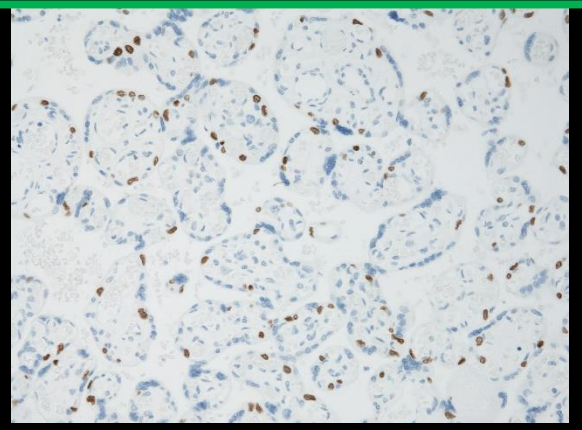


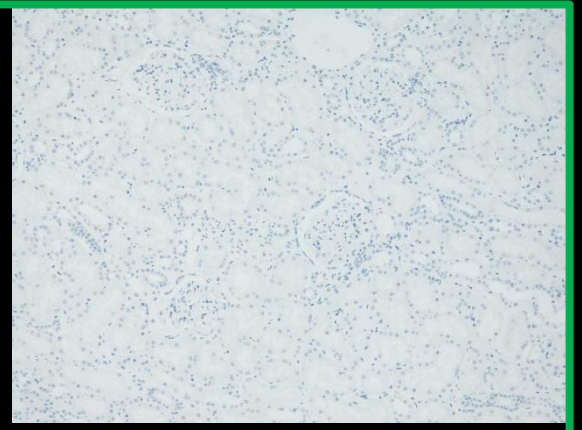




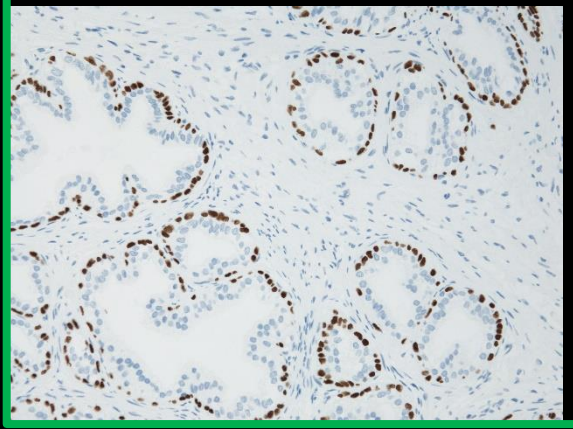
P40 - Standard Envision Flex+ system
(Poor HRP reagent substituted with Envision K4003)

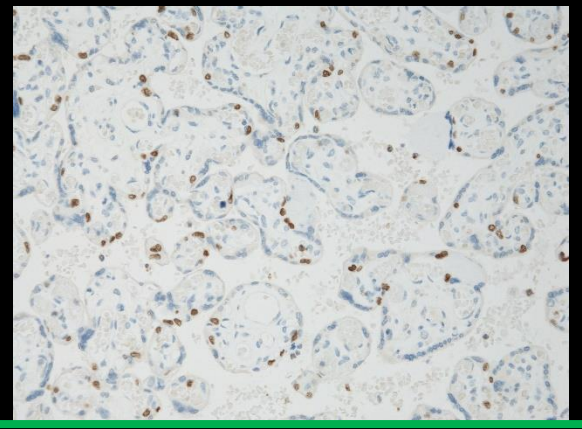


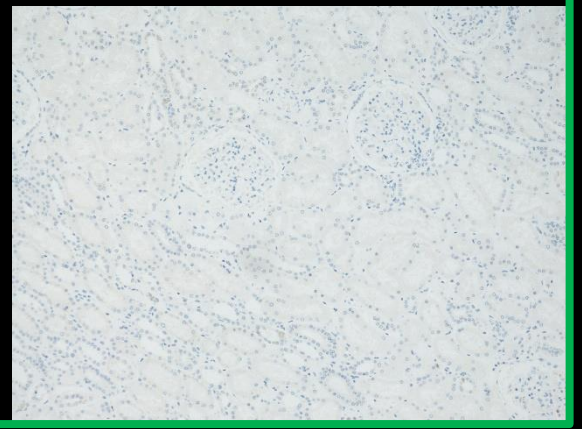




P40 - Quanto detection system (Thermo)
(Substituting all Dako`s detection reagents)



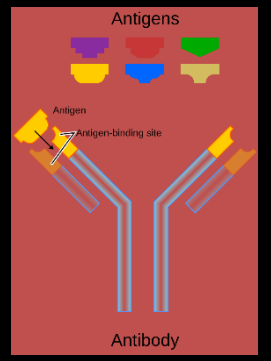




Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Parameters affecting antibody-antigen reactions in tissue

Antibody choice – Sensitivity/Specificity
Antibody Titer
Antibody performance related to the chosen automated platform
Antibody diluents



Incubation time
Incubation temperature
Sensitive to endogenous peroxidase blocking e.g., BCL6 (PG-B6p) and CD4 (1F6)

Storage of concentrated primary antibodies
Storage of diluted primary antibodies

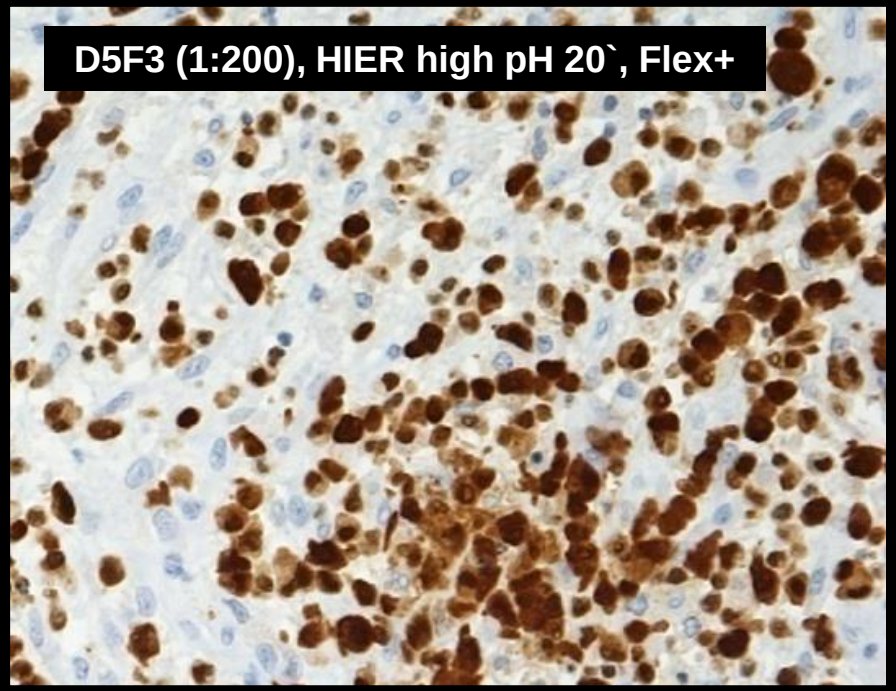
Provided that efficient antigen retrieval has been performed and a sensitive detection system has been used

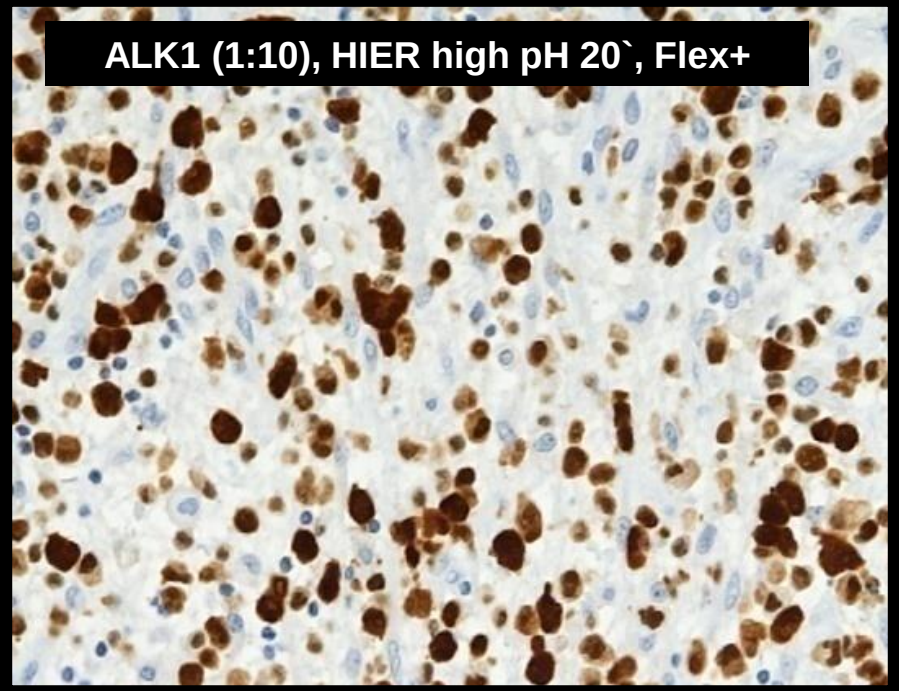
Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Primary antibody provides low sensitivity

Anaplastic lymphoma kinase (ALK)

Anaplastic large cell lymphoma (ALCL) (ALK-NPM rearrangement)

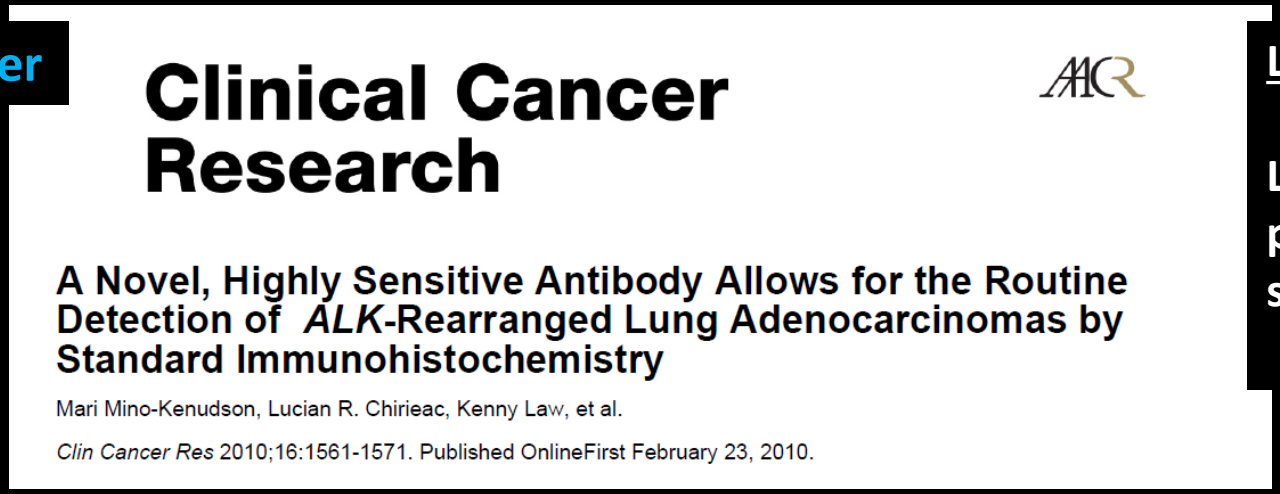




Anything wrong ?

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

IHC-Type 2 marker



Clinical Cancer Research

AACR

A Novel, Highly Sensitive Antibody Allows for the Routine Detection of *ALK*-Rearranged Lung Adenocarcinomas by Standard Immunohistochemistry

Mari Mino-Kenudson, Lucian R. Chirieac, Kenny Law, et al.

Clin Cancer Res 2010;16:1561-1571. Published OnlineFirst February 23, 2010.

Lung tumors

Low concentration of ALK Fusion protein (e.g., EML-4/ALK) = require a sensitive antibody for detection

Intended use &"fit-for-purpose"

Human Pathology (2013) **44**, 1656–1664

ELSEVIER

Human PATHOLOGY

www.elsevier.com/locate/humpath

Original contribution

Expression of anaplastic lymphoma kinase in Merkel cell carcinomas ☆

Bettina Ekvall Filtenborg-Barnkob MD*, Michael Bzorek HT*

Department of Pathology, Naestved and Slagelse Hospital, Hospital South, Denmark

Received 30 August 2012; revised 13 November 2012; accepted 13 November 2012

MCC

ALK,D5F3 = 94% pos

ALK,5A4 = 88% pos

ALK, ALK1 = 13% pos

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Table 1. **Antibodies and assessment marks for ALK (lung), run 65**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **5A4**	26 2 1 1 2 2 1	Leica Biosystems Monosan Abcam DBS Biocare Medical Zytomed Systems Invitrogen	8	9	14	4	49%	23%
mAb clone **OTI1A4***	19 1 1 1	Origene Nordic Biosite Cell Signaling Zeta Corporation	16	6	0	0	100%	73%
mAb clone **IHC509**	1	GenomeMe	0	0	1	0	-	-
rmAb clone **D5F3**	19	Cell Signaling	7	9	3	0	84%	36%
rmAb clone **ALK1**	3 1	Dako/Agilent Cell Marque	0	0	0	4	-	-
rmAb clone **QR017**	1	Quartett	0	1	0	0	-	-
rmAb clone **SP8**	1	BioGenex	0	0	0	1	-	-
rmAb clone **ZR305**	1	Zeta Corporation	0	0	1	0	-	-
Ready-To-Use antibodies								
mAb clone **5A4 PA0306**/PA0831 (VRPS)**[3]	2	Leica Biosystems	1	1	0	0	-	-
mAb clone **5A4 PA0306*/PA0831 (LMPS)**[4]	10	Leica Biosystems	4	3	2	1	70%	40%
mAb clone **5A4 API3041**	1	BioCare	0	0	1	0	-	-
mAb clone **5A4 CAM-0170**	1	Celnovte	0	1	0	0	-	-
mAb clone **5A4 MAD-001720QD**	1	Master Diagnostica	0	0	1	0	-	-
mAb clone **ALK1 GA641**	3	Dako/Agilent	0	0	0	3	-	-
mAb clone **ALK1 IR641**	4	Dako/Agilent	0	0	0	4	-	-
mAb clone **ALK1 790/800-2918 (LMPS)**[4]	10	Ventana/Roche	1	0	1	8	10%	10%
mAb clone **137E9E8 PA132**	1	Abcarta	0	0	0	1	-	-
mAb clone **OTI1A4 / 1A4 8344-C010**	1	Sakura Finetek	1	0	0	0	-	-
mAb clone **OTI1A4 / 1A4 GA785 (VRPS)**[3]	12	Dako/Agilent	12	0	0	0	100%	100%
mAb clone **OTI1A4 / 1A4 GA785 (LMPS)**[4]	4	Dako/Agilent	4	0	0	0	-	-
rmAb clone **D5F3 790-4794 (VRPS)**[3]	73	Ventana/Roche	62	7	1	3	95%	85%
rmAb clone **D5F3 790-4794 (LMPS)**[4]	48	Ventana/Roche	36	9	3	0	94%	75%
rmAb clone **SP8 RMPD007**	1	Diagnostic BioSystems	0	0	0	1	-	-
Total	256		152	46	28	30		
Proportion			59%	18%	11%	12%	77%	

1) Proportion of sufficient stains (optimal or good) (≥5 assessed protocols).
2) Proportion of Optimal Results (≥5 assessed protocols).
3) Vendor Recommended Protocol Settings (VRPS) to a specific RTU product applied on the vendor recommended platform(s) (≥5 assessed protocols).
4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product (≥5 assessed protocols).
*) OTI1A4 is called 1A4 by some vendors
**) Product no. PA0306 has been terminated and replaced by PA0831.

NQC Run 45, 51, 57 and 65 (ALK Lung)

87 protocols were based on clone ALK1:

Only two protocols (2%) were assessed as sufficient

NQC Run 65 (assessment spring/summer 2022)

1/21 protocols were assessed as sufficient

Don`t use clone ALK1 to detect ALK rearranged lung adenocarcinomas - provides too low sensitive

The clone ALK1 does not ”fit-for-purpose”

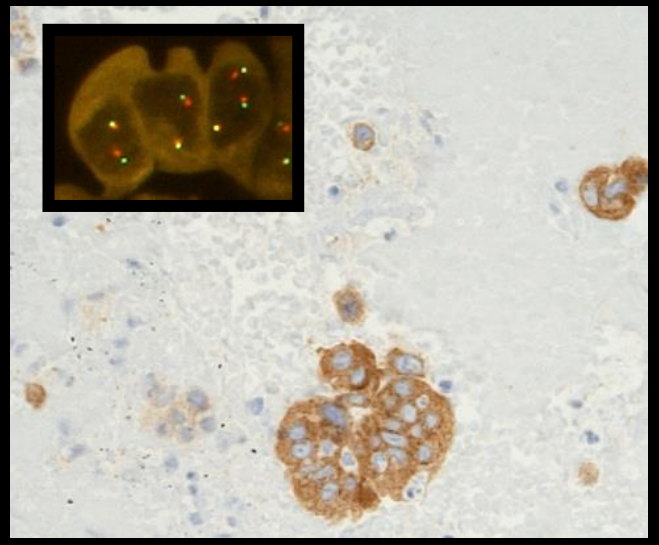
D5F3, OTI1A4, 5A4

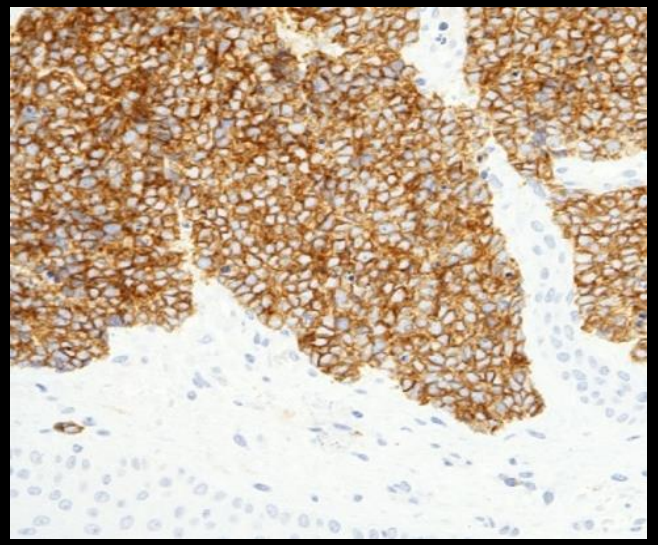
Lung AC (ALK-EML4)

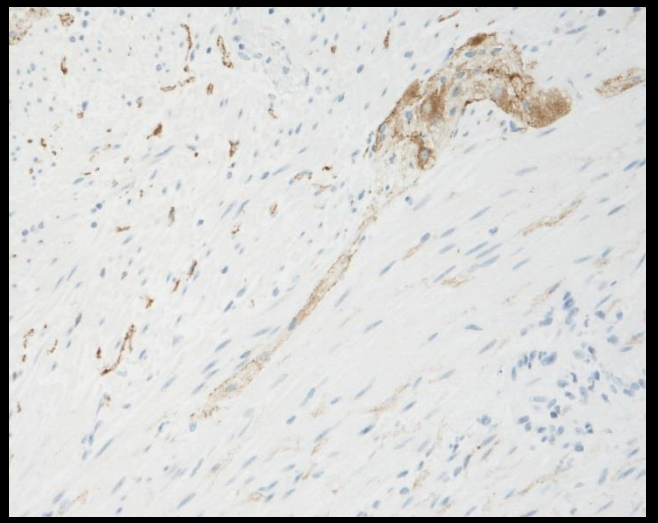
MCC (Skin)

Appendix

ALK, D5F3 (1:200)

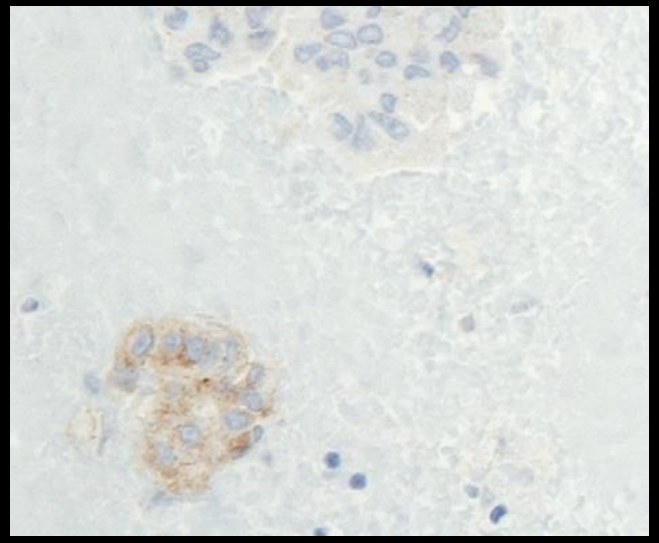


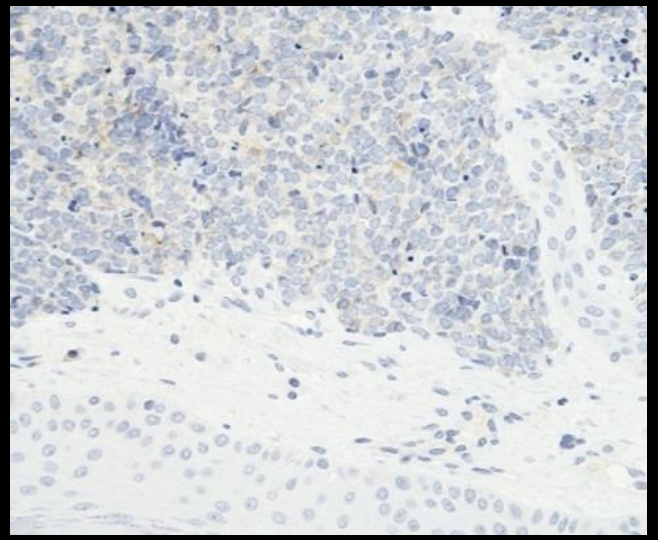


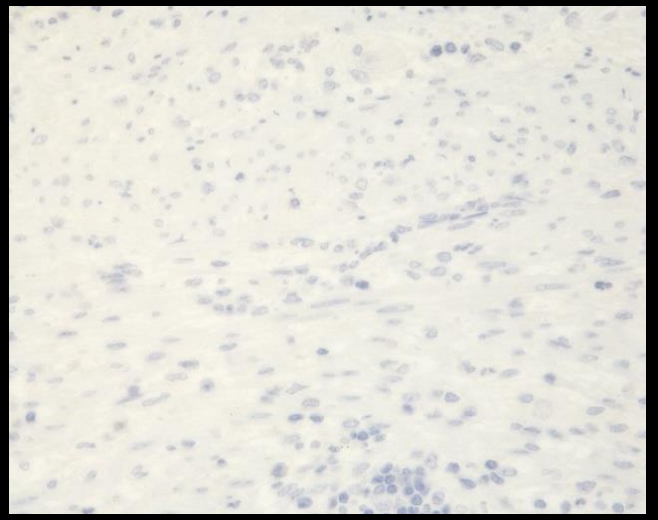


Ganglion and peripheral nerve cells

ALK, ALK1 (1:10)







HIER in high pH buffer 20`, Flex+

Clone ALK1 provides low sensitivity

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Primary antibody provides low sensitivity

URO II/III

Table 1. **Antibodies and assessment marks for URO II/III, run 59**

Concentrated antibodies	*Reactivity*	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb **BC21**	*URO II*	24 3	BioCare Medical Zytomed Systems	13	10	3	1	85%	37%
mAb **AU-1**	*URO III*	2	Cell Marque	-	-	-	2	-	-
rmAb **SP73**	*URO III*	7 1	Cell Marque Immunologic	-	-	1	7	0%	0%
rmAb **ERP18799**	*URO II*	1	ABCAM	-	1	-	-	-	-
rmAb **EP321**	*URO III*	1	Bio SB	-	-	1	-	-	-
rpAb **AB82173**	*URO III*	2	ABCAM	-	-	-	2	-	-
Ready-To-Use antibodies								Suff.[1]	OR.[2]
mAb **BC21 AVI 3051 KG**	*URO II*	1	Biocare Medical	-	1	-	-	-	-
mAb **BC21 API 3051 AA**		1	Biocare Medical	-	1	-	-	-	-
mAb **BC21 MAD-000773QD**		1	Vitro SA	1	-	-	-	-	-
mAb **BC21 MSG102**		1	Zytomed Systems	-	1	-	-	-	-
mAb **BC21+BC17 API 3094 AA**	*URO II/III*	3	Biocare Medical	-	2	1	-	-	-
rmAb **SP73 760-4533 (VRPS)[3]**	*URO III*	1	Roche/Ventana	-	-	1	-	-	-
rmAb **SP73 760-4533 (LMPS)[4]**		16	Roche/Ventana	-	-	2	14	0%	0%
rmAb **SP73 345R-17/18**		1	Cell Marque	-	-	-	1	-	-
Total		66		14	16	9	27		
Proportion				21%	24%	14%	41%	45%	

1) Proportion of sufficient stains (optimal or good). (≥5 assessed protocols).
2) Proportion of Optimal Results (≥5 assessed protocols).
3) Vendor Recommended Protocol Settings (VRPS) to a specific RTU product applied on the vendor recommended platform(s) (≥5 assessed protocols).
4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product (≥5 assessed protocols).

Urothelial carcinomas - The problem:

Abs raised exclusively against Uroplakin III show too low analytical sensitivity - detecting urothelial cancers among carcinomas of unknown origin.

RESEARCH ARTICLE

OPEN

Comparison of Antibodies to Detect Uroplakin in Urothelial Carcinomas

Heidi L. Kristoffersen, BMS, Rasmus Røge, MD, PhD, and Søren Nielsen, BMS

(*Appl Immunohistochem Mol Morphol* 2022;30:326–332)

TABLE 3. Results, H-scores

	mmAb BC21 Uroplakin II			rmAb SP73 Uroplakin III			mmAb AU-1 Uroplakin III		
	n	n%	Mean H-score	n	n%	Mean H-score	n	n%	Mean H-score
Urothelial carcinomas (n = 58)									
Positive total	40	69	72	17	29	19	11	19	4
High-expressor (H-score 150-300)	8	13	182	0	0	0	0	0	0
Medium-expressor (H-score 10-149)	23	38	60	8	13	36	2	3	10
Low-expressor (H-score 1-9)	9	15	6	9	15	3	9	15	3
Negative (H-score <1)	18	31	0	41	71	0	47	81	0
Nonurothelial carcinomas (n = 111)									
Positive total	3	3	18	0	0	0	0	0	0
High-expressor (H-score 150-300)	0	0	0	0	0	0	0	0	0
Medium-expressor (H-score 10-149)	2	2	27	0	0	0	0	0	0
Low-expressor (H-score 1-9)	1	1	2	0	0	0	0	0	0
Negative (H-score <1)	108	97	0	111	100	0	111	100	0

mmAb indicates mouse monoclonal antibody; rmAb, rabbit monoclonal antibody.

Demonstrated that UPII klon BC21 outperforms UPIII Abs and at present should be the preferred choice of UP marker.

However, should be used in a panel with other Urothelial markers as e.g., GATA3 due to moderate analytical sensitivity (and due to positivity in a minority of nonurothelial carcinomas - app. 3%)

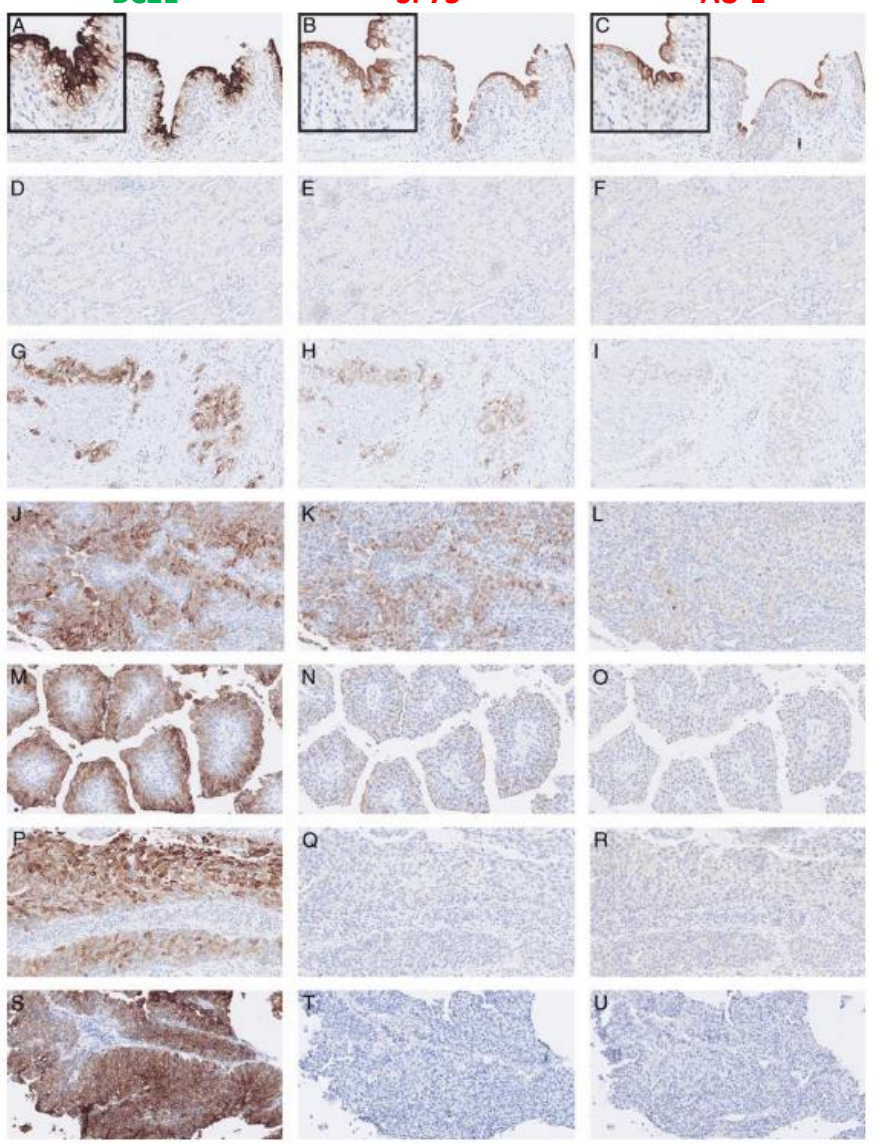
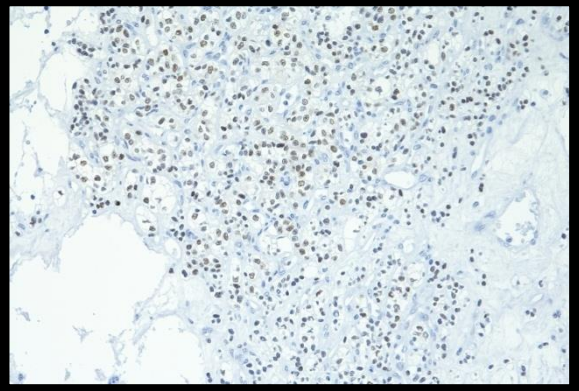
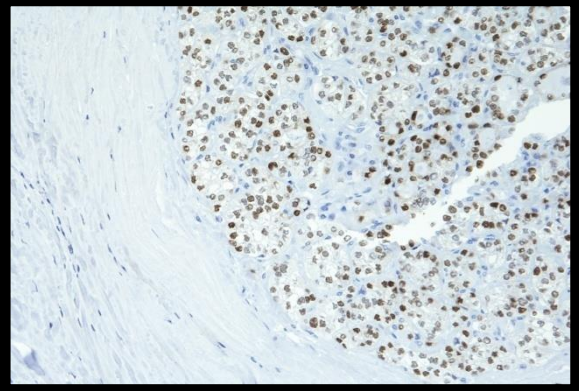
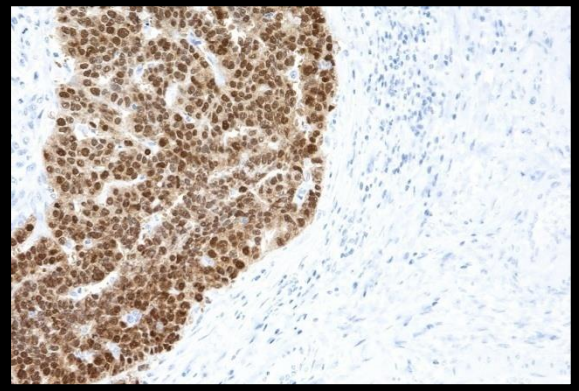
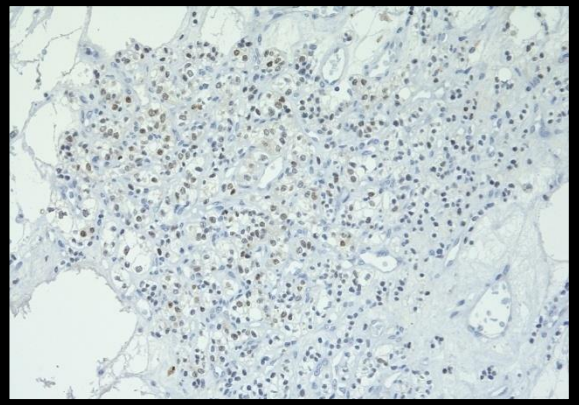
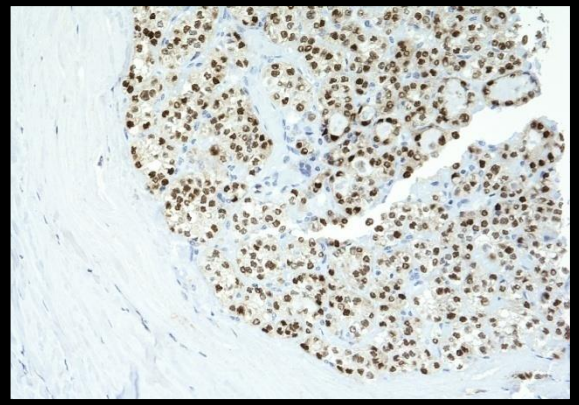
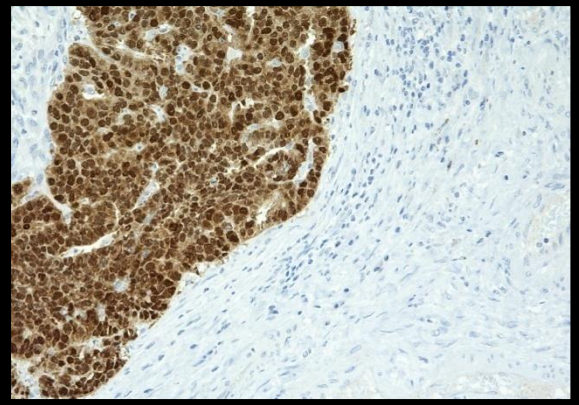


FIGURE 1. Examples of UP staining in normal tissue and UCs with various intensity using UPII mmAb clone BC21 (A, D, G, J, M, P, S), UPIII rmAb clone SP73 (B, E, H, K, N, Q, T), and UPIII mmAb clone AU-1 (C, F, I, L, O, R, U). A–C: normal urethra, insert high powerfield. D–F: normal kidney. G–I, J–L, M–O, P–R, and S–U: different UCs. mmAb indicates mouse monoclonal antibody; rmAb, rabbit monoclonal antibody; UCs, urothelial carcinomas; UP, uroplakin.

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Primary antibody provides low specificity and/or poor signal-to-noise ration

Which antibody ?

	Renal Cell Carcinoma (CC)	Thyroid Carcinoma (Pa)	Ovary Carcinoma (Se)
Pax-8, MRQ-50 (1:2000) HIER High pH/Flex+ (AS)			
Pax-8, BC12 (1:150) HIER High pH/Flex+ (AS)			

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Primary antibody provides low specificity and/or poor signal-to-noise ration

***Liau J-Y et al.: Appl Immunohistochem Mol Morphol .* 2016 Jan;24(1):57-63**

Demonstrated that neuroendocrine tumors (NET´s) from a large variety of organs were immuno-reactive with the two less specific antibodies (pAb Proteintech & mAb MRQ-50) - cross-reacting with other PAX proteins

Also, all NET`s were immuno-negative with the two monoclonal antibodies raised against the C-terminal part of PAX8 protein (PAX8R1 & BC12)

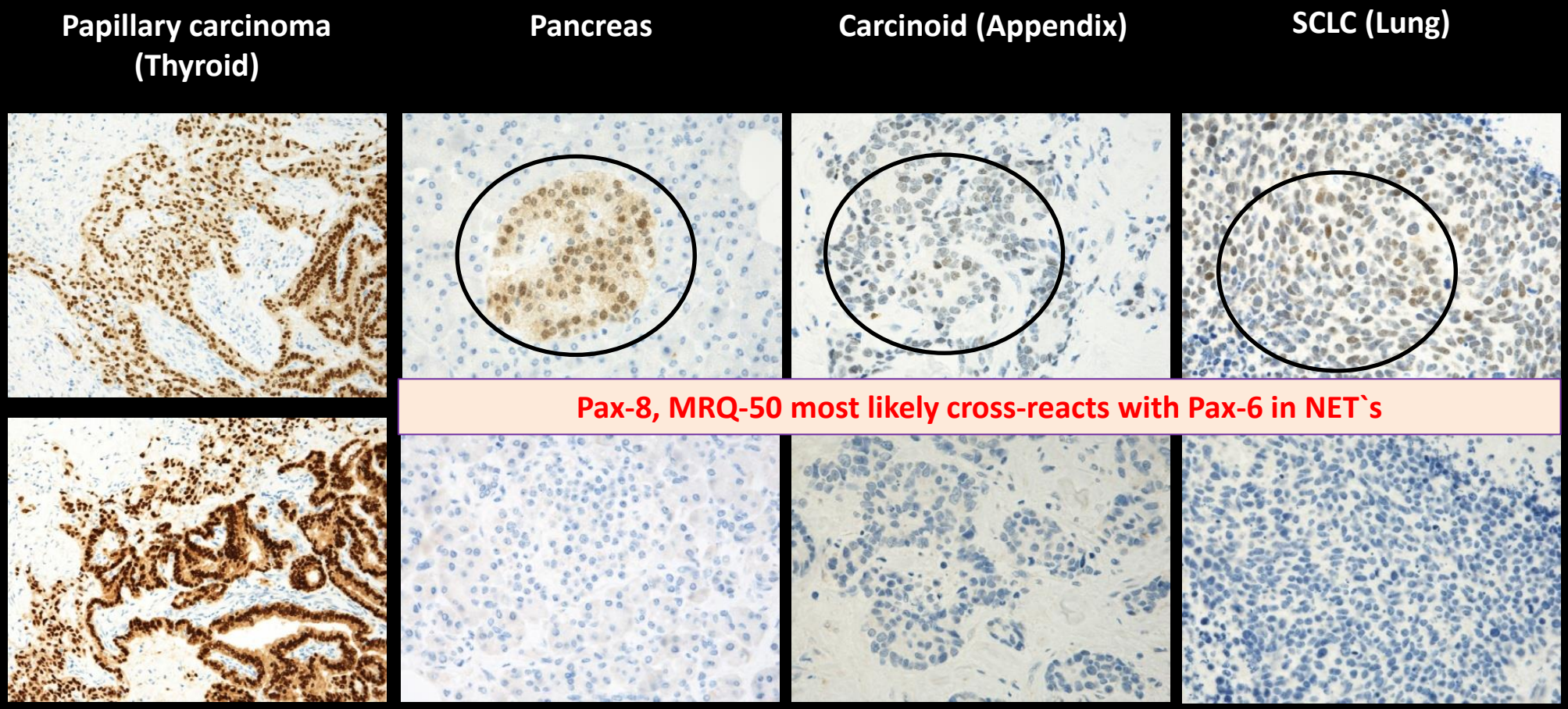
Moretti L et al. : *Mod Pathol. 2012; 25 : 231-236*

Demonstrated that an N-terminal PAX-8 polyclonal antibody cross-react with N-terminal region of PAX-5 and is responsible for reports of PAX-8 positivity in malignant lymphomas.

Also, PAX8 mRNA levels were not detected in any of the B-cell lymphoma cell lines studied. These results indicate that benign and malignant B-cells do not express PAX8.

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Primary antibody provides low specificity and/or poor signal-to-noise ration



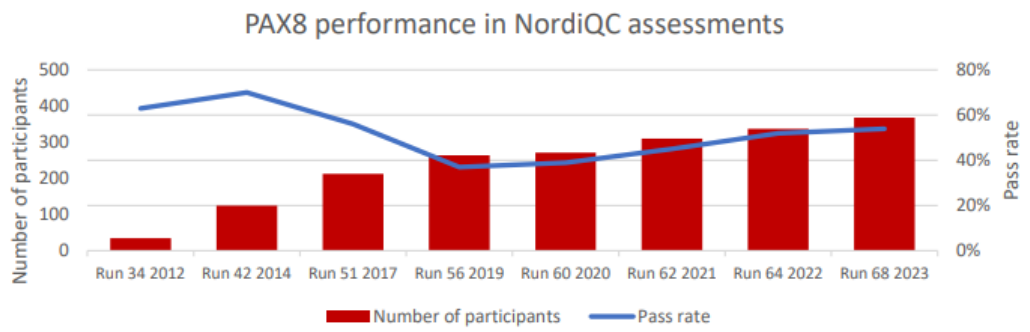
Pax8, MRQ-50 most likely raised against the N-terminal part of the PAX8 protein (cross-reacts with other PAX proteins)

Pax8, ZR1 or BC12 raised against the C-terminal part of the PAX8 protein (no cross-reacting with other PAX proteins)

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Primary antibody provides low specificity and/or poor signal-to-noise ration

Graph 1. **Proportion of sufficient results for PAX8 in the eight NordiQC runs performed**



Conclusion
Optimal staining results could be obtained with the rmAb clones **SP348**, **ZR-1**, **MXR013**, **GR002**, **OR016**, and **RM436**. Irrespective of the clone applied, efficient HIER, use of a sensitive 3-step polymer/multimer based detection system and careful calibration of the primary antibody were the most important prerequisites for an optimal staining result.
The rmAb clone **SP348** gave encouraging results and a high proportion of sufficient results on the main fully automated platforms and no cross-reaction with e.g. PAX5 was observed. No optimal results were however observed on the Bond Platform. The mAb clone **MRQ-50** provided a poor performance especially on the Ventana BenchMark and Dako Omnis platforms and at the same time also labelled PAX5 in B-cells. The **EP331** also provided a low pass rate due to aberrant nuclear staining reaction in non-PAX8 expressing cells and poor signal-to-noise ratio.

PAX8 (Run 68, 2023)

Clone MRQ-50 ?

Cross-reactivity with PAX5 resulting in a distinct nuclear staining reaction of B-cells for antibodies raised against the N-terminal part of PAX8 was seen in 34% (125/368) of the returned slides (see Figs. 5a and 5b). This reaction applied for all polyclonal Abs and mAb clones MRQ-50, C12A32, IHC008, H5A8, PAX8R1 and rmAb 2774R. Within the last couple of years well-performing rmAbs without cross reactivity has been introduced to the market (see Table 1). Based on this, cross-reactivity with PAX5 was downgraded due to the risk of misinterpretation in the diagnostic work-up of CUP. The diagnostic challenges and different reaction profiles related to the choice of PAX8 Ab has e.g. been described by Kamaljeet Singh et al.; *AIMM 2020, Aug;28(7):558-561; Comparison of PAX8 Expression in Breast Carcinoma Using MRQ-50 and BC12 Monoclonal Antibodies* and Tacha D et al., *AIMM 2013, Jan;21(1):59-63; PAX8 mouse monoclonal antibody [BC12] recognizes a restricted epitope and is highly sensitive in renal cell and ovarian cancers but does not cross-react with b cells and tumors of pancreatic origin*.

81% (101/125) of the protocols (Conc/RTU formats) showing cross-reactivity with PAX5 was based on the mAb clone MRQ-50

- 20% (20/101) of the protocols provided sufficient result – none being optimal

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Primary antibody provides low specificity and/or poor signal-to-noise ration

Table 1. **Antibodies and assessment marks for SATB2, run 58**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **CL0276**	5 2 1	Atlas Antibodies Sigma Aldrich Novus Biologicals	0	0	0	8	0%	0%
mAb clone **CL0320**	1	Atlas Antibodies	0	0	1	0	-	-
mAb clone **SATBA4B10**	3 2 2	Abcam Santa Cruz Zytomed Systems	0	0	2	5	0%	0%
mAb clone **OTI5H7**	1	ZSBio	1	0	0	0	-	-
rmAb clone **EP281**	30 12 1 1 1 1	Epitomics Cell Marque Immunologic BioSB Biocare Medical Unknown	22	14	4	6	78%	82%
rmAb clone **SP281**	4 1	Abcam Spring Bioscience	2	1	1	1	60%	40%
rmAb clone **ZR167**	1	Nordic Biosite	1	0	0	0	-	-
rmAb clone **EPNCIR130A**	5	Abcam	0	0	0	5	0%	0%
pAb **HPA001042**	5	Sigma Aldrich	0	0	2	3	0%	0%
pAb **Ab69995**	1	Abcam	0	0	0	1	-	-
Ready-To-Use antibodies							Suff.[1]	OR[2]
rmAb clone **EP281 384R-17/18**	19	Cell Marque	7	10	1	1	89%	37%
rmAb clone **EP281 PR/HAR239**	2	PathnSitu	2	0	0	0	-	-
rmAb clone **EP281 API3225**	1	Biocare Medical	0	1	0	0	-	-
rmAb clone **EP281 MAD-000747QD**	1	Máster Diagnostica	0	0	1	0	-	-
rmAb clone **EP281 BSB3199**	2	BioSB	0	0	0	2	-	-
Total	105		35	26	12	32	-	
Proportion			33%	25%	11%	31%	58%	

1) Proportion of sufficient staining (optimal or good) (≥5 assessed controls)

FP/FN

FP/FN

Inferior clones (run 58): Display same reaction patterns in run 64

Table 1. **Antibodies and assessment marks for SATB2, run 64**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **SATBA4B10**	1 3 3	Abcam Santa Cruz Zytomed Systems	0	1	6	0	14%	0%
rmAb clone **EP281**	13 66 1 3 1 5 3	Epitomics Cell Marque Diagnostic BioSystems BioSB Biocare Medical Gennova Scientific Zeta Corporation	48	28	8	8	83%	52%
rmAb clone **SP281**	4 1	Abcam Zytomed Systems	0	4	0	1	80%	0%
rmAb clone **QR023**	1	Quartett	1	0	0	0	-	-
rmAb clone **ZR167**	1	Zeta Corporation	0	0	0	1	-	-
pAb **HPA001042**	4	Sigma Aldrich	0	1	1	2	-	-
pAb **Ab69995**	1	Abcam	0	0	0	1	-	-

FN

FP/FN + aberrant cytoplasmic staining

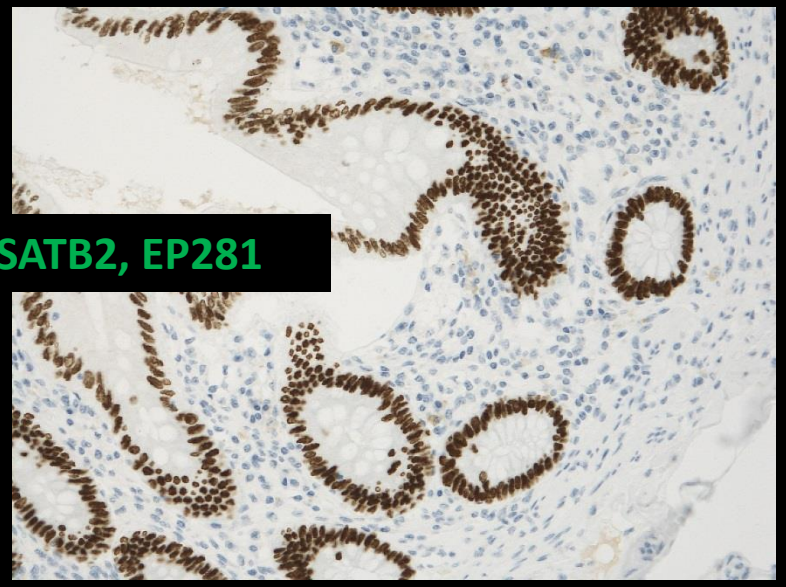
FN + aberrant cytoplasmic staining

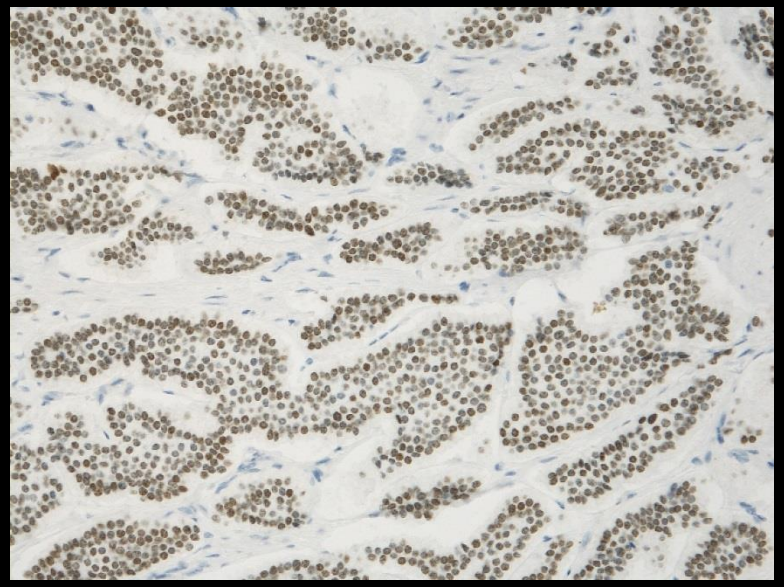
Impossible to calibrate correctly

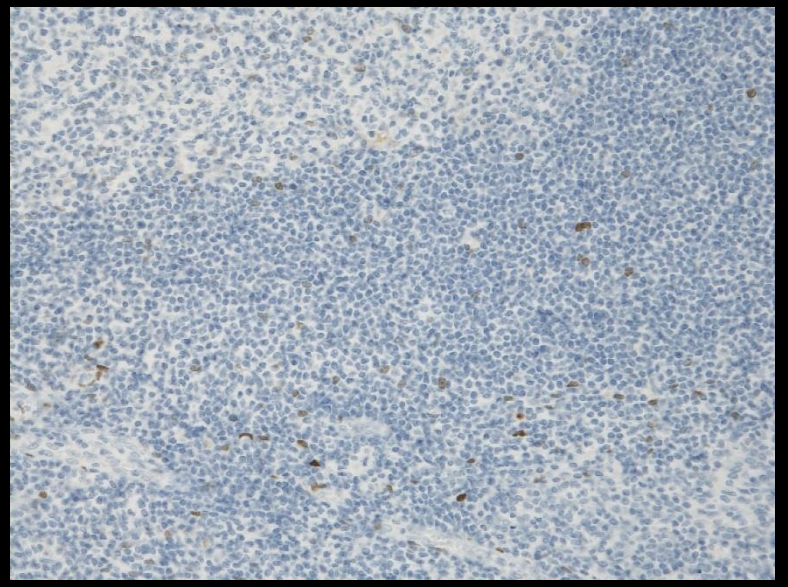
Appendix

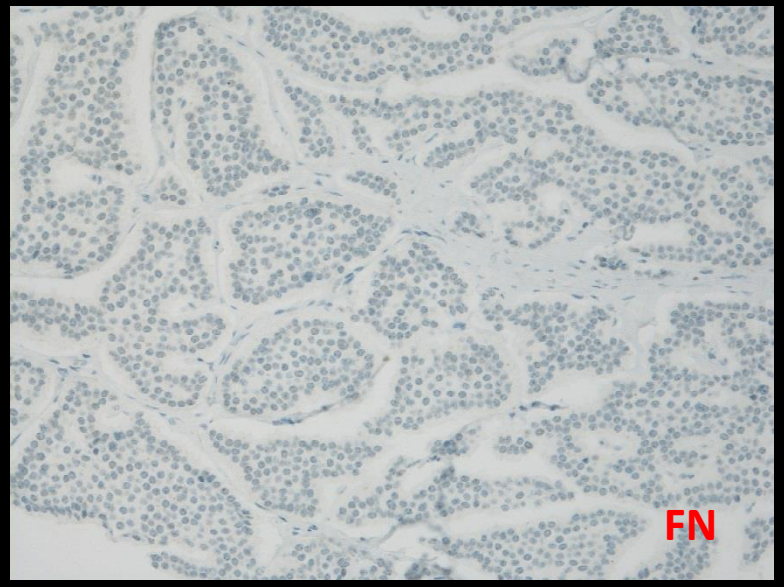
Neuroendocrine tumour (colon)

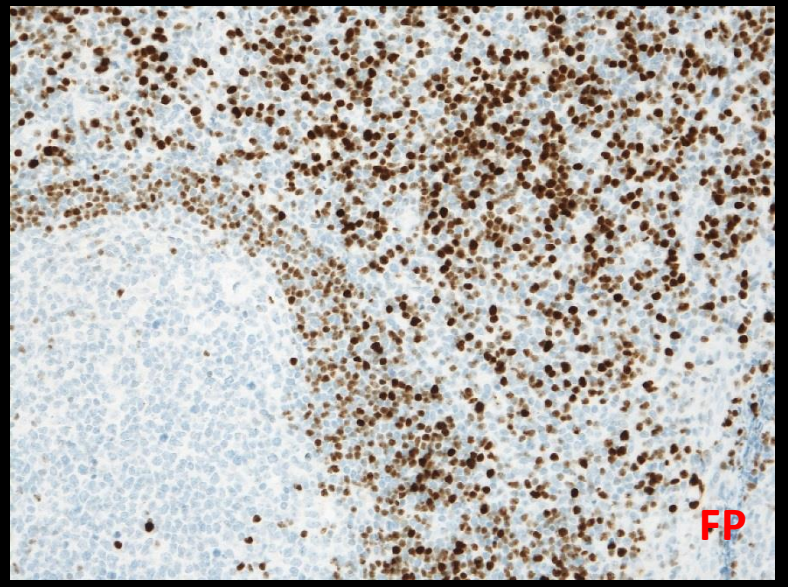
Tonsil









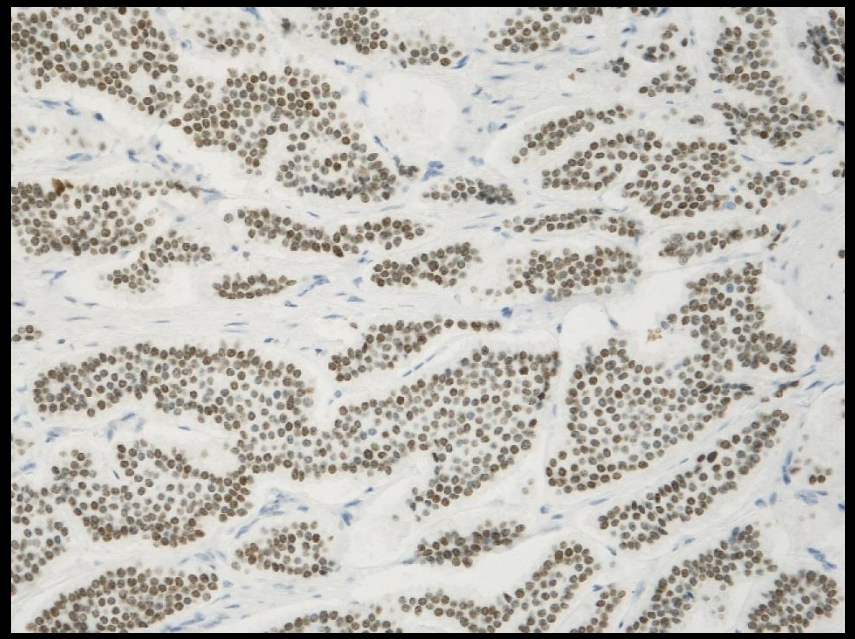


NordiQC results

Appendix

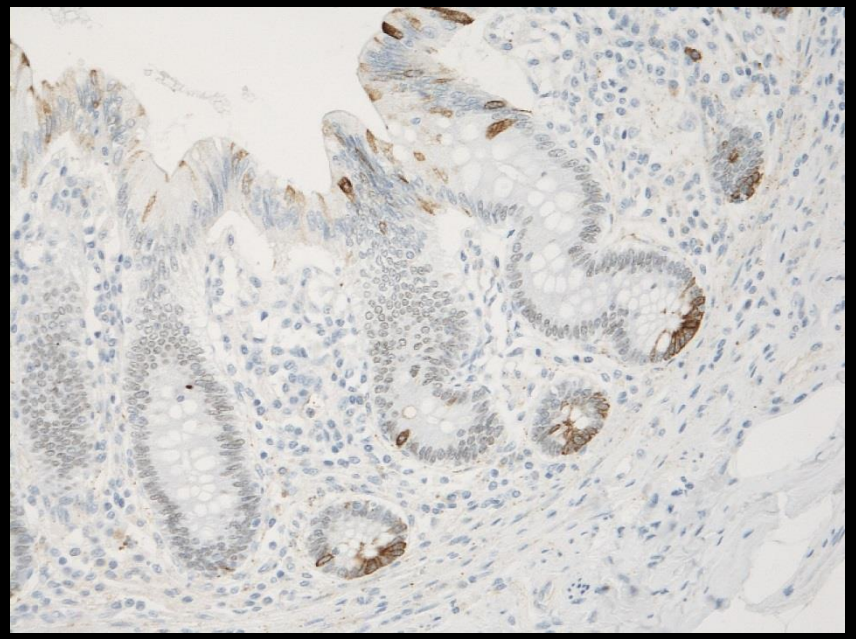
Neuroendocrine tumour (colon)

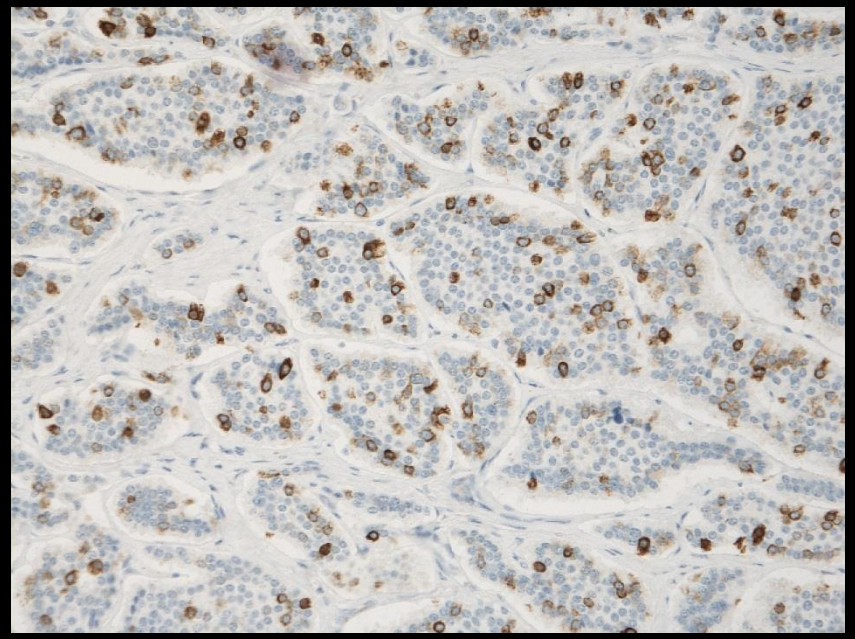
SATB2, EP281



SATB2, pAb 69995

Micro anatomic localization





NordiQC results

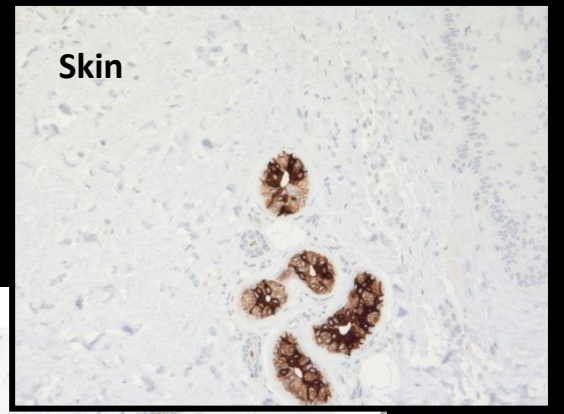
Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Antibody choice: Sensitivity & Specificity

NordiQC results (2018-2024)

Abs providing low sensitivity	Abs providing low specificity and/or aberrant staining
SATB2 clone CL0276 & SATBA4310	SATB2 clone CL0276 & SATBA4310
SATB2 clone EPNCIR130A	PAX8 clone EP331 & MRQ-50
Uroplakin II+III clone AU-1 & SP73 & EP321 (all = Uro III)	MUM1 clone MRQ-43 & BC5
P16 clone G175-405	CK-HMW clone 34βE12
TTF1 clone 8G7G3/1	PR clone 1E12
ERG (Ets-Related-Gene) clone 9FY	ECAD clone EP700Y
ALK clone ALK1	PAX5 clone SP34
CK8/18 clone DC10 & C51 & CY90 (all = CK18)	SMAD4 clone RBT-SMAD4 & SP306
CK-PAN clone MNF116	MLH1 clone M1 & G168-728
CEA clone II-7	CD79a clone HM57
CGA clone DAK-A3 & 5H7	MSH6 clone 44
P63 clone 7JUL	Many pAbs (e.g., P40 and SOX10)
CD79a clone HM57 & 11E1	……
ALK clone ALK1	
Many pAbs	
……	

Technical aspects of immunohistochemistry & pitfalls - Analytical phase



Problem: Poorly calibrated primary antibody (false negative or false positive)

The right primary antibody

The right protocol (AR procedure and detection system)

Poorly calibrated primary Ab ?

Tissue controls are important

Normal skin is the preferred positive control for GCDFP-15. The epithelial cells of the eccrine sweat glands must show an as strong as possible positive cytoplasmic staining reaction, while all other cells should be negative.

Normal breast tissue can also be used as control in which epithelial cells of the ductal glands must show an as strong as possible staining reaction.

Background staining may be seen in vicinity of highly positive tissue structures (e.g. eccrine sweat glands)

Gross cystic disease fluid protein-15 (GCDFP-15)

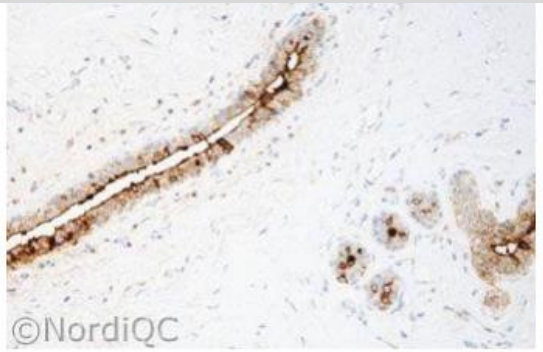


Fig. 1a
Optimal staining for GCDFP-15 of the breast hyperplasia using the mAb clone 23A3 optimally calibrated as a concentrate, HIER in an alkaline buffer and a polymer based detection system. The majority of the ductal epithelia cells show a distinct moderate to strong cytoplasmic staining reaction. Also compare with Fig. 2a – same protocol.

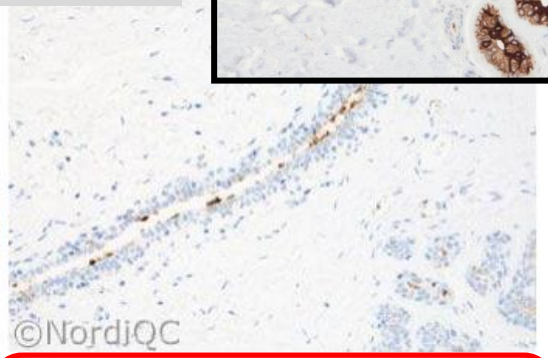


Fig. 1b
Insufficient staining for GCDFP-15 of the breast hyperplasia applying the mAb clone 23A3 as a concentrate using exactly the same protocol settings as used in Fig 1a, except for a 20 fold dilution of the primary antibody. The proportion and the intensity of the cells demonstrated are significantly reduced compared to the result in Fig. 1a. Also compare with Fig. 2b – same protocol.

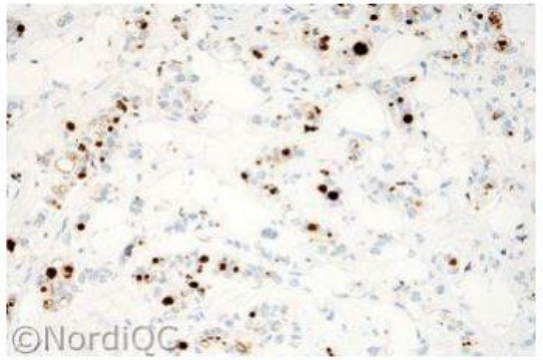


Fig. 2a
Optimal staining for GCDFP-15 of the breast carcinoma no. 5 using same protocol as in Fig. 1a.
The majority of the neoplastic cells show a moderate to strong dot-like cytoplasmic staining reaction.

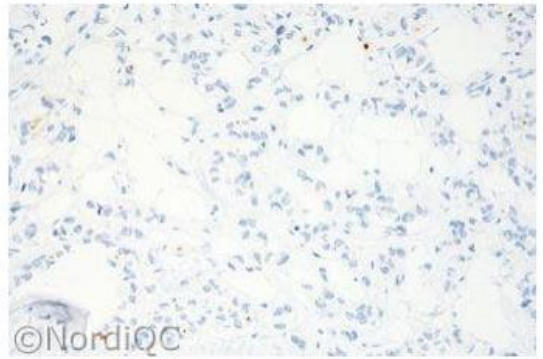
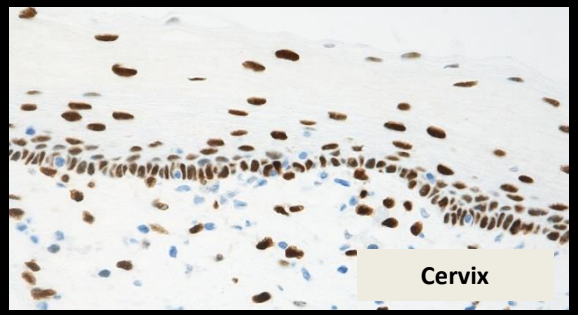
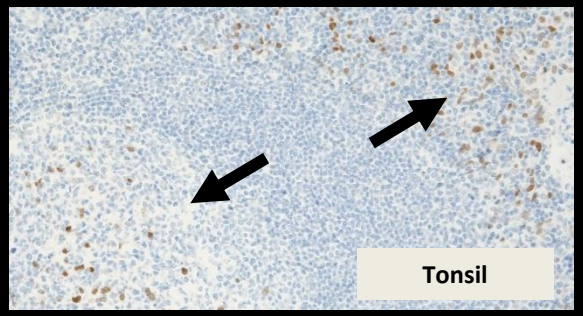
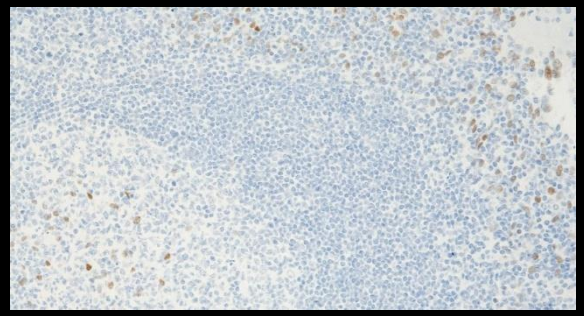
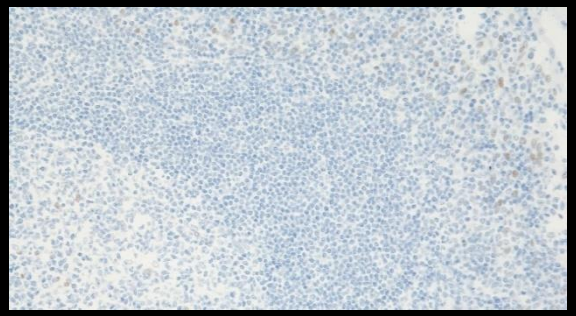
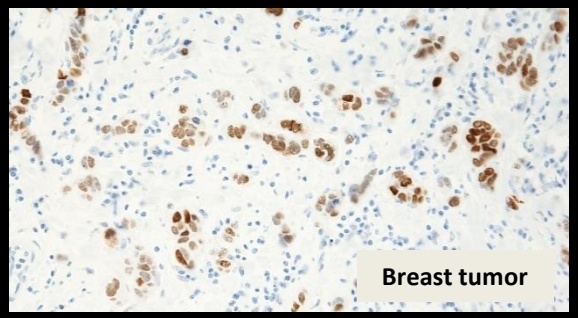
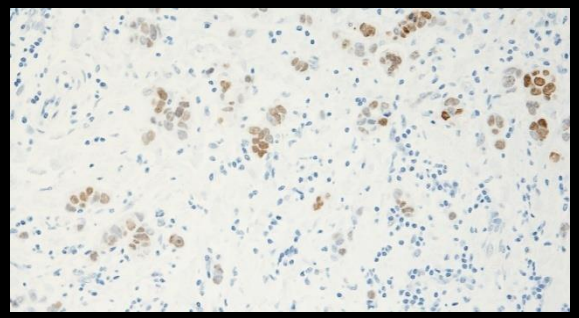
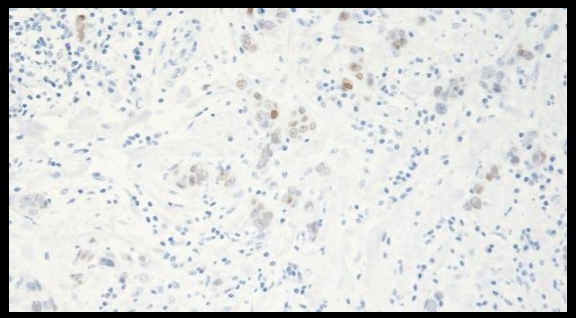


Fig. 2b
Insufficient staining GCDFP-15 of the breast carcinoma no. 5 using same protocol as in Fig. 1b. - same field as in Fig. 2a.
Only scattered neoplastic cells show a faint dot-like reaction.

Antibody-Antigen reaction - Antibody Titer & Dilutions

ER clone 6F11 / 1:100	ER clone 6F11 / 1:200	ER clone 6F11 / 1:400
		
		
		

Cervix

Tonsil

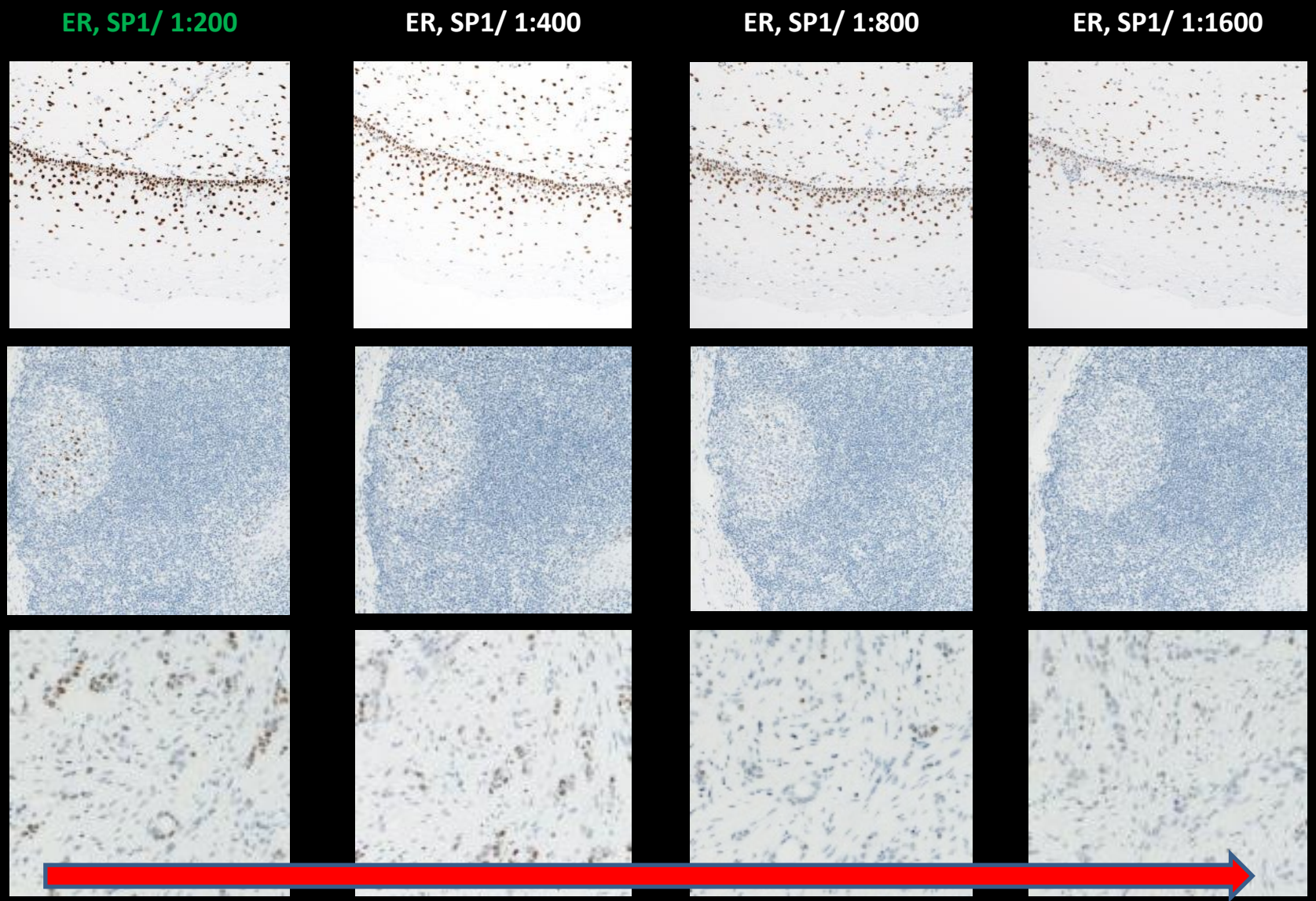
Breast tumor

Staining indicators are extremely important - helping us to calibrate the IHC assay correctly

High pH 20`, Flex+Mouse

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Primary antibody poorly calibrated (false negative)

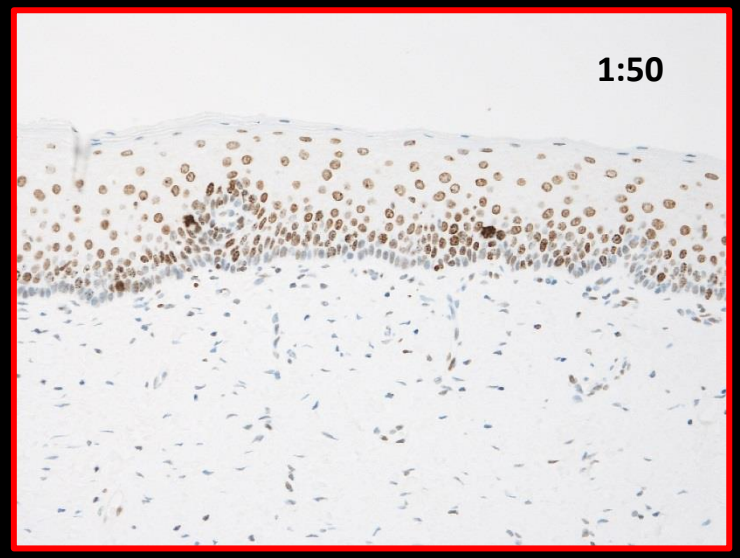


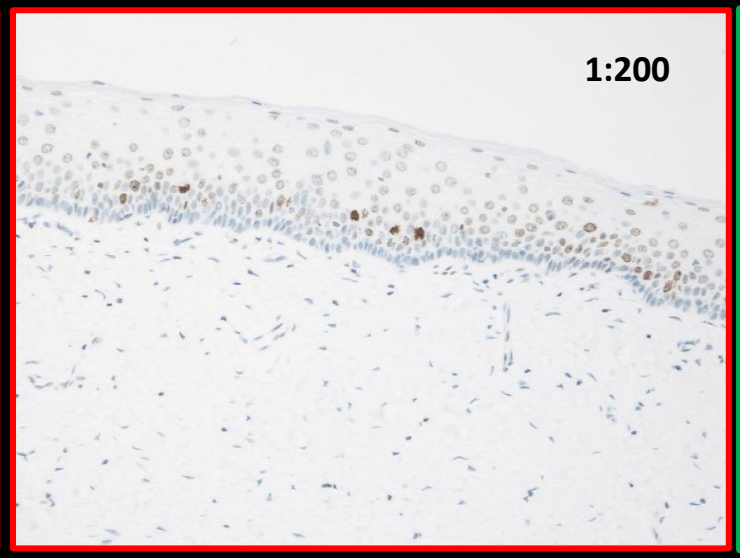
High pH 24`, Flex+Rabbit

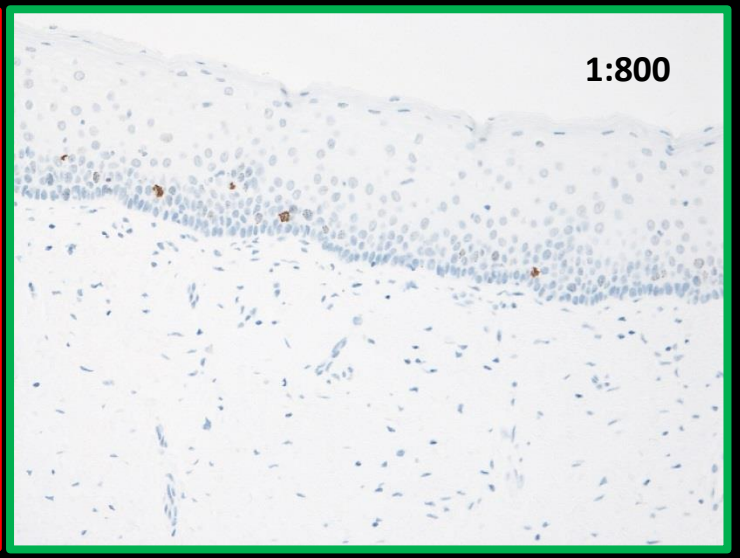
Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Primary antibody poorly calibrated (false positive)

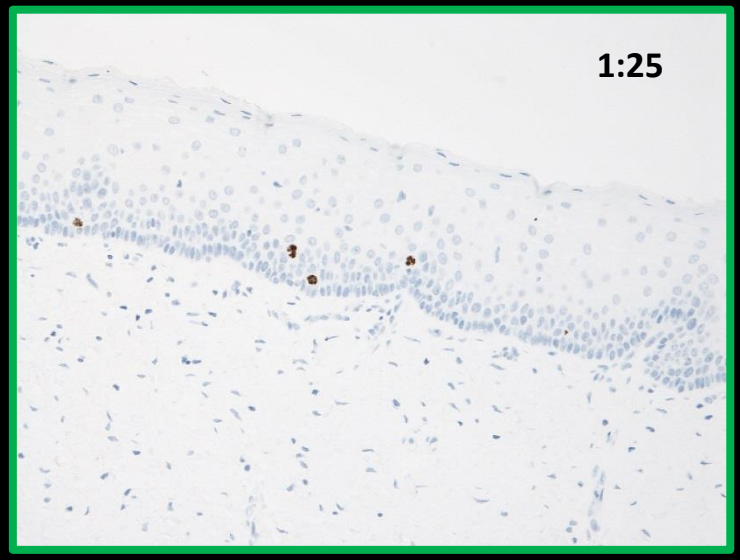
PHH3, poly (Cell Marque)

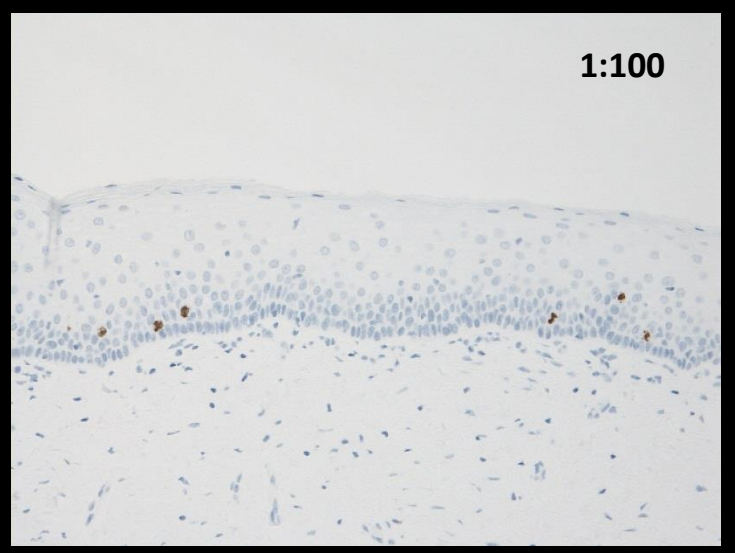


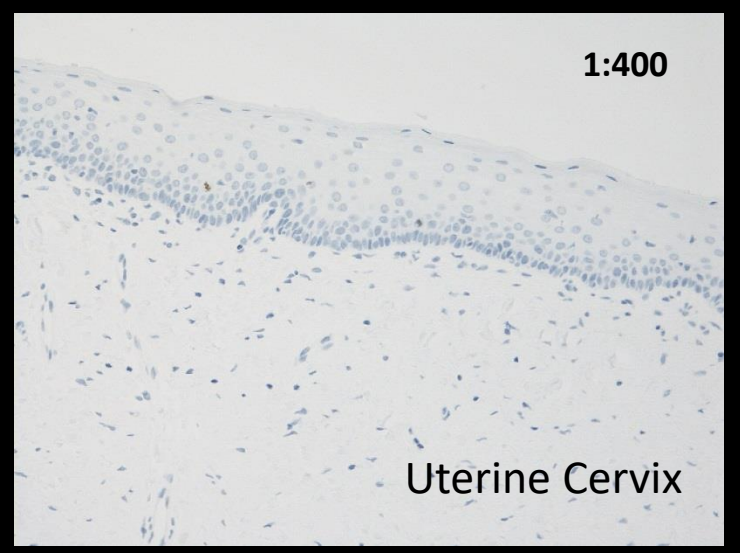




PHH3, BC37 (Biocare)





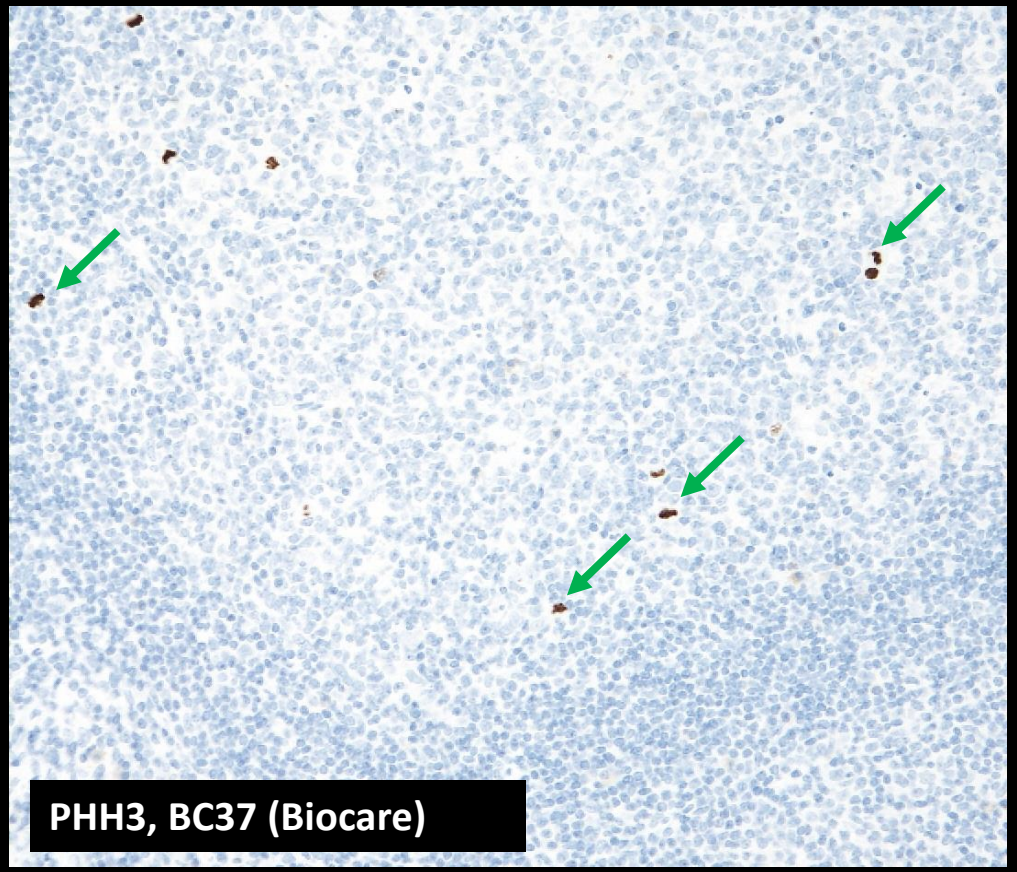


HIER Low pH/Flex+

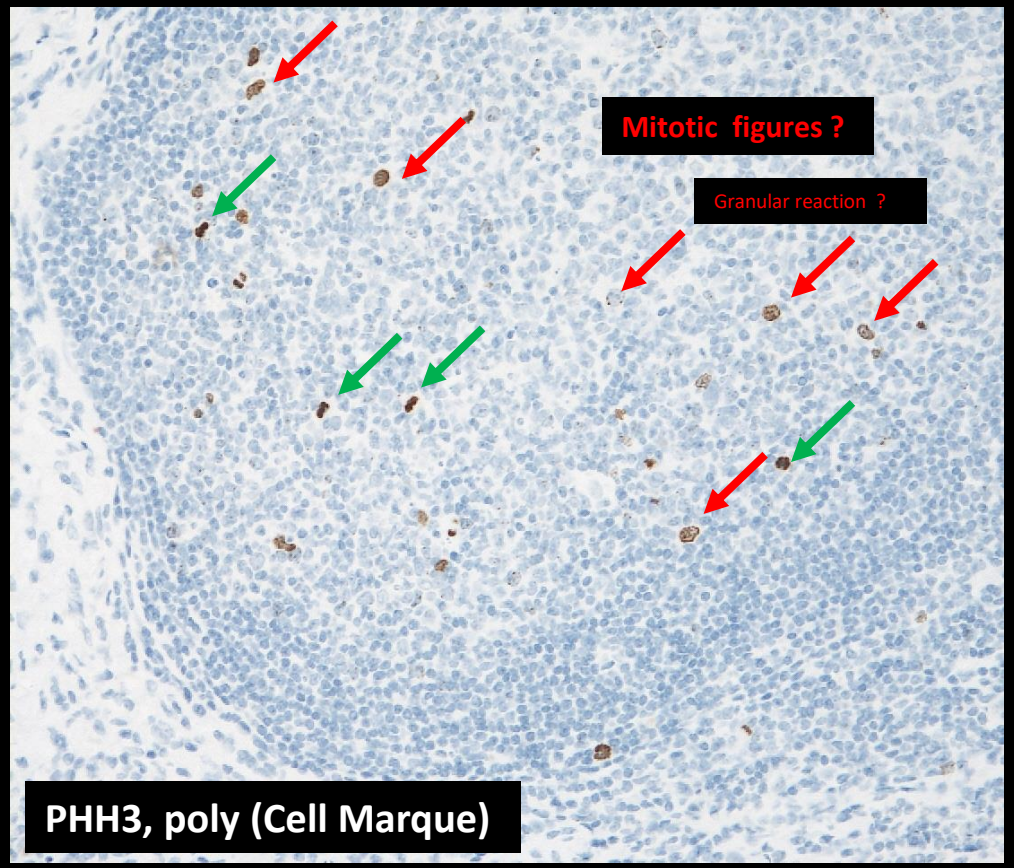
PHH3 should only be positive in cells in the late G2 and M phase (mitotic cells)

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Primary antibody poorly calibrated (false positive)



PHH3, BC37 (Biocare)



PHH3, poly (Cell Marque)

PHH3, poly might not be completely phospho-specific and might cross-reacts with cells that has not entered th late G2/M phase

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Primary antibodies sensitive to the chosen platform

Implementing a new platform has been a challenge

ALK clone D5F3 or 5A4
HCL, clone DBA44
GATA3, clone L50-823
MART-1/Melan A, clone 103
PAX 8, clone BC12
SMAD4, clone B8
WT1, clone WT49
MMR

……

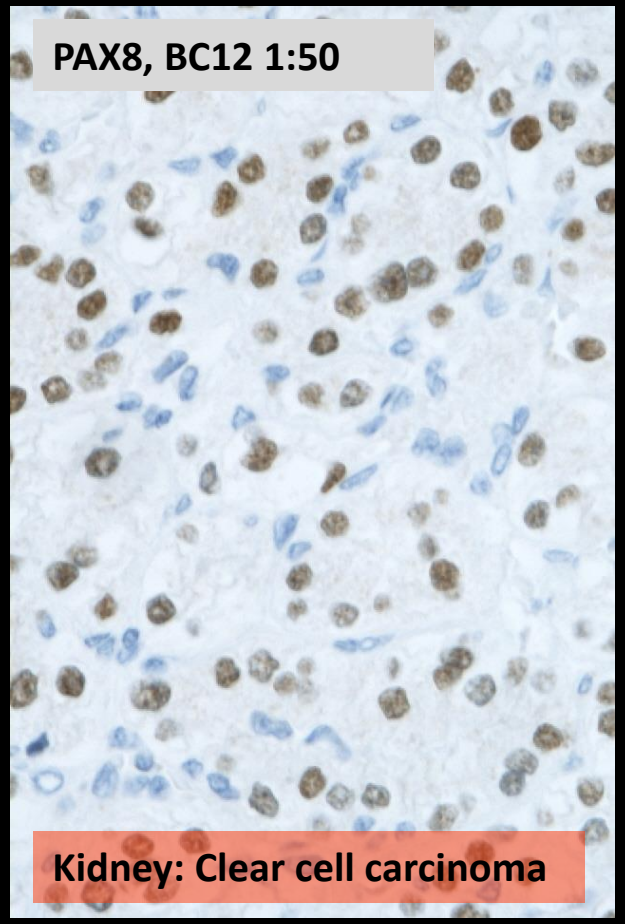
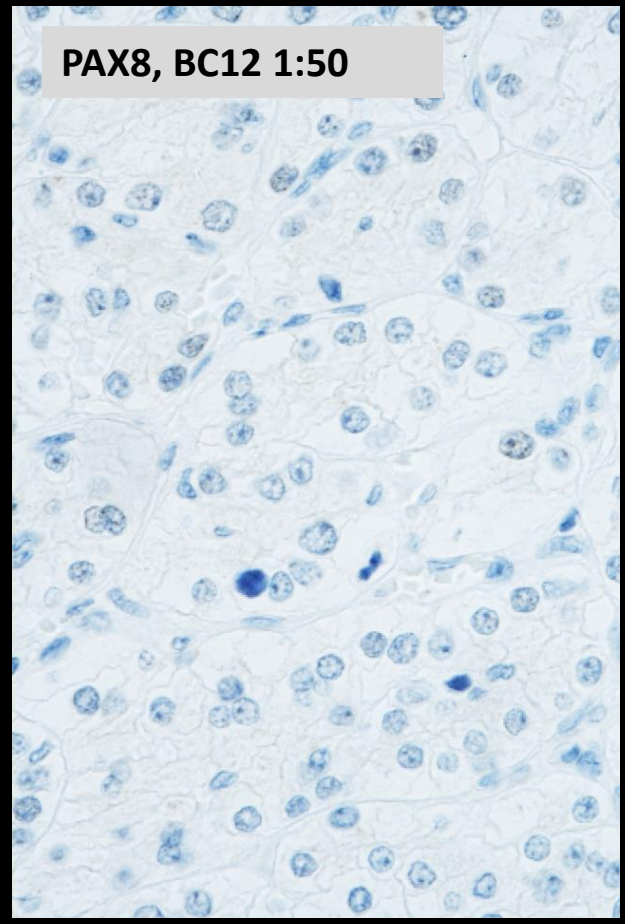
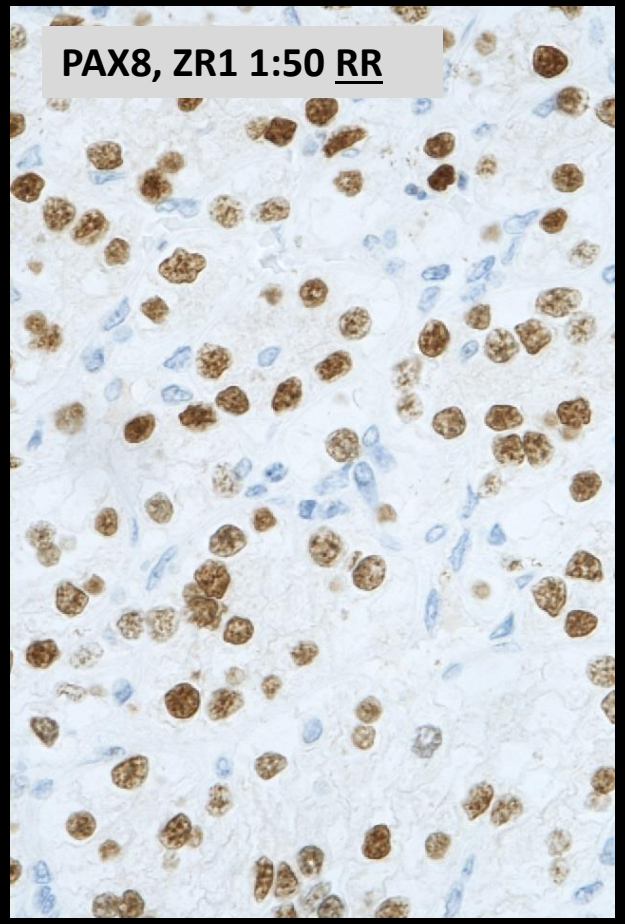
Substituting the primary Ab

Changing Ab-Ag reaction microenvironment (Diluent)

Low affinity primary antibodies

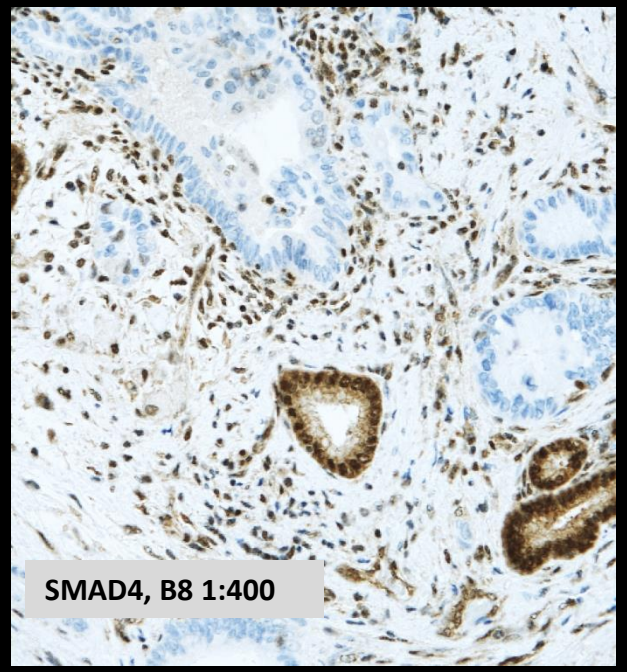
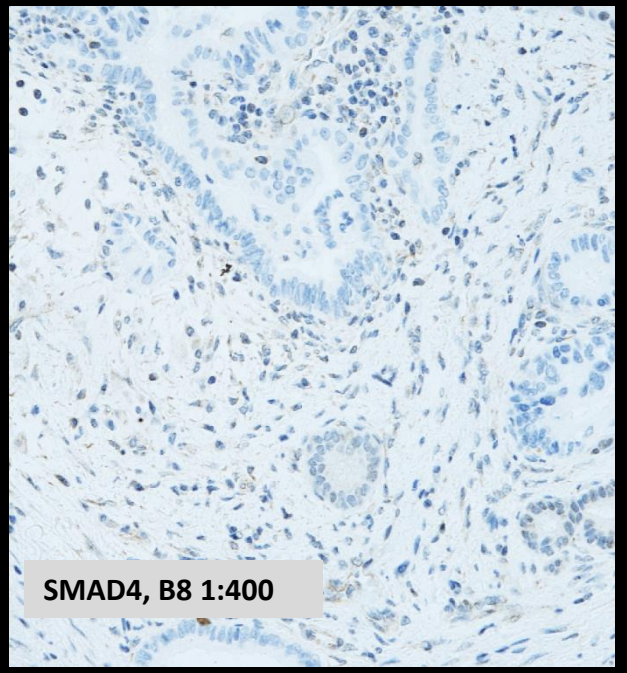
Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Primary antibodies sensitive to the chosen platform

Autostainer	Omnis	Omnis
		
PAX8, BC12 1:50	PAX8, BC12 1:50	PAX8, ZR1 1:50 RR
Kidney: Clear cell carcinoma		
HIER High pH 20`, Flex+ (10+20)		HIER High pH 48`, Flex+ (10+20)

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Primary antibodies sensitive to the chosen platform

Autostainer	Omnis	Omnis
		
SMAD4, B8 1:400	SMAD4, B8 1:400	SMAD4, EP168Y 1:2000 RR
HIER High pH 20`, Flex+ (10+20)		HIER High pH 24`, Flex+ (10+20)

Pancreatic Adenocarcinoma

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Platform dependent antibodies (difficult markers)

NordiQC results (2018-2024)

Antibody	Clone	Platform(s)
CD4	4B12	BenchMark (Ventana), Omnis (Dako) and "BOND III (Leica)"
CD56	123C3	BenchMark (Ventana) and Omnis (Dako)
PMS2	A16-4	BenchMark
Melan A	A103	Benchmark and "Omnis"
P16 (RTU format`s Ventana)	E6H4	Omnis
Alpha Smooth Muscle Actin	1A4	Benchmark
SMAD4	B8	Benchmark and Omnis
CK8/18	5D3	Benchmark
EPCAM	BER-EP4	Benchmark and BOND (Leica)
BRAFmut	VE1	In general challenging on most platforms except for the Benchmark Ultra (Ventana)
CR	DAK-Calret1	Benchmark and Omnis
Desmin	D33	Omnis
Use of RTU-formats "Off-label"	E.g., Myosin, smooth muscle heavy chain	Often seen on the Omnis (RTU portfolio: IR vs GA products ?)

Problem: Does the automatic platform come with appropriate reagents fulfilling purpose and intended use of the assay e.g., Antigen Retrieval Solutions, Antibody diluents, Detection systems…….

NordiQC

Assessment Run 56 2019

Epithelial cell-cell adhesion molecule (Ep-CAM)

Table 1. **Antibodies and assessment marks for Ep-CAM, run 56**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **BS14**	10	Nordic Biosite	9	1	0	0	100%	100%
mAb clone **Ber-Ep4**	69 6 1	Dako Cell Marque Diagnostic Biosystems	14	13	21	28	36%	93%
mAb clone **MOC-31**	23 5 1	Dako Cell Marque Diagnostic Biosystems	10	10	7	2	69%	71%
mAb clone **VU-1D9**	5 3 1 1	Thermo Scientific Merck Millipore Immunologic Novus Biologicals	9	0	1	0	90%	100%
rmAb clone **EPR20532-225**	1	Abcam	0	0	0	1	-	-
Ready-To-Use antibodies								
mAb clone **Ber-Ep4 760-4383**	16	Ventana/Cell Marque	1	6	6	3	44%	100%
mAb clone **Ber-Ep4 248M-98**	49	Cell Marque	5	13	16	15	37%	-
mAb clone **Ber-Ep4 IR/IS637**	18	Dako	5	9	3	1	78%	87%
mAb clone **Ber-Ep4 IR/IS637**[3]	6	Dako	1	2	2	1	-	-
mAb clone **Ber-Ep4 GA637**	27	Dako	26	1	0	0	100%	100%

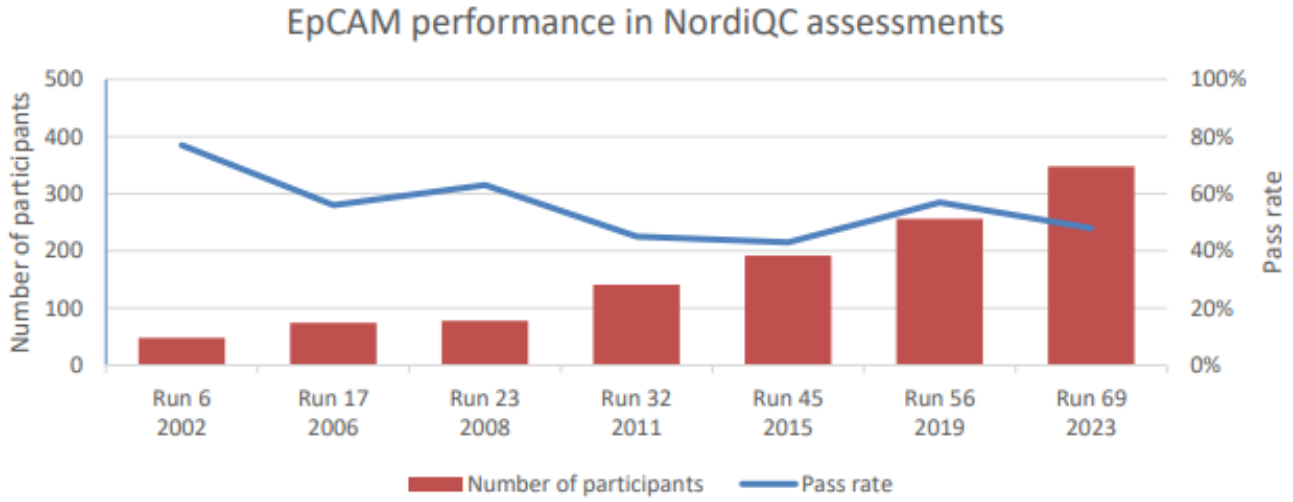
OPS: Based on HIER in mod. Low pH buffers (Dako or Biocare)

Omitted from HIER in mod. Low pH buffers - these buffers are not part of the reagent portfolio from the vendor

BS14 or VU-1D9 could be good alternatives compared to Ber-EP4 (and MOC-31) on platforms excluded from the use of modified low pH buffers as Diva Decloaker pH 6.2 (Biocare) or TRS pH 6.1 (Dako)

Performance history
This was the seventh NordiQC assessment of EpCAM and, as shown in Graph 1, the pass rate decreased compared to the latest run 56, 2023.

Graph 1. **Proportion of sufficient results for EpCAM in the seven NordiQC runs performed**



Latest assessment for EpCAM clone Ber-Ep4 - still a problem

Substitute mAb clone Ber-Ep4 or apply modified Low pH buffer ?

Protocols mostly based on VRPS: 2-step detections system

NordiQC

Assessment Run 69 2023

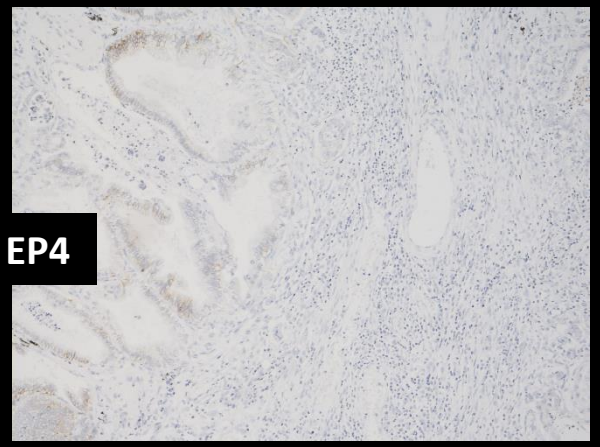
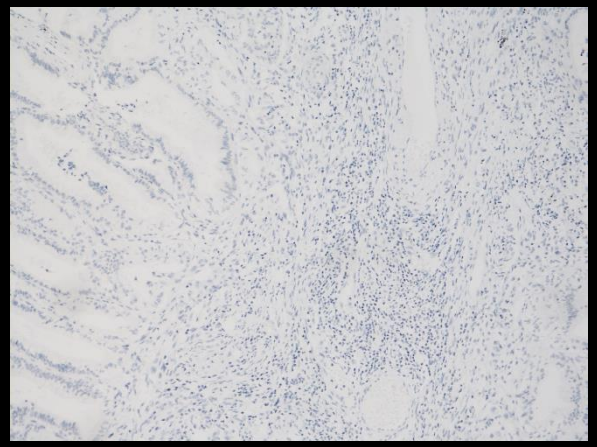
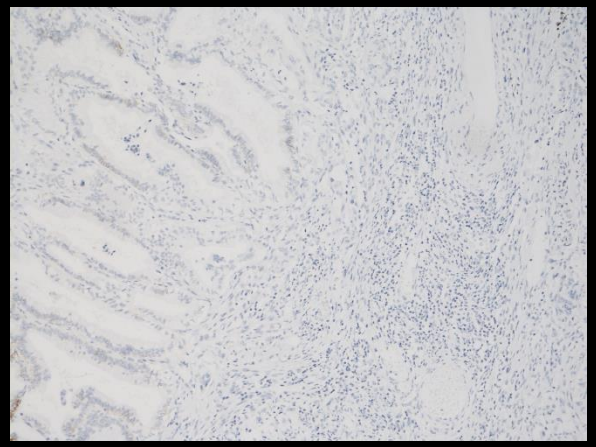
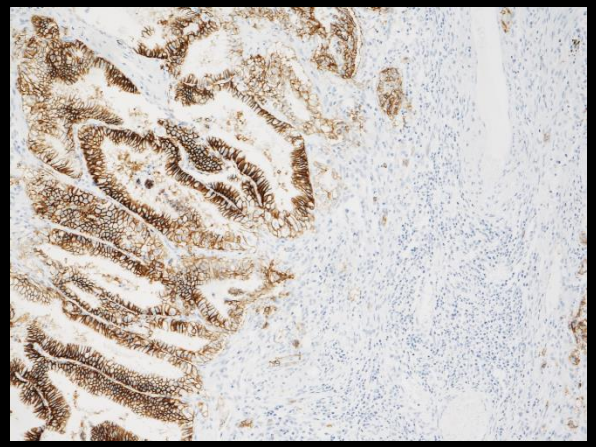
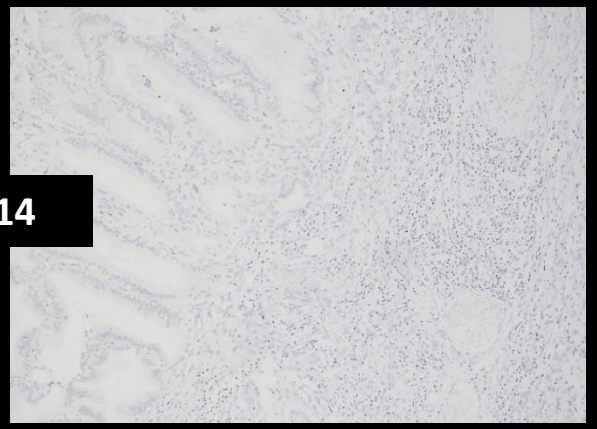
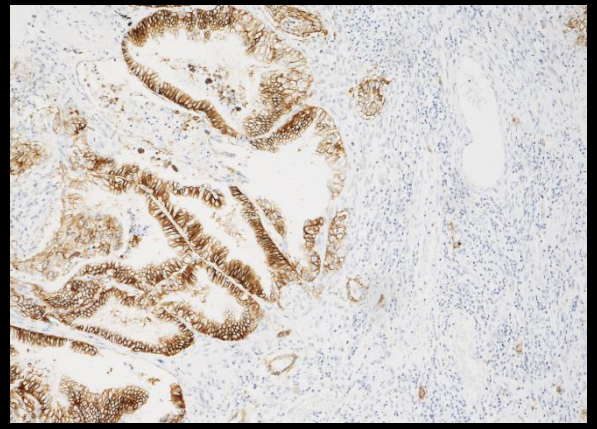
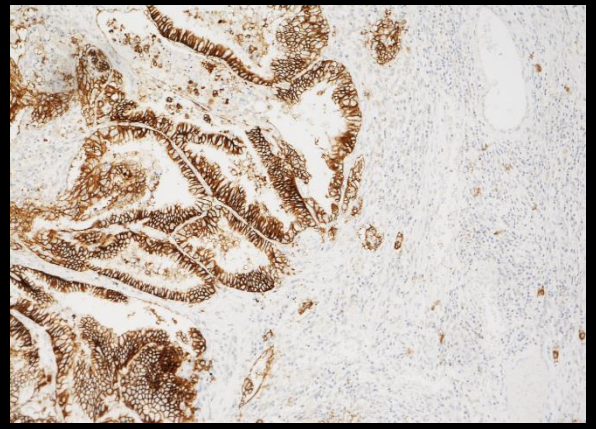
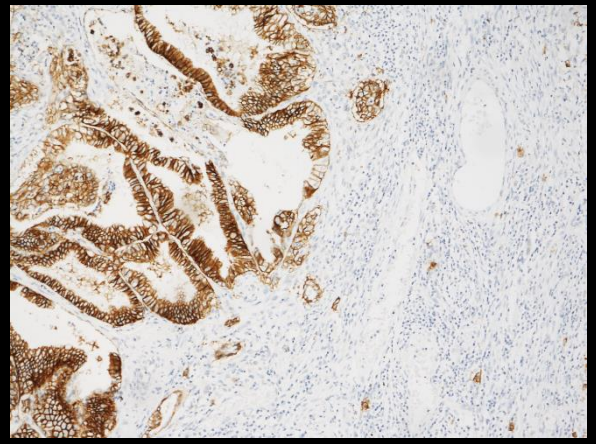
Epithelial cell-cell adhesion molecule (EpCAM)

Table 1. **Antibodies and assessment marks for EpCAM, run 69**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **BS14**	25	Nordic Biosite	19	6	0	0	100%	76%
mAb clone **Ber-Ep4**	40 8 1	Dako/Agilent Cell Marque Thermo Scientific	5	11	30	3	33%	10%
mAb clone **MOC-31**	16 1 2 1	Dako/Agilent Biocare Medical Cell Marque Leica Biosystems	1	14	4	1	75%	5%
mAb clone **VU-1D9**	9 1 4 1	Thermo Scientific Merck Millipore Diagnostic Biosystems Monosan	4	8	2	1	80%	27%
mAb clone **C-10**	2	Santa Cruz	0	0	2	0	-	-
mAb clone **ZM131**	1	Zeta Corporation	0	0	0	1	-	-
Ready-To-Use antibodies								
mAb clone **Ber-Ep4 760-4383**[3]	13	Ventana/Roche	0	0	13	0	0%	0%
mAb clone **Ber-Ep4 760-4383**[4]	86	Ventana/Roche	1	20	60	5	24%	1%
mAb clone **Ber-Ep4 248M-98**	15	Cell Marque	0	1	13	1	7%	0%
mAb clone **Ber-Ep4 IR/IS637**[3]	1	Dako/Agilent	0	0	1	0	-	-
mAb clone **Ber-Ep4 IR/IS637**[4]	15	Dako/Agilent	3	2	10	0	33%	20%
mAb clone **Ber-Ep4 GA637**[3]	44	Dako/Agilent	12	30	2	0	95%	27%
mAb clone **Ber-Ep4 GA637**[4]	16	Dako/Agilent	3	8	5	0	69%	19%
mAb clone **Ber-Ep4 PM107**	3	Biocare Medical	0	0	1	2	-	-
mAb clone **Ber-Ep4 MAD-001709QD**	3	Master Diagnostica	0	0	2	1	-	-
mAb clone **Ber-Ep4 PDM131**	2	Diagnostic Biosystems	0	0	0	2	-	-
mAb clone **Ber-Ep4 P-E002**	1	Quartett	0	0	0	1	-	-
mAb clone **Ber-Ep4 BMS048**	1	Zytomed Systems	0	1	0	0	-	-
mAb clone **Ber-Ep4 GM080402**	2	Gene Tech	0	1	1	0	-	-

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

EPCAM clone Ber-EP4 vs BS14

	Proteinase K 1:4 (Dako) / 5`	Citrate buffer pH 6 (Dako) / 20`	High pH buffer (Dako) / 20`	TRS Low pH (Dako, S1699/S1700) /20`
Ber- EP4				
BS14				

EPCAM, BS14 (Nordic Biosite) is a better alternative compared to EPCAM Ber-EP4 (or MOC31) for automated platforms excluded from use of mod. low pH buffers.

Colon tumor

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Platform dependent antibodies (difficult markers)

NordiQC results (2018-2024)

Antibody	Clone	Platform(s)
CD4	4B12	BenchMark (Ventana), Omnis (Dako) and "BOND III (Leica)"
CD56	123C3	BenchMark (Ventana) and Omnis (Dako)
PMS2	A16-4	BenchMark
Melan A	A103	Benchmark and "Omnis"
P16 (RTU format`s Ventana)	E6H4	Omnis
Alpha Smooth Muscle Actin	1A4	Benchmark
SMAD4	B8	Benchmark and Omnis
CK8/18	5D3	Benchmark
EPCAM	BER-EP4	Benchmark and BOND (Leica)
BRAFmut	VE1	In general challenging on most platforms except for the Benchmark Ultra (Ventana)
CR	DAK-Calret1	Benchmark and Omnis
Desmin	D33	Omnis
Use of RTU-formats "Off-label"	E.g., Myosin, smooth muscle heavy chain	Often seen on the Omnis (RTU portfolio: IR vs GA products ?)

Off-Label use: RTU product used on a platform to which the format has not been validated

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Use of RTU-formats "Off-Label" exemplified by Myosin, smooth muscle heavy chain (SMH) NQC assessment Run 66

Autostainer (SMH, product IR066):

VRPS: Pass rate of 73% (18% optimal)

LMPS: Pass rate of 86% (57% optimal)

Omnis (SMH, Product IR066, Off-Label use)

Pass rate of 15% (no optimal results)

All the insufficient results were commented as weak, false negative or poor-signal-to-noise-ratio characterized by extensive background reaction combined with **difficulties to demonstrate low-level antigen expressing structures.** In this context, it was more successful to use the concentrated format of mAb clone SMMS-1 on Dako Omnis (see Table 2) as alternative to the off-label use of a **RTU format not being developed and validated for the respective IHC platform.**

Dako RTU IR-products providing poor results on the Omnis (2022-2024):

Desmin, D33/IR606 (Run 64, 2022)
Calretinin, DAK-Calret1/IR627 (Run 64, 2022)
CD56, 123C3/IR628 (Run 64, 2022)
CD4, 4B12/IR649 (Run 67, 2023)
CD5, 4C7/IR082 (Run 69, 2023)

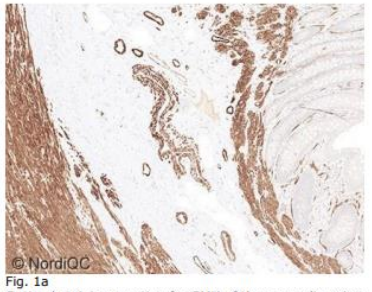


Fig. 1a
Optimal staining reaction for SMH of the appendix using the mAb clone SMMS-1 RTU (Ventana/Roche, 760-2704) within laboratory modified protocol settings, using OptiView as detection system.
Virtually all smooth muscle cells in vessels and lamina muscularis show a moderate to strong cytoplasmic staining reaction.
Also compare with Figs. 2a – 5a, same protocol.

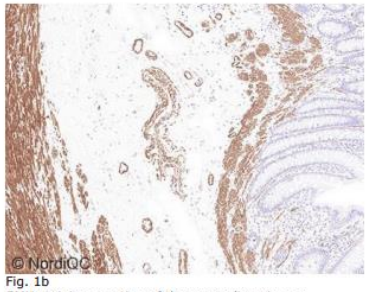


Fig. 1b
SMH staining reaction of the appendix using an insufficient protocol based on the mAb clone SMMS-1 RTU (Dako/Agilent, IR066) developed for the Autostainer, but applied on the Dako Omnis platform.
In appendix – same field as Fig. 1a, a weak to strong staining reaction is seen in virtually all smooth muscle cells as expected.
Also compare with Figs. 2b – 5b, same protocol.

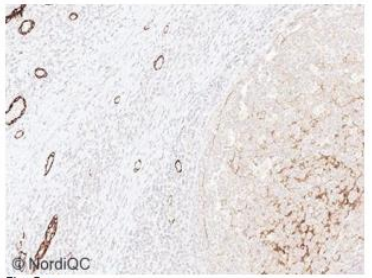


Fig. 2a
Optimal SMH staining reaction of the tonsil using same protocol as in Fig. 1a.
A weak to moderate staining reaction is seen in the follicular dendritic network in the germinal center.
A high signal-to-noise ratio is observed.

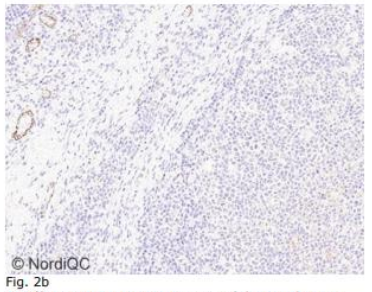


Fig. 2b
Insufficient SMH staining reaction of the tonsil using same protocol as in Fig. 1b - same field as Fig. 2a.
The follicular dendritic network in the germinal center is virtually negative and only the smooth muscle cells of large vessels are demonstrated.

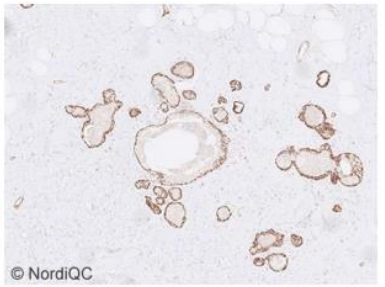


Fig. 3a
Optimal SMH staining reaction of the breast hyperplasia using same protocol as in Figs. 1a and 2a.
A moderate and distinct staining reaction is seen in virtually all myoepithelial cells lining the breast glands.

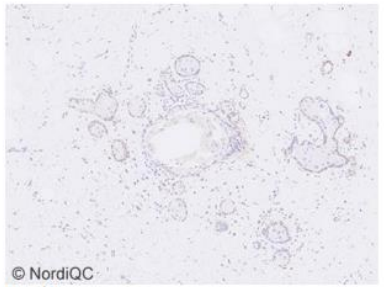


Fig. 3b
Insufficient SMH staining reaction of the breast hyperplasia using same protocol as in Figs. 1b and 2b – same field as Fig. 3a.
Only a faint staining reaction is seen in few myoepithelial cells lining the breast glands.

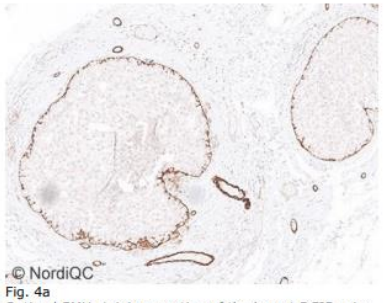


Fig. 4a
Optimal SMH staining reaction of the breast DCIS using same protocol as in Figs. 1a - 3a.
A moderate, distinct and continuous staining reaction is seen in the myoepithelial cells lining the breast DCIS component.

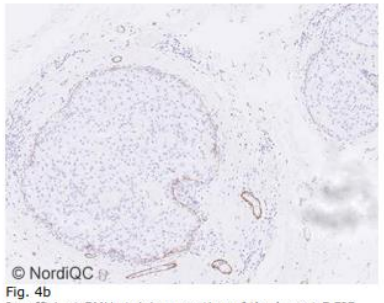


Fig. 4b
Insufficient SMH staining reaction of the breast DCIS using same protocol as in Figs. 1b – 3b – same field as Fig. 4a.
Only a weak and disrupted staining reaction is seen in the myoepithelial cells lining the breast DCIS component.

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Primary antibodies sensitive to the chosen platform

Implementing a new platform has been a challenge

ALK clone D5F3 or 5A4
HCL, clone DBA44
GATA3, clone L50-823
MART-1/Melan A, clone 103
PAX 8, clone BC12
SMAD4, clone B8
WT1, clone WT49
MMR
ASMA, 1A4
……

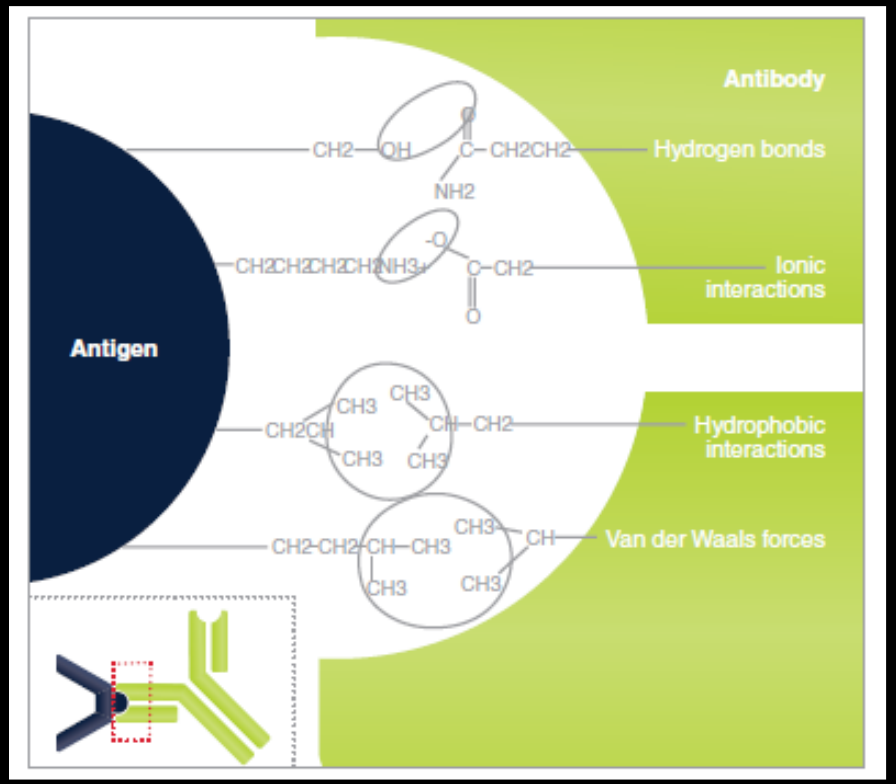
Changing the primary Ab

Changing Ab-Ag reaction microenvironment (Diluent)

Low affinity primary antibodies

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Antibody Diluents: Antigen-Antibody reactions



The strength by which the primary Ab binding site binds to an antigenic epitope is called affinity

Antibodies are attracted initially through electrostatic interactions, and subsequently through weak forces

- Hydrogen bonds
- Hydrophobic interactions
- Van der Waals forces

Antibody diluent formulations can significantly alter stability and binding properties of antibodies affecting both epitope specificity and non-specific interactions

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Applied Immunohistochemistry & Molecular Morphology 9(2): 176–179, 2001

Formalin-Fixed and Heat-Retrieved Tissue Antigens: A Comparison of Their Immunoreactivity in Experimental Antibody Diluents

Thomas Boenisch, M.S.

Antibody diluents

Demonstrated that:

pH of the antibody diluent had a high impact of the final IHC result

Addition of NaCL (increasing the ionic strength) to the diluent negates most of the sensitivity gained through Antigen Retrieval (Table 3).

TABLE 3. *Comparison of staining scores of 13 optimally diluted antibodies as a function of antigen retrieval at pH 9.9, use of 0.05 M Tris (TB), pH 6.0 and 8.6, or Tris-buffered 0.15 M NaCl (TBS) of pH 6.0 and 8.6, and 0.02 M phosphate-buffered 0.15 M NaCl of pH 7.5 (PBS)*

		TB		TBS		PBS
Clone	pH	6.0	8.6	6.0	8.6	7.3
BLA.36		2	4	1	2	1
UCHL1		4	3	2	1	1
L26		4	3	3	3	2
PC10		4	3	4	4	3
N10/2		3	2	1	2	1
V9		4	3	4	4	2
TAL1B5		4	2	3	2	2
ER-PR-8		4	3	2	1	2
Ber-H2		4	3	ND	ND	0
4KB5		4	2	4	2	4
DF-T1		4	2	2	0	1
PD7/26		4	3	ND	ND	3
C3D-1		4	2	ND	ND	1

ND, not done.

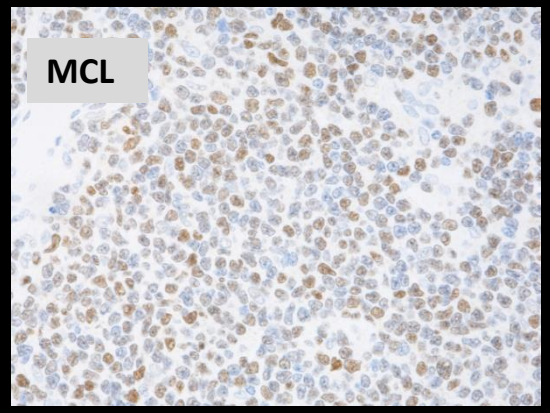
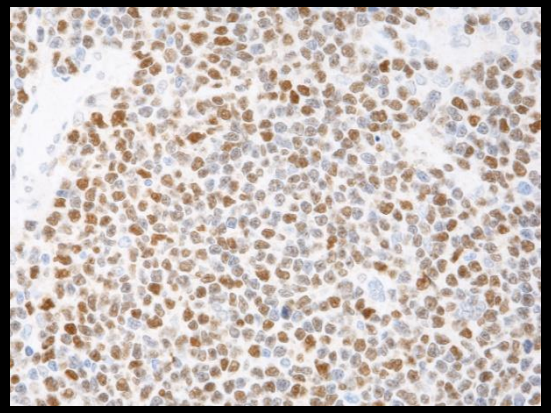
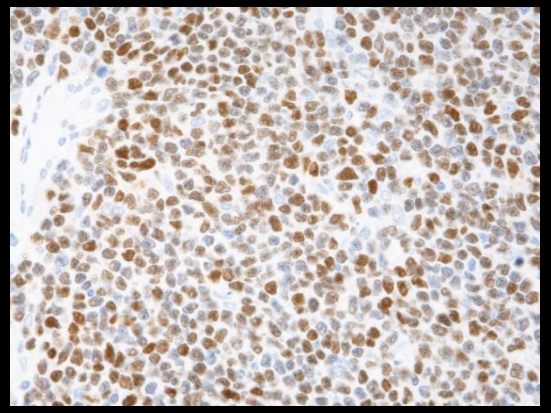
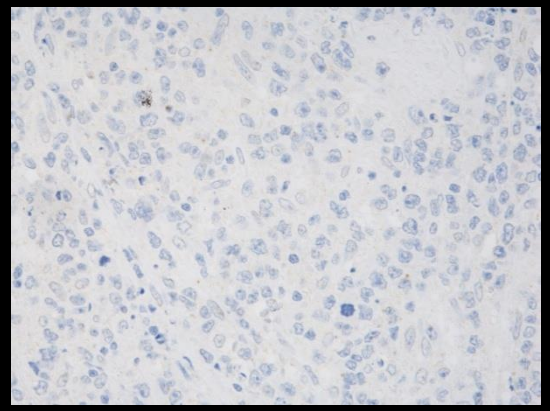
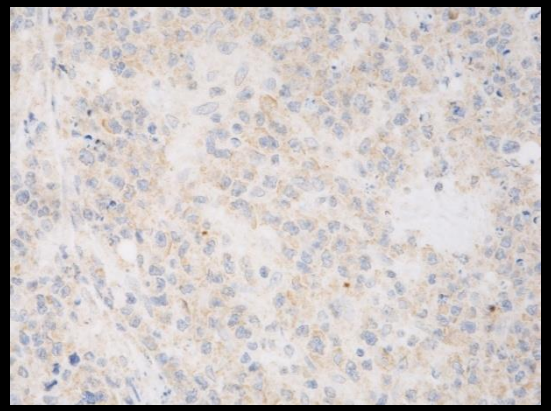
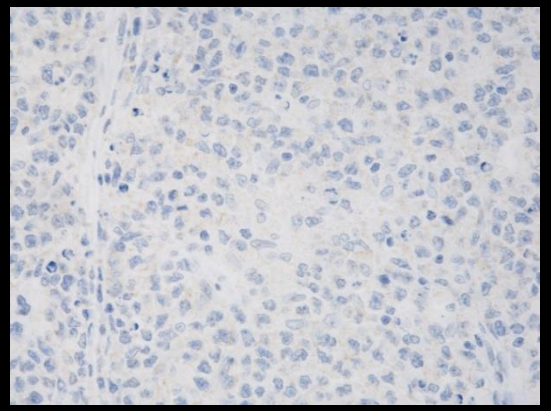
Antibody-Antigen reaction – Commercial Antibody Diluents

Biocare Medical's Antibody Diluent Offering

Antibody Diluent	Catalog Number	pH	Composition	Note
Da Vinci Green Diluent	PD900	7.3	Phosphate-based	May act as a "universal" diluent
Monet Blue Diluent	PD901	7.9	Tris-based	Uses Assure Technology with pH indicator. A change in diluent color indicates a change in pH, meaning optimal staining may be compromised.
Renaissance Background Reducing Diluent	PD905	7.3	Proprietary	Has potent background reducing agents; For antibodies with non-specific background
Renoir Red Diluent	PD904	6.0	Tris-based	Designed to work with Tris-based antibodies, rinsing buffers and/or detection systems that use alkaline phospha-tase
Van Gogh Yellow Diluent	PD902	6.0	Phosphate-based	Specifically formulated to enhance certain types of antibodies and may increase titers up to 2 to 4 times
Fluorescence Antibody Diluent	FAD901	7.3	Phosphate-based	Protease free and stabilizes fluorescent dyes for up to one month in a prediluted format
Revival Series Sampler	PD912H4	N/A	N/A	Includes 25 mls of each Da Vinci Green, Renoir Red, Van Gogh Yellow, Monet Blue

Standard Diluent pH 7.3 (Dako, K8006)

SOX11 clone MRQ-58 (1:200)

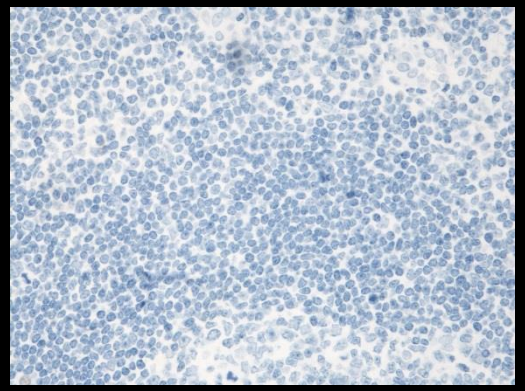
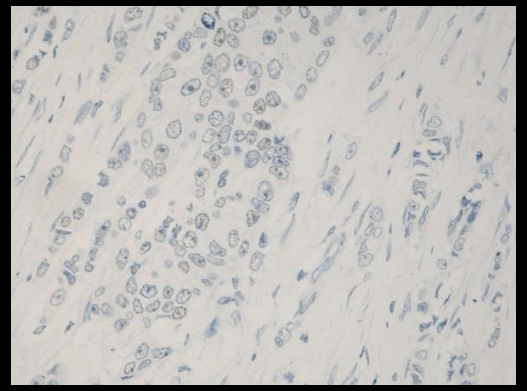
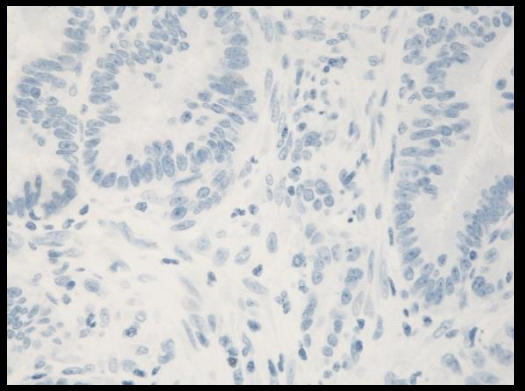
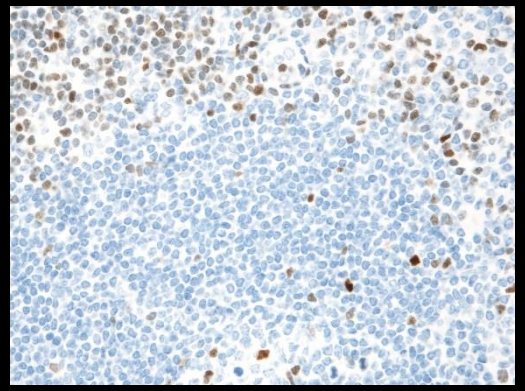
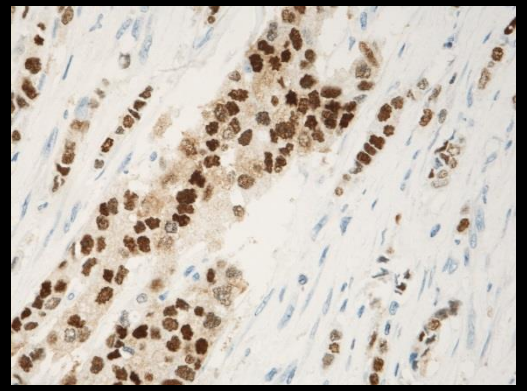
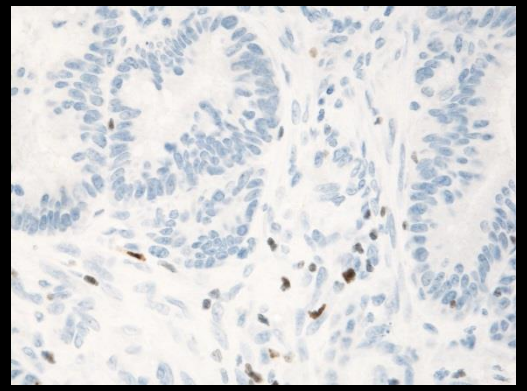
	Dako dil. pH 7.3	Van Gogh pH 6.0	Renoir Red pH 6.2
MCL			
DLBCL, NOS			

Omnis: HIER/HIGH pH 24` at 97°C, Flex+ Mouse (10+20`)

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Antibody diluents

GATA3, L50-823 (Biocare) 1:800

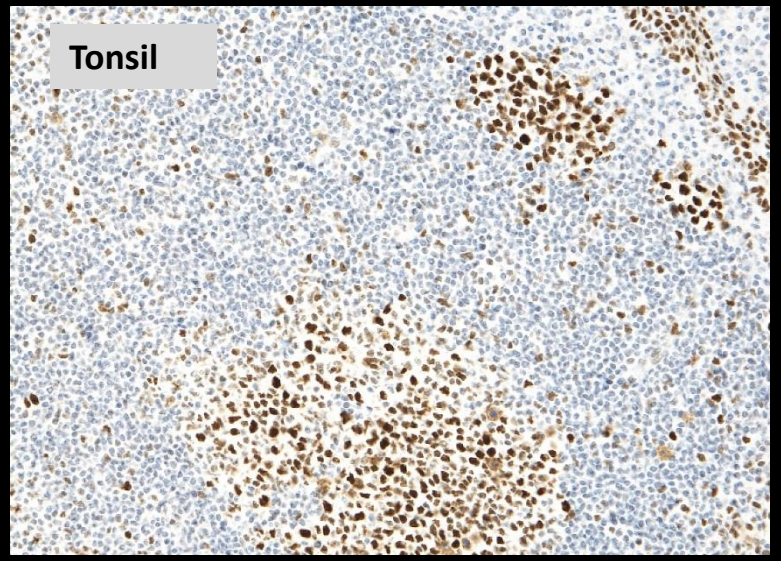
	Tonsil	Breast adenocarc.	Colon adenocarc.
Dako Dil. pH 7.3			
Biocare Renoir Red pH 6.2			

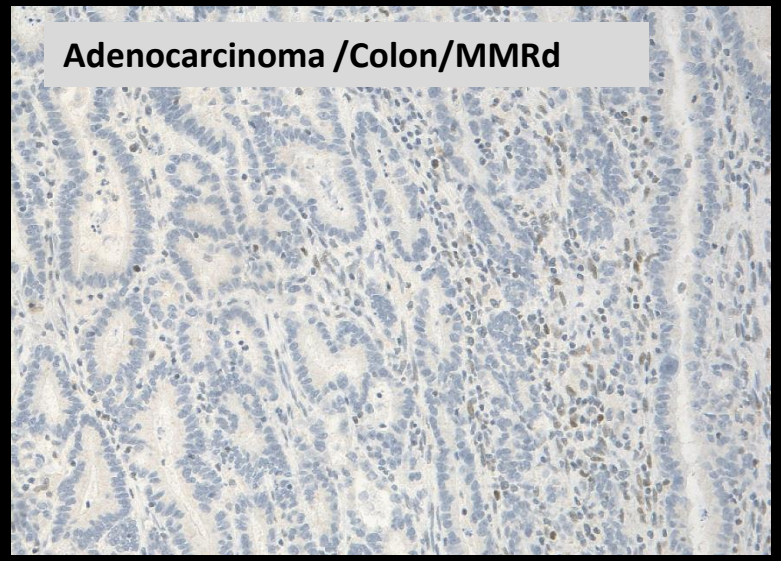
Omnis: HIER/HIGH pH 24` at 97°C, Flex+ Mouse (10+20`)

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Antibody diluents

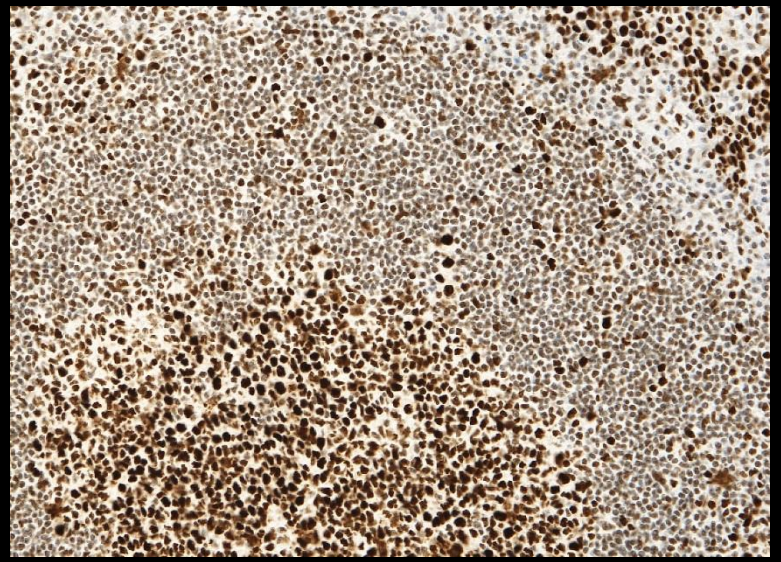
MSH2, FE11 1:50
Dako dil. pH 7.3

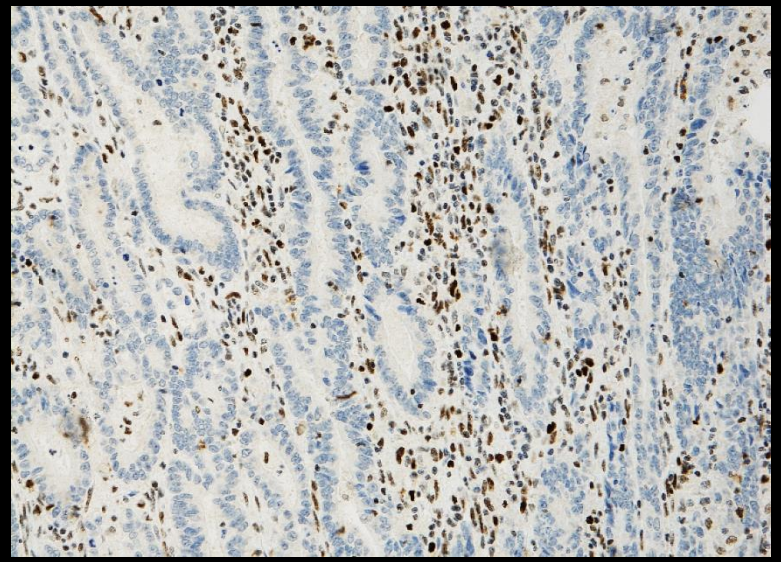




Omnis: HIER/HIGH pH 24`, Flex+ Mouse (10+20`)

MSH2, FE11 1:50
Renoir Red pH 6.2

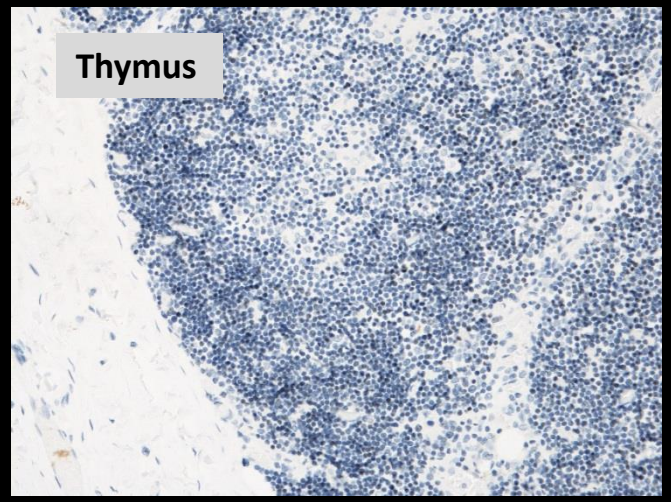


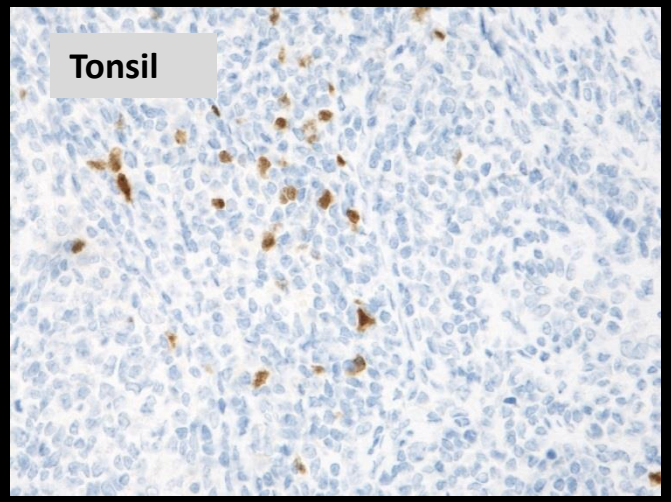


Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Antibody diluents

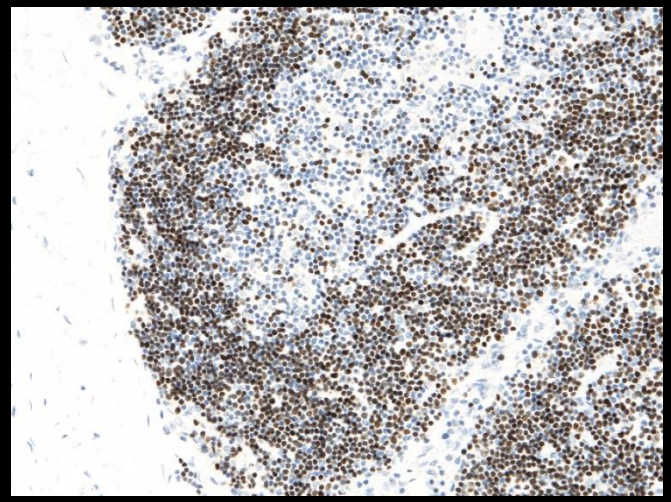
TdT, SEN28 1:50
Dako dil. pH 7.3

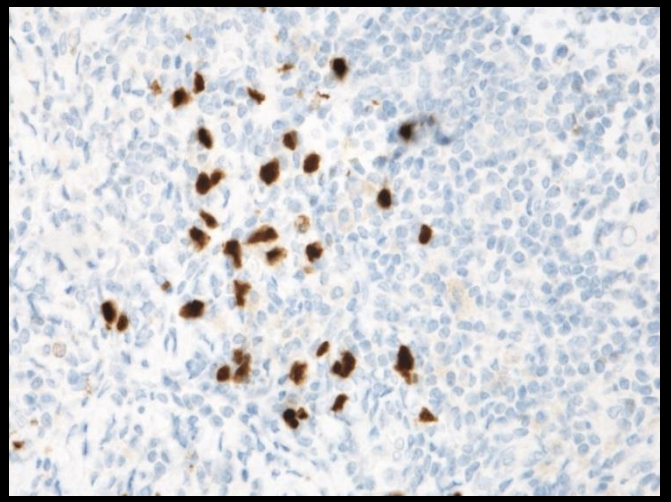




Omnis: HIER/HIGH pH 24`, Flex+ Mouse (10+20`)

TdT, SEN28 1:50
Renoir Red pH 6.2



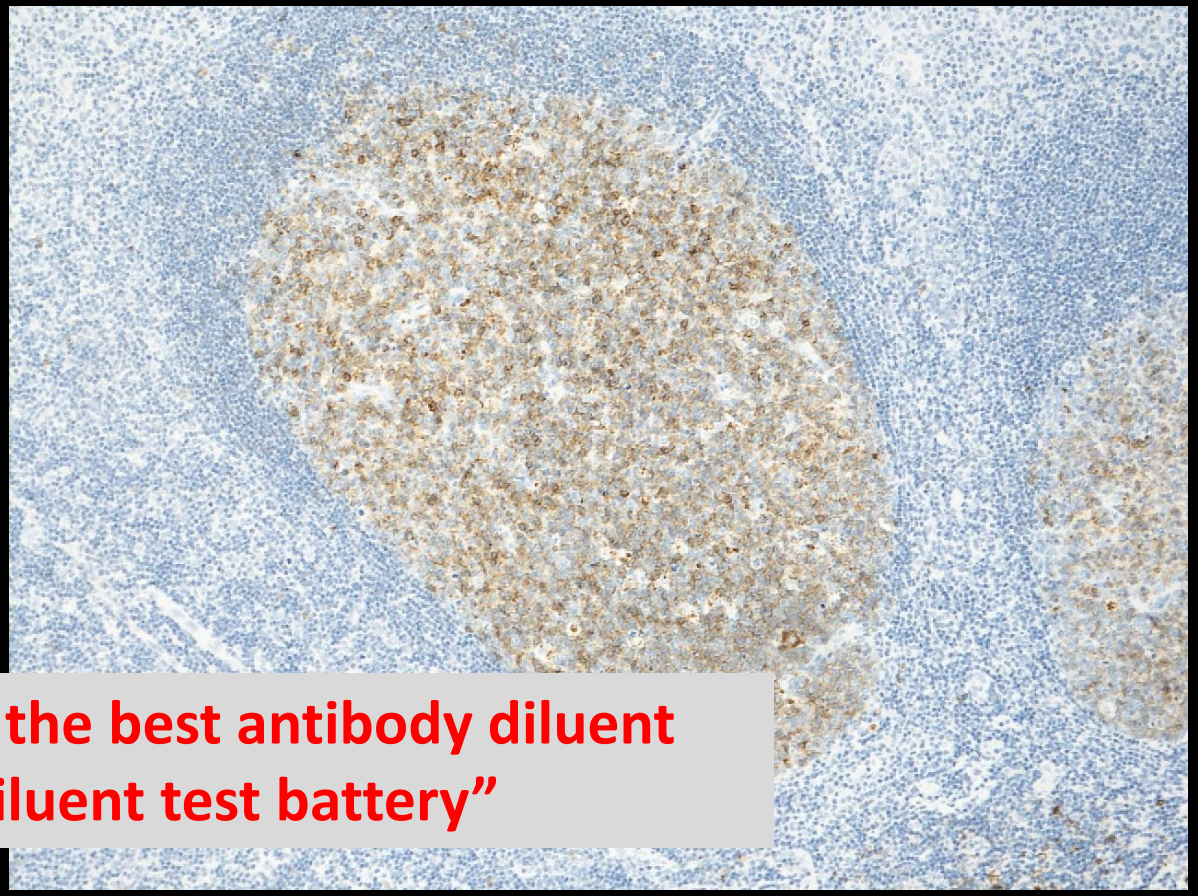


Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Antibody diluent

CD10 clone 56C6 (1:50)/Dako Diluent pH 7.3

CD10 clone 56C6 (1:50)/Renoir Red pH 6.2



Renoir Red is not always the best antibody diluent
Use a "antibody diluent test battery"

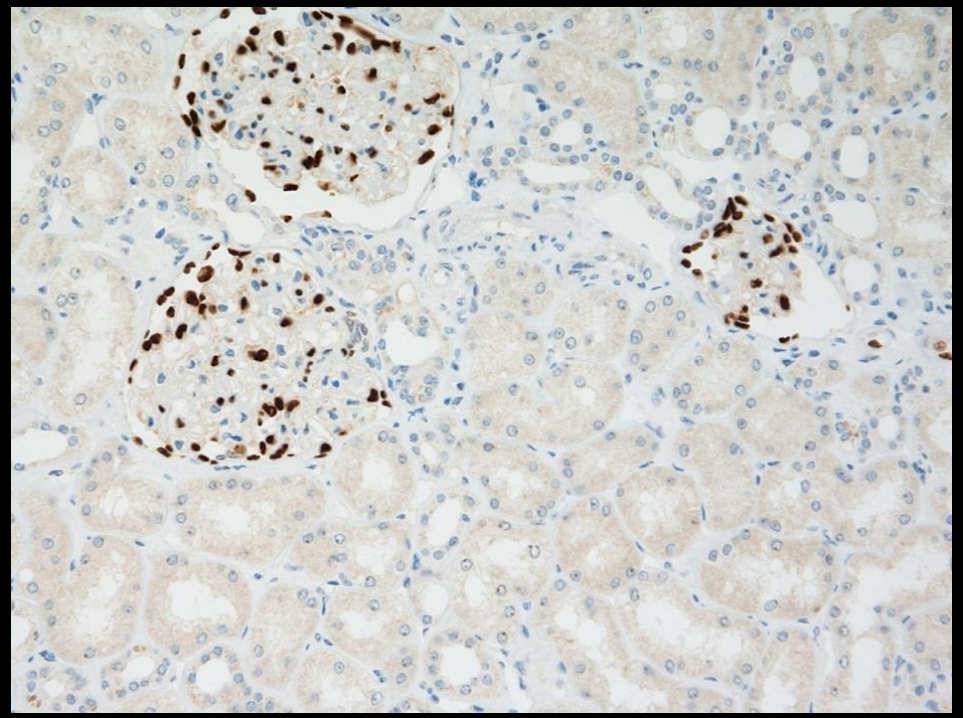
Omnis: HIER/HIGH pH 24`, Flex+ Mouse (10+20`)

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

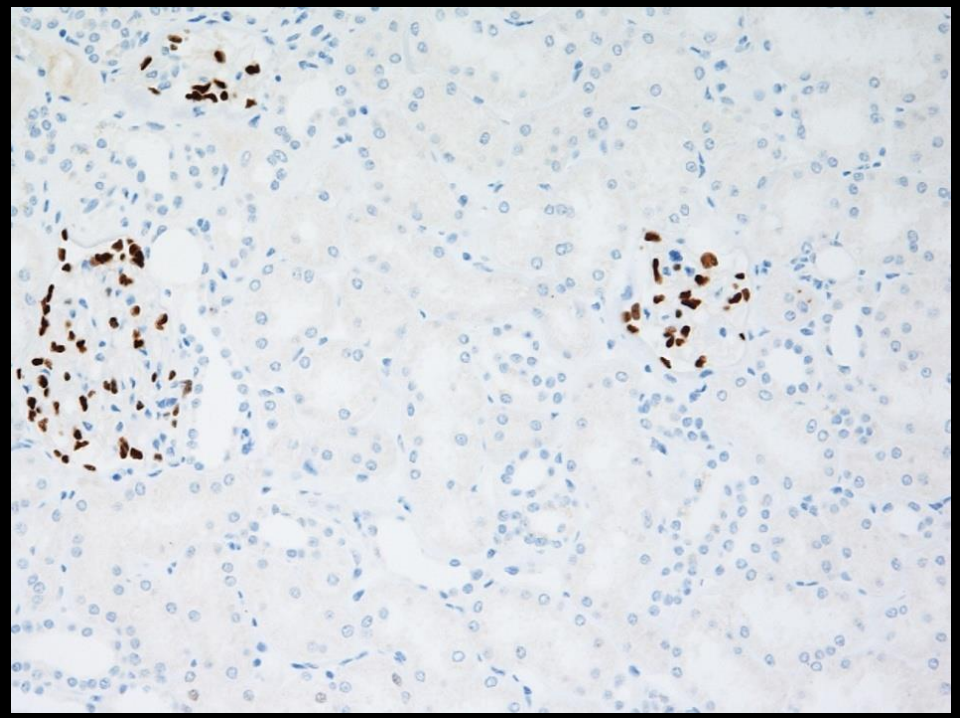
Antibody diluents

Kidney

WT1, EP122 1:25
Renoir Red (Biocare)



WT1, EP122 1:25
Background Sniper (Biocare)



The choice of antibody diluent can suppress unwanted/unspecific background staining

HIER TRS pH9 (24` /97°C) + Pep © (3`)

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

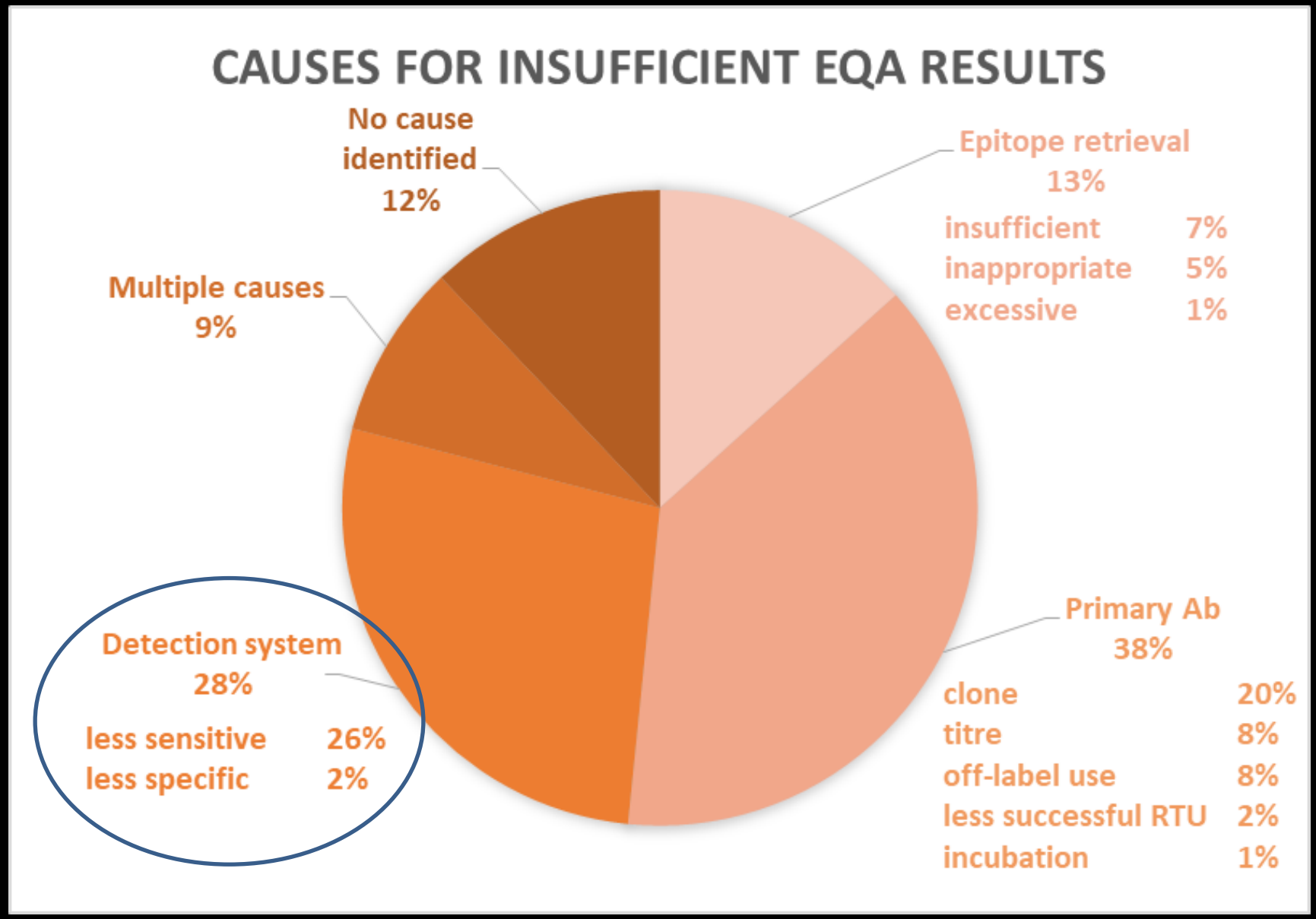
Antibody diluents (Experience from Dept. of surgical Pathology, Region Zealand, Denmark on the Omnis)

Antibody/Antigen reactions benefitting from diluting the primary Ab in Renoir Red pH 6.2 (improving signal):
ALK (1A4), CR (CAL6), CD4 (EP204), CD5 (SP19), CMYC (EP121), GATA3 (L20-823), GPC3 (1G12), IMP3 (69.1), MLH1 (BC23, ES05 & GM011), MSH2 (G219-1129), MSH2 (FE11), MSH6 (EP49), NKX 3.1 (poly), SALL4 (6E3), PAX8 (ZR1), PMS2 (EP51), SOX10 (EP268), SOX11 (C1 & MRQ58), TdT (SEN28 & EP266), UP-II (BC21), WT1 (WT49) and …….

Antibody/Antigen reactions unaffected from diluting the primary Ab in Renoir Red pH 6.2 and compared to Dako diluent pH 7.3:
BCL2 (124), BCL6 (LN22 & PG-B6p & GI191E/A3), CR (DAK-Calret1), CD10 (56C6), CD163 (MRQ26), CD21 (2G9), CD5 (4C7), ER (SP1), HHV8 (13B10), Mammaglobin (304-1A5), MUC5AC (CLH2), MUC6 (CLH5), and …….

Antibody/Antigen reactions benefitting from diluting the primary Ab in Background sniper (reduces background problems):
Spirochete (poly), BORR (poly), WT1 (EP122) and …….

NordiQC External Quality Assurance program



App. 20-30% of all results are evaluated as insufficient

79 % of insufficient results are related to the choice/use of:

- **Epitope retrieval procedure**
- **Primary Ab (including stainer platform)**
- **Detection system**

False positive and/or negative results

Courtesy of Birgit Truumees et al (NordiQC): Poster presented at the annual USCAP meeting 2024

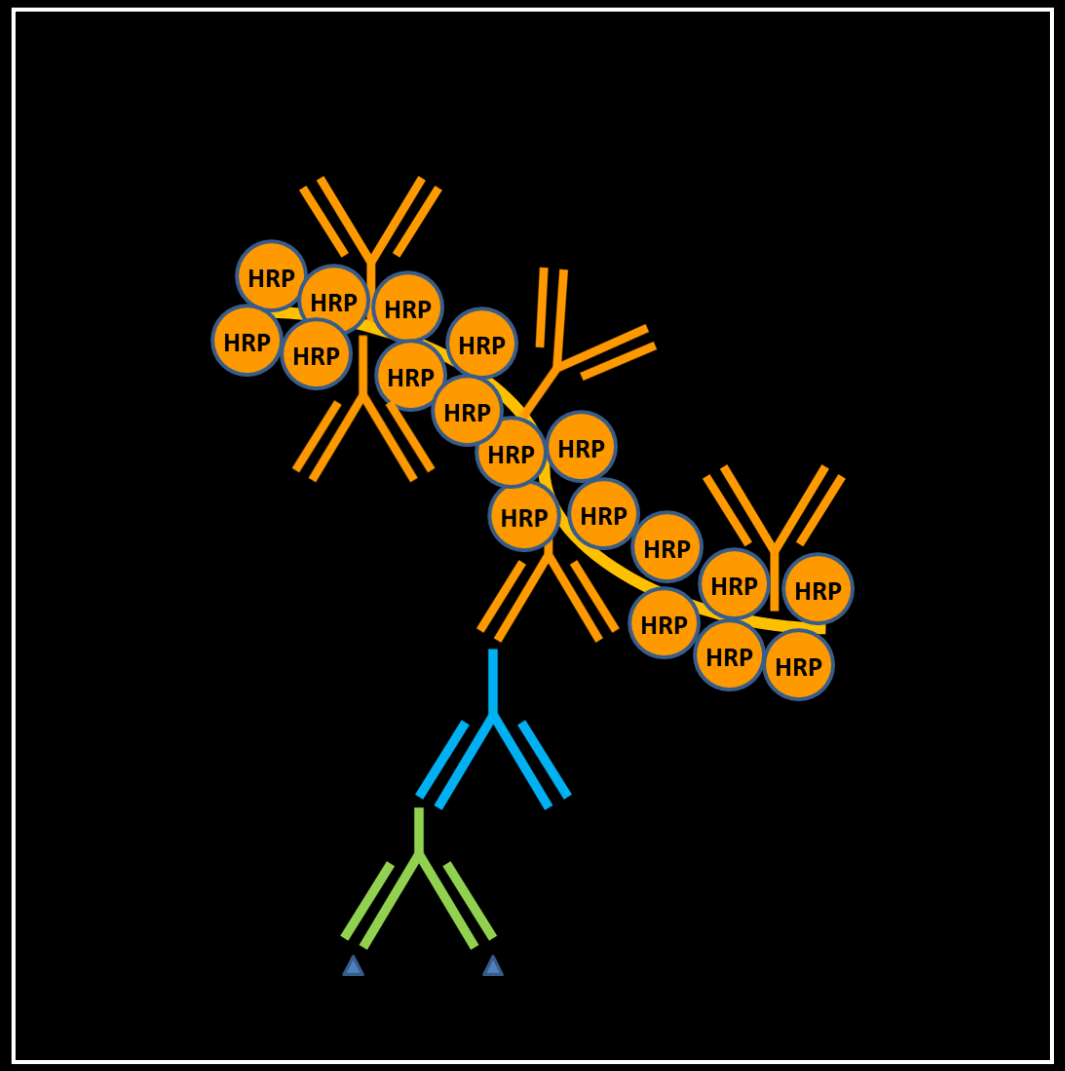
Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Considerations related to the choice of detection system:

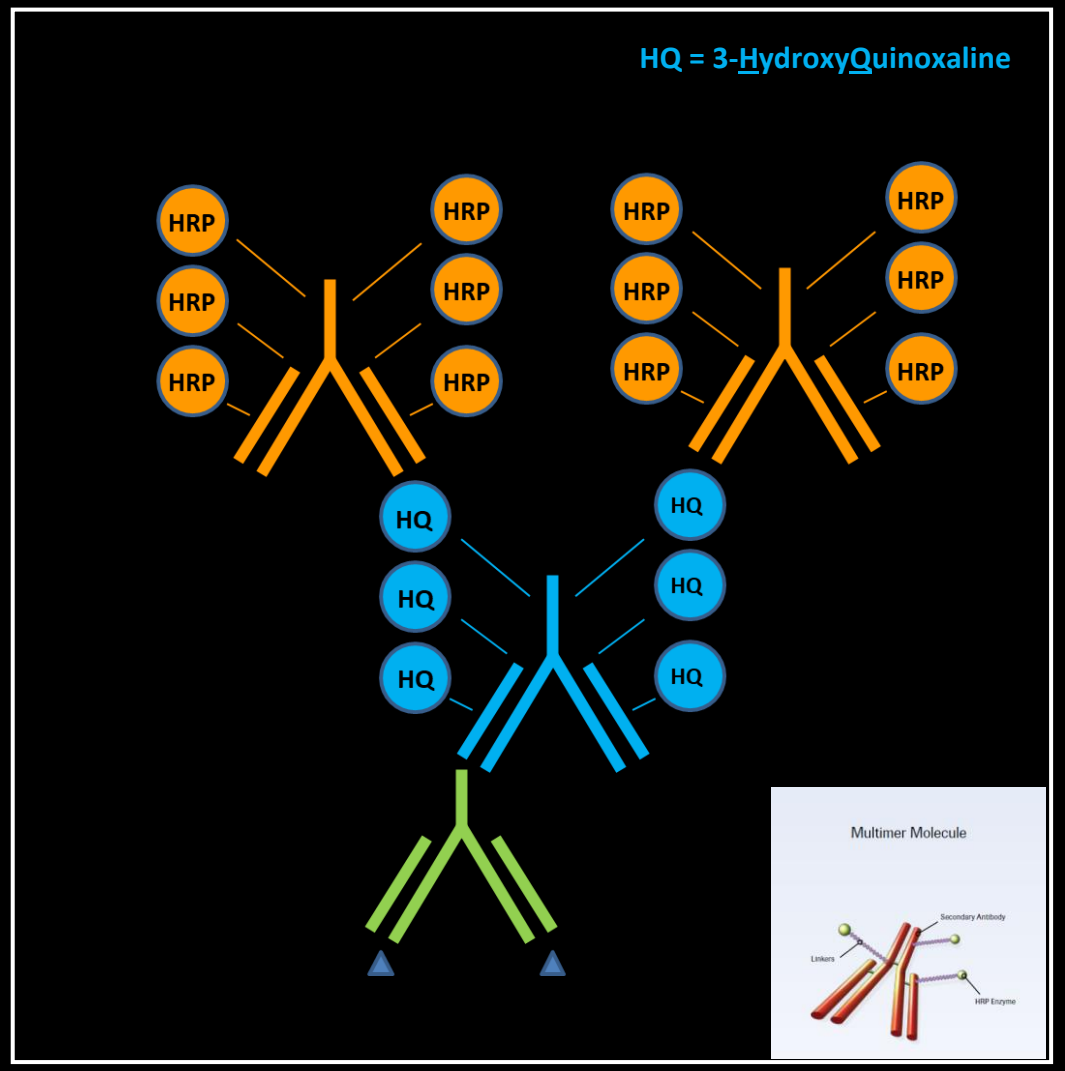
- ❑ Sensitivity
- ❑ Specificity
- ❑ Enzyme conjugate (HRP or AP)
- ❑ Blocking of endogenous activity (PAX5 clone 1EW, CD4 clone 1F6, BCL6 clone PG-B6p ……..)
- ❑ Turn around time (TAT)
- ❑ Automatic platform (open or closed system)
- ❑ Price

Virtually all NordiQC participants use a polymer/multimer based detection systems

NordiQC: Virtually all laboratories use a polymer or multimer detections system

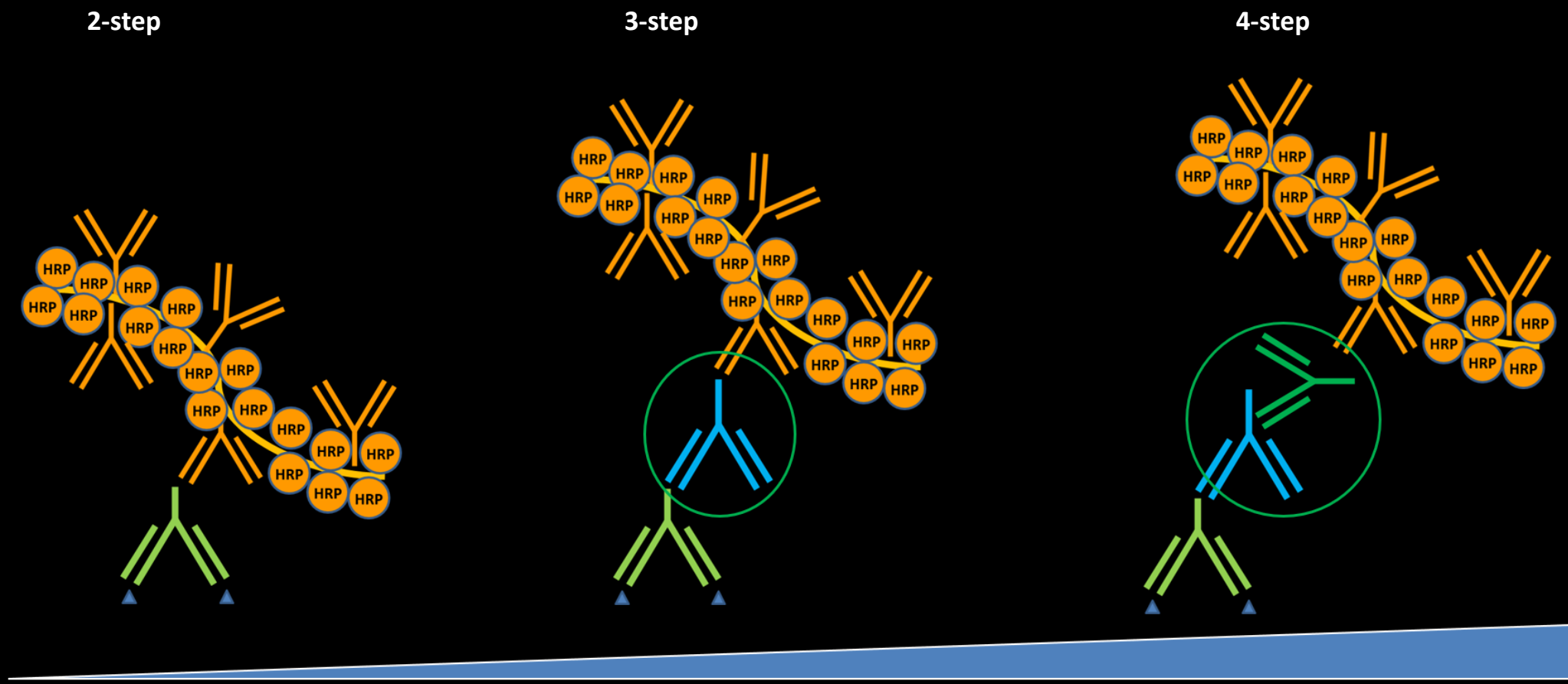


Polymer detection (e.g., Bond Refine, Envision Flex ……)



Multimer detection (Ultra View and OptiView)

Detection systems and sensitivity

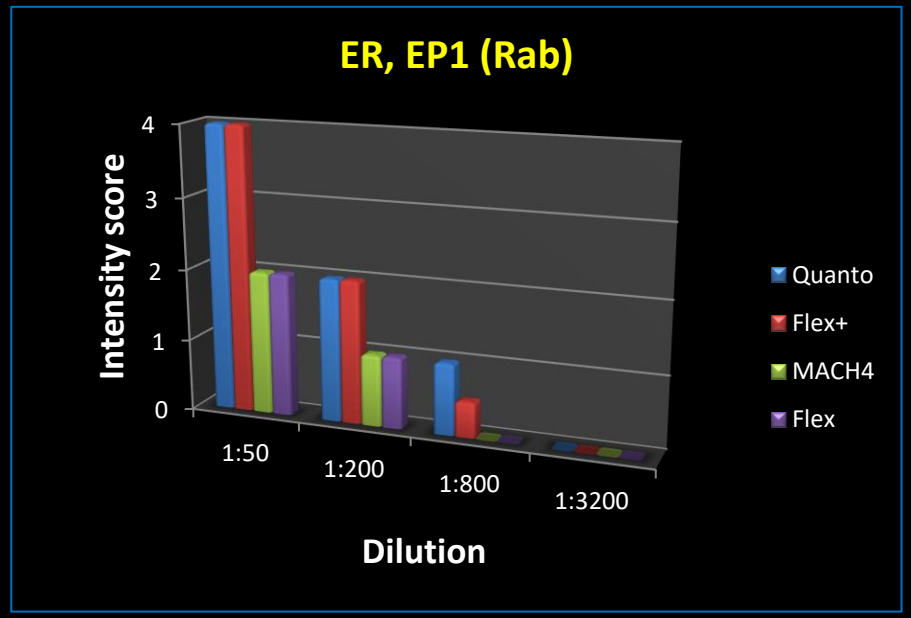


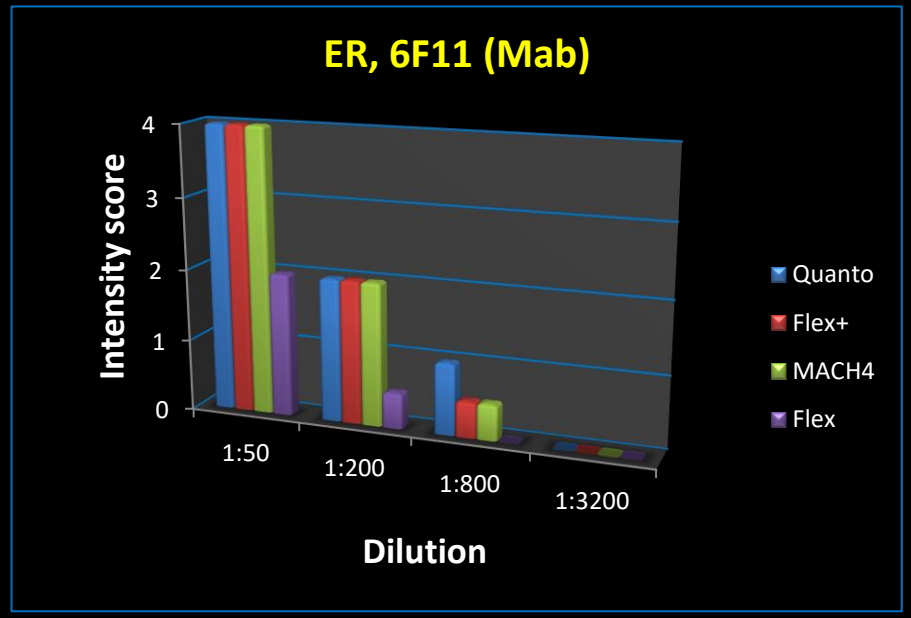
Increased sensitivity: Amplier A/B, HQ-Linker, Linker (Mouse/Rabbit), Enhancer, Universal Linker, Post Blocking .....

Know your detection system - strength and weakness

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Detection systems - Performance Testing





ER - Endpoint titration (some general remarks and important issues):

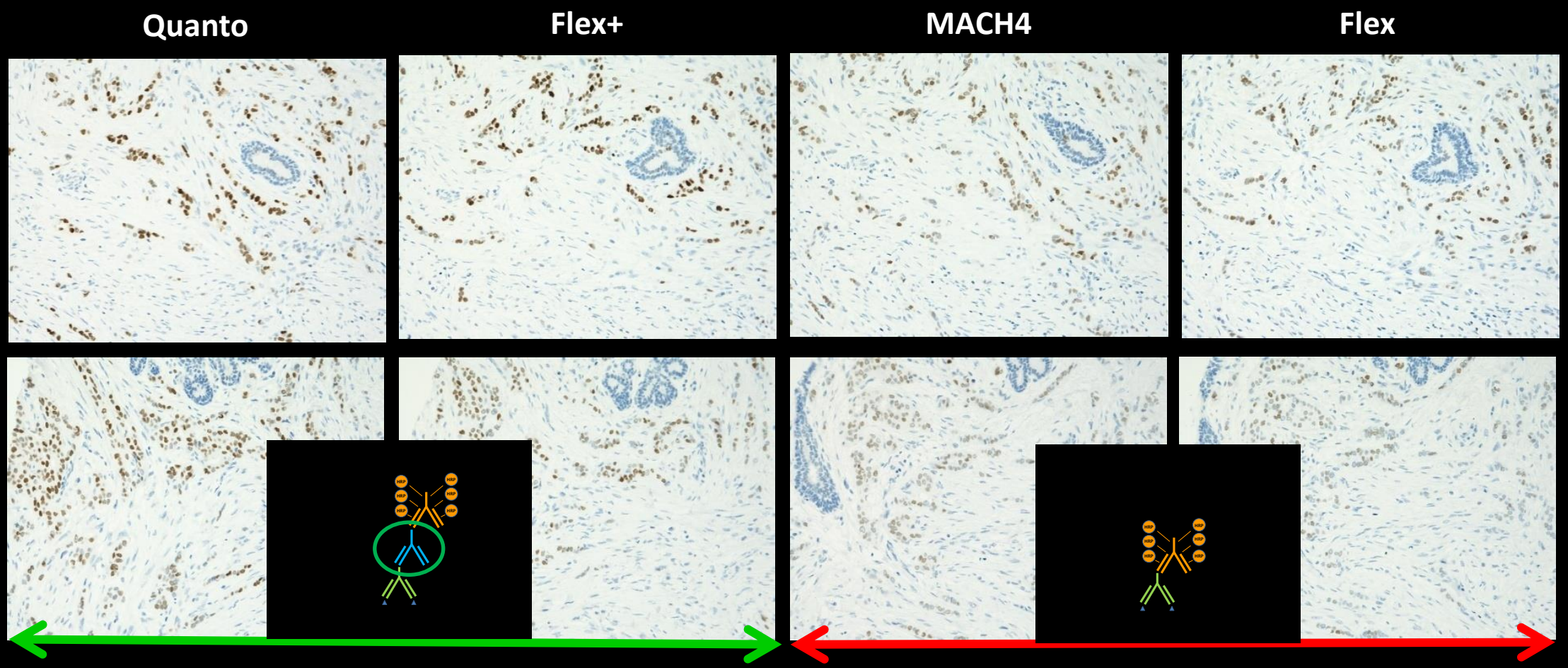
- **The 3-step polymer detection systems Quanto and Flex+ - produced the overall highest intensity.**
- **The 3-step polymer detection system MACH4 only enhances reactions with mouse monoclonal Abs (ER,6F11).**
- **"Optimal staining" was highly influenced by the concentration of the primary Abs and the nature of detection system.**

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Performance testing of detection systems (Vendor recommended protocol settings)

Breast tumor

ER, EP1 (Rab)

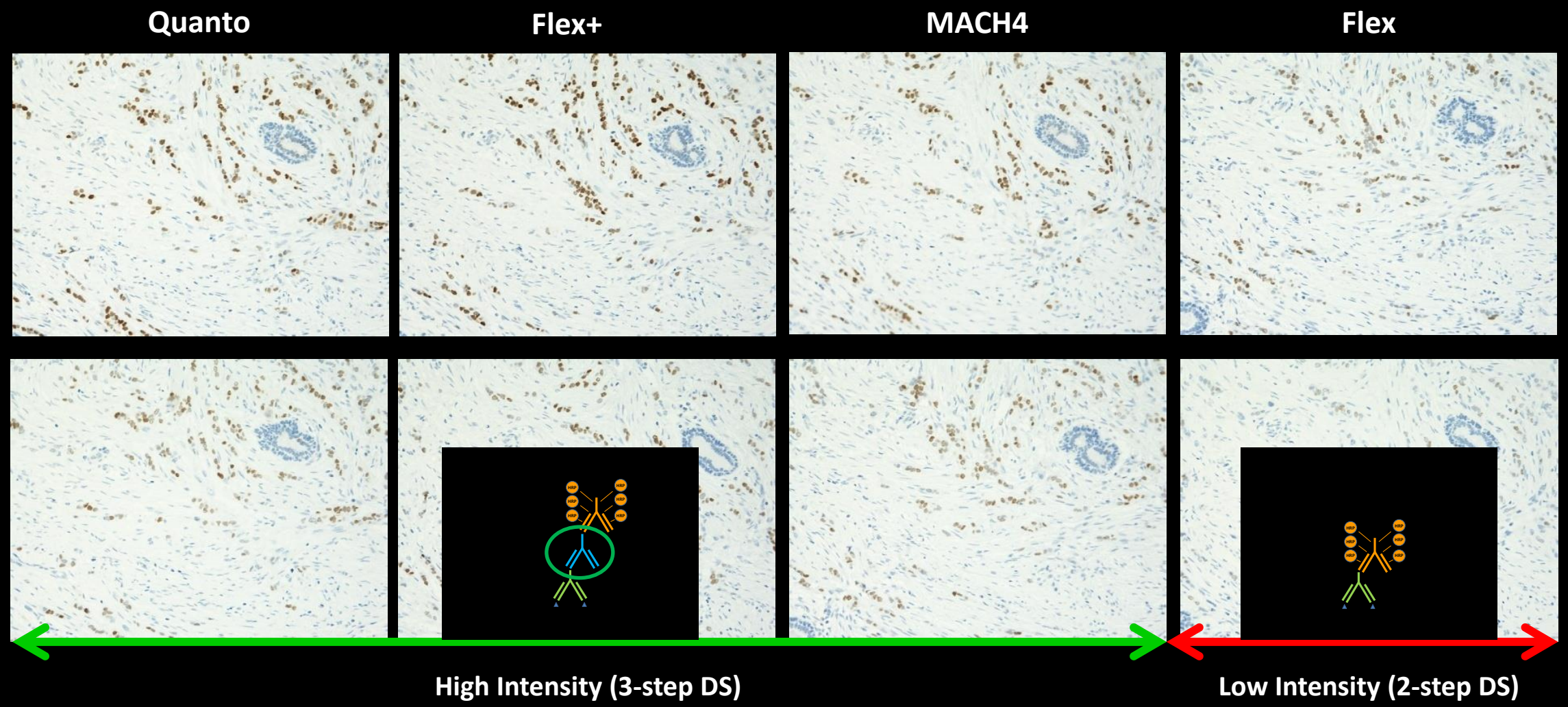


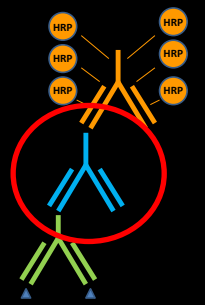
The technical test approach – Analytical phase

Performance testing of detection systems (Vendor recommended protocol settings)

Breast tumor

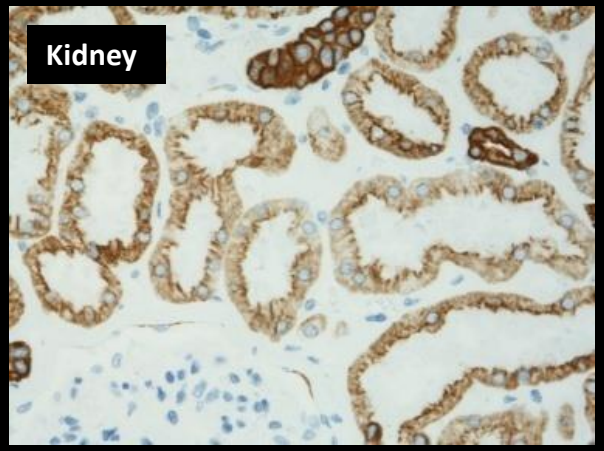
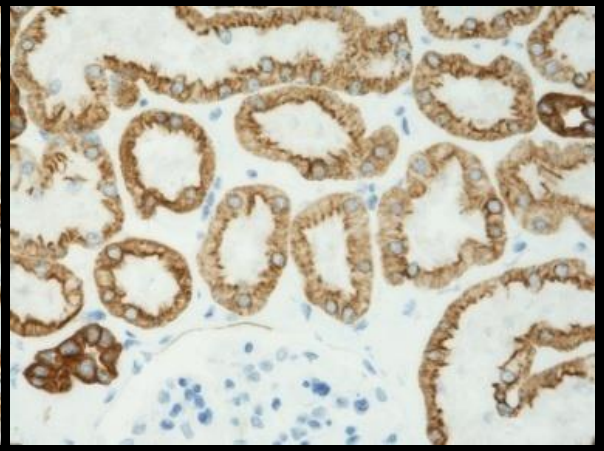
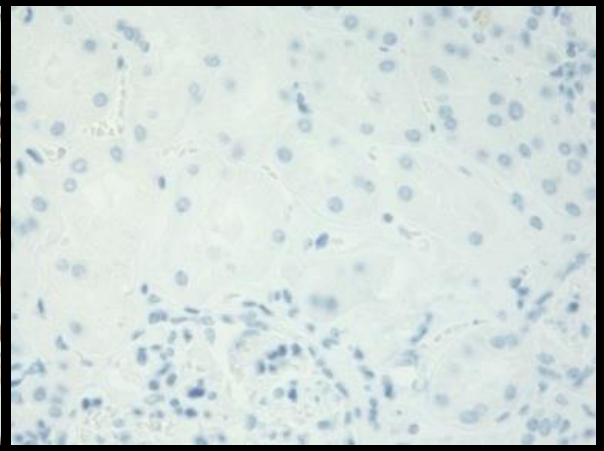
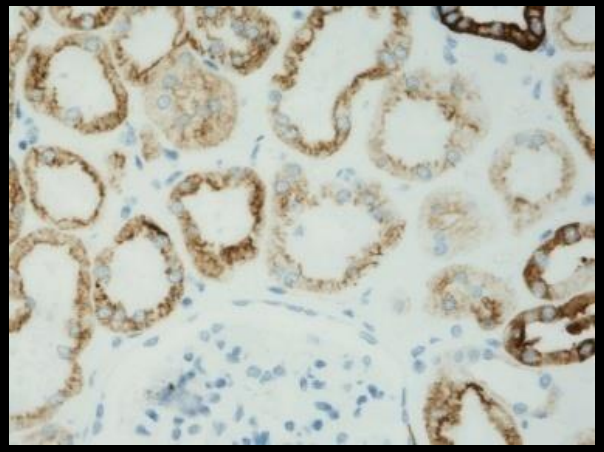
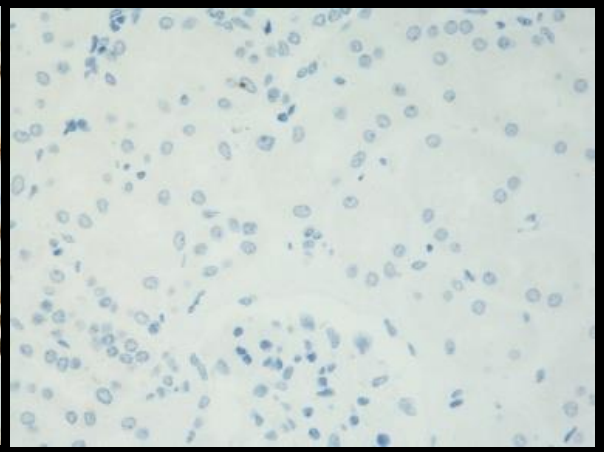
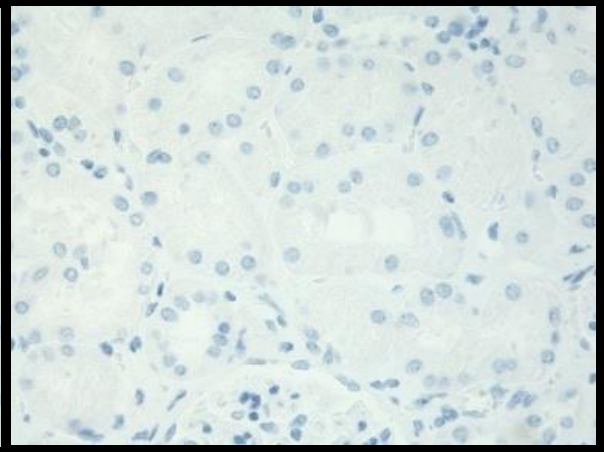
ER, 6F11 (Mab)

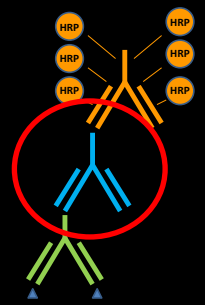




Polymer based detection systems

Without enhancement (second step)

	EnVision Flex (- Linker)	MACH4 (- Mouse probe)	Quanto (- Amplifier)
CK8, EP1628Y (rabbit Ab)			
CK8, TS1 (mouse Ab)			



Polymer based detection systems (Næstved LAB)

Expected reaction patterns

	Quanto LabVision TL-125-QHD	Quanto - Amplifier	Flex+ Dako K8012	Flex - linker	MACH4 Biocare M4U534	MACH4 - Mouse probe
Anti-X (Mab) Mouse primary Ab	+	-	+	(+)	+	-
Anti-X (Rab) Rabbit primary Ab	+	-	+	(+)	(+)	(+)

Detection system	Second step	HRP-Polymer	Enhances signal of
Quanto (LabVision)	Amplifier anti-Mab/Rab or Hapten conjugate ?	Quanto polymer (anti-unknown species or Hapten)	Both primary Mab or Rab
Flex+ (Dako)	Linker Mouse (Rab anti Mab) Linker Rabbit (Mab anti Rab)	Flex polymer anti-Mouse/Rabbit	Both primary Mab or Rab
MACH4 (Biocare)	Mouse probe anti-Mouse (Rab)	MACH4 polymer Anti-Rabbit	Only primary Mab
Flex*	-	Flex polymer anti-Mouse/Rabbit	-
* 2-step polymer system			

Know your detection system - strength and weakness

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Less sensitive detection systems

CYCD1, RUN 47 (2016)

Table 3. **Proportion of optimal results for CyD1 for the most commonly used antibodies as concentrate on the 3 main IHC systems***

Concentrated antibodies	Dako Autostainer / Omnis		Ventana BenchMark XT / Ultra		Leica Bond III / Max	
	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	ER2 pH 9.0	ER1 pH 6.0
rmAb clone **EP12**	4/5** (80%)	-	3/5 (60%)	-	1/2	-
rmAb clone **SP4**	20/41** (64%)	0/1	11/31 (49%)	-	2/15 (13%)	0/1

* Antibody concentration applied as listed above, HIER buffers and detection kits used as provided by the vendors of the respective systems.
** (number of optimal results/number of laboratories using this buffer)

Bond™ Polymer Refine Detection
Catalog No: DS9800

Intended Use

This detection system is for *in vitro* diagnostic use.

Bond Polymer Refine Detection is a biotin-free, polymeric horseradish peroxidase (HRP)-linker antibody conjugate system for the detection of tissue-bound mouse and rabbit IgG and some mouse IgM primary antibodies. It is intended for staining sections of formalin-fixed, paraffin-embedded tissue on the Bond™ automated system.

The clinical interpretation of any staining or its absence should be complemented by morphological studies and proper controls. They should be evaluated within the context of the patient's clinical history and other diagnostic tests by a qualified pathologist.

The Bond Polymer Refine Detection Kit must be used with laboratory best practice in the use of tissue controls. For assurance, laboratories should stain each patient sample in conjunction with positive, negative, and other tissue specific controls as needed.

Summary and Explanation

Immunohistochemical techniques can be used to demonstrate the presence of antigens in tissue and cells (see "Using Bond Reagents" in your Bond user documentation).

Bond Polymer Refine Detection utilizes a novel controlled polymerization technology to prepare polymeric HRP-linker antibody conjugates. The detection system avoids the use of streptavidin and biotin, and therefore eliminates non-specific staining as a result of endogenous biotin.

Bond Polymer Refine Detection works as follows:

- The specimen is incubated with hydrogen peroxide to quench endogenous peroxidase activity.
- A user-supplied specific primary antibody is applied.
- Post Primary IgG linker reagent localizes mouse antibodies.
- Poly-HRP IgG reagent localizes rabbit antibodies.
- The substrate chromogen, 3,3'-Diaminobenzidine tetrahydrochloride hydrate (DAB), visualizes the complex via a brown precipitate.
- Hematoxylin (blue) counterstaining allows the visualization of cell nuclei.

Using Bond Polymer Refine Detection in combination with the Bond automated system reduces the possibility of human error and inherent variability resulting from individual reagent dilution, manual pipetting and reagent application.

The detection system Bond Refine acts by nature as a 2-step polymer system for detection of rabbit polyclonal or rabbit monoclonal primary antibodies

Only enhances reactions with mouse primary antibodies due to the Post Primary IgG linker (Rabbit antibody)

NQC Run 66, 2022: Synaptophysin clone DAK-Synap (RTU GA660)

The choice of the detection system can significantly influence the result

Synaptophysin (RTU GA660, DAK-SYNAP)	Pass rate / Optimal results
VRPS (FLEX+ with linker)	100% (34/34) / 85%
LMPS (FLEX without linker)	0% (13/13) / 0%

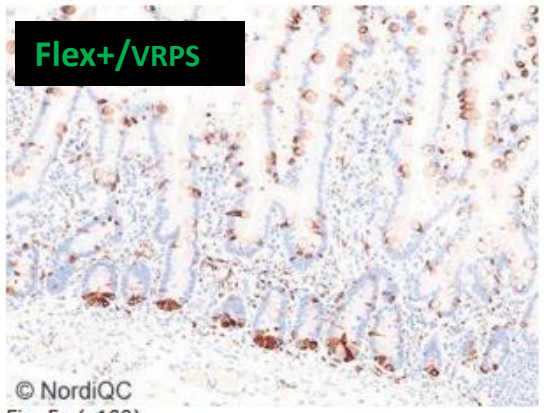


Fig. 5a (x100)
Optimal staining reaction for SYP in the duodenum using the mAb clone DAK-SYNAP (RTU GA660, Dako/Agilent), with vendor recommended protocol settings. The Paneth cells in the bottom of the crypt of Lieberkühn display a strong staining reaction. The goblet cells both in the mucin contained in the cytoplasm and in the cell membrane surrounding the mucin display a moderate to strong staining reaction without any staining of the other epithelia cells. Same protocol used in Fig. 6a.

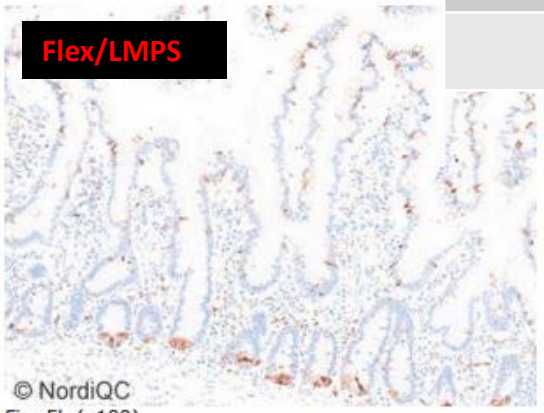


Fig. 5b (x100)
Insufficient staining reaction for SYP in the duodenum using the mAb clone DAK-SYNAP (RTU GA660, Dako/Agilent), with the same settings as in Fig. 5a but without linker. The Paneth cells in the bottom of the crypt of Lieberkühn display a weak to moderate staining reaction and only few goblet cells in the bottom of the crypts are weakly stained.
Same protocol used in Fig. 6b – same field as 1a- 1b and 5a.

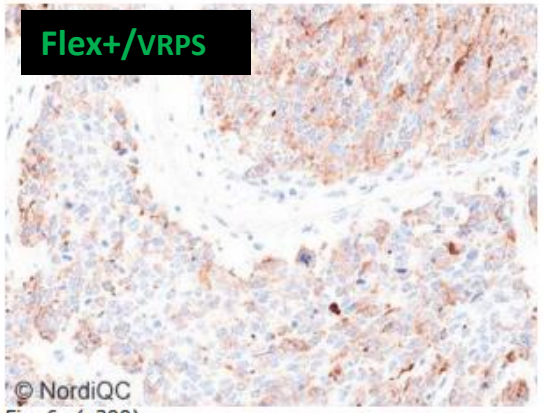


Fig. 6a (x200)
Optimal staining reaction of SYP in the SCLC using same protocol as in Fig. 5a. All majority of the neoplastic cells show a weak to strong cytoplasmic staining reaction.

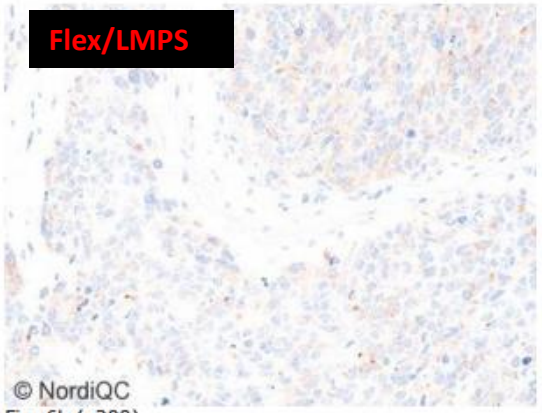


Fig. 6b (x200)
Insufficient staining reaction of SYP in the SCLC using same protocol as in Fig. 5b – same field as Figs. 2a- 2b and 6a. The neoplastic cells display a too weak staining intensity with large parts of the tumor completely negative.

NQC Run 70, 2024: GATA3 clone L50-823 (RTU 760-4897)

The choice of the detection system can significantly influence the result

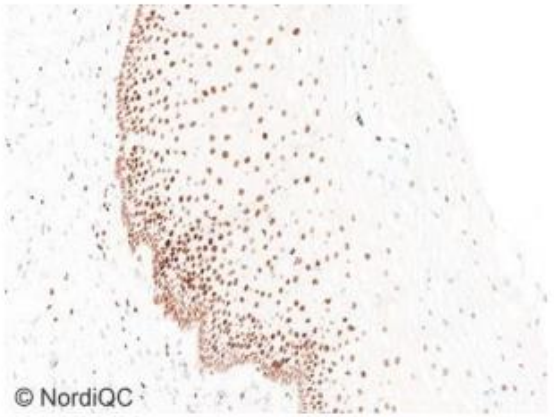


Fig. 1a (x100)
Optimal GATA3 staining reaction of the uterine cervix using the RTU system 760-4897 (Ventana/Roche), based on the mAb clone L50-823, applying vendor recommended protocol settings and OptiView as detection system. The squamous epithelial cells in the basal and intermediate layer of the surface epithelium display a weak to moderate, but distinct nuclear staining reaction, whereas the nuclei of superficial layers and stroma cells are negative. Same protocol settings as in Figs. 2a-4a.

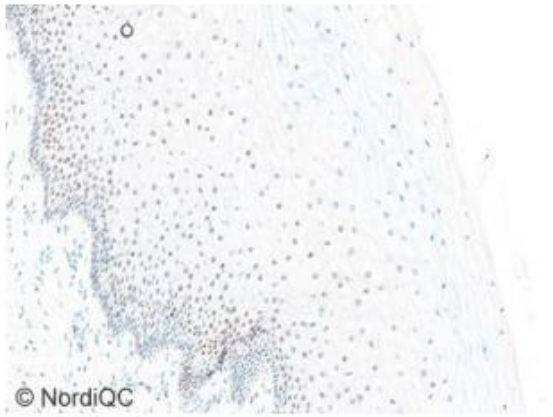


Fig. 1b (x100)
Insufficient GATA3 staining reaction of the uterine cervix using the same RTU system as in Fig. 1a, but with the vendor recommended protocol settings based on UltraView as the detection system. The proportion and intensity of cells expected to be demonstrated is significantly reduced, displaying only faint or false negative staining reaction. Same protocol settings as in Figs. 2b-4b. Same field as Fig 1a.

RTU system 760-4897 (Ventana/Roche) applying VRPS

OptiView vs UltraView ?

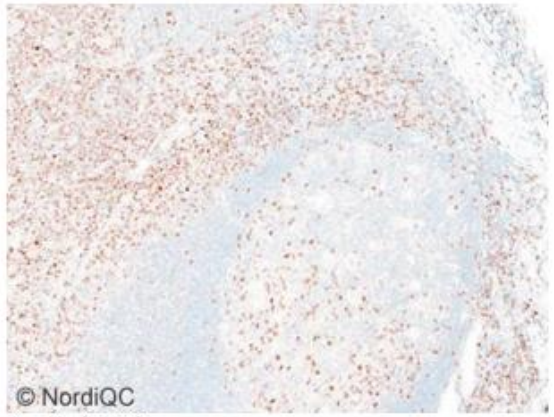


Fig. 2a (x100)
Optimal GATA3 staining reaction of the tonsil using the same protocol as in Figs. 1a-4a. The vast majority of T helper cells (Th2) display a moderate but distinct nuclear staining reaction, whereas the B-cells are negative.

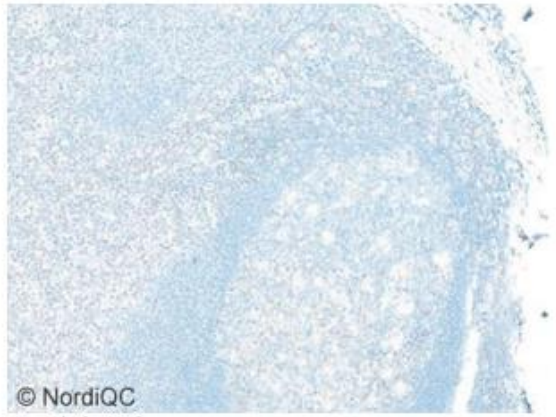


Fig. 2b (x100)
Insufficient GATA3 staining reaction of the tonsil using the same protocol as in Figs. 1b-4b. The vast majority of T helper cells (Th2) are false negative and only a fraction of germinal centre T-cells are weakly demonstrated – compare with Fig. 2a, same field.

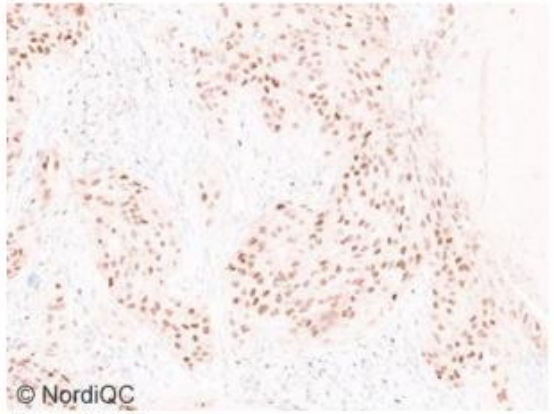


Fig. 3a (x100)
Optimal GATA3 staining reaction of the breast carcinoma using same protocol settings as in Figs. 1a-4a. A weak to strong nuclear staining reaction of virtually all neoplastic cells are seen.

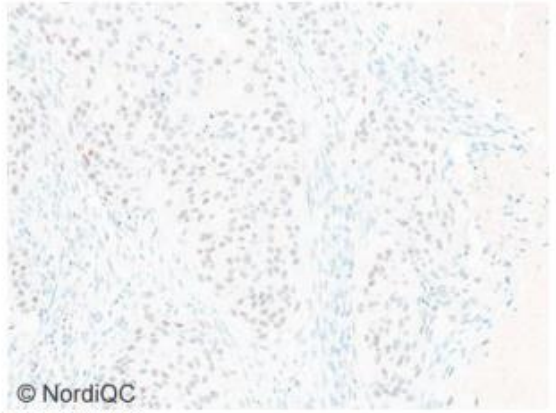
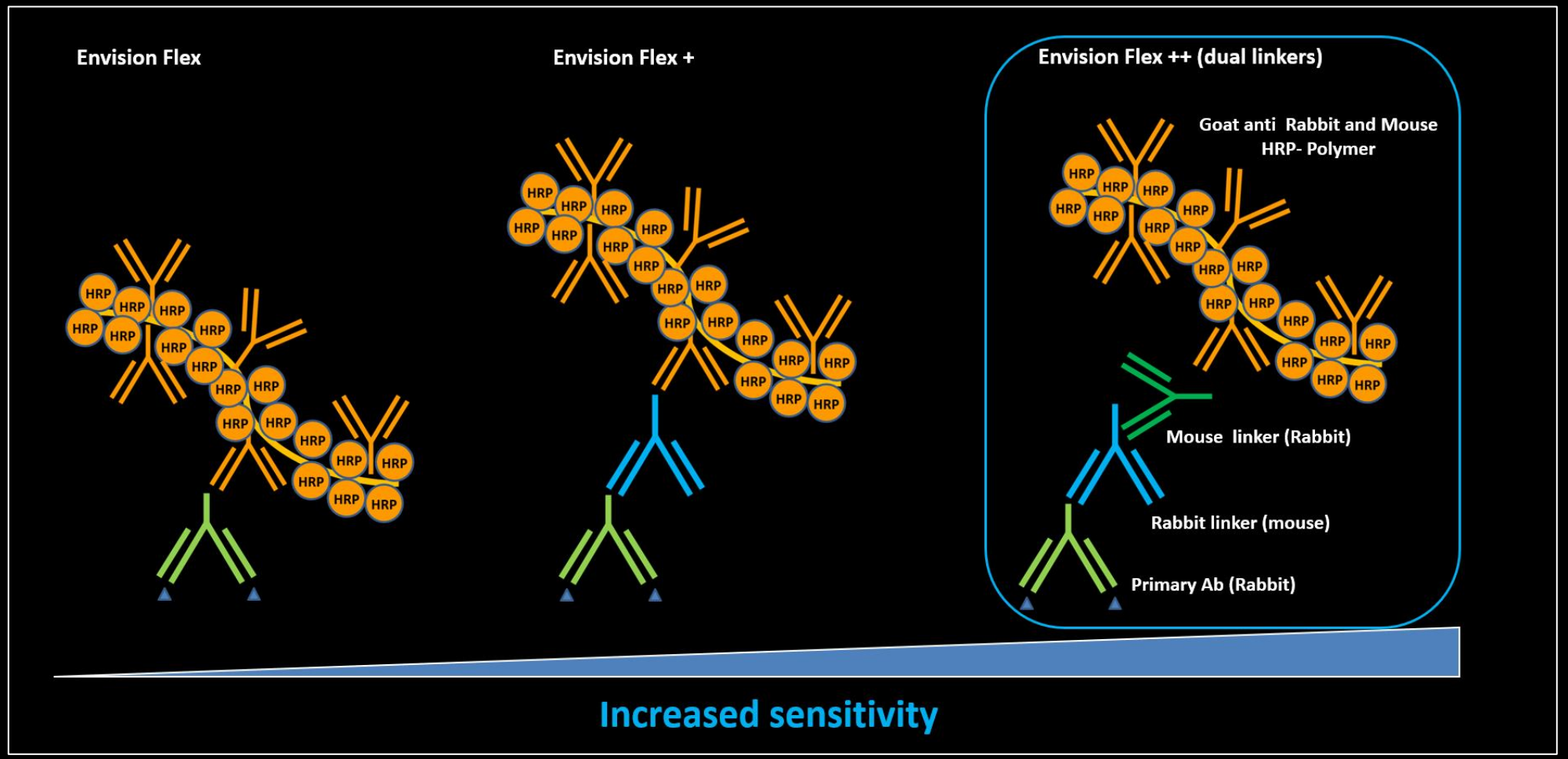


Fig. 3b (x100)
Insufficient GATA3 staining reaction of the breast carcinoma using the same protocol settings as in Figs. 1b-4b. Many of the neoplastic cells are false negative – compare with Fig. 3a

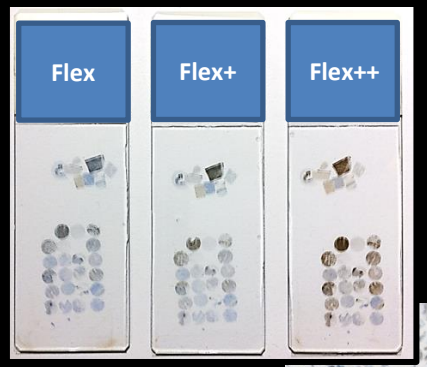
Technical aspects of immunohistochemistry & pitfalls - Analytical phase



"New option" on the Omnis: Use of dual linkers

In general, works well with rabbit primary Abs but less efficient with primary mouse Abs

Omnis: PMS2 clone EP51 (1:300 RR) /High pH 48`



FLEX (2-step)

FLEX+ (3-step)

FLEX++ (4-step)

Colon tumor

MMRd

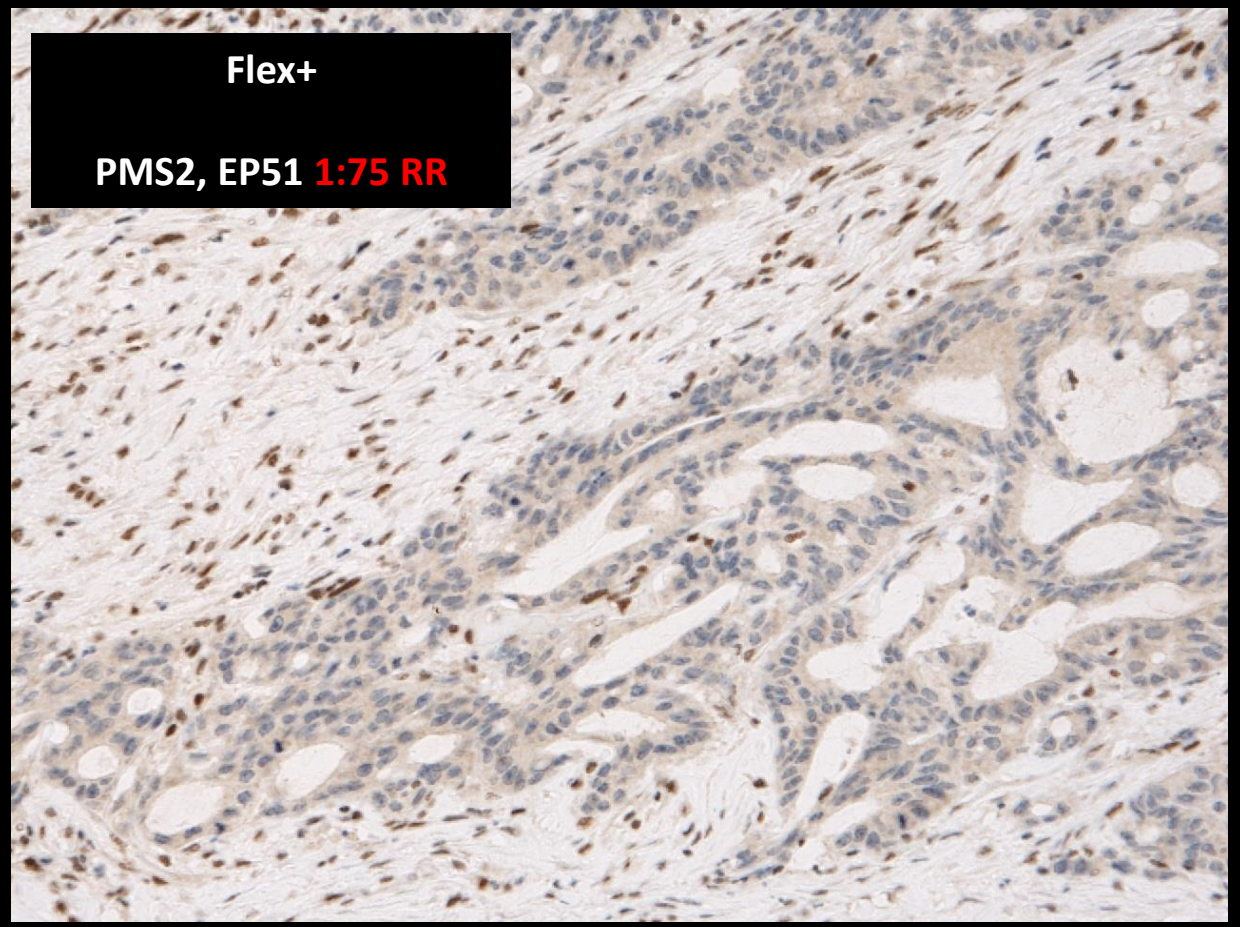
Sensitivity

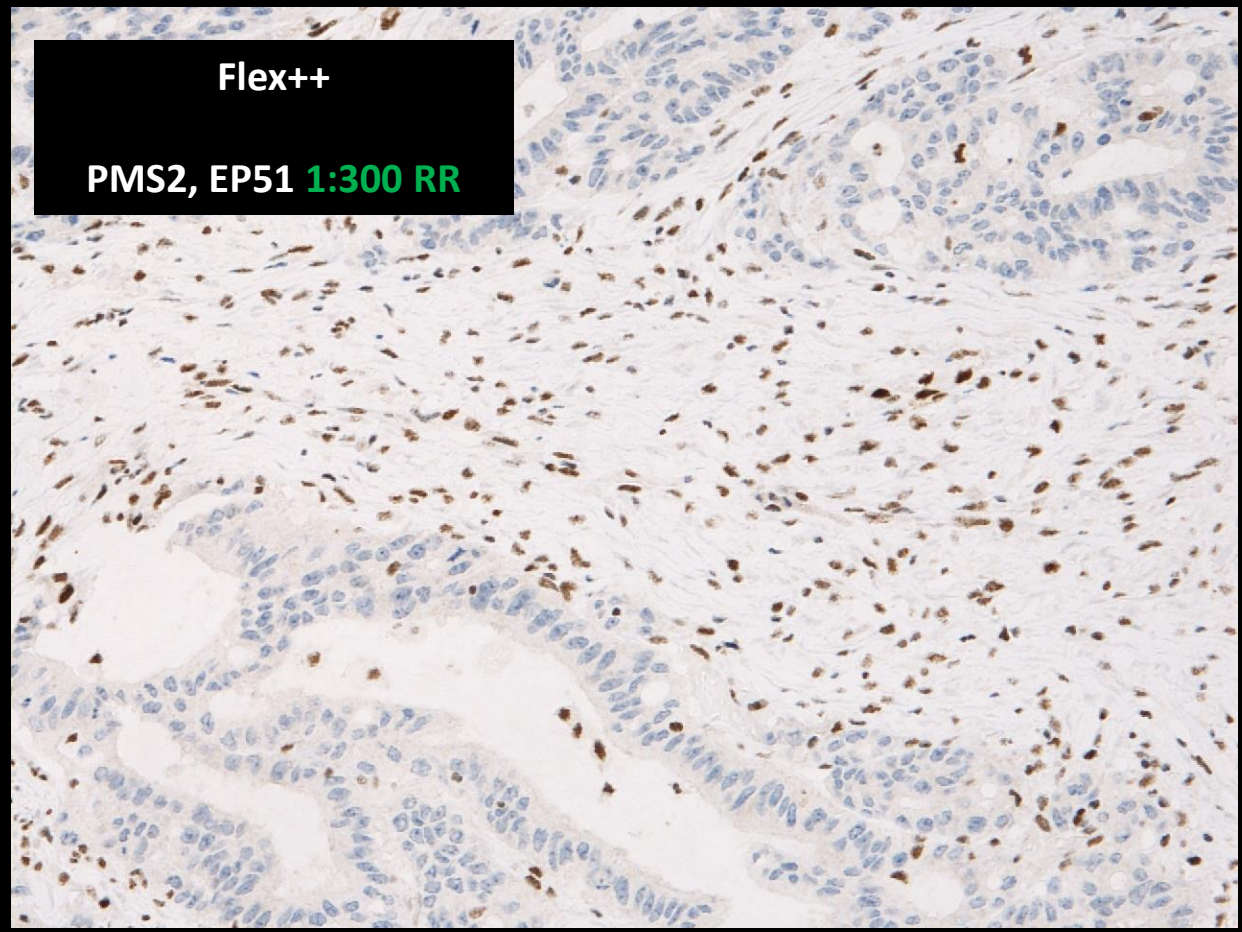
Colon tumor

MSS

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Colon tumor with loss of PMS2: Improves staining quality due to reduced background/noise





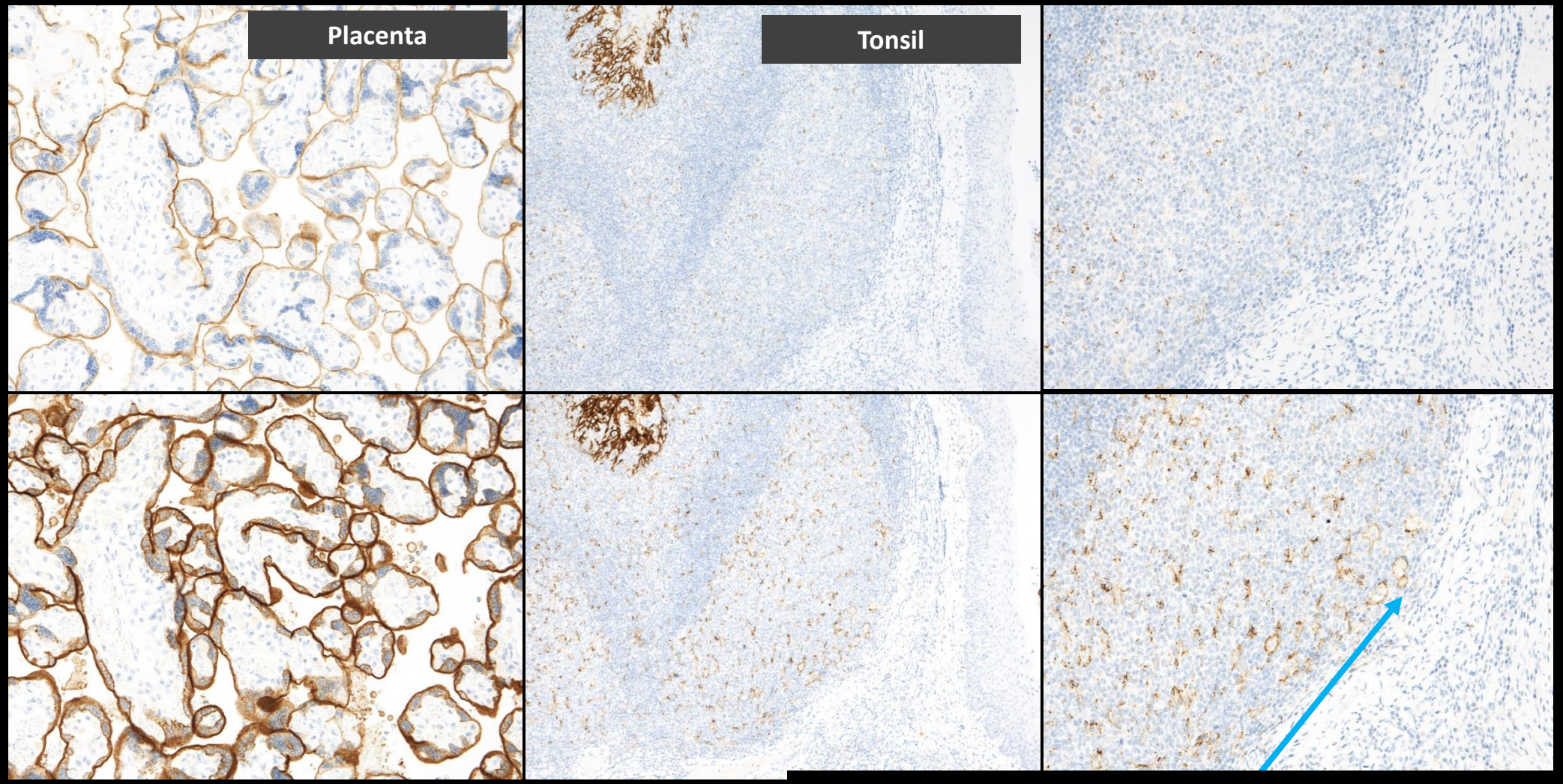
Omnis: HIER High pH 48`

Flex++ : 4-step polymer detection system (30-10-10-20`/Omnis)

rmAb PD-L1, CAL10 (rmAb 1:30RR); HIER in High pH 48`

Flex+

Flex++

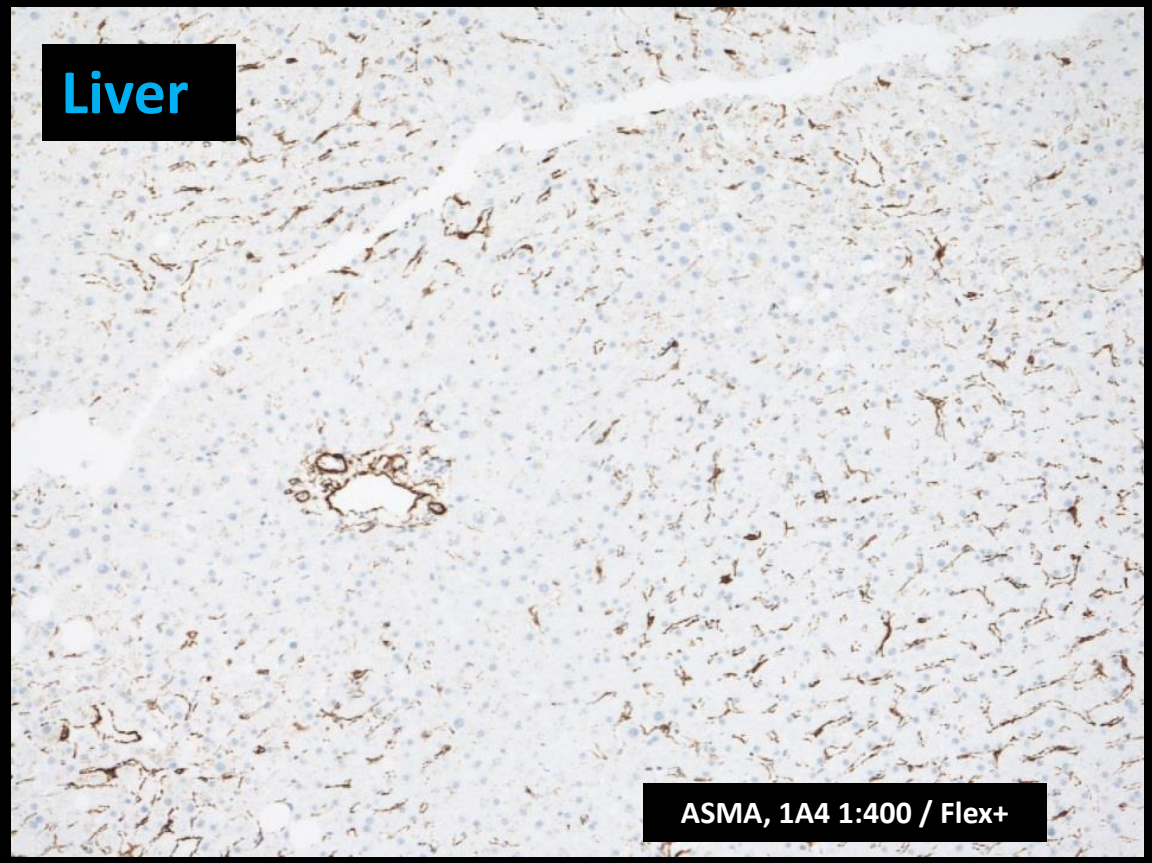


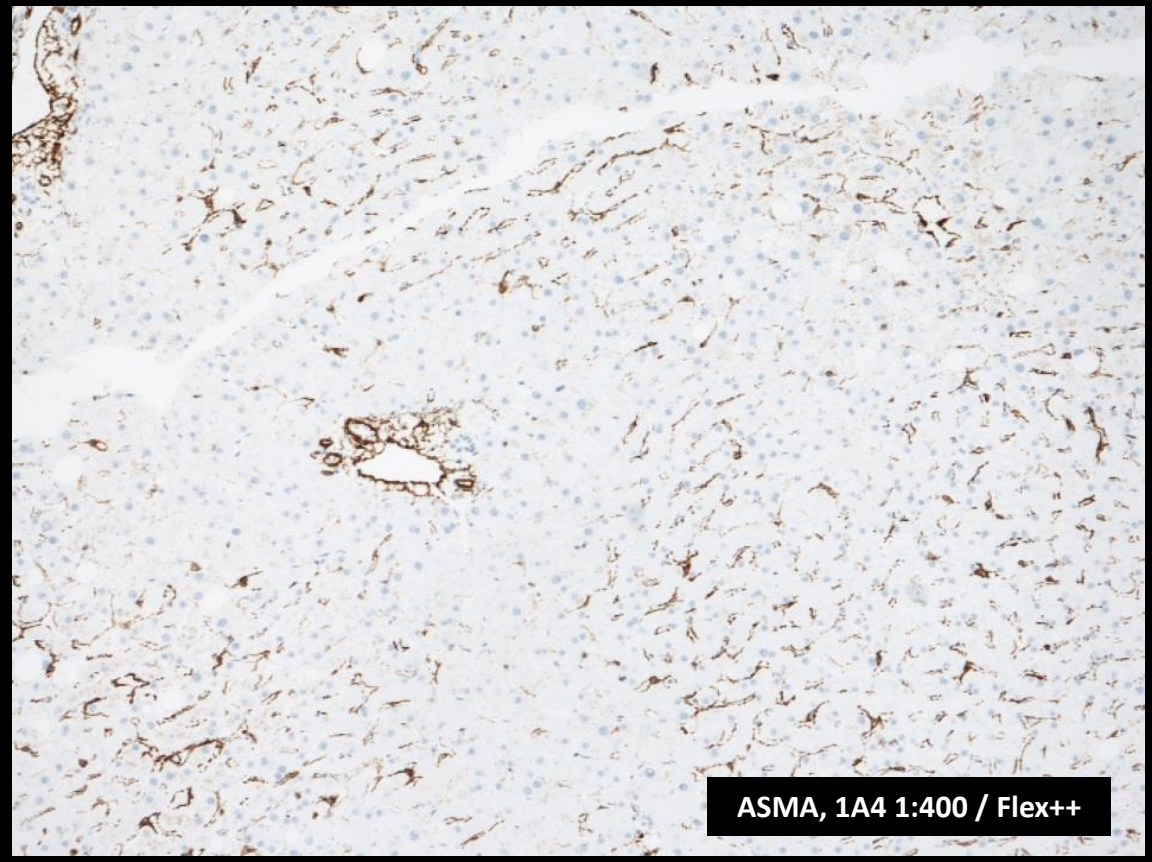
rmAbs: Dual linkers enhances the sensitivity of the assay

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Flex++ : 4-step polymer detection system (Omnis)

mAb ASMA clone 1A4; HIER in High pH 24`





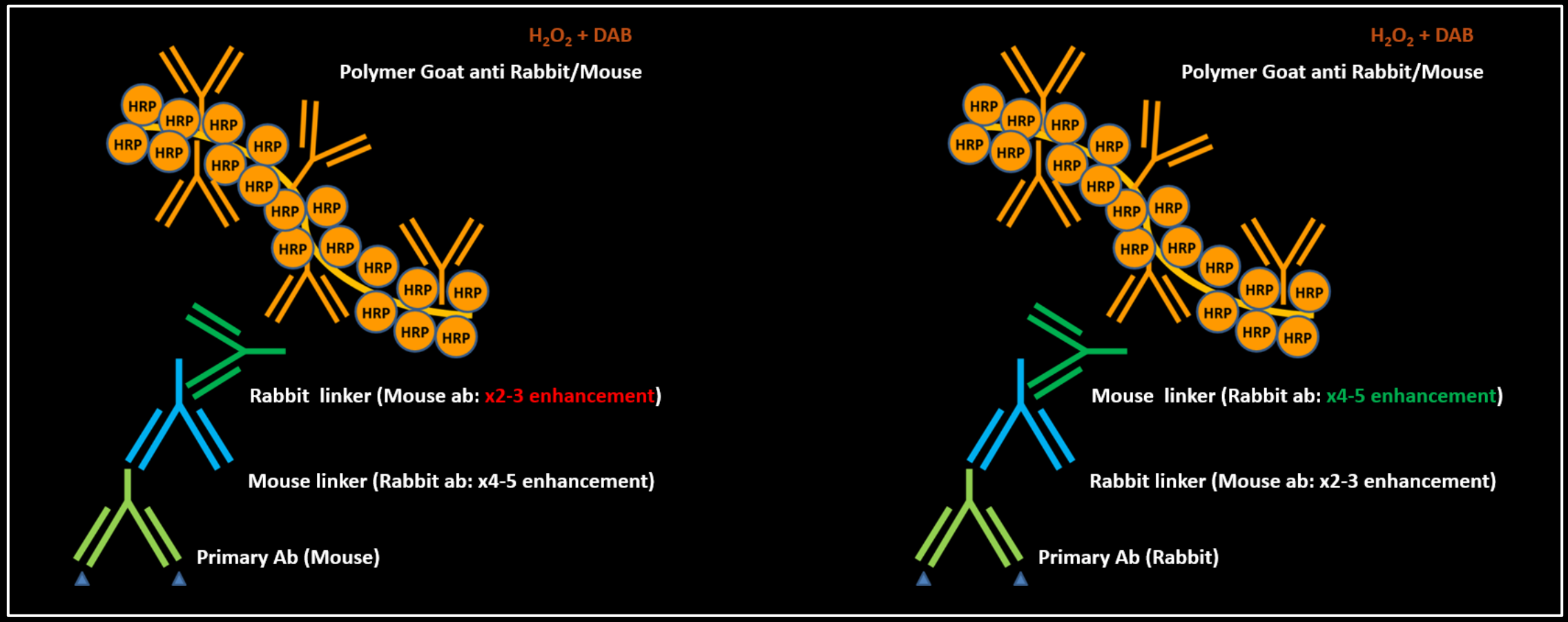
Mouse monoclonal antibodies: In most cases, no improvement in signal intensity using Flex++ compared to Flex+

Envision Flex with Dual Linkers: Mouse versus Rabbit primary Abs

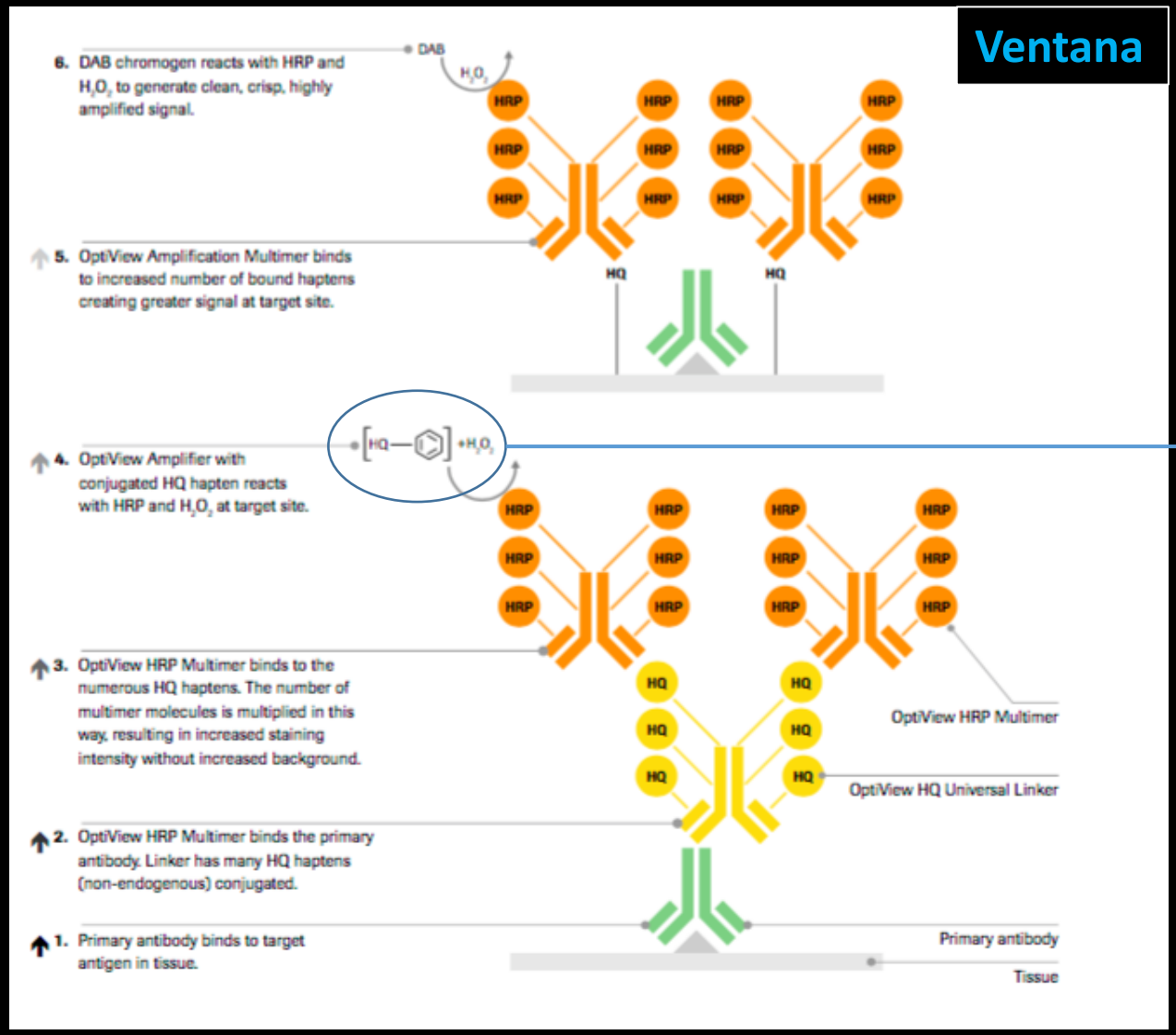
EnVision FLEX+ Rabbit (LINKER) (Code K8009) may be applied for an optional signal amplification of rabbit primary antibodies. For signal amplification when using mouse primary antibodies, EnVision FLEX+ Mouse (LINKER) (Code K8021 or included in Code K8002) may be applied. As a guideline: EnVision FLEX+ Rabbit (LINKER) provides 2–3 fold signal amplification and EnVision FLEX+ Mouse (LINKER) provides 4–5 fold signal amplification.

EnVision FLEX /HRP visualization reagent (SM802) in the kit consists of a dextran backbone to which a large number of peroxidase (HRP) molecules and secondary antibody molecules have been coupled. A unique chemistry is used for the coupling reaction, which permits the binding of up to 100 HRP molecules and up to 20 antibody molecules per backbone.

Agilent/Dako (product insert K8000)



Technical aspects of immunohistochemistry & pitfalls - Analytical phase



Tyramide Signal Amplification

Mechanism of Tyramide amplification:

- Introducing HRP (Optiview)
- Incubation with HQ-labelled Tyramide + H2O2

Tyramide, a phenolic compound, is converted into an short-lived extremely reactive intermediate

- Deposit HQ molecules in close vicinity of Ab/Ag reactions

Intermediates covalently binds rapidly to electron rich regions of adjacent proteins (esp. tyrosine)

- Detection of HQ with anti-HQ / HRP Multimer
- Visualization with DAB

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Tyramide Signal Amplification (TSA)

Table 1. **Antibodies and assessment marks for ALK (lung), run 65**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **5A4**	26 2 1 1 2 2 1	Leica Biosystems Monosan Abcam DBS Biocare Medical Zytomed Systems Invitrogen	8	9	14	4	49%	23%
mAb clone **OTI1A4***	19 1 1 1	Origene Nordic Biosite Cell Signaling Zeta Corporation	16	6	0	0	100%	73%
mAb clone **IHC509**	1	GenomeMe	0	0	1	0	-	-
rmAb clone **D5F3**	19	Cell Signaling	7	9	3	0	84%	36%
rmAb clone **ALK1**	3 1	Dako/Agilent Cell Marque	0	0	0	4	-	-
rmAb clone **QR017**	1	Quartett	0	1	0	0	-	-
rmAb clone **SP8**	1	BioGenex	0	0	0	1	-	-
rmAb clone **ZR305**	1	Zeta Corporation	0	0	1	0	-	-
Ready-To-Use antibodies								
mAb clone **5A4 PA0306**/PA0831 (VRPS)**[3]	2	Leica Biosystems	1	1	0	0	-	-
mAb clone **5A4 PA0306*/PA0831 (LMPS)**[4]	10	Leica Biosystems	4	3	2	1	70%	40%
mAb clone **5A4 API3041**	1	BioCare	0	0	1	0	-	-
mAb clone **5A4 CAM-0170**	1	Celnovte	0	1	0	0	-	-
mAb clone **5A4 MAD-001720QD**	1	Master Diagnostica	0	0	1	0	-	-
mAb clone **ALK1 GA641**	3	Dako/Agilent	0	0	0	3	-	-
mAb clone **ALK1 IR641**	4	Dako/Agilent	0	0	0	4	-	-
mAb clone **ALK1 790/800-2918 (LMPS)**[4]	10	Ventana/Roche	1	0	1	8	10%	10%
mAb clone **137E9E8 PA132**	1	Abcarta	0	0	0	1	-	-
mAb clone **OTI1A4 / 1A4 8344-C010**	1	Sakura Finetek	1	0	0	0	-	-
mAb clone **OTI1A4 / 1A4 GA785 (VRPS)**[3]	12	Dako/Agilent	12	0	0	0	100%	100%
mAb clone **OTI1A4 / 1A4 GA785 (LMPS)**[4]	4	Dako/Agilent	4	0	0	0	-	-
rmAb clone **D5F3 790-4794 (VRPS)**[3]	73	Ventana/Roche	62	7	1	3	95%	85%
rmAb clone **D5F3 790-4794 (LMPS)**[4]	48	Ventana/Roche	36	9	3	0	94%	75%
rmAb clone **SP8 RMPD007**	1	Diagnostic BioSystems	0	0	0	1	-	-
Total	256		152	46	28	30		
Proportion			59%	18%	11%	12%	77%	

1) Proportion of sufficient stains (optimal or good) (≥5 assessed protocols).
2) Proportion of Optimal Results (≥5 assessed protocols).
3) Vendor Recommended Protocol Settings (VRPS) to a specific RTU product applied on the vendor recommended platform(s) (≥5 assessed protocols).
4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product (≥5 assessed protocols).
*) OTI1A4 is called 1A4 by some vendors
**) Product no. PA0306 has been terminated and replaced by PA0831.

Lu-ALK

For certain type of markers, the TSA system provides very good results but……..

Ready-To-Use antibodies and corresponding systems

mAb clone **5A4,** product no. **PA0306/PA0831**, Leica Biosystems, Bond III / Max:
Protocols with optimal results were based on HIER using BERS2 (efficient heating time 20-40 min. at 99-100°C), 15-30 min. incubation of the primary Ab and Bond Polymer Refine (DS9800) as detection system. Using these protocol settings, 7 of 8 (88%) produced a sufficient staining result (optimal or good).

mAb clone **OTI1A4,** product no. **GA785**, Dako/Agilent, Omnis:
Protocols with optimal results were based on HIER using TRS High pH (efficient heating time 30 min. at 97-99°C), 20-30 min. incubation of the primary Ab and EnVision Flex (GV800/GV823+GV821) as detection system. Using these protocol settings, 16 of 16 (100%) produced a sufficient staining result.

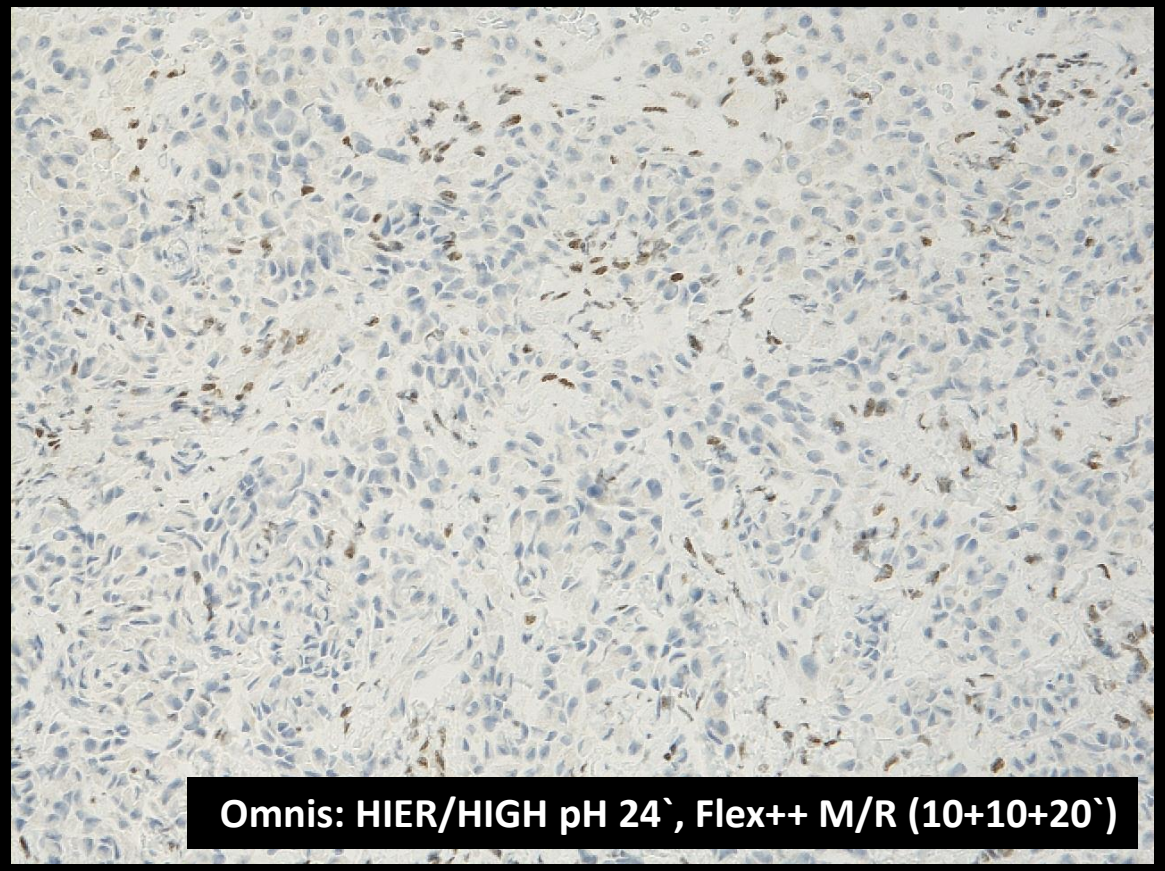
rmAb clone **D5F3** product no. **790-4794**, Ventana/Roche, BenchMark GX, XT and Ultra:
Protocols with optimal results were typically based on HIER using CC1 (efficient heating time 92 min.), 16 min. incubation of the primary Ab. and OptiView (760-700) + amplification kit (760-099) as detection system. Using these protocol settings, 110 of 117 (94%) laboratories produced a sufficient staining result.

TSA

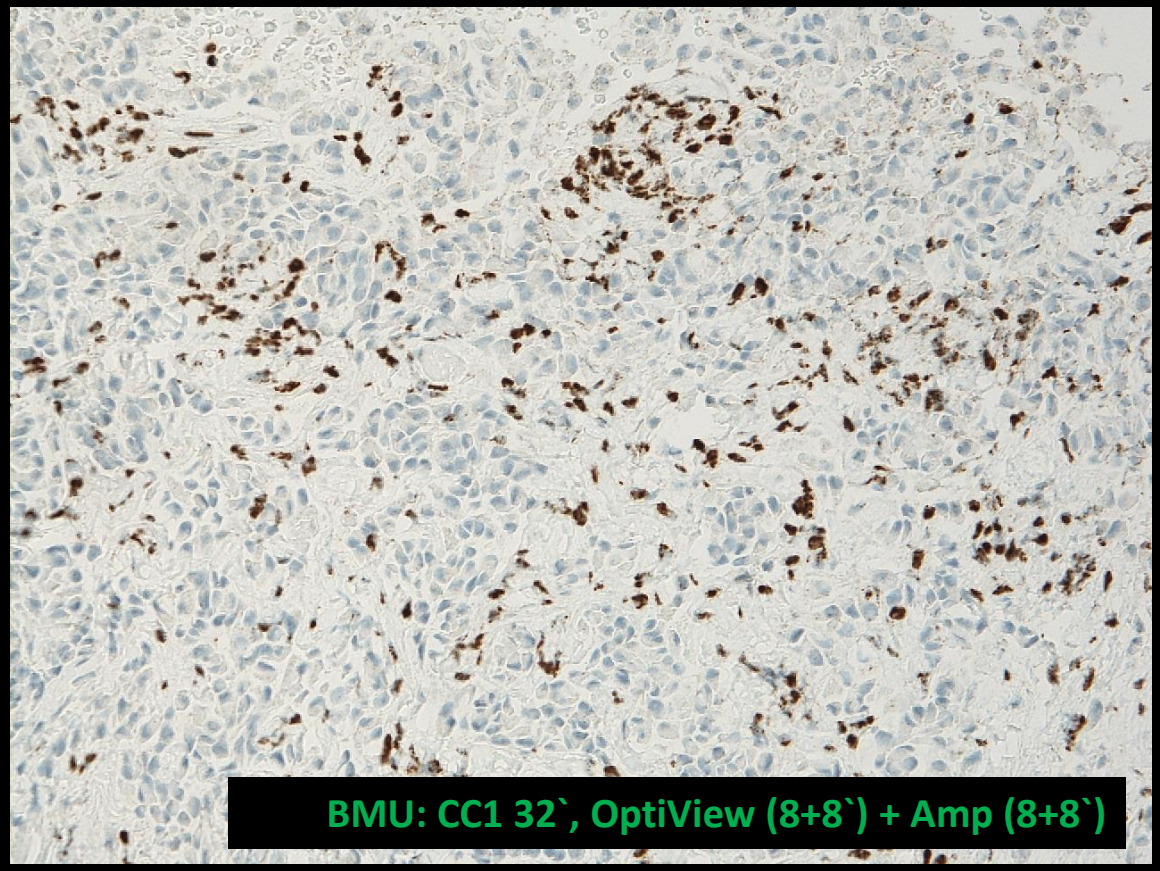
Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Detection System

BAP1 clone C-4 (1:100) / Mesothelioma



Omnis: HIER/HIGH pH 24`, Flex++ M/R (10+10+20`)



BMU: CC1 32`, OptiView (8+8`) + Amp (8+8`)

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

The Histochemical Journal **31:** 195–200, 1999.

Concentration dependent and adverse effects in immunohistochemistry using the tyramine amplification technique

Michael Mengel[1], Martin Werner[2] & Reinhard von Wasielewski[1,*]
[1]*Institut für Pathologie der Medizinischen Hochschule Hannover, Carl Neuberg-Str. 1, D-30625 Hannover, Germany*
[2]*Institut für Pathologie der Technischen Universität München, Germany*
**Author for correspondence*

Received 8 September 1998 and in revised form 1 December 1998

Summary

Although the tyramine amplification technique to enhance sensitivity in immunohistochemistry has been described in numerous methodological papers, it has not yet gained access to diagnostic immunohistochemistry. This is mainly due to problems and pitfalls occurring in adaptation of this method to routine application.

In this study a monoclonal antibody and a polyclonal antiserum (pan-cytokeratin and anti-myoglobin) were tested in tissues with different amounts of epitopes, using a checkerboard table and testing a total of 133 different dilution combinations of both the tyramide solution and the primary antibodies.

The specific tissue investigated, i.e. the amount of accessable epitope to be detected and the applied concentration of the tyramide solution mainly influenced the staining reaction. Several pitfalls such as an uneven distribution of the staining or dramatic overstaining (paradoxical overstaining) must be considered to achieve optimal results.

In conclusion, our data confirm methodological studies that the tyramine amplification technique is a powerful method to enhance immunohistochemical sensitivity. However, for reliable daily practice several pitfalls of the technique have to be circumvented.

TSA enhancement

Difficult to control causing:

- False positive staining

- Weak or false negative staining, unbalanced reaction of primary Ab and target epitopes giving a yes or no answer

- Uneven distribution of the TSA reaction product

NQC Run 66 (Napsin A)

Problem with TSA Amp and false positive reactions (SCC of the lung)

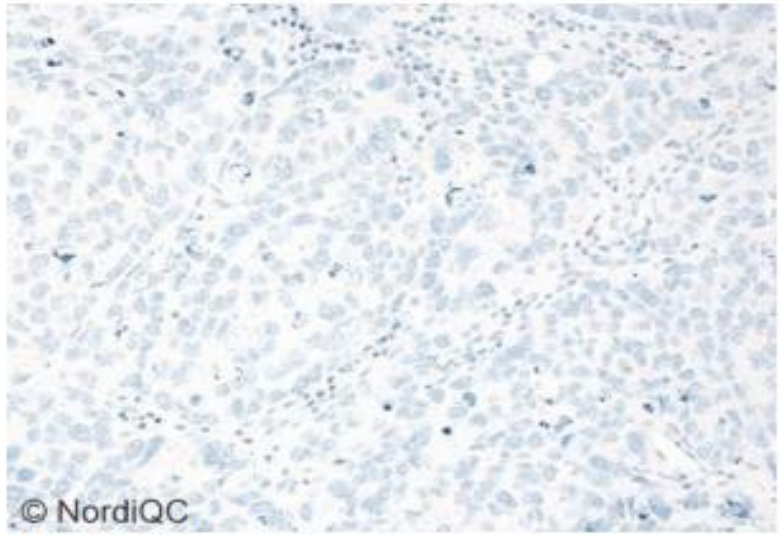


Fig. 5a (x200)
Optimal staining reaction for Napsin A of the lung squamous cell carcinoma using the RTU system 760-4867 (Benchmark Ultra, Ventana/Roche) based on the mAb clone MRQ-60. Vendor recommended protocol settings were applied and OptiView was used as the detection system. All (8/8) protocols based on the same conditions, gave an optimal result. The protocol also provided the same reaction patterns as illustrated in Fig 1a-3a. and as expected, a negative result in the neoplastic cells of the lung squamous cell carcinoma.

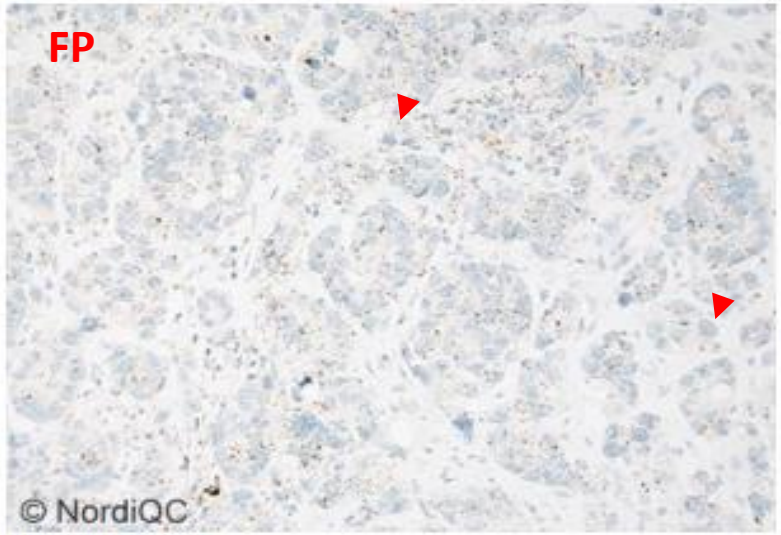


Fig. 5b (x200)
Insufficient staining reaction for Napsin A of the lung squamous cell carcinoma using the same RTU product as in Fig. 5a, but with extended HIER time in CC1 (64 min. at 98°C) extended incubation time in primary Ab (32 min.) and OptiView with amplification as detection system. The neoplastic cells display a false positive and granular staining reaction. This problem was often seen with tyramide signal amplification. Laboratories should be cautious using this amplification step due to the granular deposit of the reaction product, that erroneously can be interpreted as a specific signal for Napsin A - compare with Fig 5a.

Adenocarcinoma of the lung)

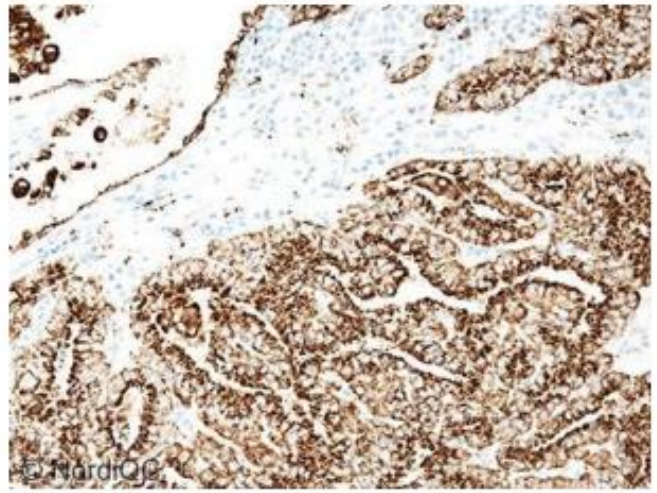


Fig. 3a (x200)
Optimal staining reaction for Napsin A staining of the lung adenocarcinoma (tissue core 3) using same protocol as in Figs. 1a and 2a. All the neoplastic cells display a strong and distinct granular cytoplasmic staining reaction.

TSA: Granular specific reactions vs granular unspecific reactions ?

PMS2

Fig. 1a (x100)
Optimal PMS2 staining reaction of the tonsil using the GA087 RTU system for the Dako Omnis platform based on rmAb clone EP51, following the recommended protocol settings using dual linker. Virtually all mantle zone B-cells show a moderate and distinct nuclear staining reaction, while the germinal centre B-cells show a strong nuclear staining reaction. Also compare with Figs. 2a - 4a, same protocol.

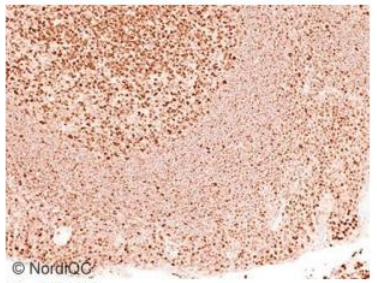


Fig. 1b (x100)
Insufficient PMS2 staining reaction of the tonsil using the mAb clone A16-4 on the Ventana Benchmark platform following the vendor recommended protocol settings with tyramide amplification same field as in Fig. 1a.
Virtually all mantle zone B-cells show a moderate but granulated staining reaction of both nuclei and cytoplasm, while the germinal centre B-cells show a strong nuclear staining reaction.
Also compare with Figs. 2b – 4b, same protocol.

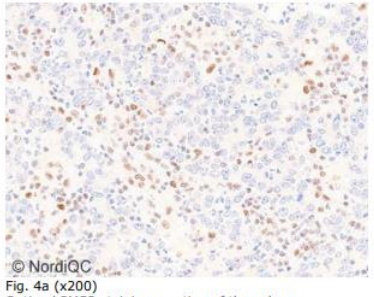


Fig. 4a (x200)
Optimal PMS2 staining reaction of the colon adenocarcinoma, tissue core no. 5, with loss of PMS2 expression using same protocol as in Figs. 1a – 3a. The neoplastic cells are negative, while stromal cells show a distinct nuclear staining reaction serving as internal positive tissue control.

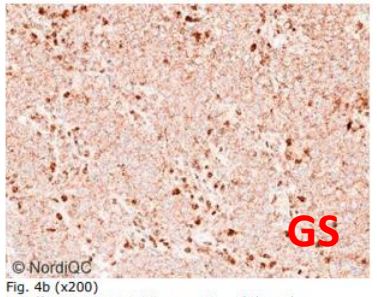


Fig. 4b (x200)
Insufficient PMS2 staining reaction of the colon adenocarcinoma, tissue core no. 5, with loss of PMS2 expression using same protocol as in Figs. 1b – 3b, same field as in Fig. 4a. A diffuse granular staining reaction is seen in both the cytoplasmic and nuclear compartment complicating the interpretation of the neoplastic cells expected to be negative.

OptiView + TSA

CD4

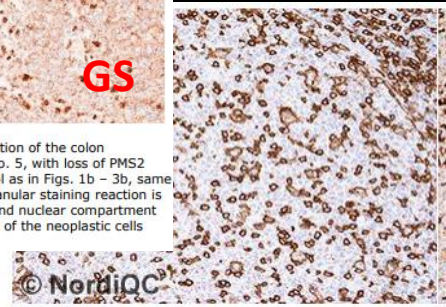


Fig. 1a (x200)
Optimal CD4 staining of the tonsil using the rmAb clone SP35 as a concentrate, HIER in an alkaline buffer (CC1 pH 8.5) and a 3-step multimer based detection system (OptiView, Ventana) - same protocol used in Figs. 2a - 4a. The inducer/helper T-cells show a strong staining reaction, while the germinal centre macrophages show a moderate and distinct membranous staining reaction - compare with Fig.1b.

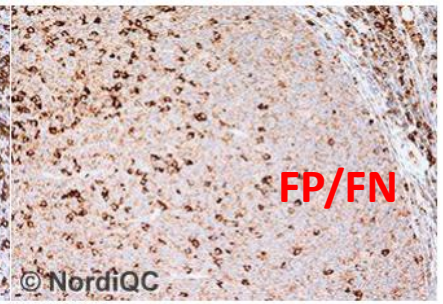


Fig. 1b (x200)
Insufficient CD4 staining of the tonsil using the mAb 4B12 as a concentrate, HIER in an alkaline buffer (CC1 pH 8.5) and a multimer based detection system (OptiView with Tyramide amplification, Ventana) -same protocol used in Figs. 2b-4b. The protocol provided a too low sensitivity, but also poor signal-to-noise ratio and false positive staining. No staining of germinal centre macrophages is seen and simultaneously B-cells are labelled. The pattern of too weak staining reaction was observed with all protocols based on the mAb 4B12 performed on the Ventana BenchMark platform - compare with Fig. 1a (same field).

MSH6

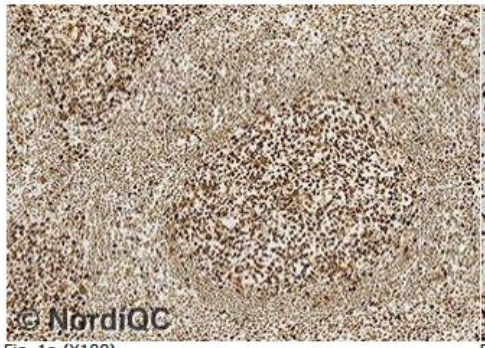


Fig. 1a (X100)
Optimal MSH6 staining reaction of the tonsil using the rmAb clone EP49, optimally calibrated, HIER in an alkaline buffer and a 3-step multimer based detection system (OptiView, Ventana). Virtually all mantle zone B-cells show a moderate and distinct nuclear staining reaction, while the germinal centre B-cells show a strong nuclear staining reaction. Also compare with Figs. 2a - 4a, same protocol.

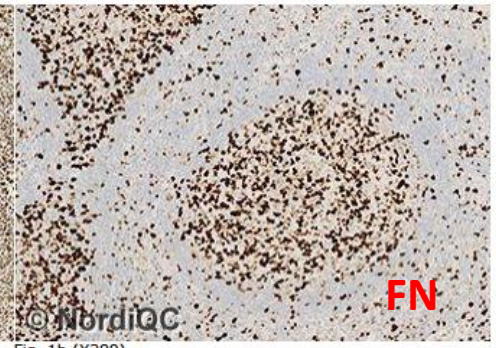


Fig. 1b (X200)
Insufficient MSH6 staining reaction of the tonsil using the rmAb clone EP49 with a protocol providing a too low sensitivity- same field as in Fig. 1a.
Only the germinal centre B-cells are distinctively demonstrated, while mantle zone B-cells expressing low level MSH6 virtually are unstained. The protocol was based on OptiView + amplification (Ventana) and the combination of a too low titre of the primary Ab and the use of tyramide based amplification provided an inadequate balance of the staining reaction.
Also compare with Figs. 2b - 4b, same protocol.

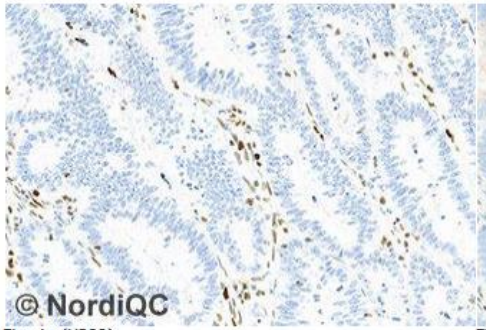


Fig. 4a (X200)
Optimal MSH6 staining reaction of the colon adenocarcinoma no. 4 with loss of MSH6 expression using same protocol as in Figs. 1a - 3a. The neoplastic cells are negative, while stromal cells show a distinct nuclear staining reaction serving as internal positive tissue control.

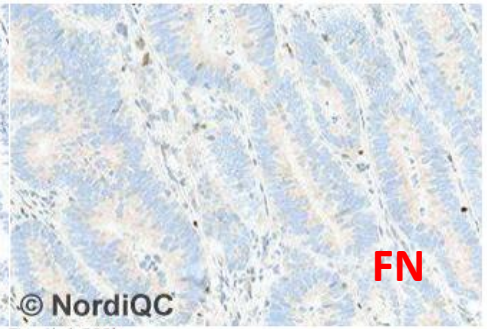
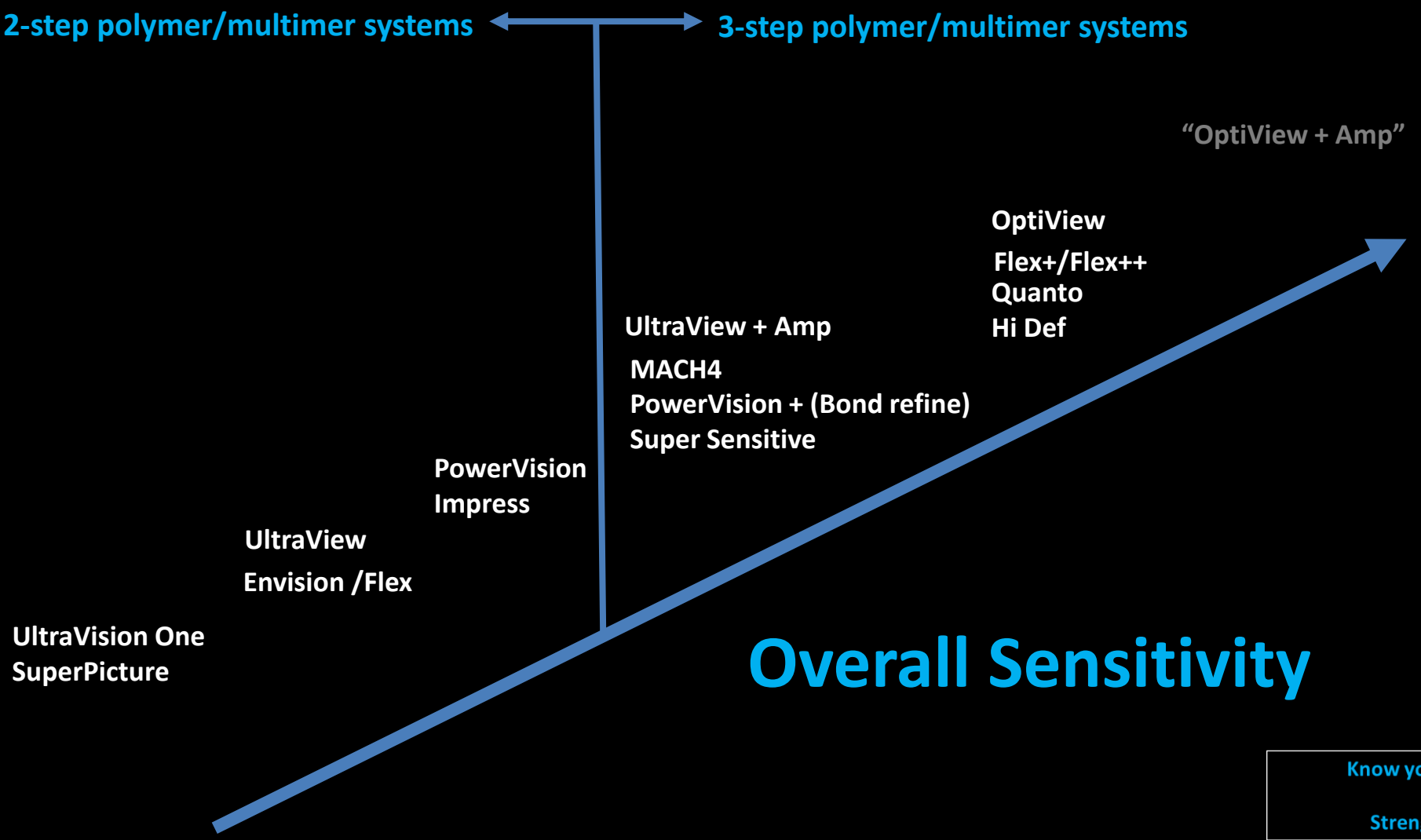


Fig. 4b (x200)
Insufficient MSH6 staining reaction of the colon adenocarcinoma no. 4 with loss of MSH6 expression using same protocol as in Figs. 1b - 3b – same field as in Fig. 4a. No nuclear staining reaction in the neoplastic cells is seen, but as only an equivocal nuclear staining reaction in the normal stromal cells is present, the staining pattern cannot reliably be interpreted. In addition a weak aberrant cytoplasmic staining reaction is seen.

The TSA detection system is not without problems and may provided either false positive or negative results.

Detection systems - Tested in Dept. of Pathology, Naestved, DK



Know your detection system

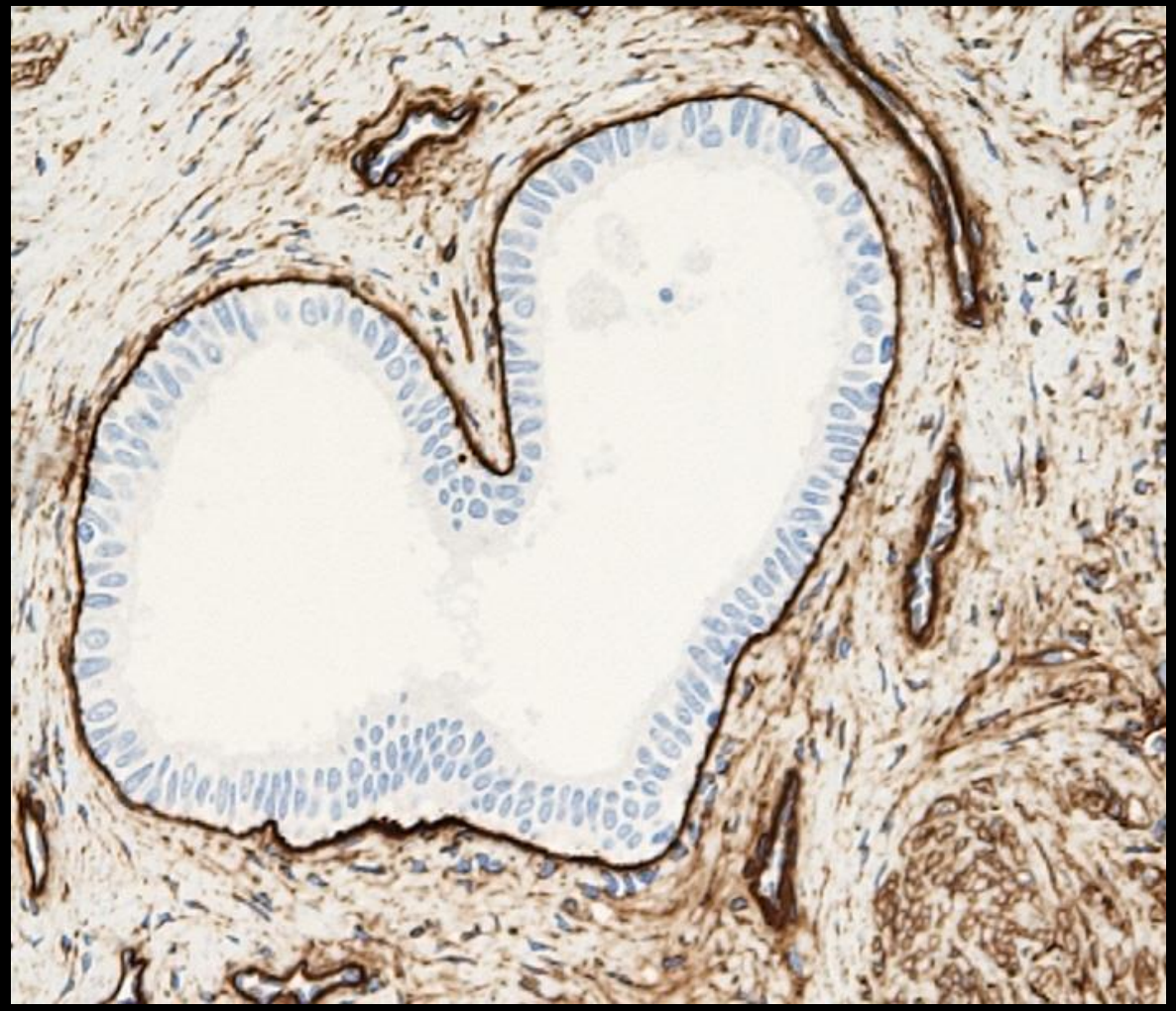
Strength and weakness

The basal fundament for a technical optimal performance is :

- **Appropriate tissue fixation and processing**
- **Appropriate and efficient epitope retrieval**
  - 97-98% of the primary Abs require HIER and app. 85-90% prefer high pH (alkaline) retrieval buffers.
  - Use efficient HIER temperature and time (app. 100°C for 20 - 40min).
- **Appropriate choice of antibody / clone, diluent and dilution**
  - Compare different clones / Abs against the selected antigen of interest before implementation
  - Calibrate the Ab concentration carefully
- **Appropriate and specific / sensitive detection system**
  - Use of a 3-step multimer/polymer system is preferable to a 2- step multimer/polymer system
  - Don`t use biotin-based detection systems
- **Appropriate choice of control material**
  - Focus on Immunohistochemistry Critical Assay Performance Controls (iCAPCs)

Courtesy of Søren Nielsen (NordiQC, slide modified)

Thank you for your attention

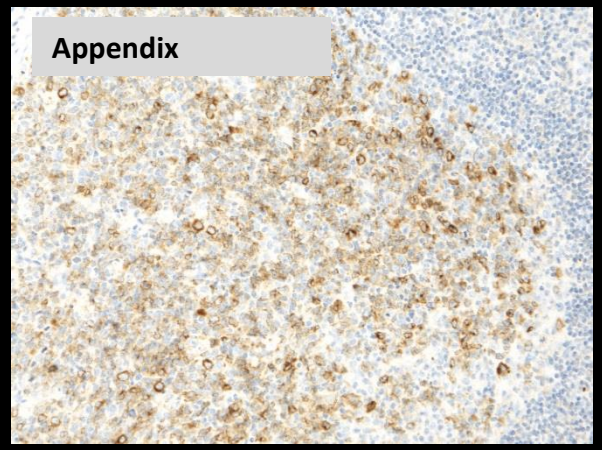


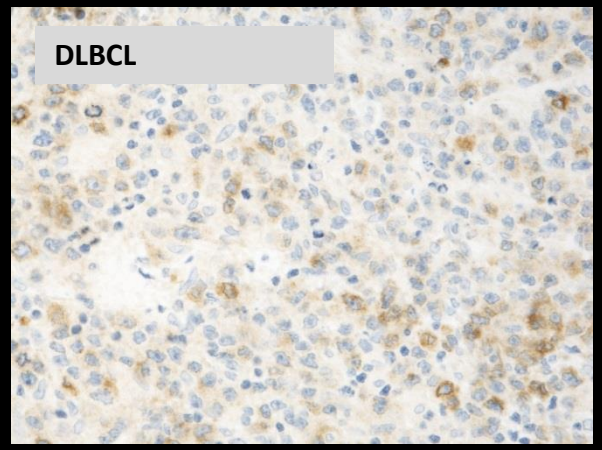
Technical aspects of immunohistochemistry & pitfalls - Analytical phase

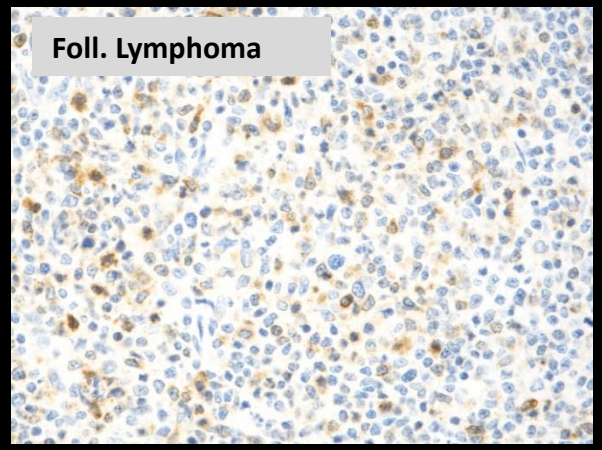
Problem: Detection systems

Serpin A9/GCET1 (clone 585302/1:2000RR or RAM341/1:200)

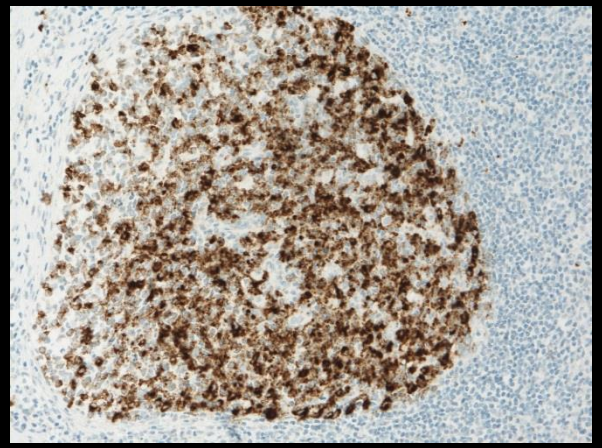
Omnis
HIER High pH (24`), Flex++ (30-10-10-20`)

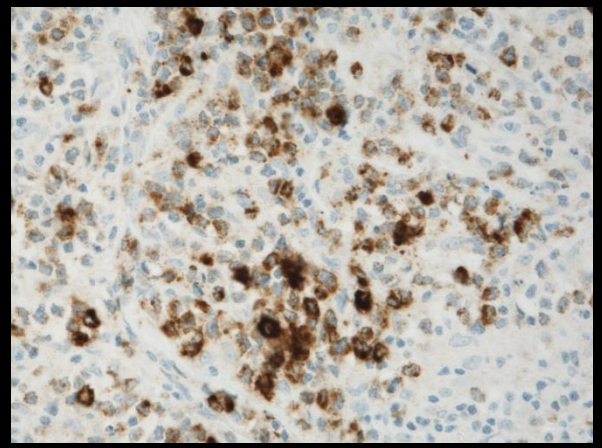


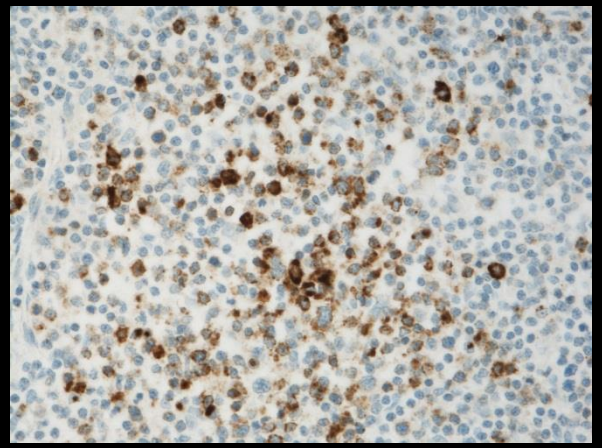




Benchmark Ultra
P3 (4`), CC1 (32`), OV+A (16-(8-8)-(4-4)`)







Data & method kindly provided by Ole Nielsen, Dept. of Pathology, Odense, Denmark

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Less sensitive detection systems

CD10, RUN 39 (2013)

Table 2. **Optimal results for CD10 using concentrated antibodies on the 3 main IHC systems***

Concentrated antibodies	Dako Autostainer Link / Classic		Ventana BenchMark XT / Ultra		Leica Bond III / Max	
	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	ER2 pH 9.0	ER1 pH 6.0
mAb clone **56C6**	64 % 14/22**	0 % 0/1	67 % 35/52	-	95 % 19/20	0 % 0/1

* Antibody concentration applied as listed above, HIER buffers and detection kits used as provided by the vendors of the respective platforms.
** (number of optimal results/number of laboratories using this buffer)

Protocols based on 2-step polymer/multimer detection systems, provided a sufficient staining result in 91% (n=42/46), and in 52% (n=24/46) an optimal result was obtained. If the same protocol settings were applied with a 3-step polymer/multimer based detection system (EnVision FLEX+ (Dako), Optiview (Ventana) or Bond Refine (Leica)), sufficient staining results were seen in 100% (n=58/58), and 86% (n=50/58) was evaluated as optimal. This is also reflected in the high proportion of optimal results (95 %) using the mAb clone 56C6 as concentrate on the Leica IHC platforms (table 2), on which a 3-step polymer based detection system is used as standard.

2 & 3-step detection system (Flex/Flex+ or UV/OV

BOND refine (3-step detection system)

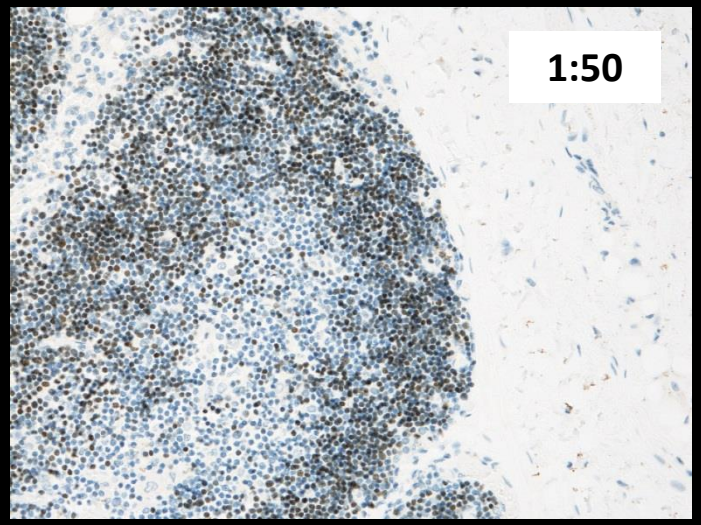
LD assay (mmAb clone 56C6) HIER in alkaline buffer and optimal dil. range	Detection system	Pass Rate`s (%)	Optimal (%)
2-step polymer/multimer system	Flex (Dako) or UltraView (Ventana)	91 (42 of 46)	52 (24 of 46)
3-step polymer/multimer system	Flex+ (Dako), OptiView (Ventana) or BOND Refine (Leica)	100 (58 of 58)	86 (50 of 58)

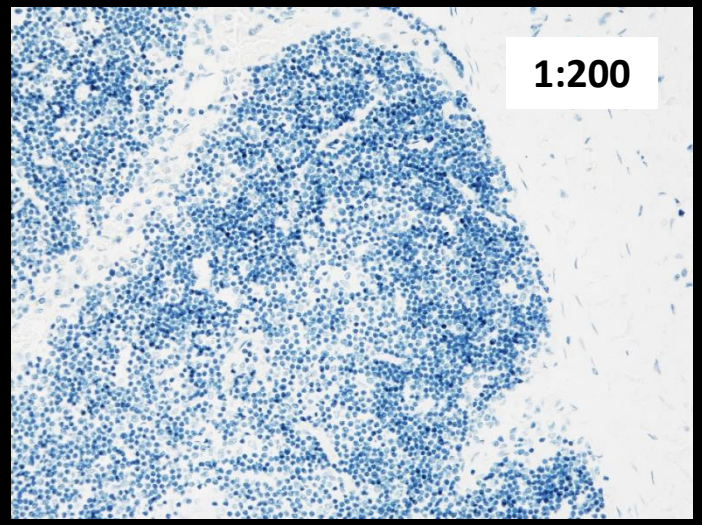
Antibody-Antigen reaction – Antibody Diluents

Thymus

TdT, Clone EP266

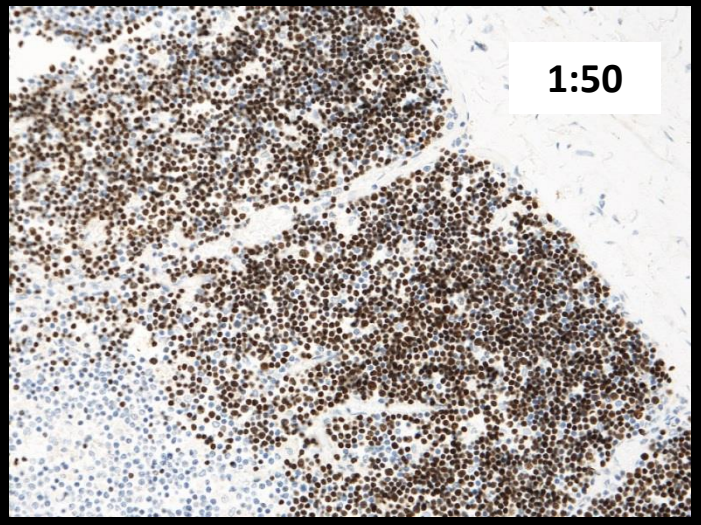
TdT, EP266
Dako dil. pH 7.3

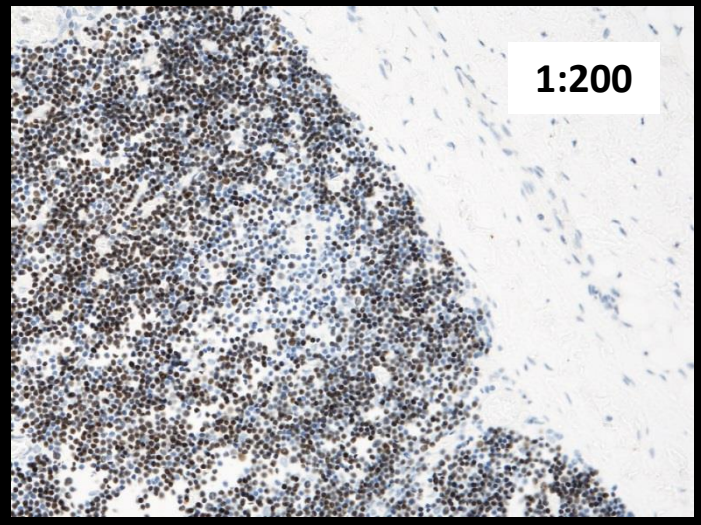




Omnis: HIER/HIGH pH 24`, Flex+ Rabbit (10+20`)

TdT, EP266
Renoir Red pH 6.2

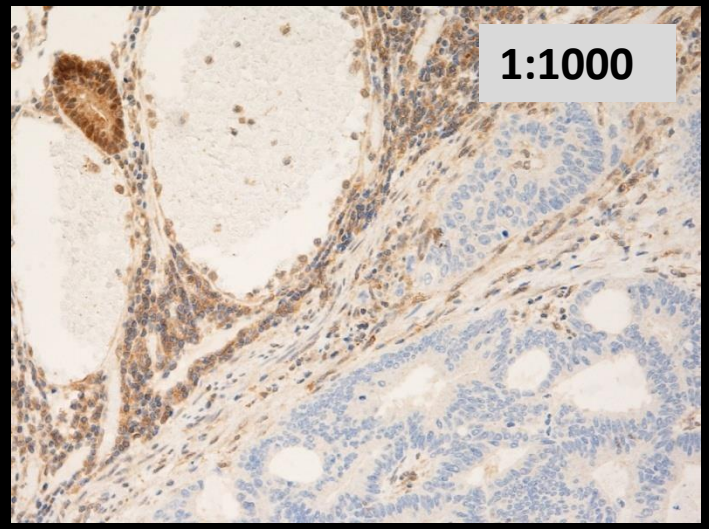
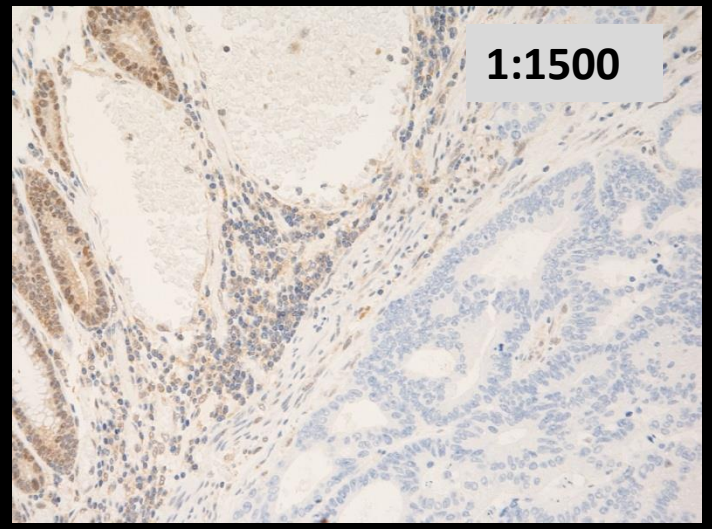
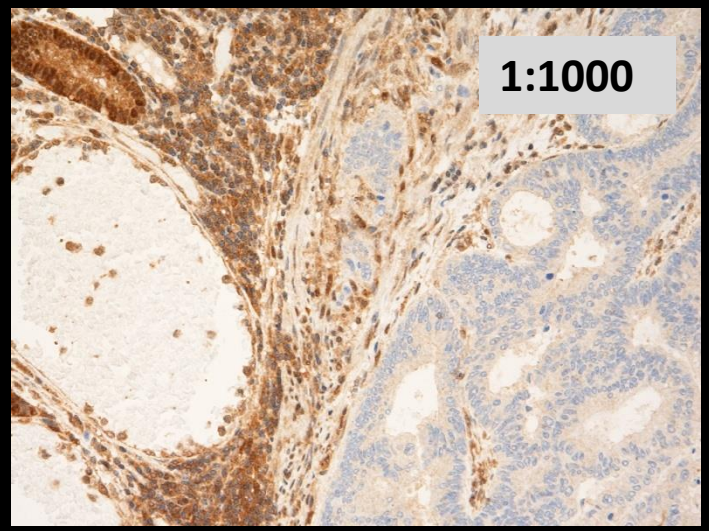
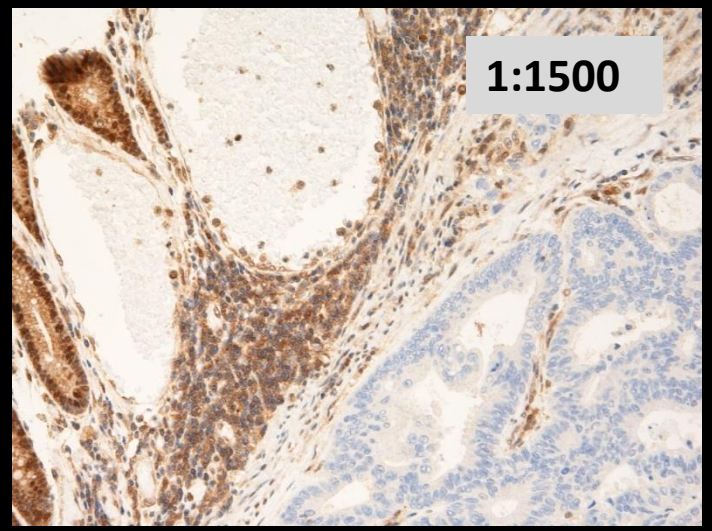




Antibody diluents

SMAD4, Clone EP168Y

Colon Adenocarcinoma

Dako dil. pH 7.3		
Renoir Red pH 6.2		

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

RESEARCH ARTICLE

Performance of 4 Immunohistochemical Phosphohistone H3 Antibodies for Marking Mitotic Figures in Breast Cancer

Cornelia M. Focke, MD,† Kai Finsterbusch, MSc,* Thomas Decker, MD,* and Paul J. van Diest, MD, PhD†*

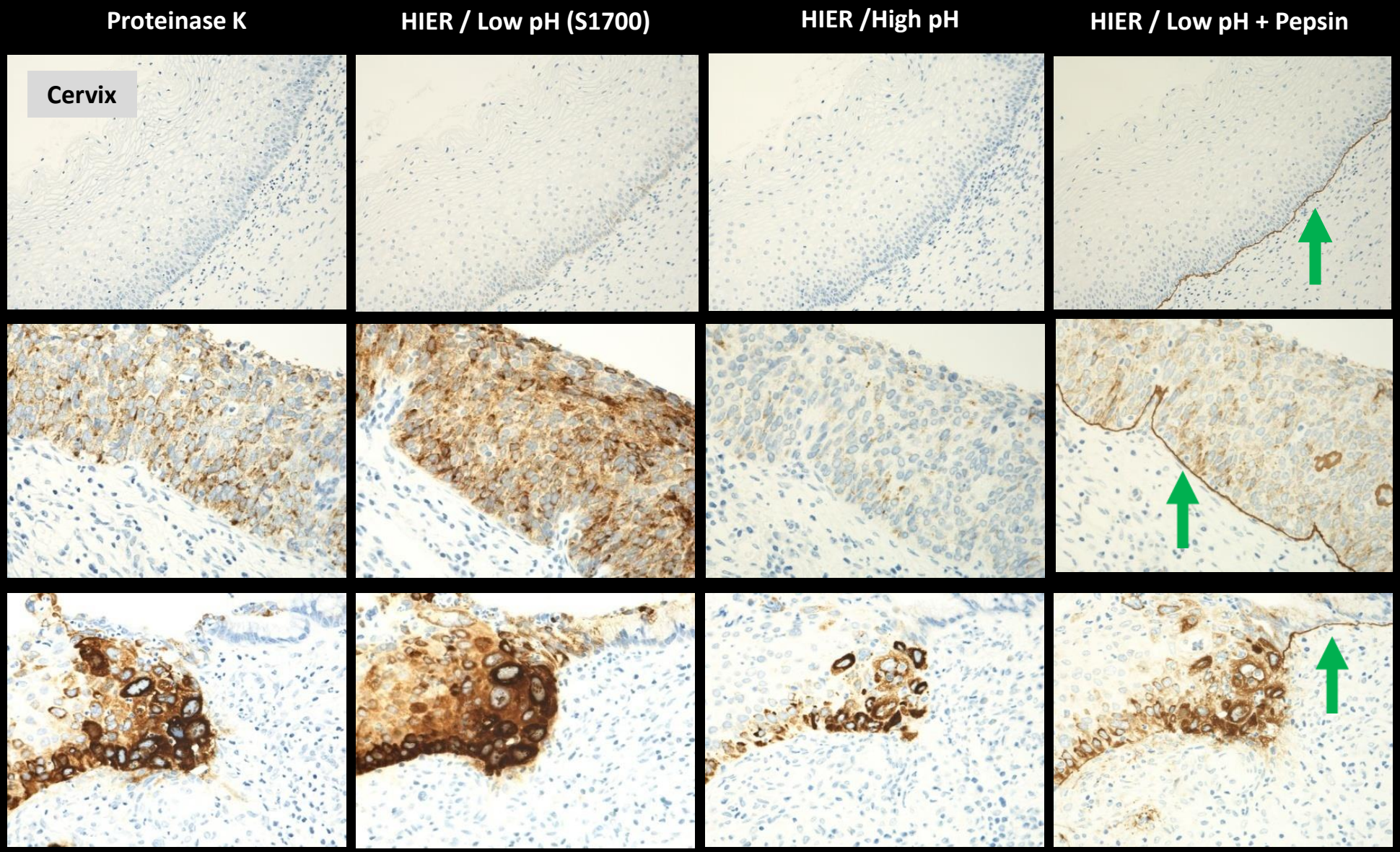
Conclusions: Performance and reliability varied significantly between the 4 tested antibodies. For faster identification of mitotic hot spots and as potential marker in digital image analysis, the Merck antibodies seem to be most suitable.

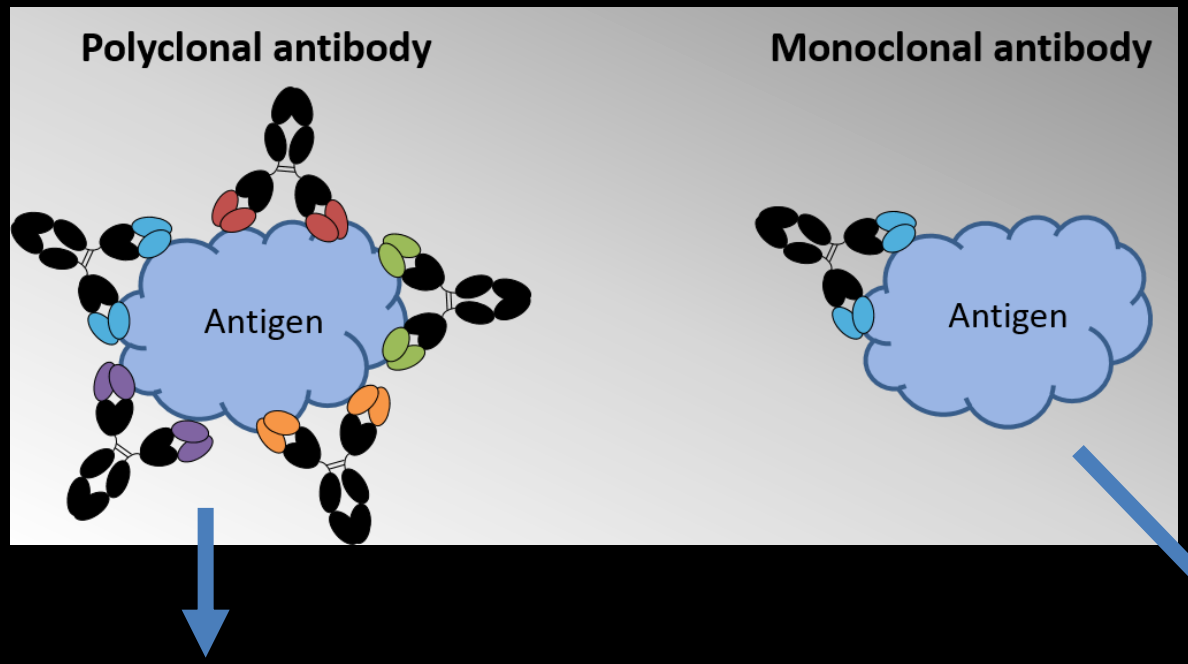
Key Words: PHH3, breast cancer, proliferation, mitotic activity, phosphohistone H3

(*Appl Immunohistochem Mol Morphol* 2016;00:000–000)

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

LAM-5 (γ2), D4B5





Antibodies bind to antigen through the variable regions of the antibody.

The strength of the binding of an antibody to a specific epitope is called affinity.

High affinity antibodies will bind larger amounts of antigen in a given period of time and can be used at higher dilutions.

Heterogeneous population of antibodies reacting with different epitopes of an antigen

Not epitope specific

Might cross-reactivity with similar antigens

Background noise

Batch/lot variability

Many host species options: Normally rabbit

Homogeneous population of a specific antibody reacting with one epitope on a particular antigen

Epitope specific

No cross-reactivity with similar antigens

Less background noise

Less batch/lot variability

Host species options: Normally rabbit and mouse