

Workshop in Diagnostic Immunohistochemistry
Aalborg University Hospital, September/October 2021

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

Michael Bzorek

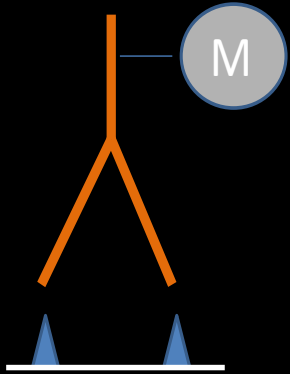
Histotechnologist


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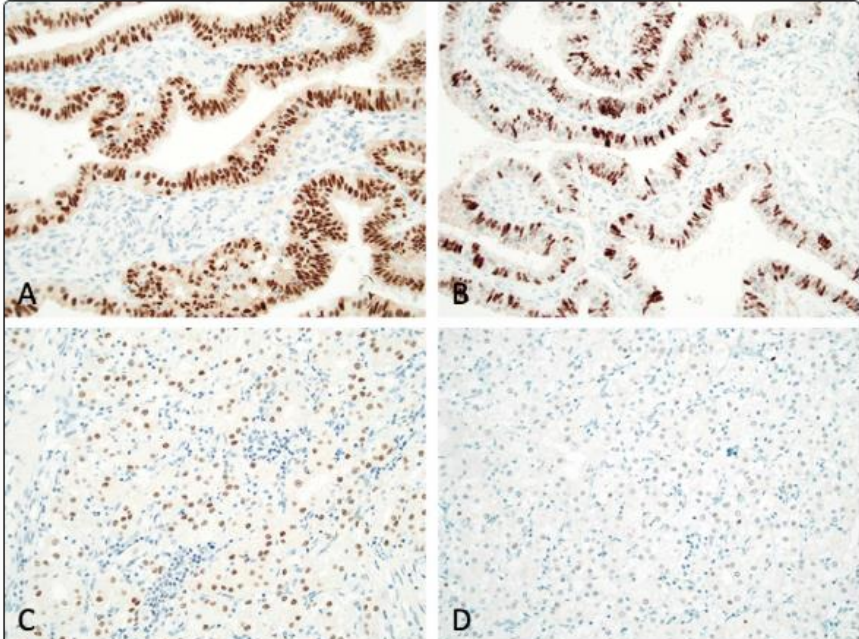
University Hospital, Region Zealand, Denmark

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Immunohistochemistry – A simple technique ?



 [Info](#) [Modules](#) [Assessments](#) [Protocols](#) [Controls](#) [Events](#) [Login](#)



A **B** **C** **D**

IHC for PAX8 in two laboratories: Lab 1 (A/C): Optimal results in Fallopian tube and Renal clear cell carcinoma. Lab 2 (B/D): Insufficient result in same tissues, especially characterized by false negative reaction in the neoplastic cells of the Renal clear cell carcinoma.

Events

[NordiQC Workshop in Diagnostic Immunohistochemistry](#)
2-4 Oct 2019: Aalborg, DK

[6th Academy of Diagnostic Immunohistochemistry](#)
9-11 Oct 2019: Krakow, Poland

4th NordiQC Conference on Applied Immunohistochemistry
2-5 Jun 2020: Aalborg, Denmark

Important dates

[Run 57, B28, H16, C6](#)
Protocol submission deadline
4 Sep 2019
Slide circulation
10 Sep 2019
Slide return deadline
11 Oct 2019
Publication of results
6 Dec 2019

Questions

Check out our [FAQ](#) (Frequently asked questions) or [contact us](#)

External Quality Assurance programs

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

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

IHC-type 1 markers (Diagnostic)

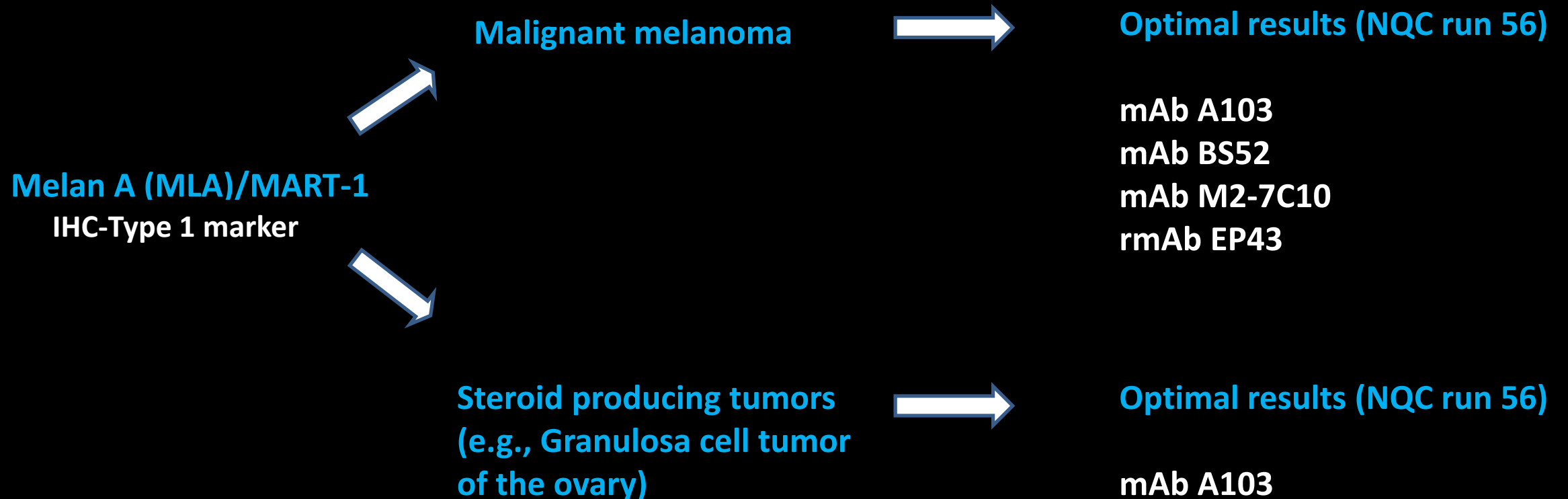
Often calibrated to produced appropriate level of sensitivity and specificity (positive versus negative)

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



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

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/IS633³	29	Dako/Agilent	9	12	8	0	72%	-
mAb clone A103, IR633/IS633⁴	9	Dako/Agilent	1	2	5	1	-	-
mAb clone A103, PA0233	9	Novocastra/Leica	1	8	0	0	-	-
mAb clone A103, PA0233⁵	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 Omnis)

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/MART-1 (Run 56)

MLA, A103 (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 the Leica Bond, 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 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 790-2990³	3	Ventana/Roche	0	0	3	0	-	-
mAb clone A103 790-2990⁴	94	Ventana/Roche	6	74	11	3	85%	6%
mAb clone A103, IR633/IS633³	14	Dako/Agilent	1	13	0	0	100%	7%
mAb clone A103, IR633/IS633⁴	56	Dako/Agilent	12	36	7	1	85%	21%
mAb clone A103, PA0233/PA0044³	7	Leica Biosystems	2	5	0	0	100%	29%
mAb clone A103, PA0233/PA0044⁴	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 assessed 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) (≥5 assessed protocols).

4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product applied either on the vendor recommended platform(s) or on validated semi/fully automatic systems or used manually (≥5 assessed 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

MLA Run 56, Mab clone A103

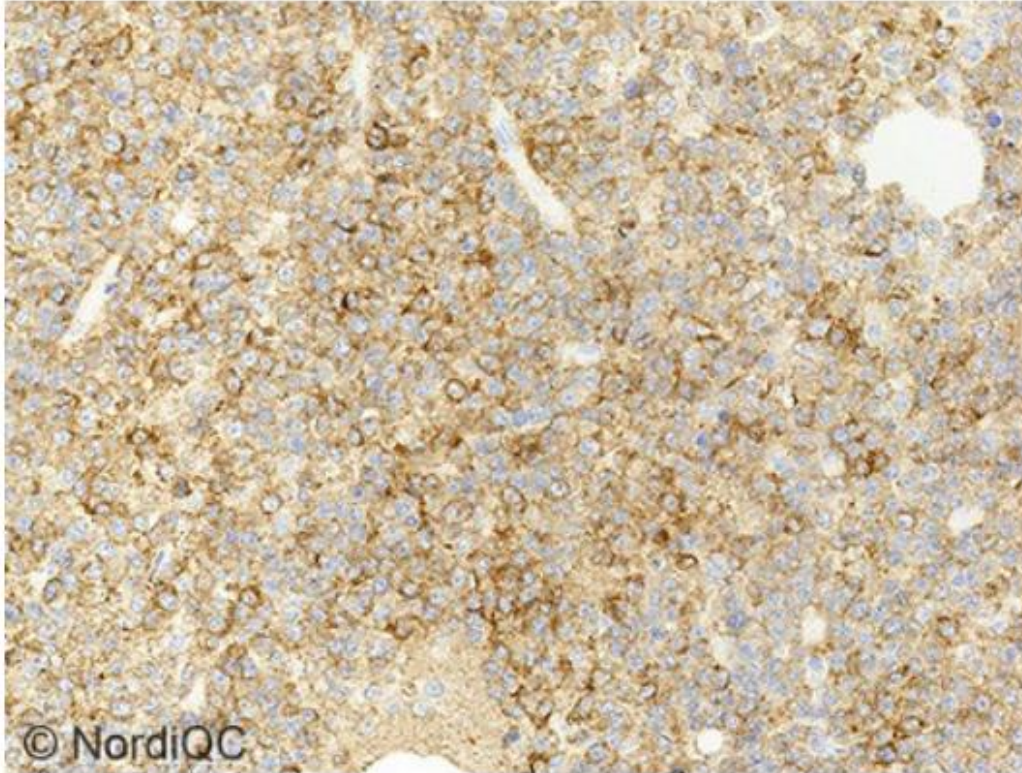


Fig. 8a
Optimal MLA staining of the malignant melanoma tissue core no. 4 (low-level expressor) using same protocol as in Figs. 7a and 7b. The majority of neoplastic cells show a weak to moderate cytoplasmic staining reaction. Compare with Figs. 7a and 8b, same protocol. Also compare with Figs. 5a and 5b - same field.

MLA Run 56, Rab clone EP43

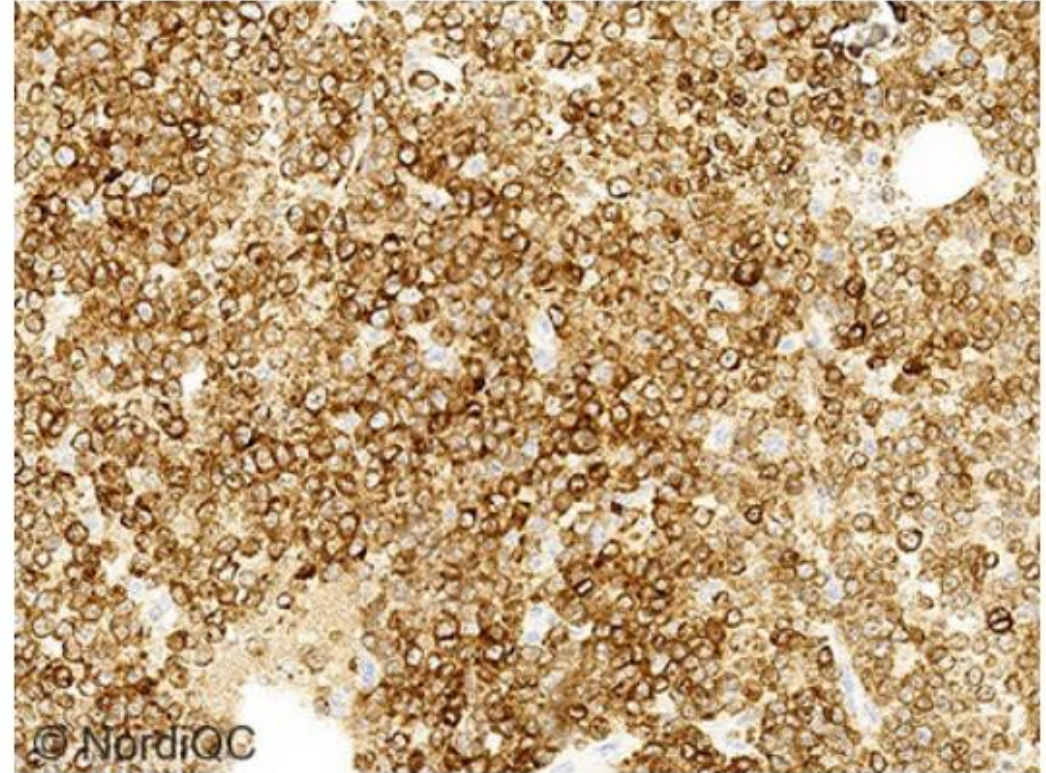


Fig. 8b
Optimal MLA staining of the malignant melanoma tissue core no. 4 (low-level expressor) using **rmAb EP43** in similar protocol settings as in Fig. 8a (and 7a and 7b) All neoplastic cells show a moderate to strong cytoplasmic staining reaction. In melanomas, rmAb EP43 produce very strong reactions. Compare with mAb A103 in Fig. 8a - same field.

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Melan A /MART1

Melanoma (Sentinel node)

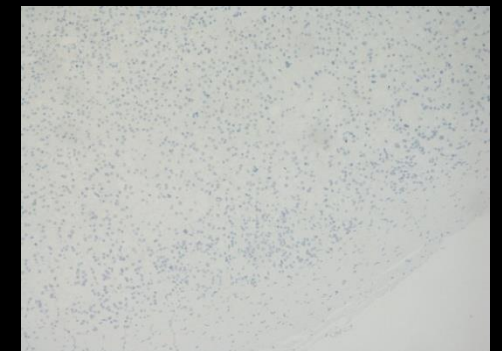
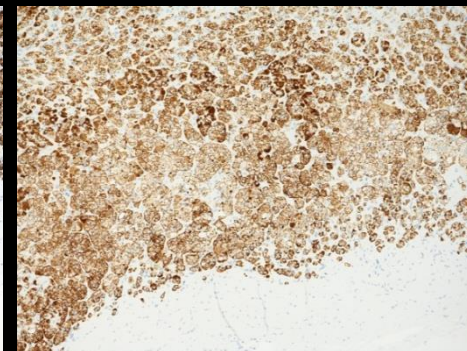
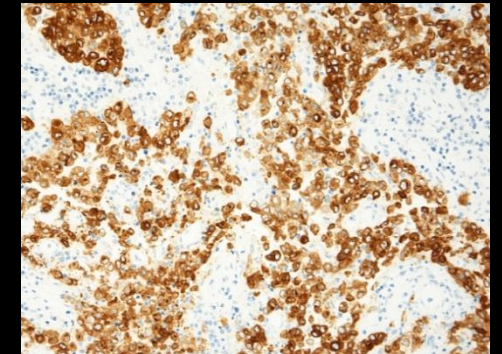
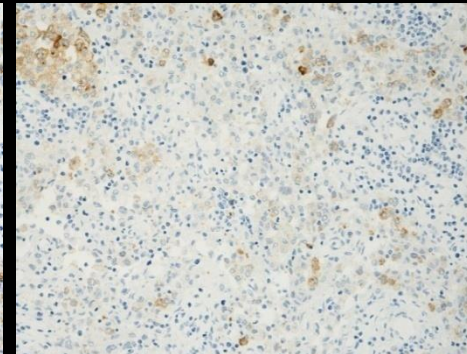
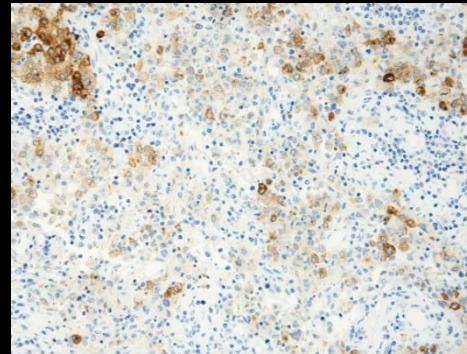
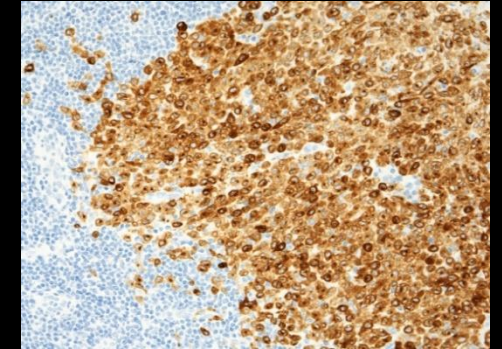
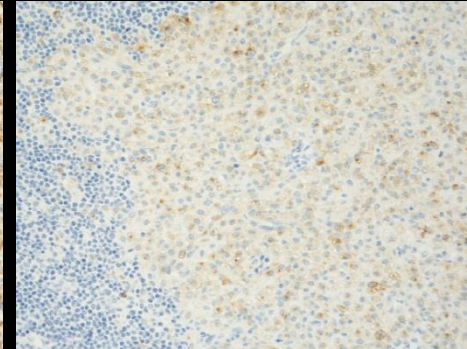
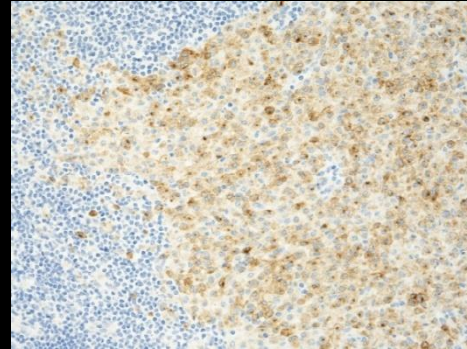
Melanoma (Lymph node)

Adrenal gland

MLA, A103 1:25
AutoStainer

MLA, A103 1:25
Omnis

MART1, EP43 1:30
Omnis



TRS High pH/Flex+ protocols

Technical aspects of IHC and pitfalls – Analytical phase

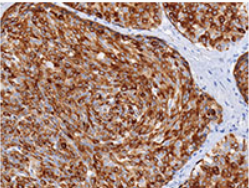
Optimization of the IHC assay – issues to be addressed

- 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



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

Roche/ Ventana/ Cell Marque

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, paraffin-embedded tissues using standard test methods. This antibody is used as an aid in the diagnosis of thyroid medullary carcinoma within the clinical history, and other diagnostic tests determined.

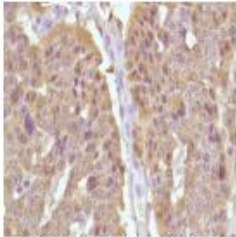
Recommended staining protocol with ultraView	
Procedure Type	Method
Deparaffinization	Selected
Cell Conditioning (Antigen Unmasking)	Cell Conditioning 1, Mild
Enzyme (Protease)	Not required
Antibody (Primary)	BenchMark ULTRA instrument: 16 minutes, 36°C
	BenchMark XT instrument: 16 minutes, 37°C
	BenchMark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

Optimizing an assay can be confusing
(Vendor recommendations)



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:	Rabbit 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

Optimizing IHC test (IHC-type 1) - parameters to consider

Use a “Test battery approach” (pre-treatment and dilution range)

Test more than one antibody clone against an antigen of interest before implementation in the routine

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

Laboratory Developed Assays : Dept. of Surgical Pathology, Region Zealand, Denmark
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`)
G	Pep 6 & 10 min + TRS High *	Pep 6 & 10 min + TRS High	Pep 6 & 10 min + TRS High
H	Pepsin 20 min.	Pepsin 20 min	Pepsin 20 min

Omnis

Protocol A: 0.5 %
Protocol B: 2.0 %
Protocol C: 10.0 %
Protocol D: 83.5 %

Protocol E: 1.0 %
Protocol F: 3.0 %

Protocol G: 0 %
Protocol H: 0 %

* Off board enzymatic pre-treatment

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

TMA Normal Tissue			
Tonsil 6h	Tonsil 24-72h	Tonsil 96-168h	Liver
Colon 6h	Colon 24-72h	Colon 96-168	Placenta
Breast	Pancreas	Salivary Gl.	Skin
Kidney	Adrenal Gl.	Prostate	Brain
Cervix	Testis	Skeletal Muscle	Colon/Tonsil 24h decalc. EDTA

Protocol set-up: Evaluate analytic sensitivity and specificity

Normal tissue including fixation and decalcification controls

Identification of the best practice protocol (clone, titer, retrieval etc.)

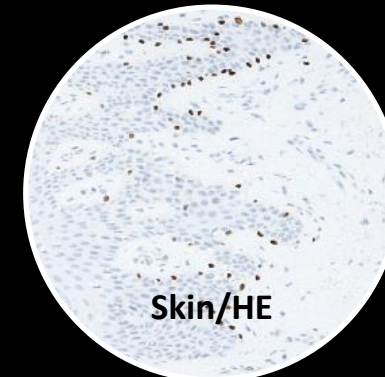
SOX10, BS7; HIER High pH 24` ; 1:350 RR; Flex+Mouse linker

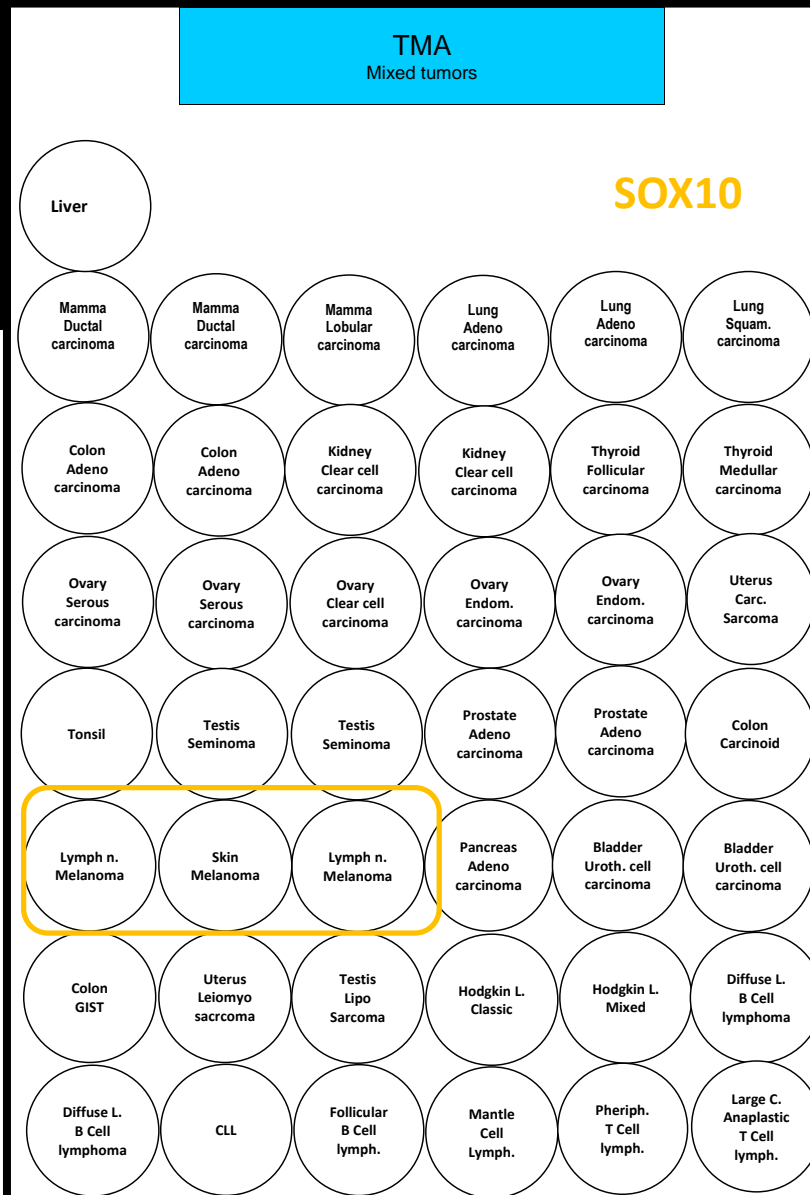
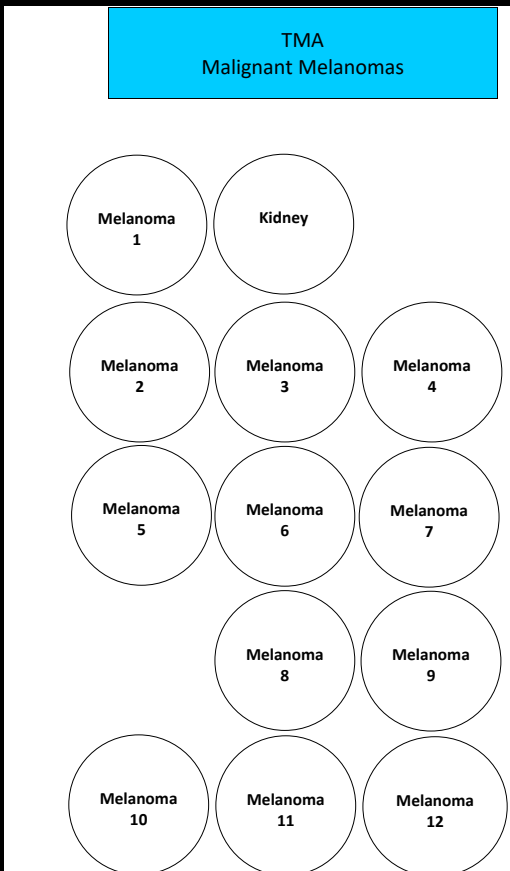
Establishing robustness of the IHC assay / pre-analytic parameter`s ?

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

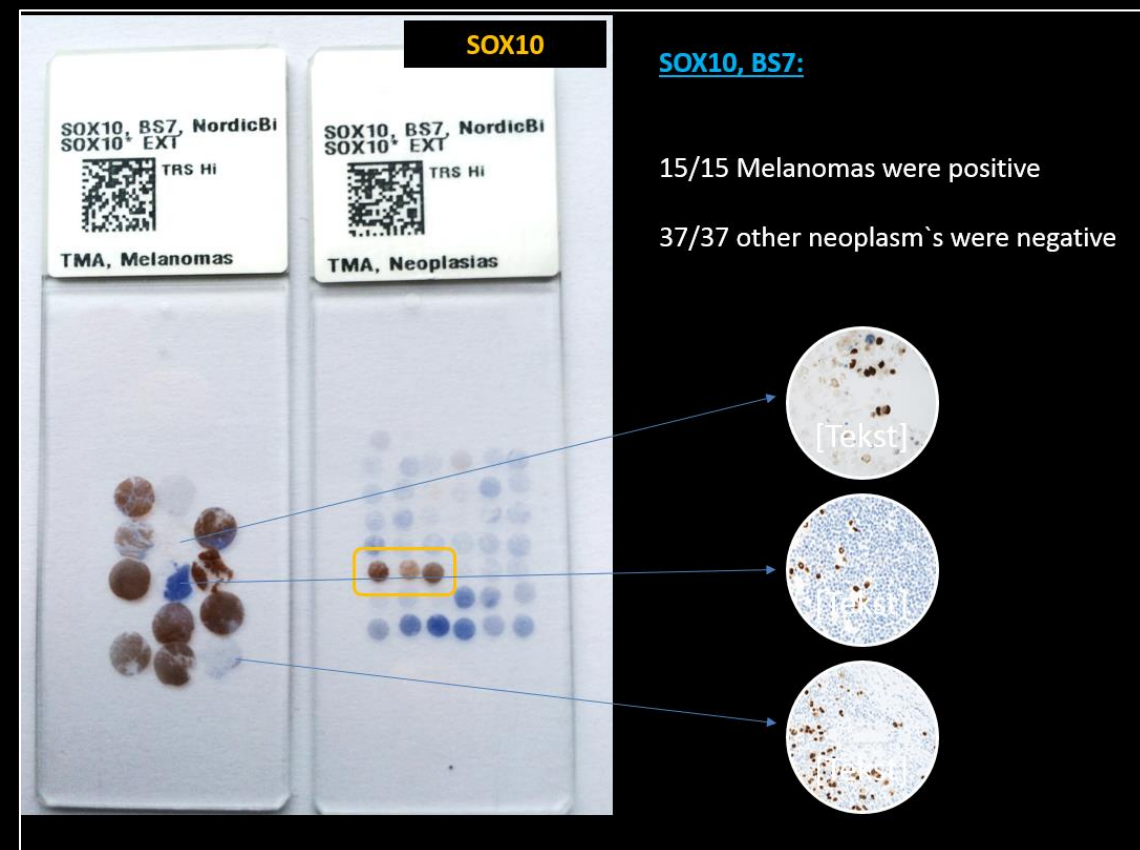
Identification of robust controls

SOX10, BS7; High, Low & Non-expressors ?



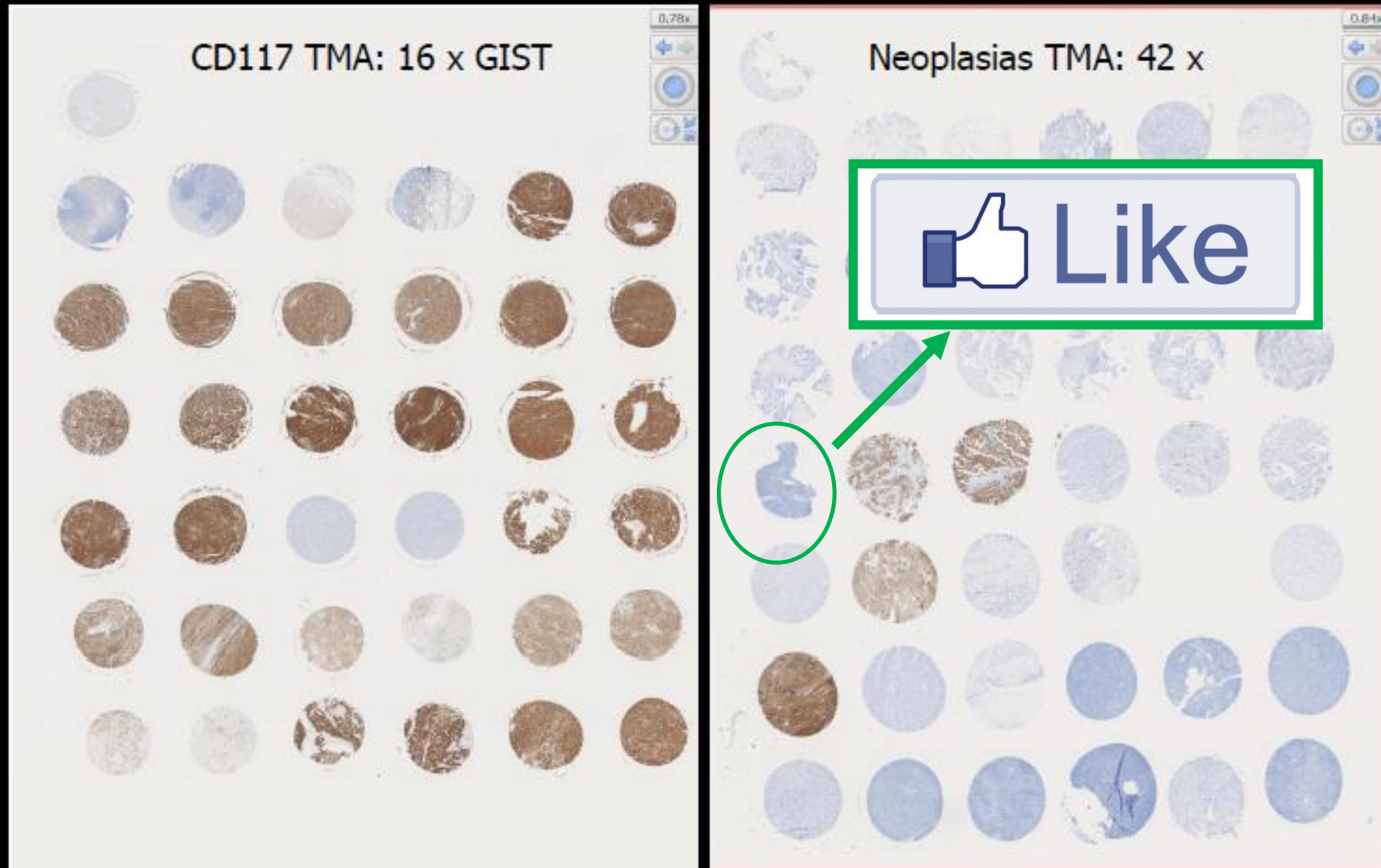


Diagnostic potential Analytical validation



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

IHC – The Technical Test Approach



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

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C. Blake Gilks, MD,‡‡ Jacqueline A. Hall, PhD,§§|| Jason L. Hornick, MD, PhD,¶¶
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Clive R. Taylor, MD,¶¶¶ Mogens Vyberg, MD,‡‡‡§§§ Xiaoge Zhou, MD,####****
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*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

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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

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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

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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

Proficiency testing in immunohistochemistry—experiences from Nordic Immunohistochemical Quality Control (NordiQC)

Mogens Vyberg^{1,2} · Søren Nielsen¹

Table 3 Major causes of insufficient staining reactions

1. Less successful antibodies (17 %)
 - a. Poor antibodies^a
 - b. Less robust antibodies^b
 - c. Poorly calibrated RTUs
 - d. Stainer platform dependent antibodies
2. Insufficiently calibrated antibody dilutions (20 %)
3. Insufficient or erroneous epitope retrieval (27 %)
4. Error-prone or less sensitive visualization systems^c (19 %)
- 5 Other (17 %)
 - a. Heat-induced impaired morphology
 - b. Proteolysis induced impaired morphology
 - c. Drying out phenomena
 - d. Stainer platform-dependant protocol issues
 - e. Excessive counterstaining impairing interpretation

^a Consistently gives false negative or false positive staining or a poor signal-to-noise ratio in one or more assessment runs

^b Frequently giving inferior staining results, e.g., due to mouse-anti-Golgi reactions or sensitive to standard operations as blocking of endogenous peroxidase

^c Biotin-based detection kit for cytoplasmic epitopes, use of detection kits providing a too low sensitivity, or use of detection kits and chromogens giving imprecise localization of the staining signals complicating the interpretation

Main causes of insufficient staining reactions are related to:

- The choice of antigen retrieval method
- The choice of primary antibody (Concentrate or RTU)
 - a) Calibration of the antibodies (dilutions)
 - b) Stainer platform dependent antibodies
- The choice of detection system

83 % of insufficient results

89 markers assessed during the period 2003-2015 and several markers have been assessed several times.
Seven runs for HER2 ISH (more than 30000 slides assessed)

Proficiency testing in immunohistochemistry—experiences from Nordic Immunohistochemical Quality Control (NordiQC)

Mogens Vyberg^{1,2} · Søren Nielsen¹

Problems related to the choice of antigen retrieval methods:

- Use of non-alkaline HIER buffer (low pH buffer)
- Use of inefficient / too short HIER period
- Use of no / enzymatic pre-treatment instead of HIER
- Use of excessive retrieval procedure → impaired morphology

False positive or false negative results

Table 3 Major causes of insufficient staining reactions

1. Less successful antibodies (17 %)
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 - b. Less robust antibodies^b
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 - d. Stainer platform dependent antibodies
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 - e. Excessive counterstaining impairing interpretation

27% insuff.

^a Consistently gives false negative or false positive staining or a poor signal-to-noise ratio in one or more assessment runs

^b Frequently giving inferior staining results, e.g., due to mouse-anti-Golgi reactions or sensitive to standard operations as blocking of endogenous peroxidase

^c Biotin-based detection kit for cytoplasmic epitopes, use of detection kits providing a too low sensitivity, or use of detection kits and chromogens giving imprecise localization of the staining signals complicating the interpretation

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

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

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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 Laboratories

Received for publication

We describe a new method for antigen retrieval in formalin-fixed, paraffin-embedded tissue sections.

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 that failed to stain with conventional methods.
- Certain antibodies which were typically unreactive with formalin-fixed tissue gave excellent staining.

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

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

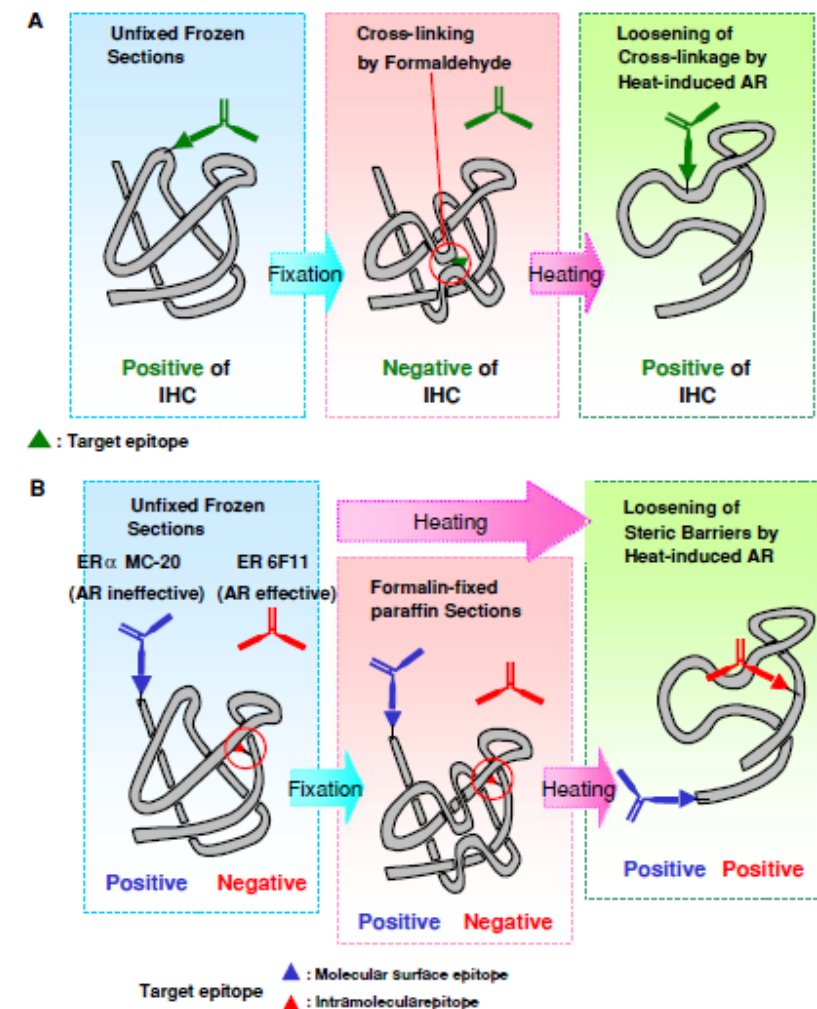
Heating tissue sections in an appropriate buffer may unmask epitopes by :

- ☐ **Hydrolysis/disruption of methylene cross-links formed by formalin fixation**
- ☐ **Extraction of diffusible blocking proteins**
- ☐ **Precipitation 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 ?**

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

Kochi Kakimoto · Susumu Takekoshi · Katsuhiko 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 ER α 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



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

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

The authors proposed that :

One of the other mechanisms for heat-induced AR is that accessibility to the target epitopes of antigenic proteins is limited by natural steric barriers even in the fresh state caused by the antigenic protein itself.

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Efficient HIER depends on:

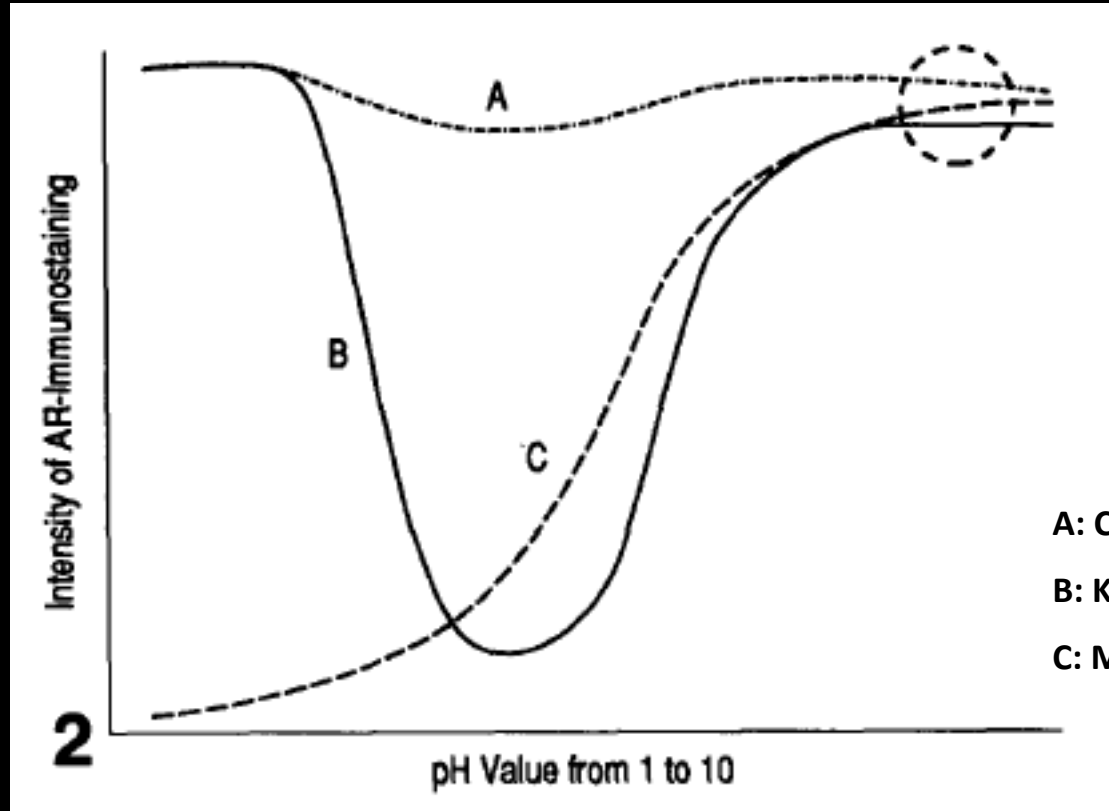
- ☐ pH of the HIER buffer
- ☐ Temperature
- ☐ Time
- ☐ Elementary nature of the HIER buffer (e.g., Citrate; TRIS; EDTA; TE)

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



A: CD20 (clone L26)

B: Ki-67 (clone MIB1)

C: MSA (clone HMB45)

Efficient HIER - Influence of pH

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

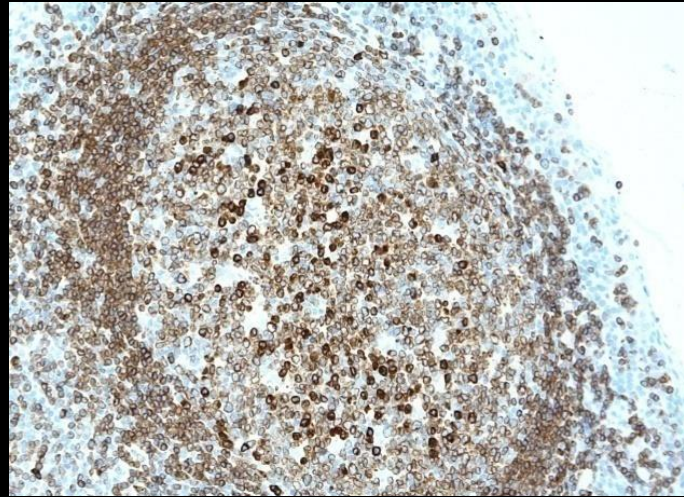
Also, the results indicate the advantage of using an AR solution of higher pH value (8-9).

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

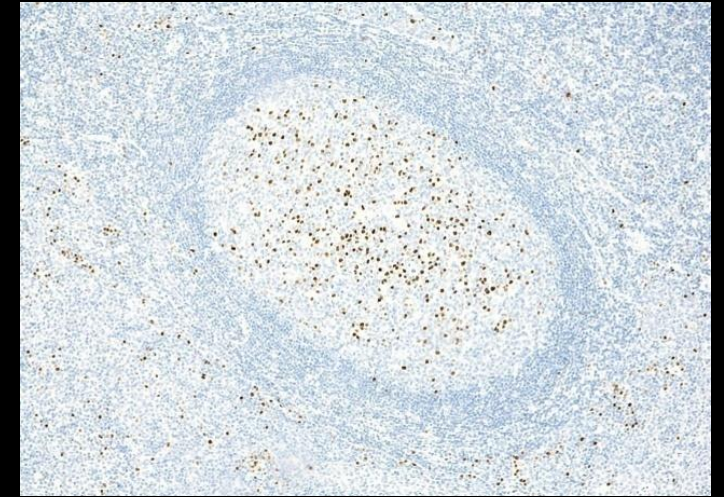
Efficient HIER - Influence of pH

HIER in TRS pH 6.1
(20 min at 97°C)

CD79, JCB117 (1:300)

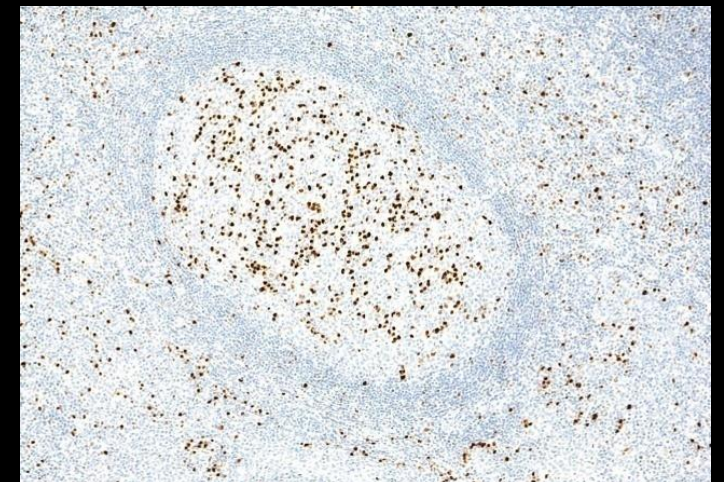
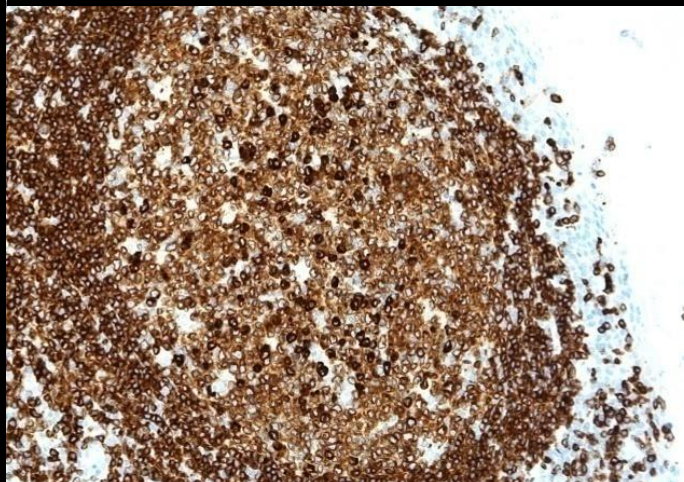


MUM-1, MUM1p (1:400)



Autostainer: Flex+ as the detection system

HIER in TRS pH 9
(20 min at 97°C)



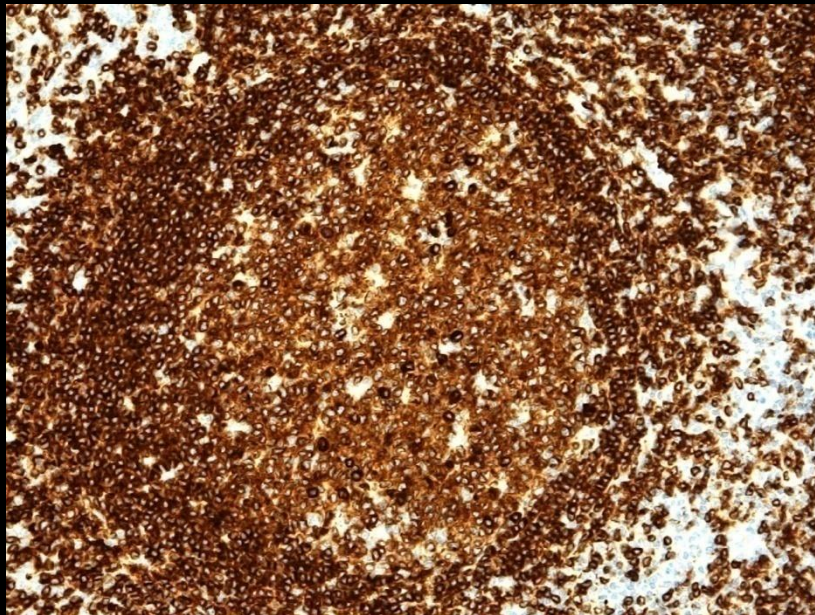
Tonsillar tissue fixed in 10% NBF (48h).

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Efficient HIER - Influence of pH

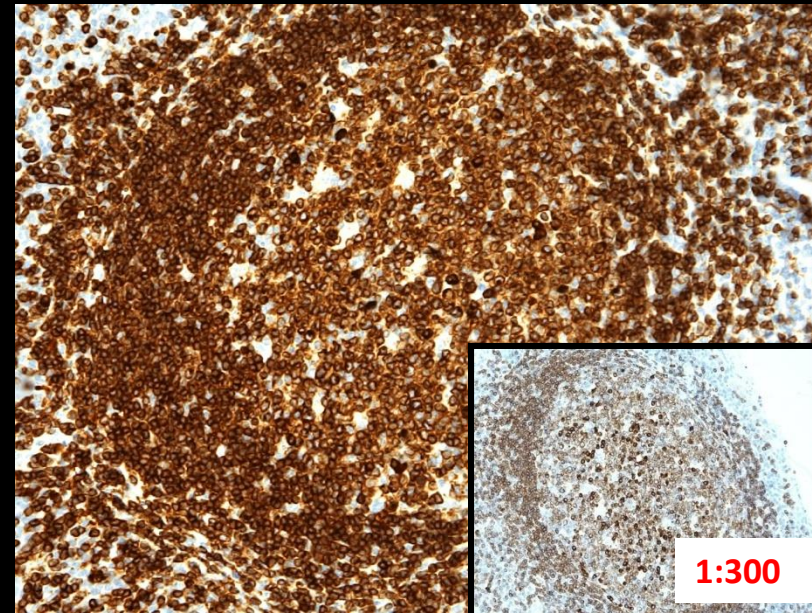
HIER in TRS pH 9

CD79, JCB117 (1:300)



HIER in TRS pH 6.1

CD79, JCB117 (1:50)



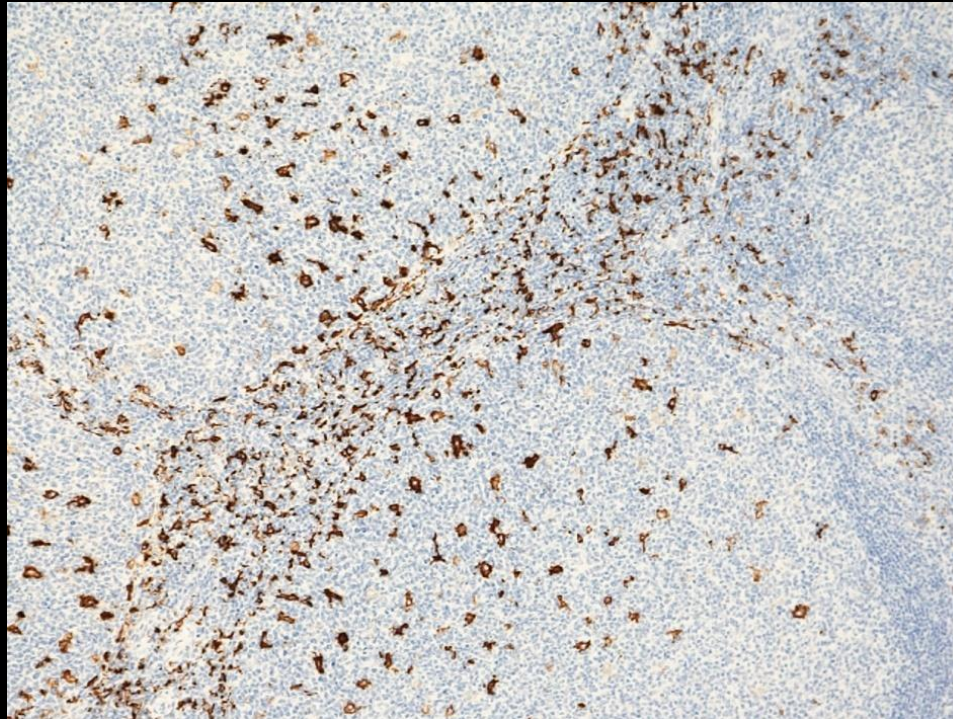
Tonsillar tissue fixed in 10% NBF (48h).
Flex+ as detection system

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Efficient HIER - Influence of pH

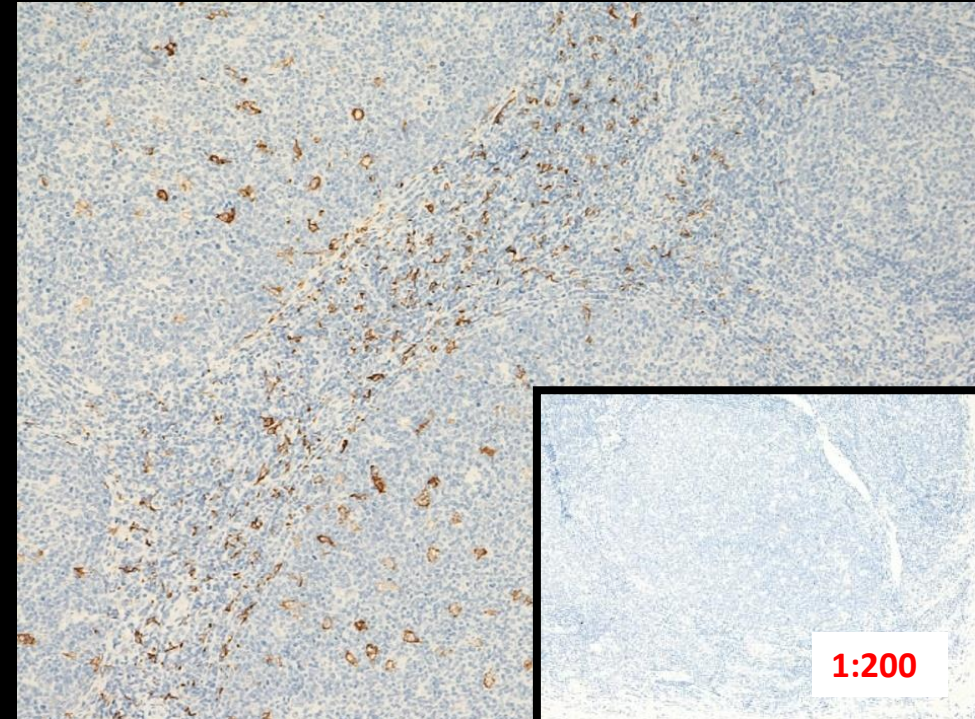
HIER in TRS pH 9

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

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:

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

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

HIER buffer - Influence of time and temperature

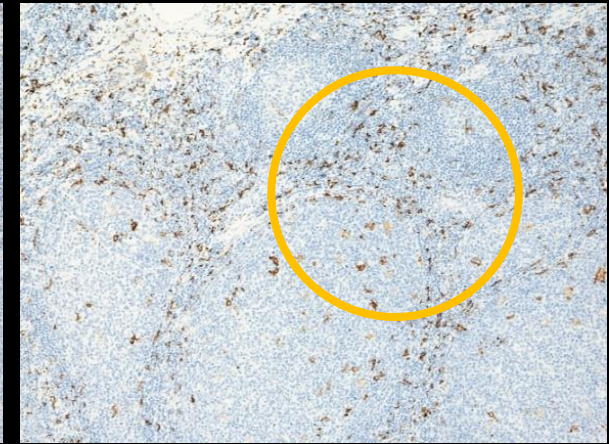
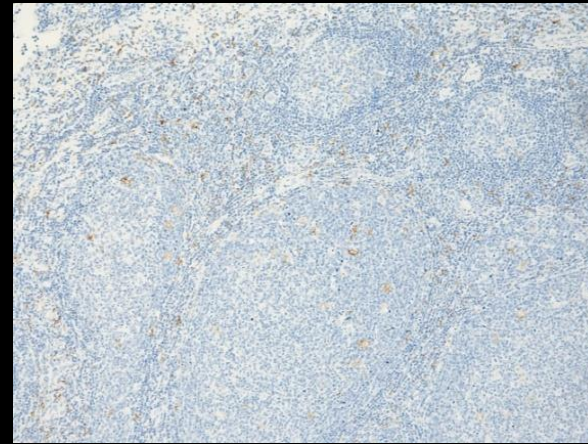
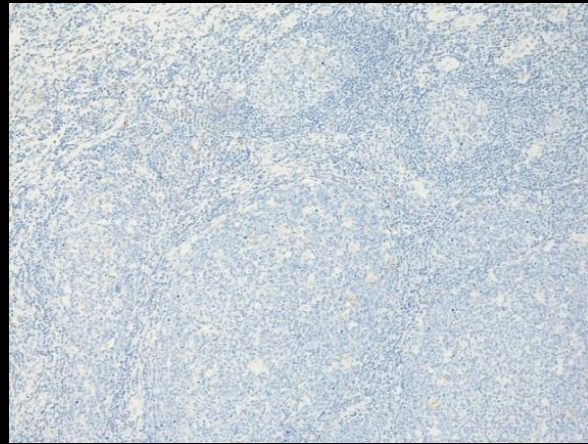
CD163, MRQ-26 (1:200)

10 min

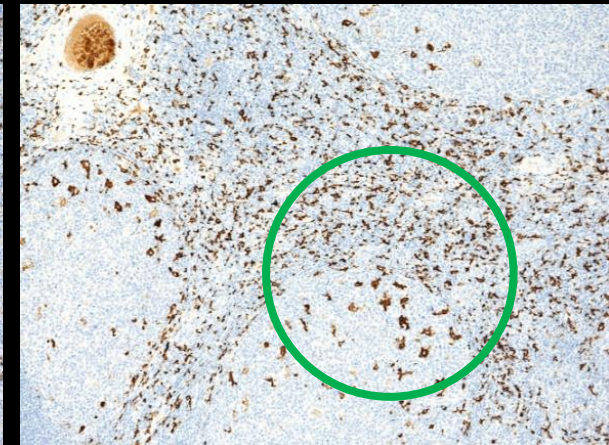
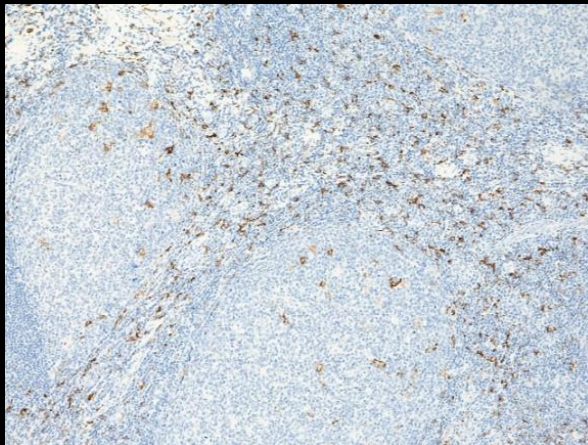
20 min

80 min

HIER at 80°C
TRS pH 9, Flex+



HIER at 97°C
TRS pH 9, Flex+



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')

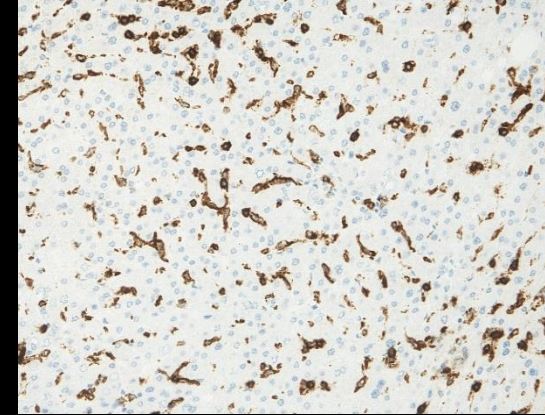
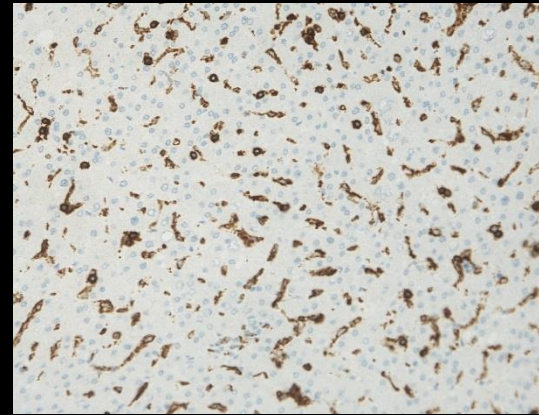
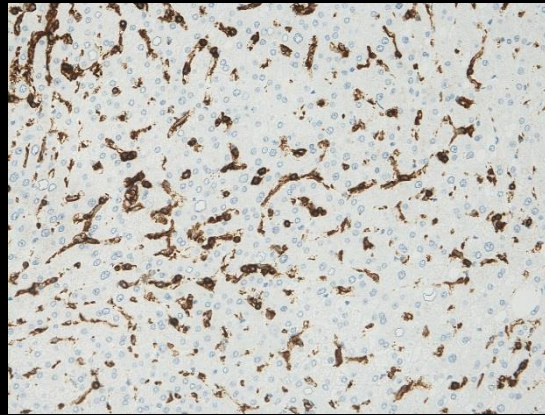
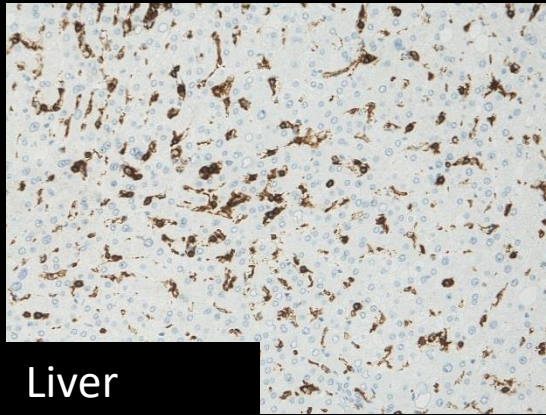
TRS pH 9 (90°C/30')

TRS pH9 (90°C/60')

TRS pH9 (97°C/24')

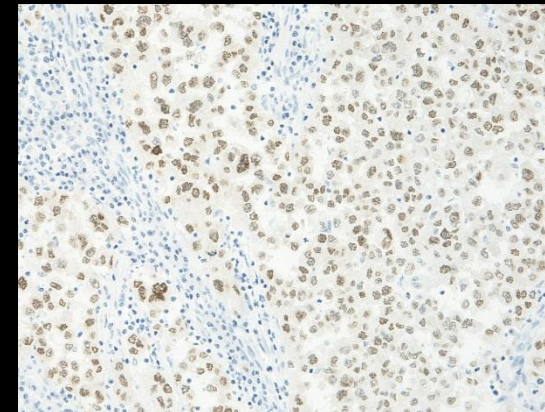
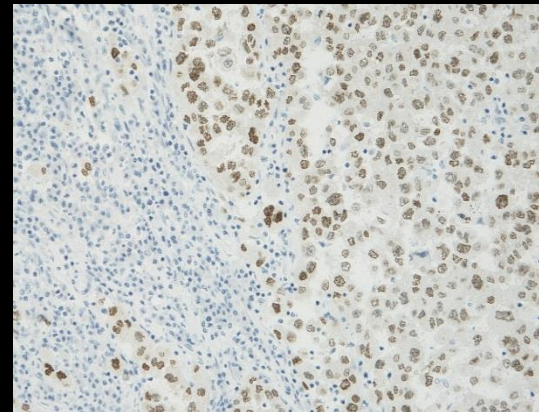
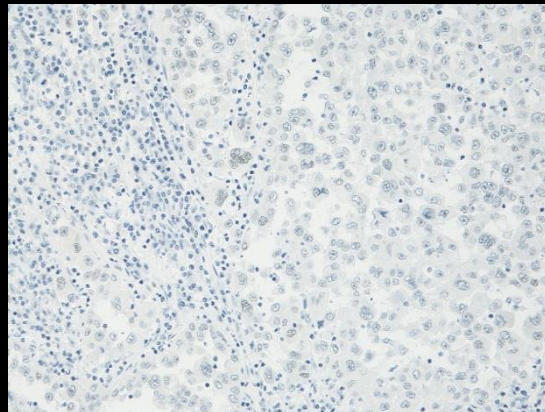
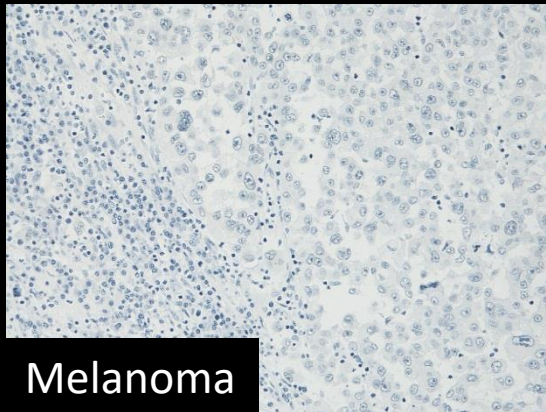
CD45/LCA
(2B11+PD7/26)

Liver



SOX10
(BS7)

Melanoma



ANTIGEN RETRIEVAL TECHNIQUES IN IMMUNOHISTOCHEMISTRY: COMPARISON OF DIFFERENT METHODS

STEFANO A. PILERI^{1*}, GIOVANNA RONCADOR¹, CLAUDIO CECCARELLI¹, MILENA PICCIOLI¹, ASPASIA BRISKOMATIS¹, ELENA SABATTINI¹, STEFANO ASCANI¹, DONATELLA SANTINI¹, PIER PAOLO PICCALUGA¹, ORNELLA LEONE¹, STEFANIA DAMIANI¹, CESARINA ERCOLESSI¹, FEDERICA SANDRI¹, FEDERICA PIERI¹, LORENZO LEONCINI² AND BRUNANGELO FALINI³

¹Second Service of Pathologic Anatomy and Haematopathology Section, Bologna University, Italy

²Institute of Pathologic Anatomy, Siena University, Italy

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Chemical composition 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	—	++++	—	—	—
MF16	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	++—	++++	—	++—	++—
MA689	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	++—	++—	++—	++++	++—
K1-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.

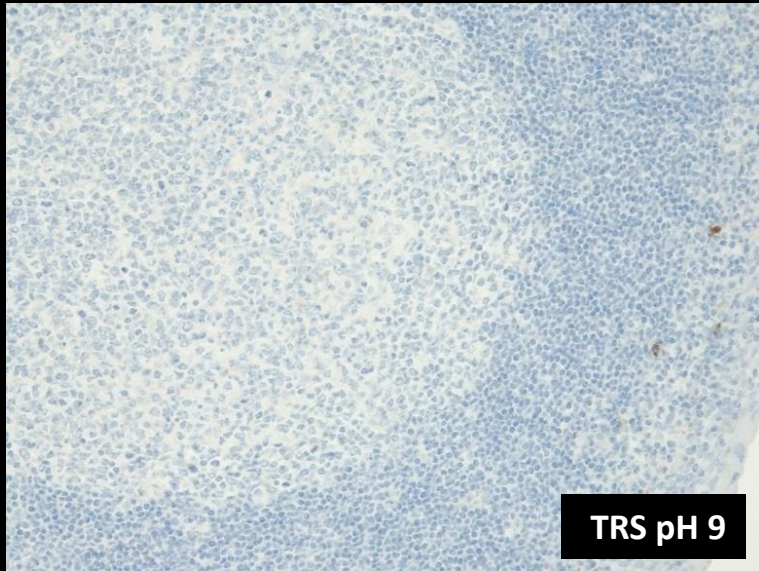
In bold: overnight incubation of the primary antibody+SABC technique.

—=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.

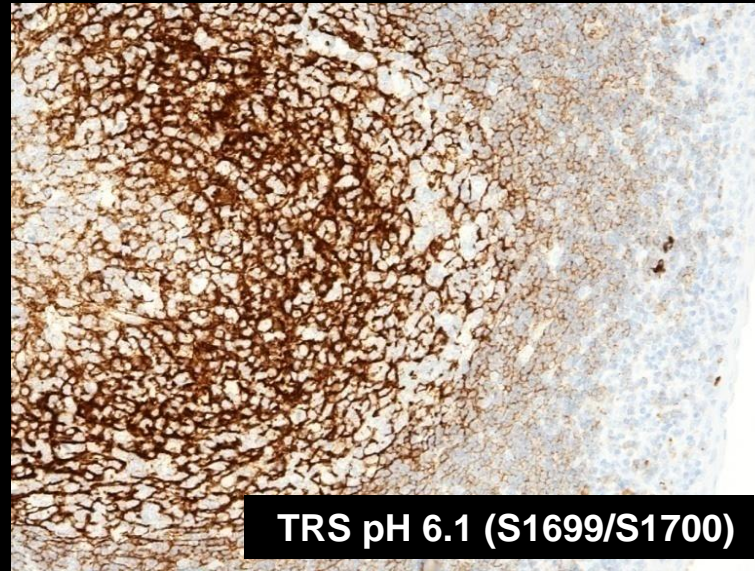
Technical aspects of immunohistochemistry & pitfalls - Analytical phase

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 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

CD30, ConD6/D5 (1:50)
(Hodgkin Lymphoma)

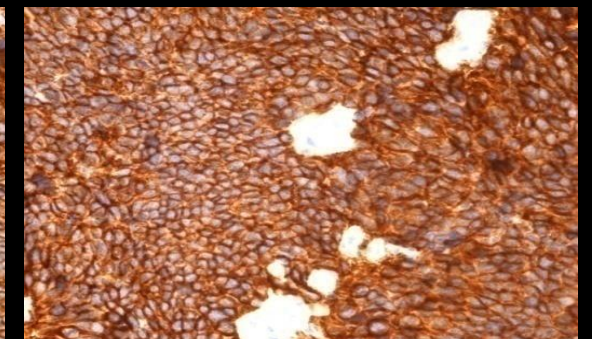
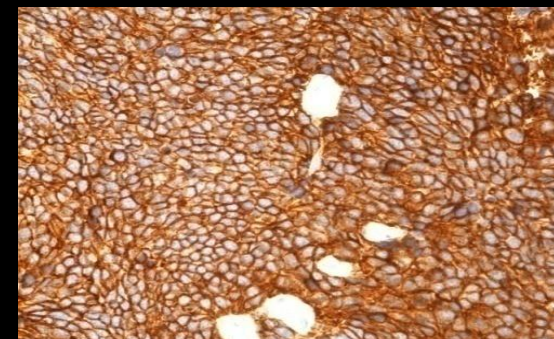
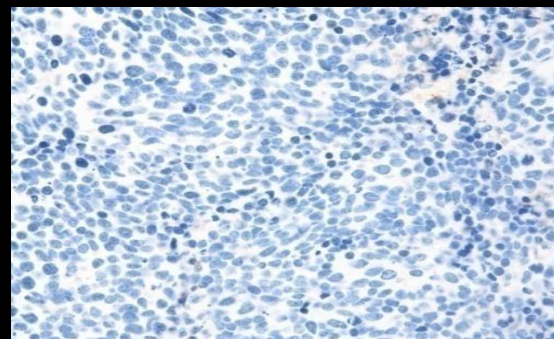
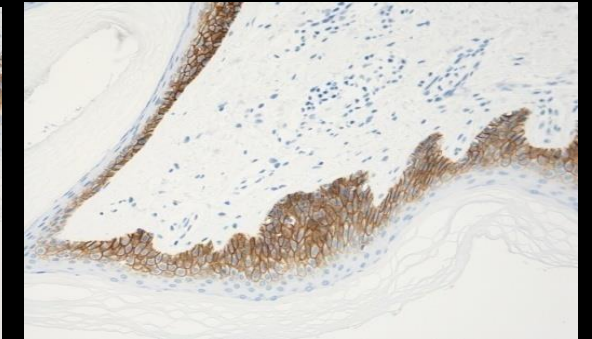
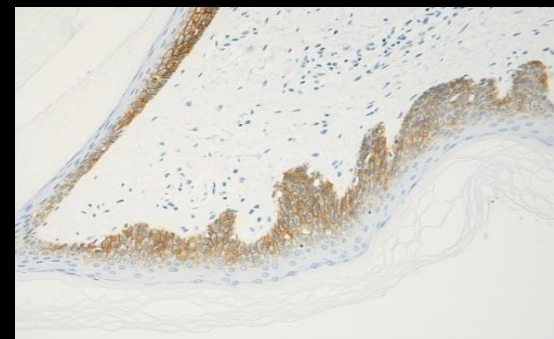
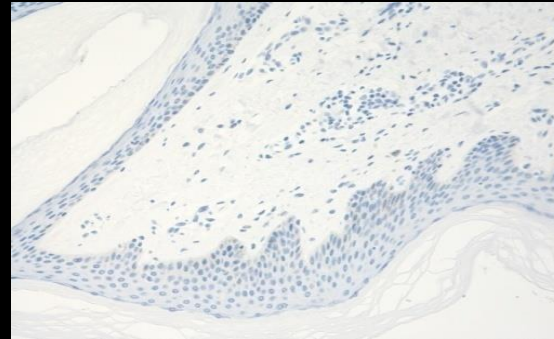
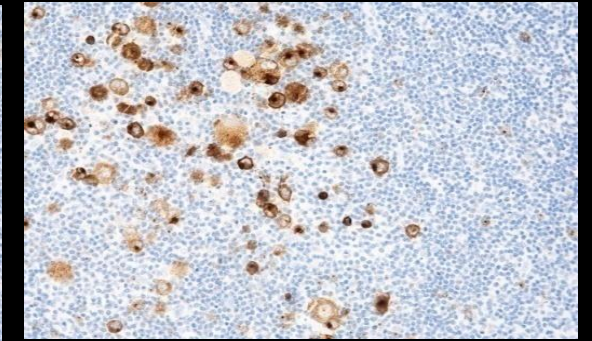
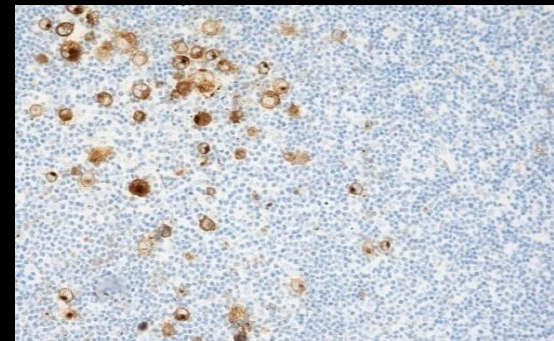
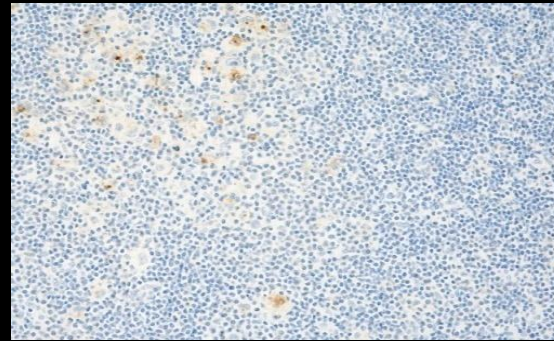
Desmoglein-3, BC11 (1:25)
(Skin)

EP-CAM, MOC-31 (1:20)/EP4
(Small cell carcinoma)

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



Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Modified low pH buffers

CD30 clone ConD6/B5

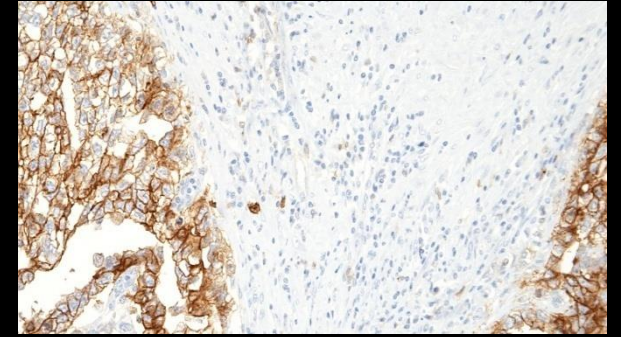
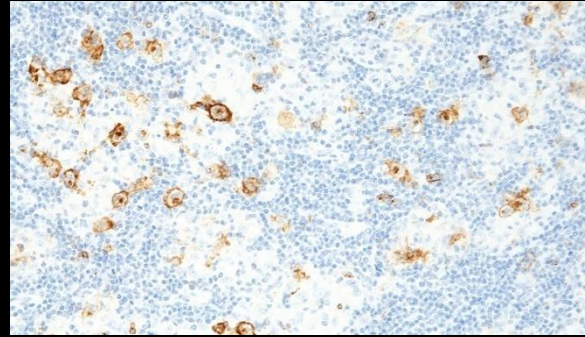
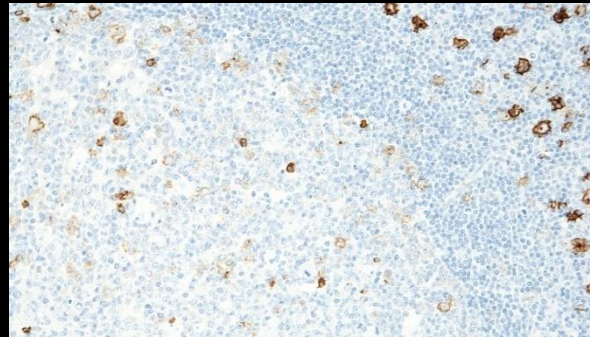
Tonsil

Hodgkin lymphoma

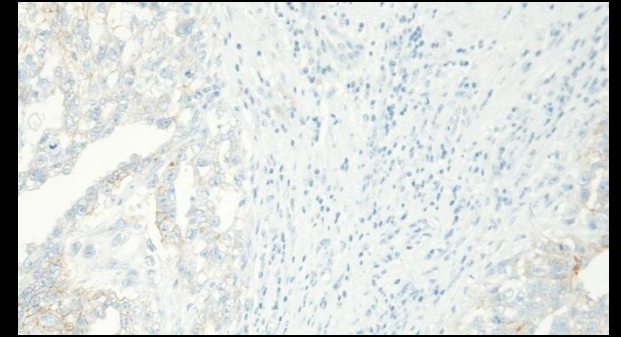
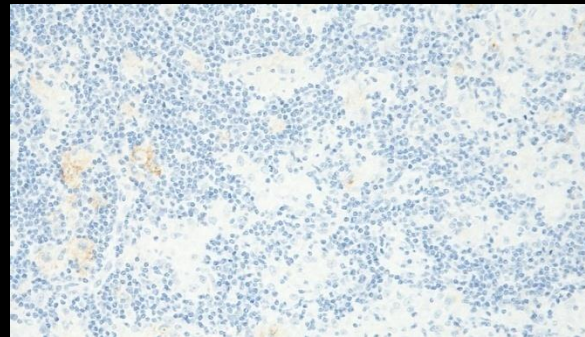
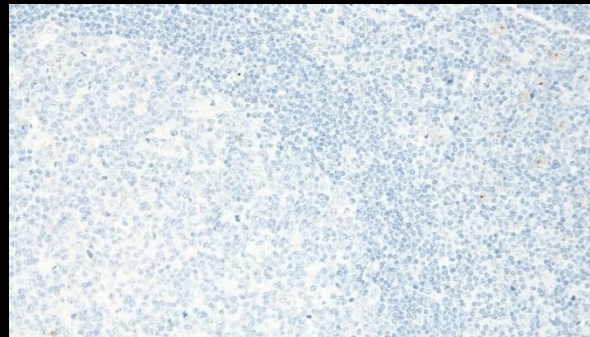
Embryonal carcinoma

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 (and not covalent cross-links formed in tissues during formalin fixation).

→ Improves penetration of reagents into the tissue structures and restore the epitopes of interest.

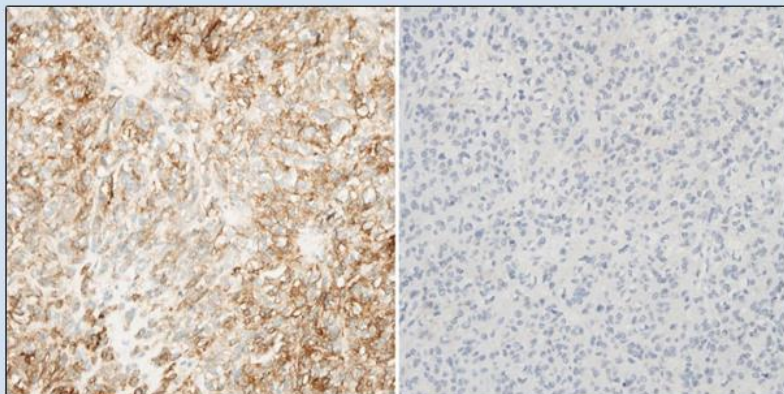
Enzymatic digestion ?

News

11-Jul-2016

↑ Top of page

Results general module - run 47



Runs 47 was accomplished April to July 2016. A very short summary of the tests is given below. Click on the epitope name to see the complete general assessment results for each marker, including recommended clones and protocols, and major causes of insufficient staining results. Individual results will be sent to participant by email.

Figure: Serial sections of GIST stained for CD117 in two labs. Left: optimal, right: false negative due to an insufficient protocol.

CK20: 284 participants, 92% sufficient, 62% optimal. Efficient HIER is recommended, proteolytic pretreatment generally gives a lower pass rate.

CK-PAN: 275 participants, 72% achieved a sufficient mark, 48% optimal. For Ab cocktails containing AE1/AE3 HIER is mandatory. mAb MNF116 requires proteolytic pretreatment but the clone performs less well than AE1/AE3.

mAb clone Ks20.8 *	Sufficient result	Optimal result
HIER in Alkaline buffer	92% (91 of 99)	47%
Enzymatic pre-treatment	75% (9 of 12)	25%

***As concentrate: App. 10 % of the protocols (12 of 126) were based on enzymatic pre-treatment**

Table 4. Pass rates for antibody cocktails combined with epitope retrieval methods in seven NordiQC runs

	Pass rate for run 15, 20, 24, 30, 36, 41 & 47							
	Total		HIER		Proteolysis		HIER + proteolysis	
	Protocols	Sufficient	Protocols	Sufficient	Protocols	Sufficient	Protocols	Sufficient
mAb AE1/AE3	752	542 (72%)	693	535 (77%)	44	5 (11%)	5	2 (40%)
mAb AE1/AE3/5D3	37	34 (92%)	36	34 (94%)	1	0	0	0
mAb AE1/AE3/PCK26	176	105 (60%)	25	13 (48%)	34	0	117	92 (79%)
mAb MNF116	91	30 (33%)	40	9 (23%)	47	21 (45%)	4	2 (50%)

AE1/AE3: App. 6 % of all protocols (44 of 742) were based on enzymatic pre-treatment (seven NQC Runs).

Problem

A significant proportion of Labs still uses enzymatic digestion for a “wide” range of markers “requiring” HIER for optimal performance

Only few markers require enzymatic digestion for routine purpose

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

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)

Short time formalin fixation = gentle proteolysis

Long time formalin fixation = prolonged proteolysis

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

Choice of proteolytic enzyme

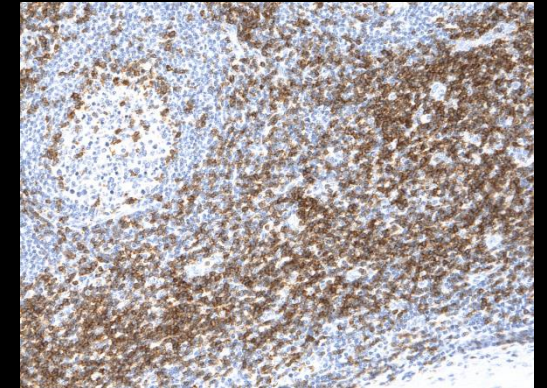
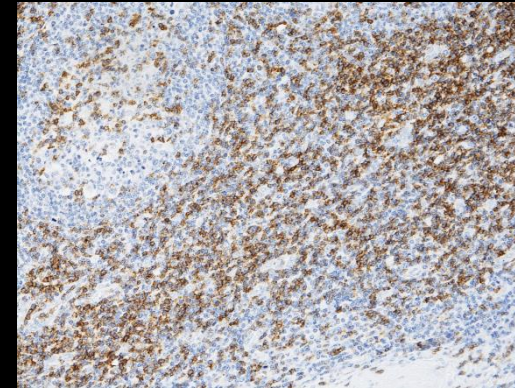
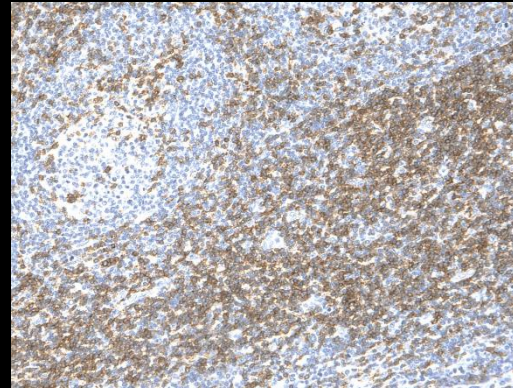
Trypsin (Biocare, RTU)
40`

Pepsin (ZytoVision, RTU-H)
15`

Proteinase K (Dako, RTU)
dil. 1:4 / 5`

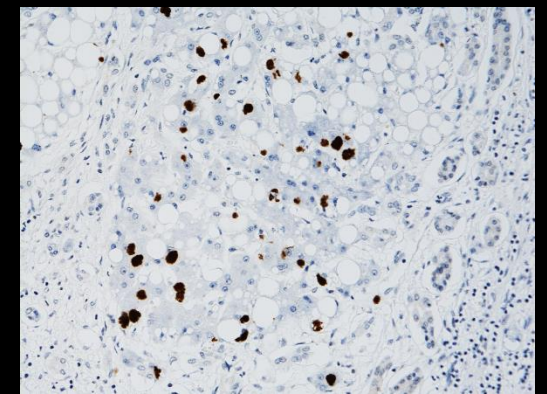
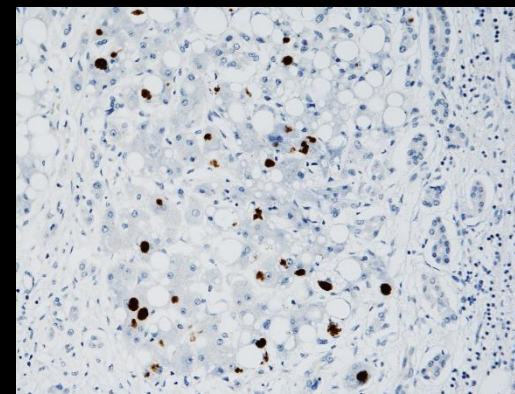
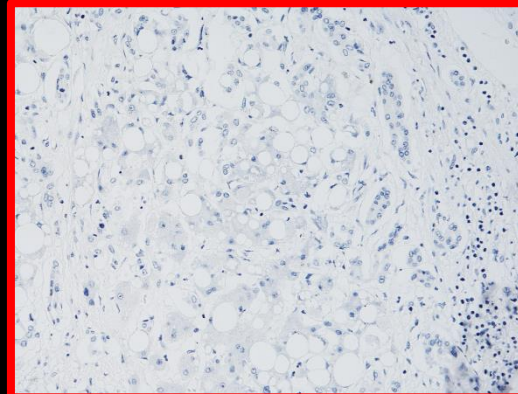
TCR- β , 8A3, 1:200 RR

Tonsil



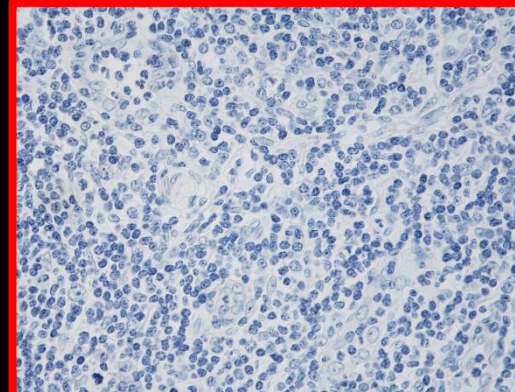
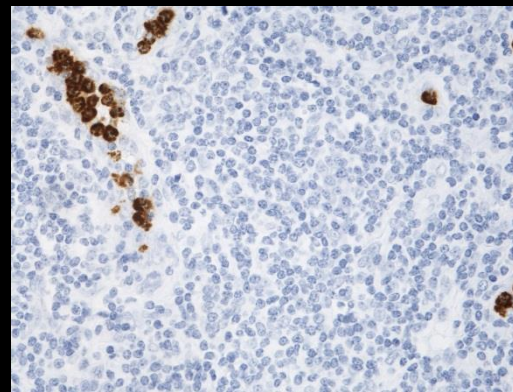
Ubiquitin, Ubi-1 1:750

Liver/ Mallory bodies



Neutrophil Elastase, NP57 1:1000

Tonsil



Proteolytic enzyme & digestion time ?

Tonsil NBF 48h

Digestion temp. 32°C

Proteinase K
(RTU S3020, Dako)

Proteinase K dil. 1:4
(RTU S3020, Dako)

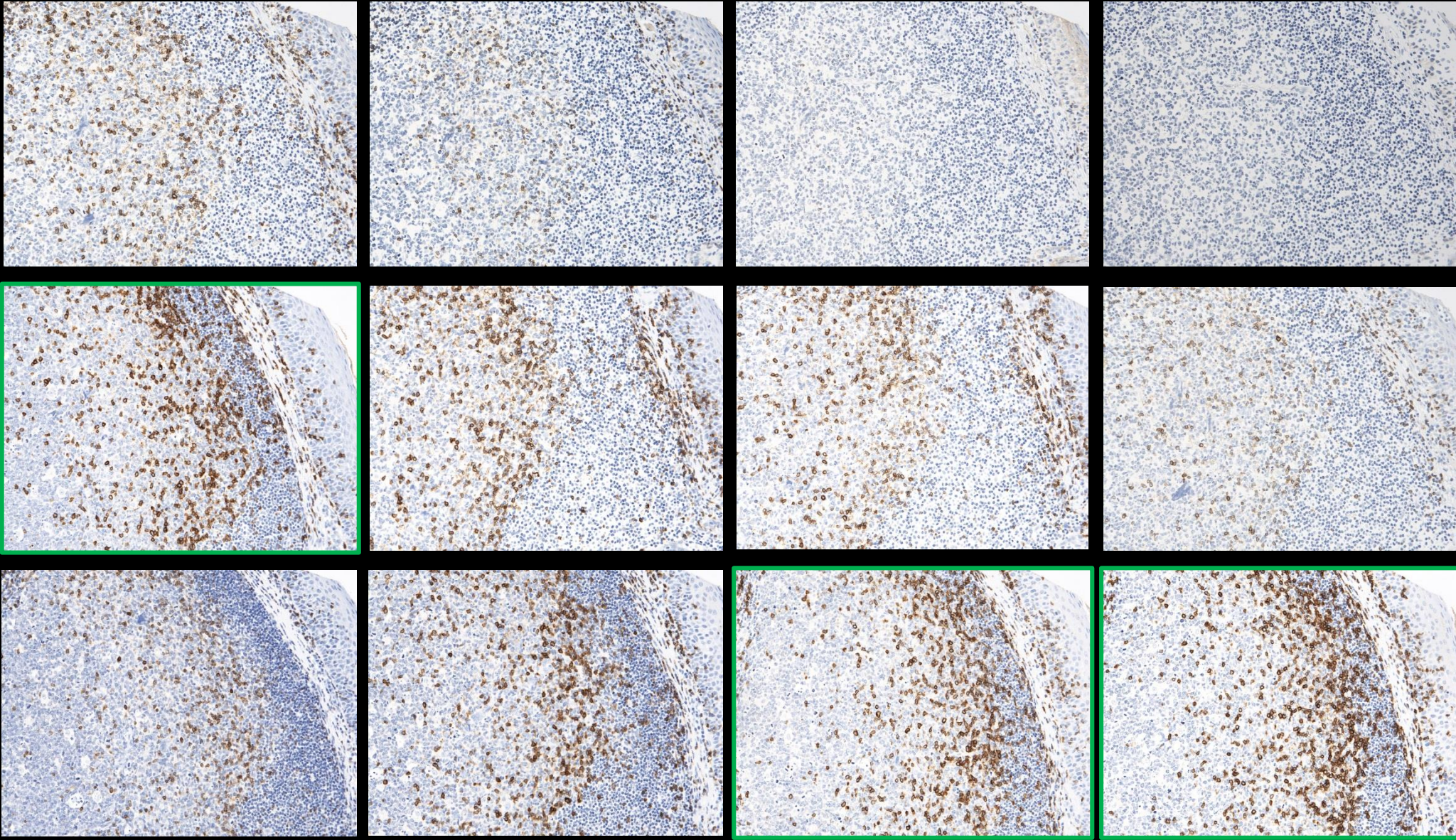
Trypsin
(RTU, Biocare)

5 min

10 min

20 min

40 min



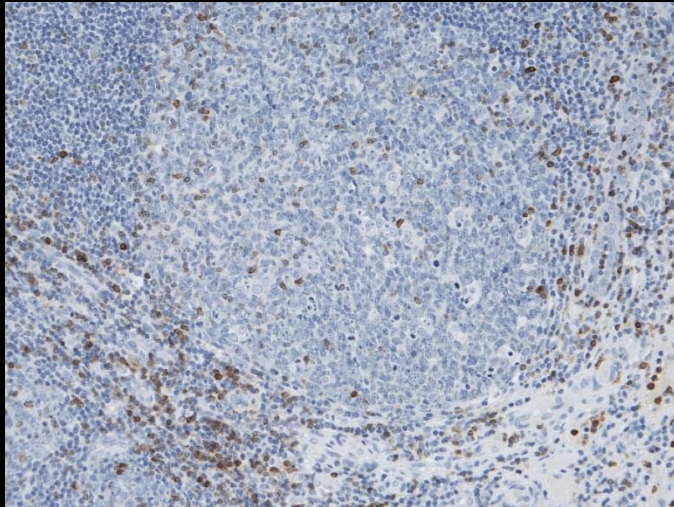
TCR-β clone 8A3 (1:200 RR) / Flex+ (Omnis)

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

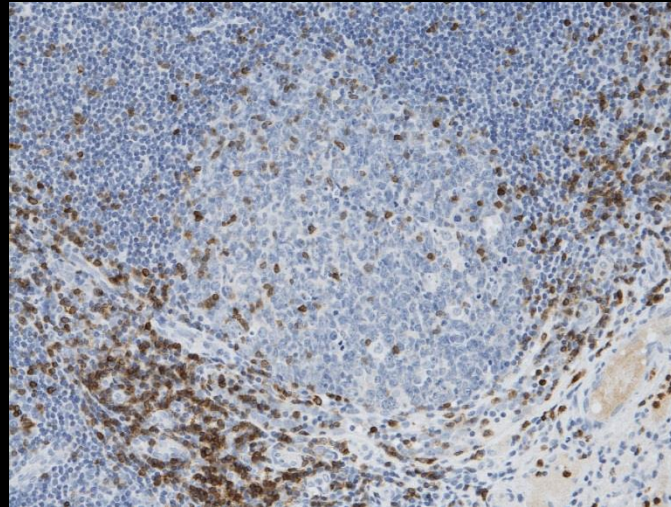
Proteolytic enzyme & digestion temperature ?

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

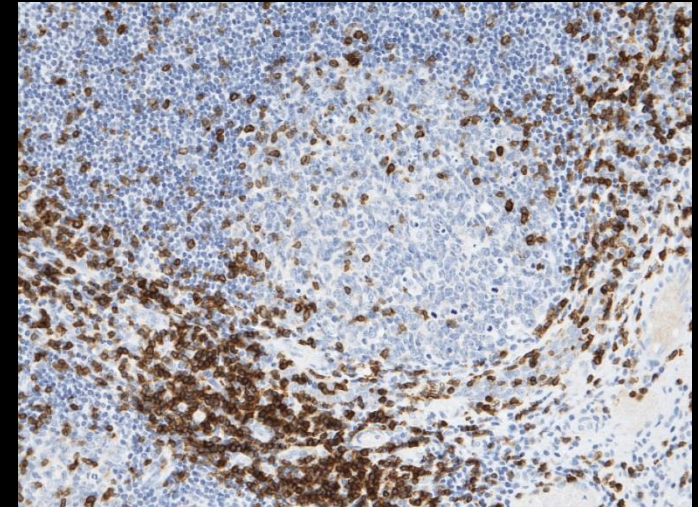
Trypsin Digestion temp. 4°C (10 `)



Trypsin Digestion temp. 24°C (10`)



Trypsin Digestion temp. 37°C (10`)



Increased intensity of TCR β positive T-cells

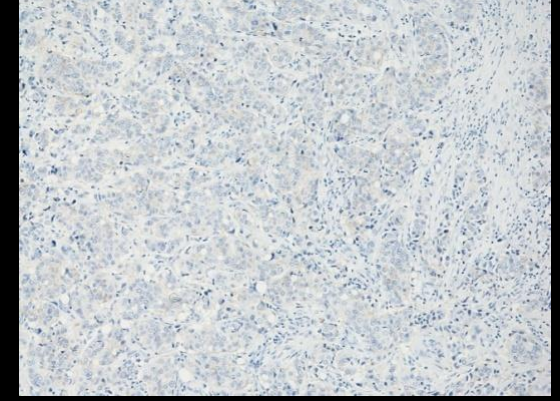
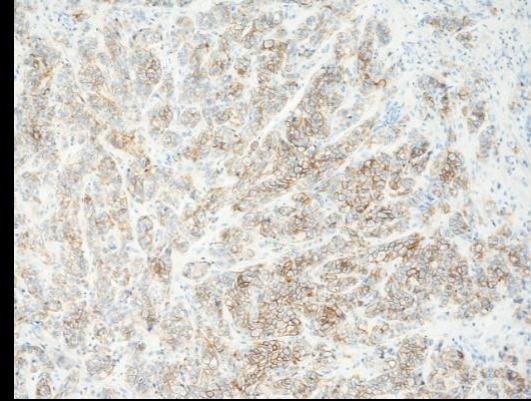
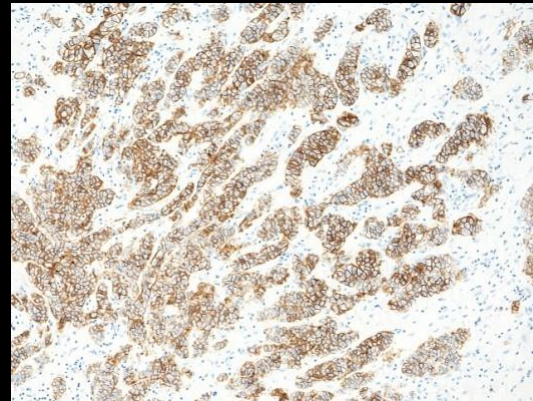
Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Enzymatic digestion (Influence of fixation time)

EPCAM, clone MOC-31, dilution 1:20

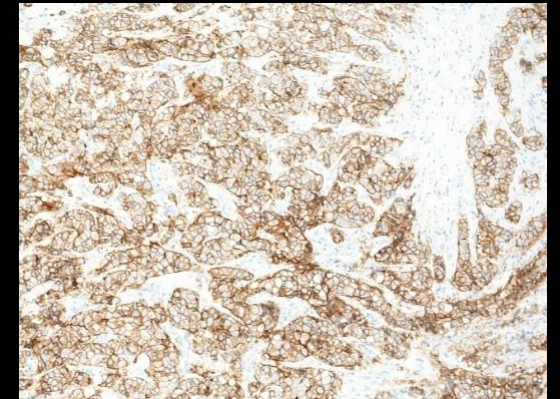
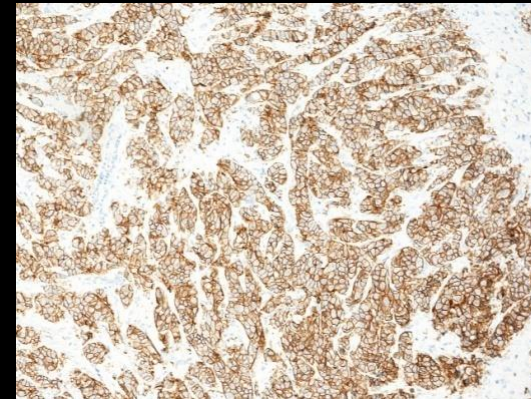
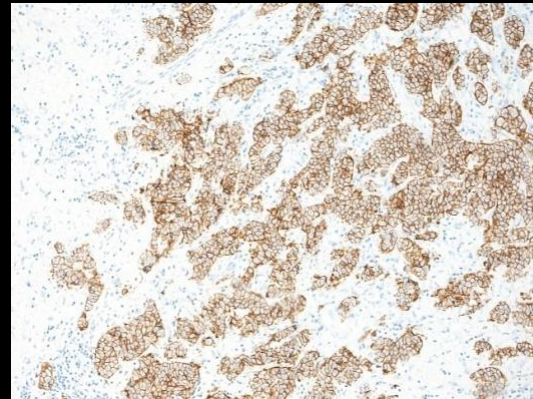
Pepsin / (Dako, S3002)

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

Proficiency testing in immunohistochemistry—experiences from Nordic Immunohistochemical Quality Control (NordiQC)

Mogens Vyberg^{1,2} · Søren Nielsen¹

Virchows Arch (2016) 468:19–29

Table 3 Major causes of insufficient staining reactions

1. Less successful antibodies (17 %)
 - a. Poor antibodies^a
 - b. Less robust antibodies^b
 - c. Poorly calibrated RTUs
 - d. Stainer platform dependent antibodies
2. Insufficiently calibrated antibody dilutions (20 %)
3. Insufficient or erroneous epitope retrieval (27 %)
4. Error-prone or less sensitive visualization systems^c (19 %)
- 5 Other (17 %)
 - a. Heat-induced impaired morphology
 - b. Proteolysis induced impaired morphology
 - c. Drying out phenomena
 - d. Stainer platform-dependant protocol issues
 - e. Excessive counterstaining impairing interpretation

^a Consistently gives false negative or false positive staining or a poor signal-to-noise ratio in one or more assessment runs

^b Frequently giving inferior staining results, e.g., due to mouse-anti-Golgi reactions or sensitive to standard operations as blocking of endogenous peroxidase

^c Biotin-based detection kit for cytoplasmic epitopes, use of detection kits providing a too low sensitivity, or use of detection kits and chromogens giving imprecise localization of the staining signals complicating the interpretation

Problems related to the choice of antigen retrieval method :

- Use of non-alkaline HIER buffer (low pH buffer)
- Use of inefficient / too short HIER period
- Use of no / enzymatic pre-treatment instead of HIER
- Use of excessive retrieval procedure → impaired morphology

False positive or false negative results

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Proteolytic pretreatment (excessive retrieval procedures)

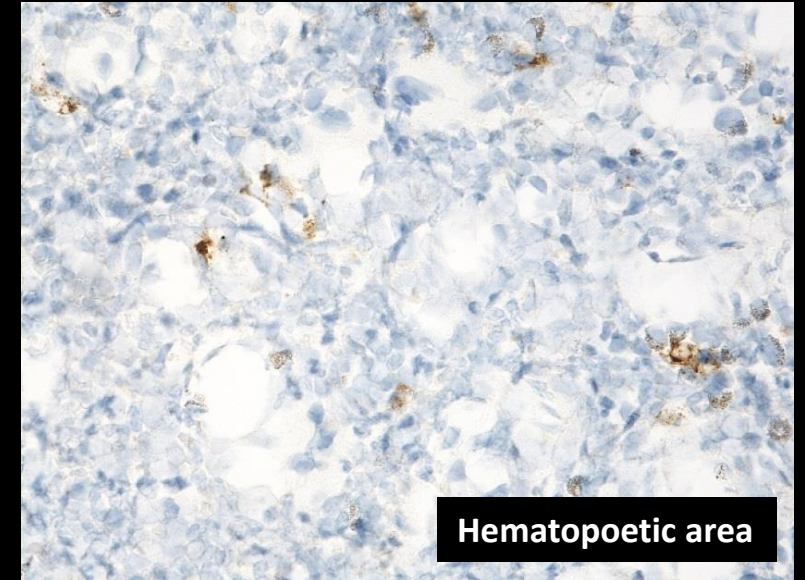
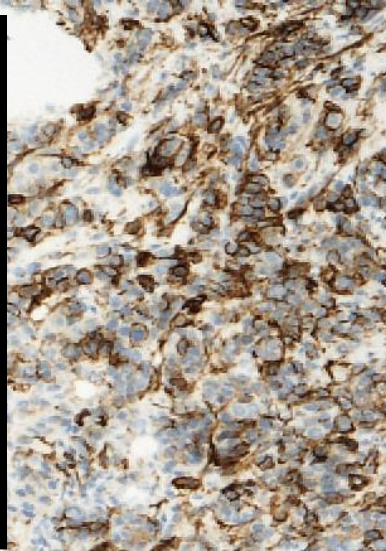
- over digestion (e.g. not calibrated to the fixation time in NBF)

HIER (excessive retrieval procedures)

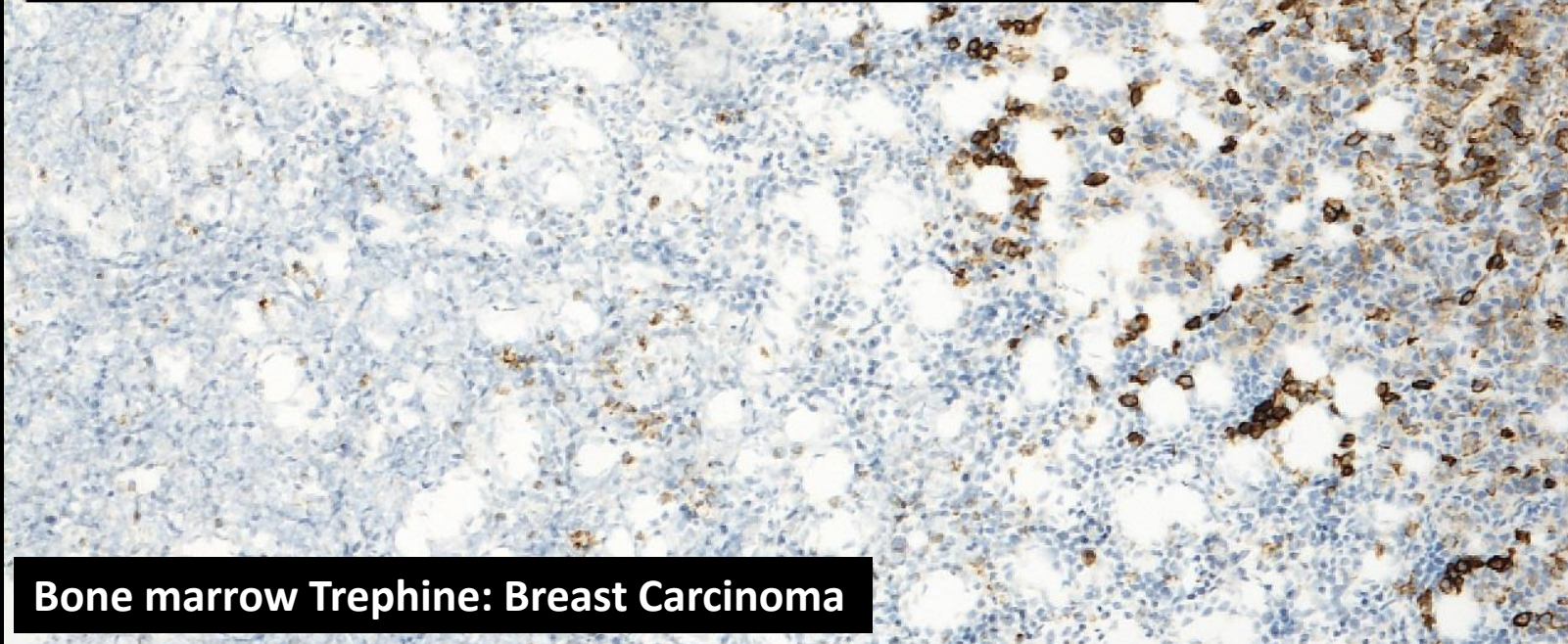
- too high temperature for too long time especially in alkaline retrieval buffers

HIER applying standard procedures (excessive retrieval procedures)

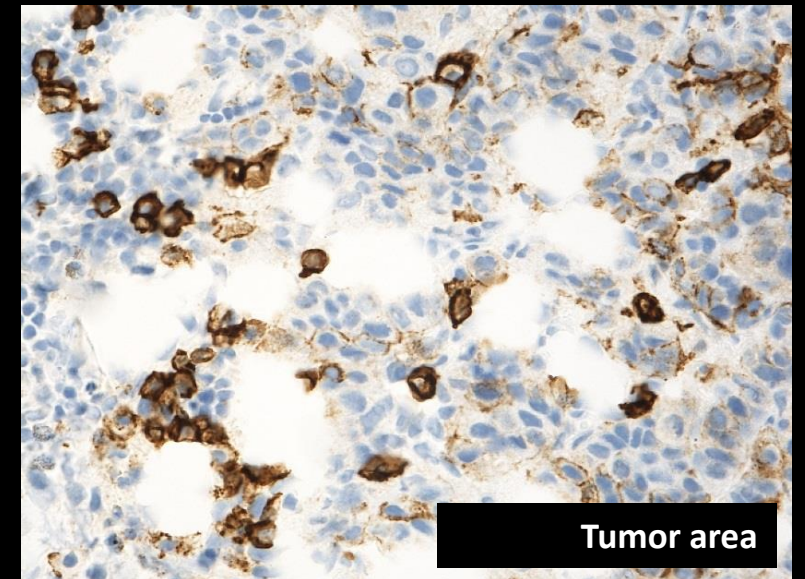
- fragile tissue/cell material (cell clot's)



Hematopoietic area



Bone marrow Trephine: Breast Carcinoma

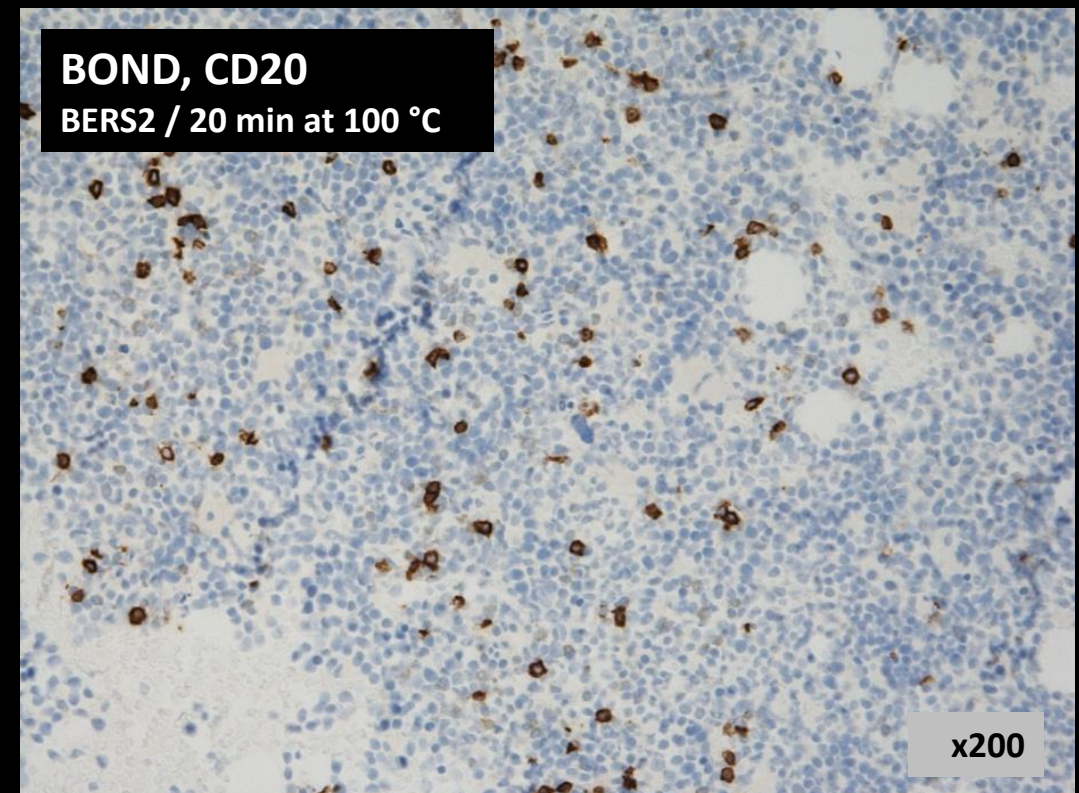
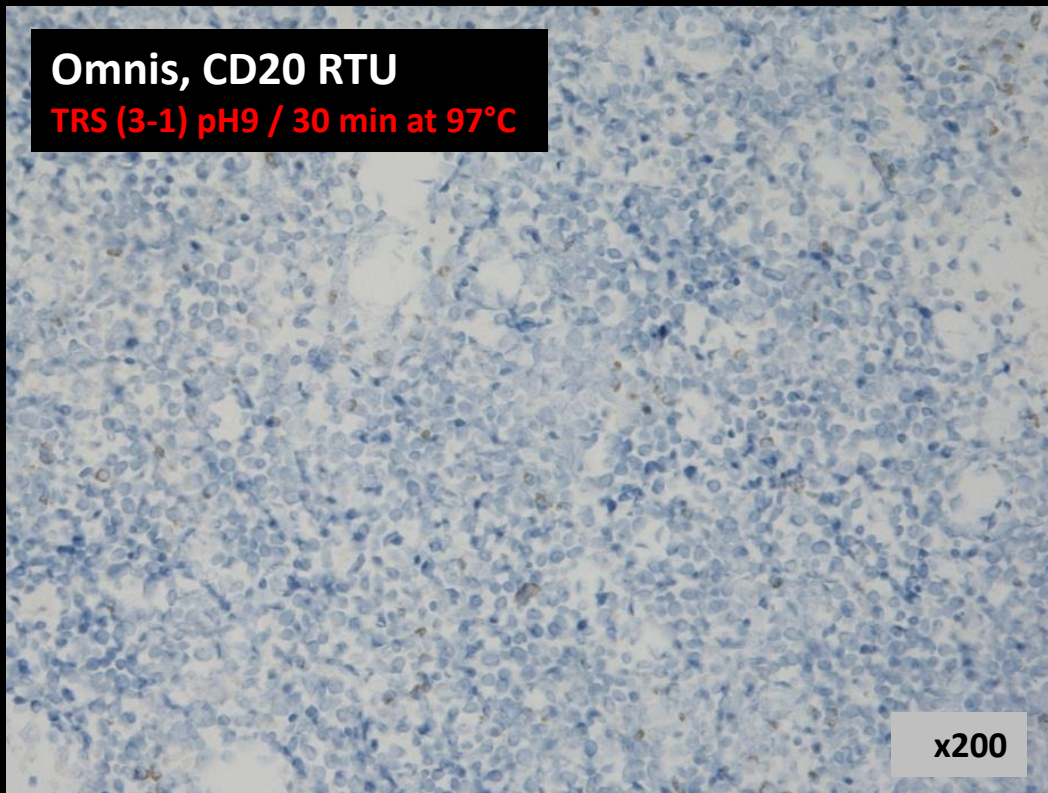


Tumor area

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

CD20 clone L26

Bone Marrow Coagulum/Clot (fixed for 24h in NBF)



HIER settings: Recommendations of the manufacturer`s

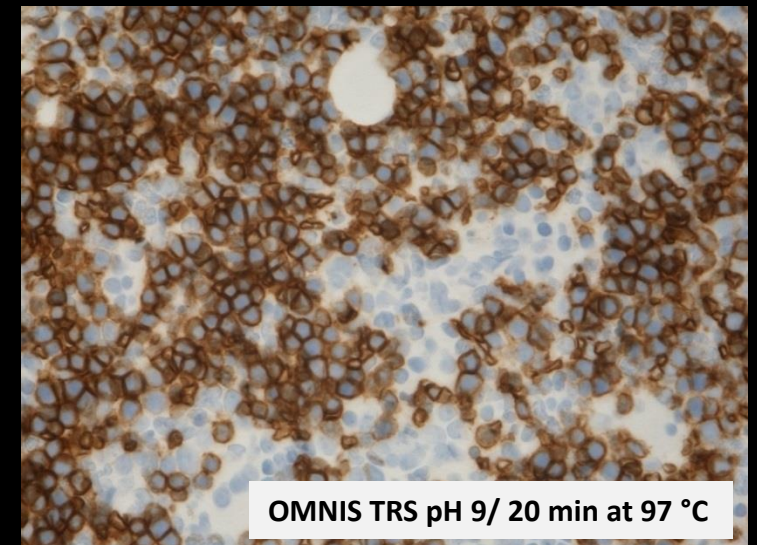
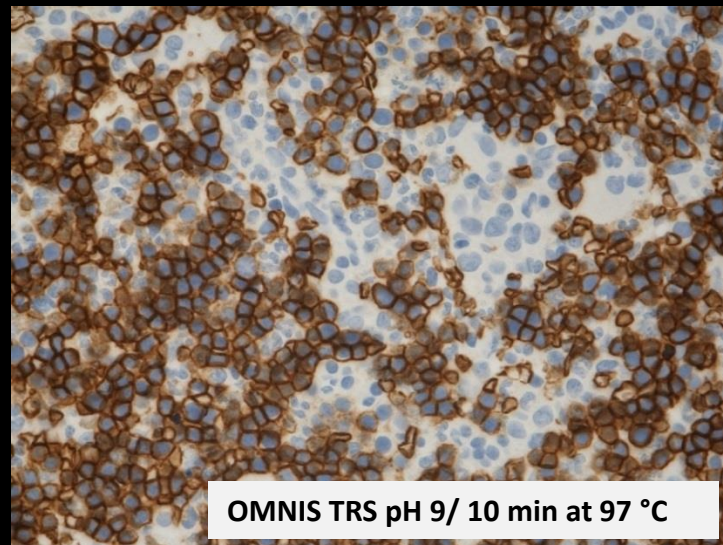
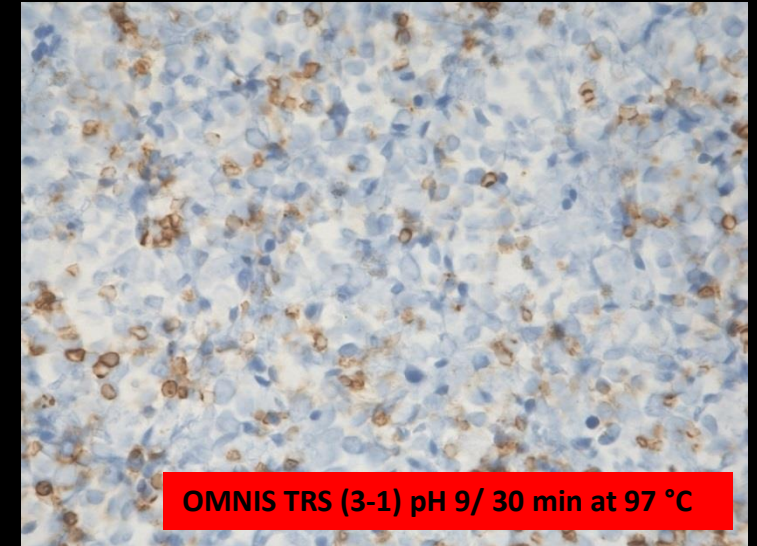
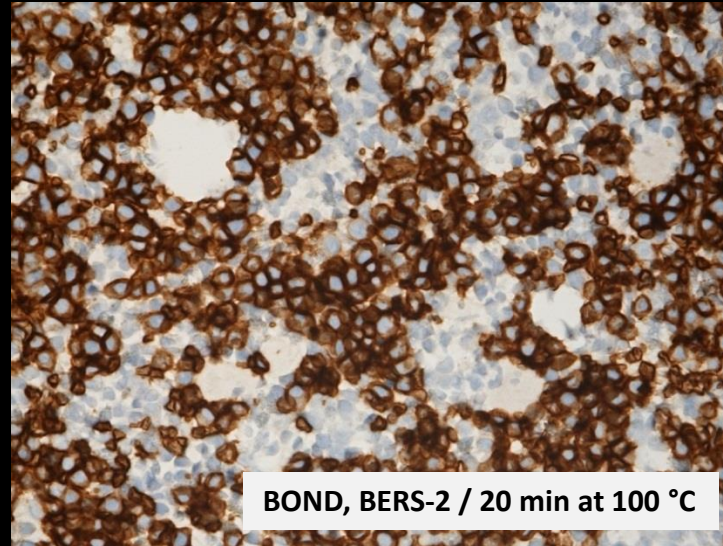
Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Bone marrow clot (NBF 24h)

Morphology ?

Glycophorin A clone JC159 (1:500)

Flex+



Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Bone Marrow cloth (NBF 24 h)

Morphology ?

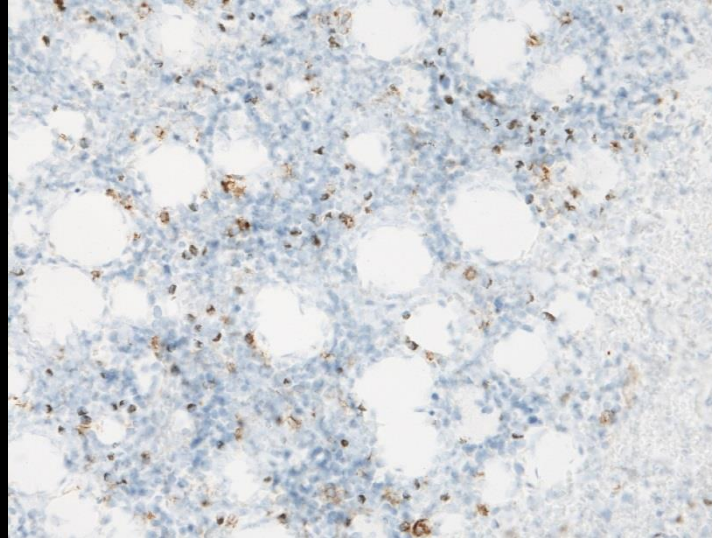
TRS (3-1) High pH 9, 24` at 97C,

Agilent/Dako (Omnis, Flex+)

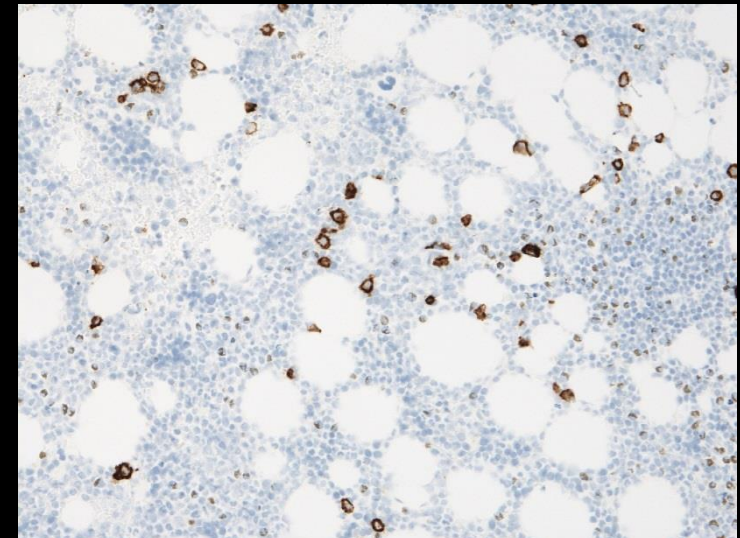
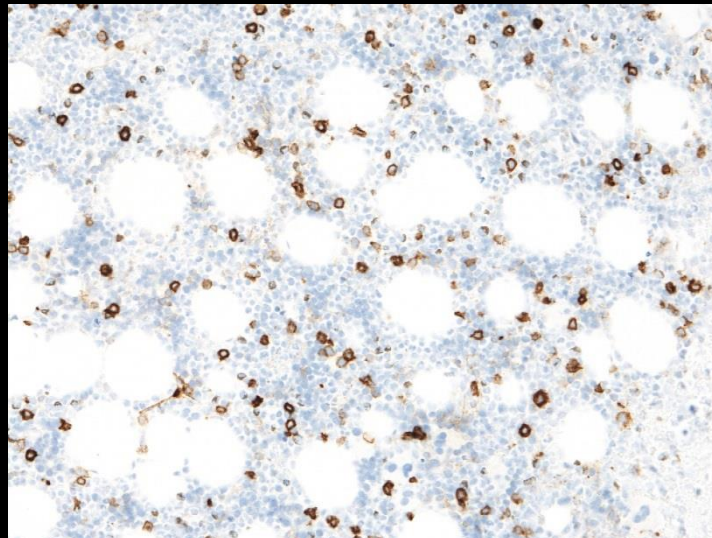
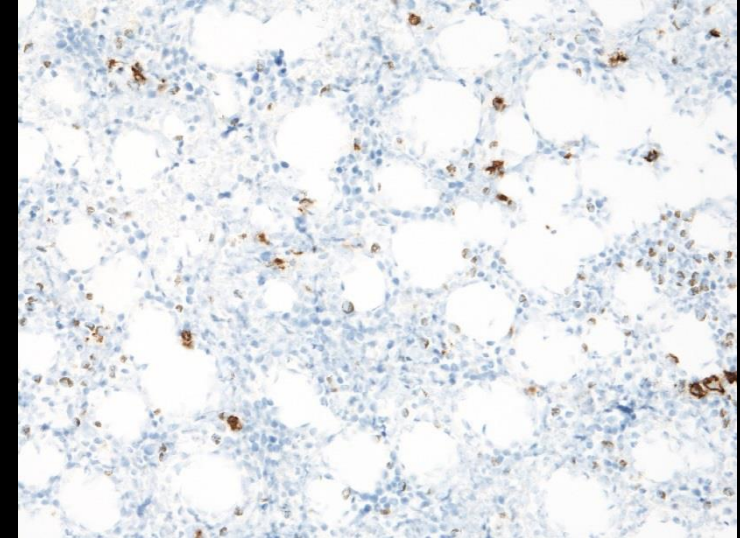
HIER buffer H, 24` at 97C

Thermo S./ LabVision (Omnis, Flex+)

CD117, EP10 (1:25 RR)



CD138, B-A38 (1:1000)



Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Bone marrow clot

AS: PT-Link, High pH buffer's at 97°C / 20`

Morphology ?

High pH (3-1) (Dako)

Recommended settings:

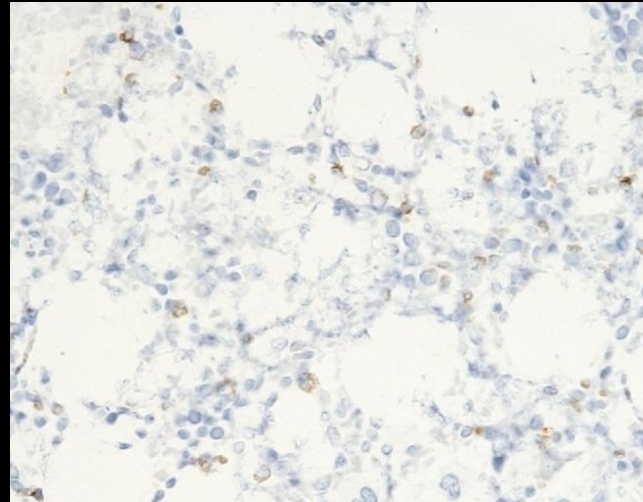
65°C

HIER buffer H (LabVision)

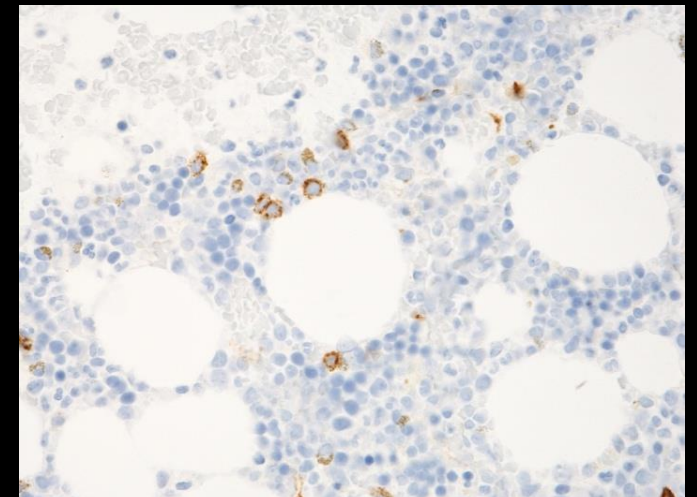
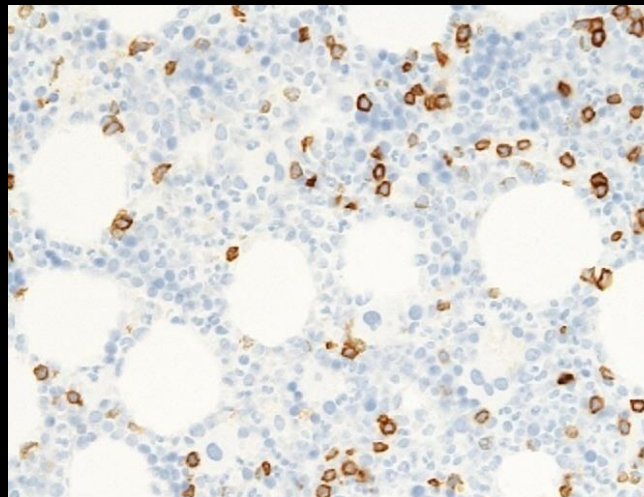
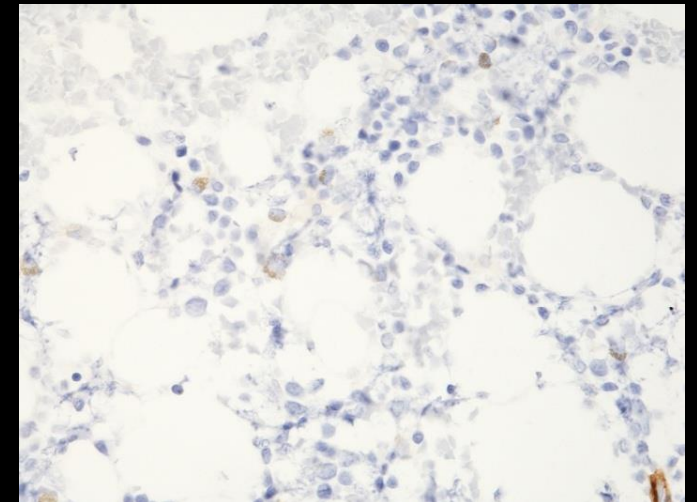
Recommended settings:

85°C

CD5 clone SP19



CD34 clone QBEND-10



BoneSTATION

Advanced system for fixation and decalcification of bone tissues

A new and innovative workstation for complete control of the all important pre-analytical step.

Provides accurate, reliable diagnostic results for morphology and molecular studies.

The BoneSTATION consists of two work platforms complete with glass modules for fixation/ decalcification.

The front platform (for the decalcification step) features a heating plate with infrared sensors for automatic temperature control up to 50°C and magnetic stirring. The rear platform (for the fixation step) has built-in magnetic stirring only.

Two user-friendly work platforms, for easy handling of bone specimens, complete the unit. The BoneSTATION station can be used with ANY type of fixative/decalcifier, even with strong mineral acids (HCl - HNO₃), as all contact surfaces are either glass or PTFE polymer. The magnetic stirrer assures homogeneity of temperature throughout the solution.

The PTFE cover condenses vapors, generating a reflux of the reagent for consistent and safe protocols at constant pH.

A touch-screen terminal allows the user to optimize, standardize and fully document all the processes.



At arrival , Fresh fixative is added to the bone marrow specimens and a post fixation step for 24-48h at 42°C has improved morphology.

However, the pre-analytic conditions is suboptimal but difficult to change in our hospital

Pause

Antibody Corporation (Anonymized) - Citation:

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.

Proficiency testing in immunohistochemistry—experiences from Nordic Immunohistochemical Quality Control (NordiQC)

Mogens Vyberg^{1,2} · Søren Nielsen¹

Virchows Arch (2016) 468:19–29

Table 3 Major causes of insufficient staining reactions

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- 5 Other (17 %)
 - a. Heat-induced impaired morphology
 - b. Proteolysis induced impaired morphology
 - c. Drying out phenomena
 - d. Stainer platform-dependant protocol issues
 - e. Excessive counterstaining impairing interpretation

37% insuff.

^a Consistently gives false negative or false positive staining or a poor signal-to-noise ratio in one or more assessment runs

^b Frequently giving inferior staining results, e.g., due to mouse-anti-Golgi reactions or sensitive to standard operations as blocking of endogenous peroxidase

^c Biotin-based detection kit for cytoplasmic epitopes, use of detection kits providing a too low sensitivity, or use of detection kits and chromogens giving imprecise localization of the staining signals complicating the interpretation

Problems related to the choice and/or use of the primary antibody:

- **Inappropriate primary antibody**
 - Provide low sensitivity/specificity
- **Appropriate primary antibody**
 - Inapp. titre (too low or too high concentration)
- **Platform (stainer) dependent antibodies**
 - Provide low sensitivity / specificity

False positive or false negative results

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

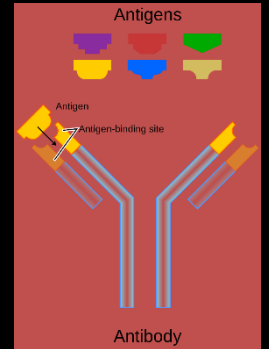
Parameters affecting antibody-antigen reactions in tissue

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

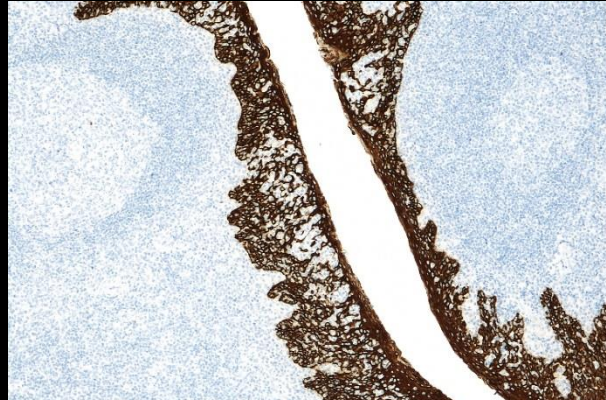
Provided that efficient antigen retrieval has been performed and a sensitive detection system has been used

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

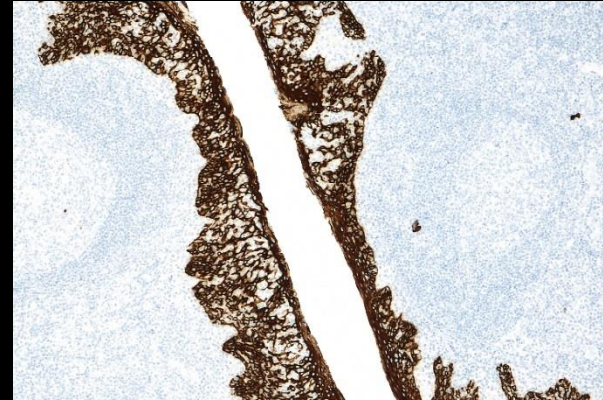
Antibody choice: Sensitivity & Specificity

Tonsil

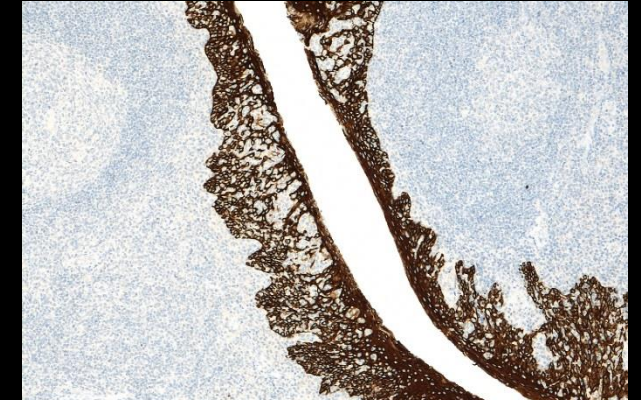
CK5 clone BSR55 (1:200)



CK5 clone XM26 (1:200)

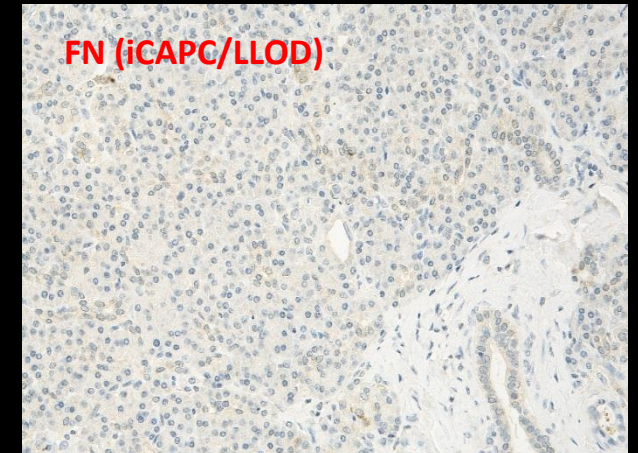
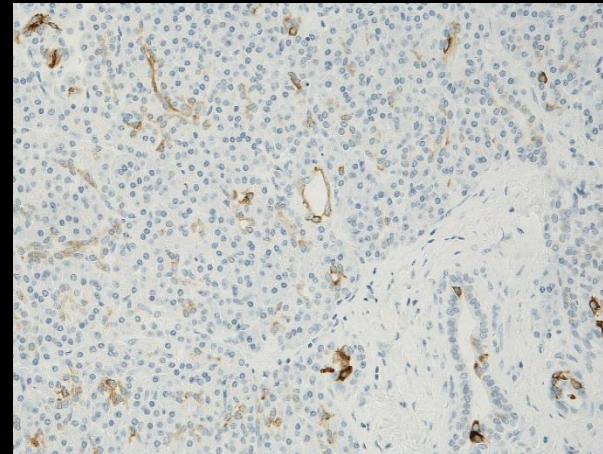
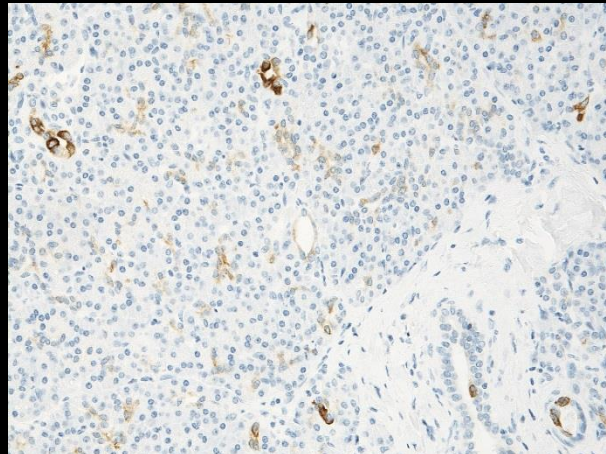


CK5 clone BS42 (1:200)



Standardization of Positive Controls in Diagnostic Immunohistochemistry: Recommendations From the International Ad Hoc Expert Committee: Appl Immunohistochem Mol Morphol 2015;23:1–18/ immunohistochemistry critical assay performance control (iCAPC)

Pancreas



FN (iCAPC/LLOD)

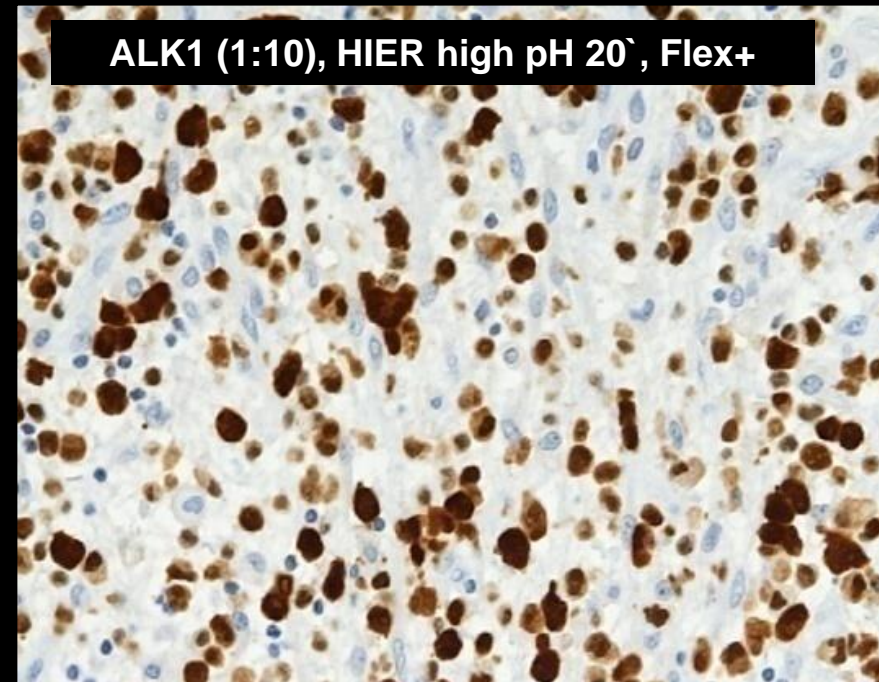
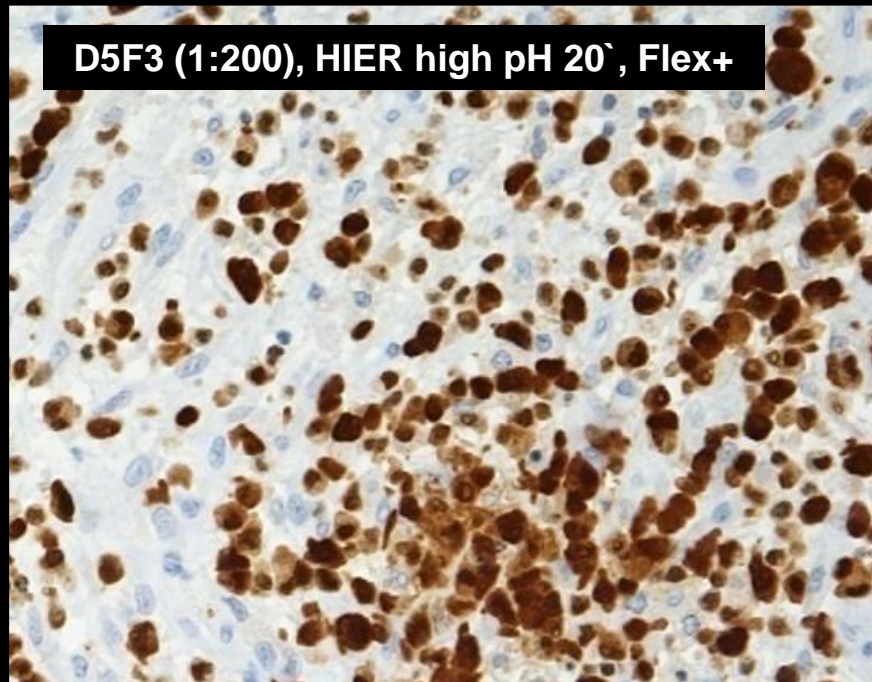
immunohistochemistry critical
assay performance control
(iCAPC)

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

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

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



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 lu-ALK, run 45

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff. ¹	Suff. OPS ²
mAb clone 5A4	46 3 2 1	Leica/Novocastra Thermo/NeoMarkers Monosan Abcam	24	16	13	1	74%	81%

Table 1. Antibodies and assessment marks for lu-ALK, run 51

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff. ¹	Suff. OPS ²
mAb clone 5A4	43 1 1 1	Leica/Novocastra Abcam Biocare Monosan	1	15	24	7	34%	22%

Table 1. Antibodies and assessment marks for lu-ALK, run 57

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff. ¹	Suff. OPS ²
mAb clone 5A4	27 2 1 1	Leica/Novocastra Monosan Abcam DBS	8	18	4	2	81%	89%

Don't use clone ALK1 to detect ALK rearranged lung adenocarcinomas

It does not "fit-for-purpose"

D5F3, OTI1A4, 5A4

IR041								
mAb clone ALK1 790/800-2918	10	Ventana	0	1	6	3	10%	-
mAb clone ALK1 204M-18	1	Cell Marque	0	0	0	1	-	-
mAb clone ALK1 CA641	1	Dako	0	0	0	1	-	-
rmAb clone D5F3 790-4794	47	Ventana	41	4	2	0	96%	96%
rmAb clone D5F3 790-4843 (CDx assay)	4	Ventana	3	0	1	0	-	-
Unknown	1	Unknown	1	0	0	0	-	-
Total	176		93	25	33	25	-	-
Proportion			53%	14%	19%	14%	67%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of sufficient stains with optimal protocol settings only, see below.

GA641								
mAb clone ALK1 790/800-2918	7	Ventana	0	0	2	5	-	-
rmAb clone SP8 AN770	1	BioGenex	0	0	0	1	-	-
rmAb clone D5F3 790-4796	70	Ventana	53	12	4	1	93%	100%
rmAb clone D5F3 790-4796³	2	Ventana	1	0	1	0	-	-
mAb clone OTI1A4 8344-C010	1	Sakura Finetek	1	0	0	0	-	-
Total	189		72	43	43	31	-	-
Proportion			38%	23%	23%	16%	61%	

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 Ventana BenchMark systems (Ultra/XT) but used by laboratories on different platforms (e.g. Dako Autostainer)

mAb clone MX064 MAB-0848	1	Maixin	0	1	0	0	-	-
mAb clone OTI1A4 / 1A4 8344-C010	1	Sakura Finetek	0	1	0	0	-	-
rmAb clone D5F3 790-4794 / 790-4796	95	Ventana	76	12	4	3	93%	
rmAb clone D5F3 790-4794³	1	Ventana	0	0	1	0	-	-
rmAb clone SP144 AN874-5M(10M)	1	BioGenex	0	0	0	1	-	-
Total	201		118	50	18	15	-	-
Proportion			59%	25%	9%	7%	84%	

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 Ventana BenchMark systems (Ultra/XT) but used by laboratories on different platforms (e.g. Dako Autostainer)

RUN 45, 51 and 57

66 protocols were based on clone ALK1:

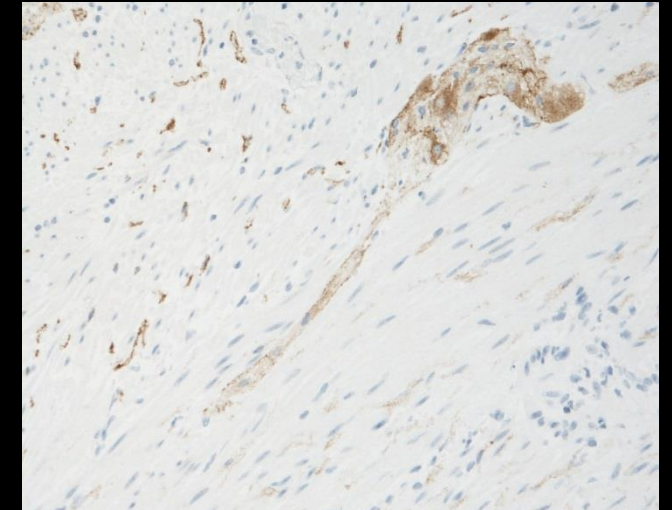
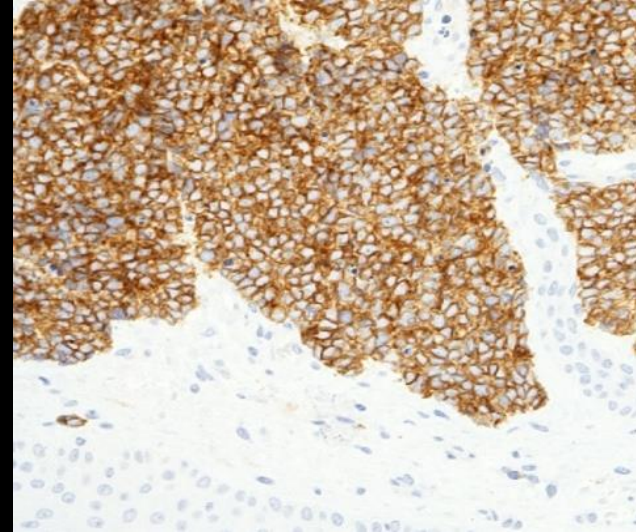
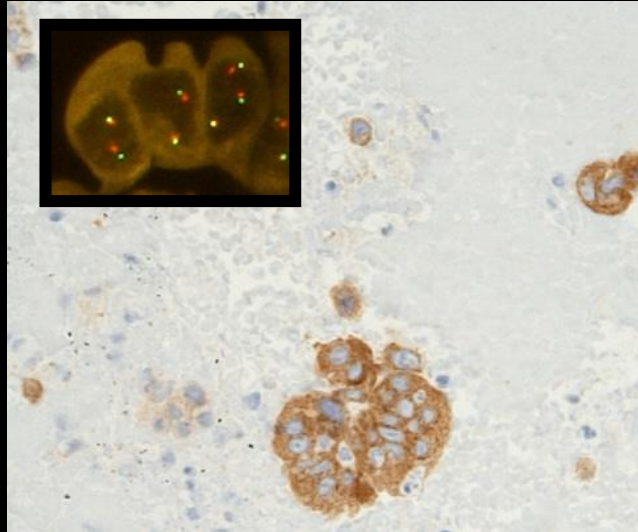
Only one protocol were assessed as sufficient, none were optimal

Lung AC (ALK-EML4)

MCC (Skin)

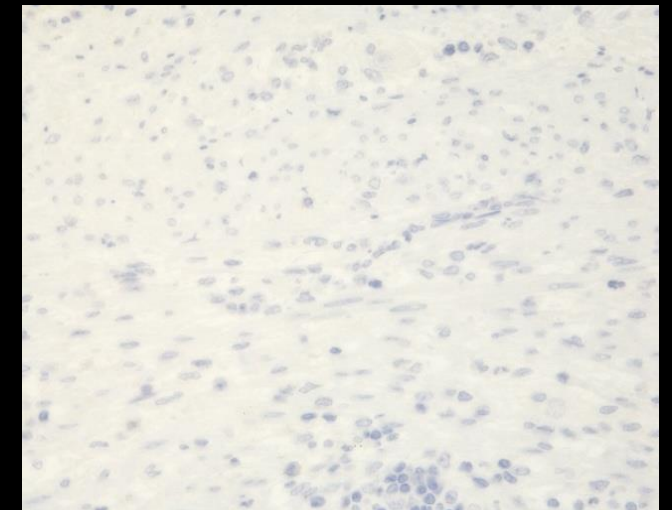
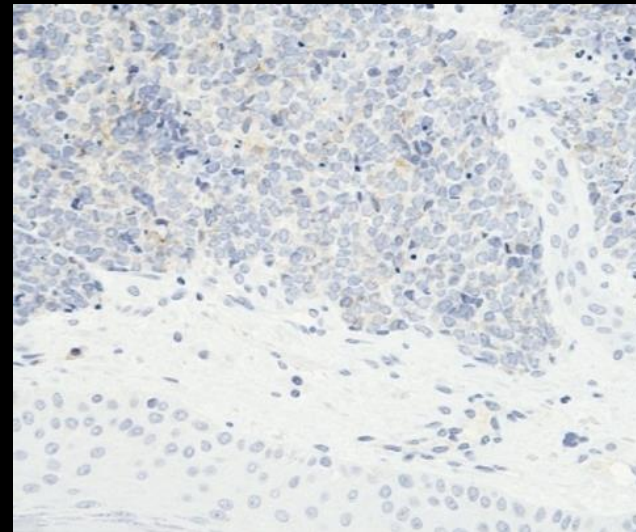
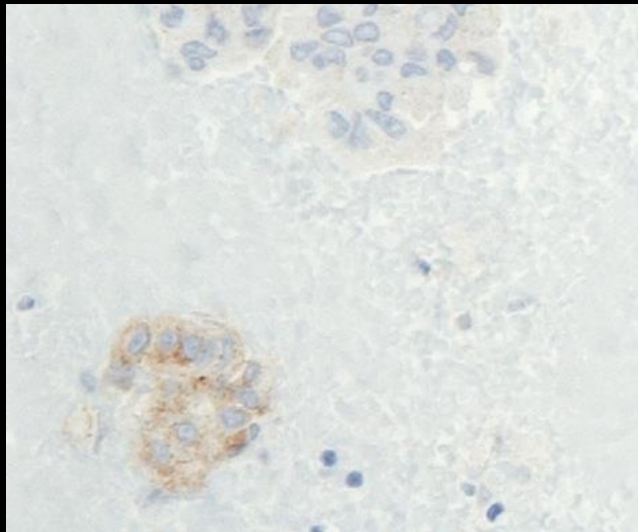
Appendix

ALK, D5F3 (1:200)



Ganglion and peripheral nerve cells

ALK, ALK1 (1:10)



Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Primary antibody provides low sensitivity

CK8/18

Table 1. Antibodies and assessment marks for CK8/18, run 57

Concentrated antibodies	reactivity	n	Vendor	Optimal	Good	Borderline	Poor	Suff. ¹	Suff. OPS ²
mAb clone 34betaH11	CK8	1	Diagnostic BioSystems	0	0	1	0	-	-
mAb clone 5D3	CK8/18	16	Leica/Novocastra	5	6	4	10	44%	-
		3	Diagnostic BioSystems						
		2	Biocare						
		2	Thermo Scientific						
		1	DCS						
		1	Monosan						
mAb clones B22.1/B23.1	CK8/18	11	Cell Marque	8	3	2	1	79%	92%
		1	Bio SB						
		1	Immunologic						
		1	Menarini						
mAb clone C51	CK18	1	Zyomed	0	0	0	1	-	-
mAb clone CAM5.2	CK8(7)	3	Zytomed	0	1	2	0	-	-
mAb clone CY90	CK18	1	Nordic Mubio	0	0	1	1	-	-
		1	Sigma						
mAb clone DC10	CK18	11	Agilent/Dako	0	0	9	7	0%	-
		3	Thermo Scientific						
		1	Biocare						
		1	Immunologic						
mAb clone TS1	CK8	2	Thermo Scientific	0	2	1	0	-	-
		1	Leica/Novocastra						
mAb clone TS1+DC10 ("homebrew")	CK8/18	1	Thermo + Dako	1	0	0	0	-	-
rmAb clone BSR15	CK8	1	Nordic Biosite	1	0	0	0	-	-
rmAb clone EP17	CK8	4	Epitomics	4	0	1	0	-	-
		1	Cell Marque						
rmAb clones EP17/EP30	CK8/18	9	Dako/Agilent	9	1	0	0	100%	-
		1	Cell Marque						
Ready-To-Use antibodies									
mAb clone DC10	CK18	5	Agilent/Dako	0	1	3	1	-	
mAb clone IR618/IS618									
mAb clone DC10	CK18	9	Agilent/Dako	0	2	6	1	-	
mAb clone GA618									

The problem: Abs raised exclusively against CK18 may show partial or complete loss of CK18 in 25% of breast carcinomas depending on their histological type (included in this run 57).

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	BioCare Medical	13	10	3	1	85%	37%
mAb AU-1	URO III	2	Cell Marque	-	-	-	2	-	-
rmAb SP73	URO III	7	Cell Marque	-	-	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									
mAb BC21	URO II	1	Biocare Medical	-	1	-	-	-	-
mAb AVI 3051 KG		1	Biocare Medical	-	1	-	-	-	-
mAb BC21		1	Biocare Medical	-	1	-	-	-	-
mAb API 3051 AA		1	Vitro SA	1	-	-	-	-	-
mAb BC21		1	Zytomed Systems	-	1	-	-	-	-
mAb MSG102	URO II/III	3	Biocare Medical	-	2	1	-	-	-
mAb BC21+BC17		3	Biocare Medical	-	2	1	-	-	-
mAb SP73	URO III	1	Roche/Ventana	-	-	1	-	-	-
mAb 760-4533 (VRPS)³		16	Roche/Ventana	-	-	2	14	0%	0%
mAb SP73		1	Cell Marque	-	-	-	1	-	-
mAb 760-4533 (LMPS)⁴	URO III	1	Cell Marque	-	-	-	1	-	-
mAb SP73		1	Cell Marque	-	-	-	1	-	-
mAb 345R-17/18	URO III	1	Cell Marque	-	-	-	1	-	-
mAb SP73		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).

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

NordiQC
CK 8/18

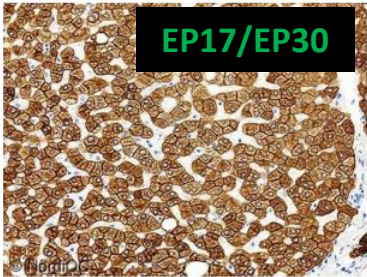


Fig. 3a
Optimal CK8/18 staining of the liver using the mAb clone cocktail EP17/EP30 as a concentrate in an optimally calibrated LD assay, performed on the Dako Omnis. The vast majority of hepatocytes show a distinct, moderate staining reaction with a membrane enhancement. Compare with Figs. 4a – 6a, same protocol.

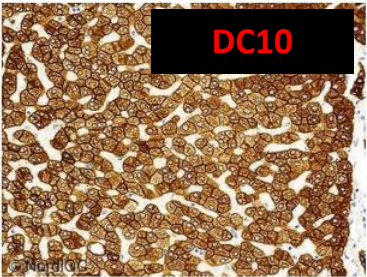


Fig. 3b
Optimal CK8/18 staining of the liver using an insufficient protocol based on the mAb clone DC10. Clone DC10 reacts with CK18 and in this (DC10) CK18-protocol the vast majority of hepatocytes show a distinct, moderate staining reaction with a membrane enhancement. Compare with Figs. 4b – 6b, same protocol. Compare also with Fig. 3a – same field.

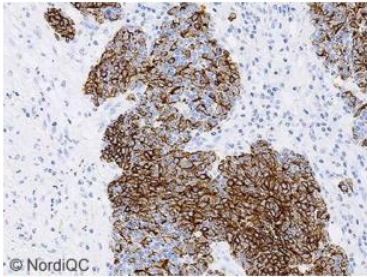


Fig. 4a
Optimal CK8/18 staining of the SCLC using same protocol as in Fig. 3a. The majority of neoplastic cells show a weak to strong and distinct staining reaction. A dot-like cytoplasmic staining reaction is observed in the weakly positive neoplastic cells. Compare with Figs. 3b, 5b and 6b, same protocol. Compare also with Fig. 4a – same field.

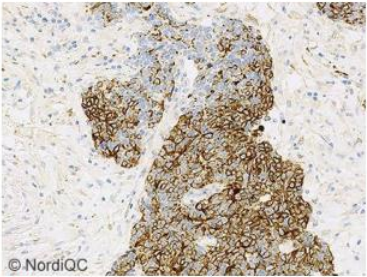


Fig. 4b
Optimal CK8/18 staining of the SCLC using the same DC10-protocol as in Fig. 3b. The majority of neoplastic cells show a weak to strong and distinct staining reaction. A dot-like cytoplasmic staining reaction is observed in the weakly positive neoplastic cells. Compare with Figs. 3b, 5b and 6b, same protocol. Compare also with Fig. 4a – same field.

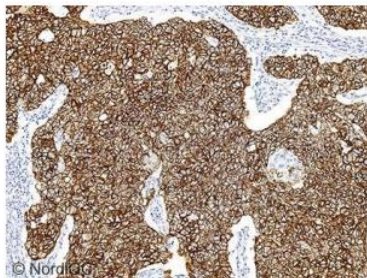


Fig. 5a
Optimal CK8/18 staining of the breast ductal carcinoma using same protocol as in Figs. 3a - 4a. Virtually all neoplastic cells show a strong and distinct cytoplasmic staining reaction. The use of a cocktail of CK8 and CK18 antibodies secures optimal staining reaction despite the apparent loss of CK18 in the tumour. Compare with Fig. 5b – same field.

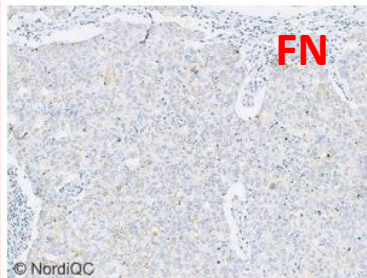


Fig. 5b
Insufficient CK8/18 staining of the breast ductal carcinoma using same protocol as in Figs. 3b – 4b. Despite producing optimal staining in liver (and the SCLC), virtually all neoplastic cells are unstained. This tumour belongs to the approx. 25% of breast carcinomas that shows partial or complete loss of CK18 expression, making CK18 antibodies like clone DC10 less suitable identifying non-squamous carcinomas including adenocarcinoma of unknown origin. Compare with Fig. 5a – same field.

NordiQC
URO II/III

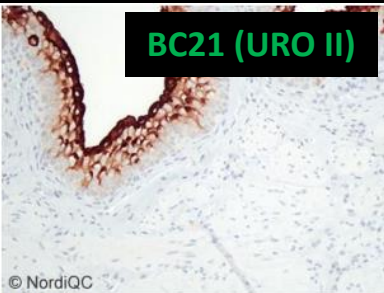


Fig. 1a (x200)
Optimal Uroplakin II/III staining of the urethra using the mAb clone BC21 - diluted, 1:25 (30 min. incubation), epitope retrieval using HIER in TRS High (25 min.) and a 3-step multimer based detection system (EnVision Flex+) performed on Autostainer (Dako). Virtually all umbrella cells display a strong, membranous and cytoplasmic staining reaction and the vast majority of intermediate urothelial cells show a weak to moderate predominantly apical but also cytoplasmic staining reaction. Same protocol used in Figs. 2a-4a.



Fig. 1a (x200)
Insufficient Uroplakin II/III staining of the Urethra using the mAb clone SP73, diluted, 1:100 (32 min. incubation), epitope retrieval using HIER in CC1 (32 min.) and a 3-step multimer based detection system (OptiView with amplification) performed on BenchMark (Ventana). The urothelium displays a strong, plaque-like membranous, predominantly apical staining reaction in all umbrella cells but no cytoplasmic or membranous staining of the intermediate urothelial cells. Same protocol used in Figs. 2b-4b.

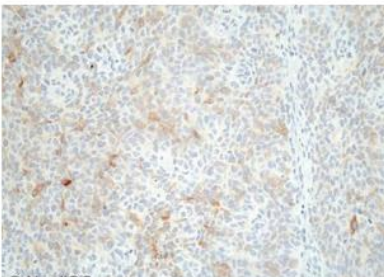


Fig. 2a (x200)
Optimal Uroplakin II/III staining of the urothelial carcinoma tissue core no 2 using same protocol as in Fig. 1a. The majority of the neoplastic cells showing a weak to moderate cytoplasmic and membranous staining reaction.

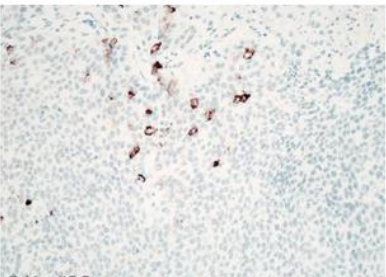


Fig. 2b (x200)
Insufficient Uroplakin II/III staining of the urothelial carcinoma tissue core no 2 using same protocol as in Fig. 1b. The vast majority of neoplastic cells are almost completely negative and only a few scattered cells display a strong staining reaction.

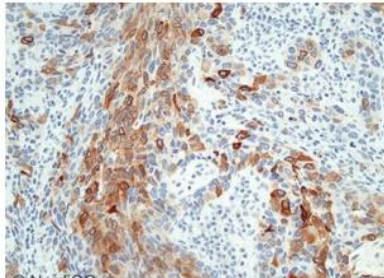


Fig. 3a (x200)
Optimal Uroplakin II/III staining of the urothelial carcinoma tissue core no 3 using same protocol as in Figs. 1a and 2a. The vast majority of the neoplastic cells display a moderate to strong cytoplasmic and membranous staining reaction.

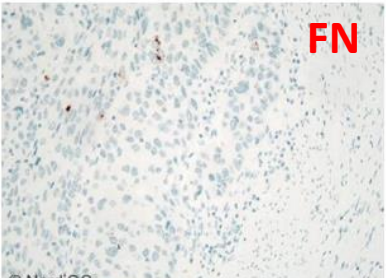


Fig. 3b (x200)
Insufficient Uroplakin II/III staining of the urothelial carcinoma tissue core no 3 using same protocol as in Figs. 1b and 2b. The neoplastic cells are negative and only a dubious dot-like reaction are seen in a few cells.

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

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

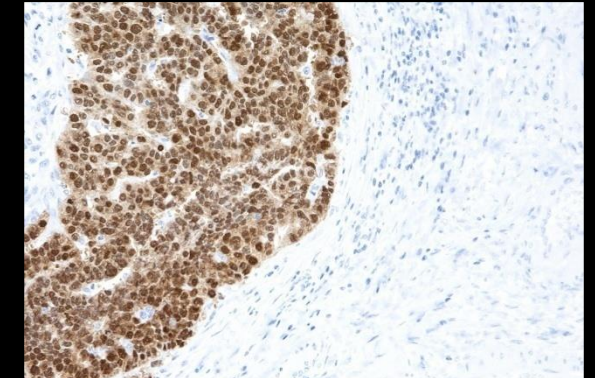
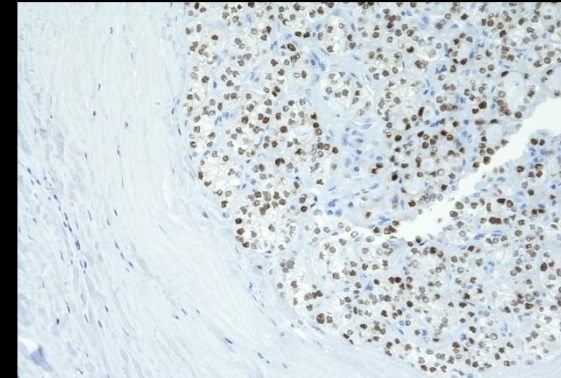
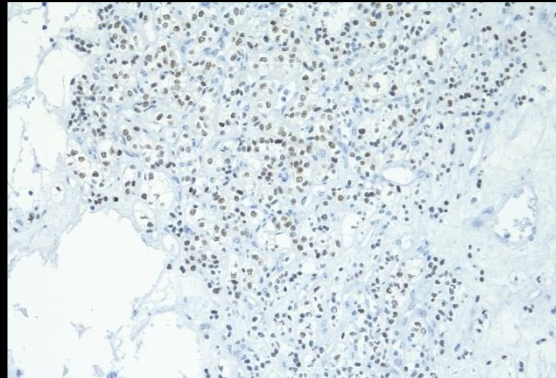
Which antibody ?

Renal Cell Carcinoma (CC)

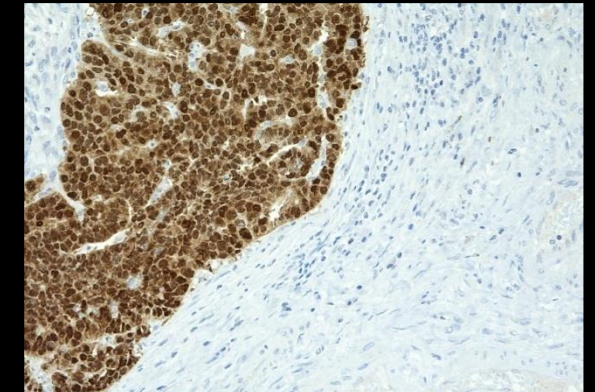
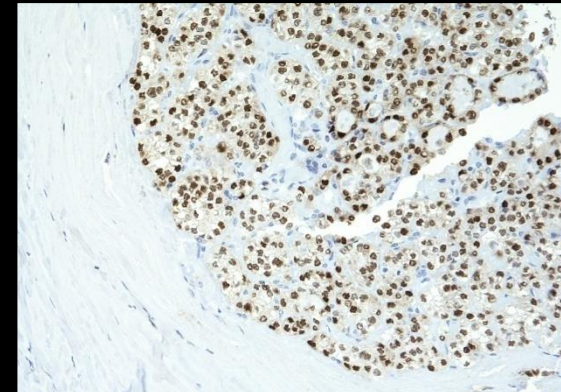
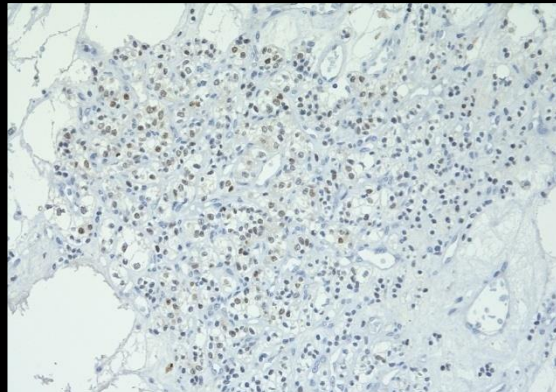
Thyroid Carcinoma (Pa)

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 ratio

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)

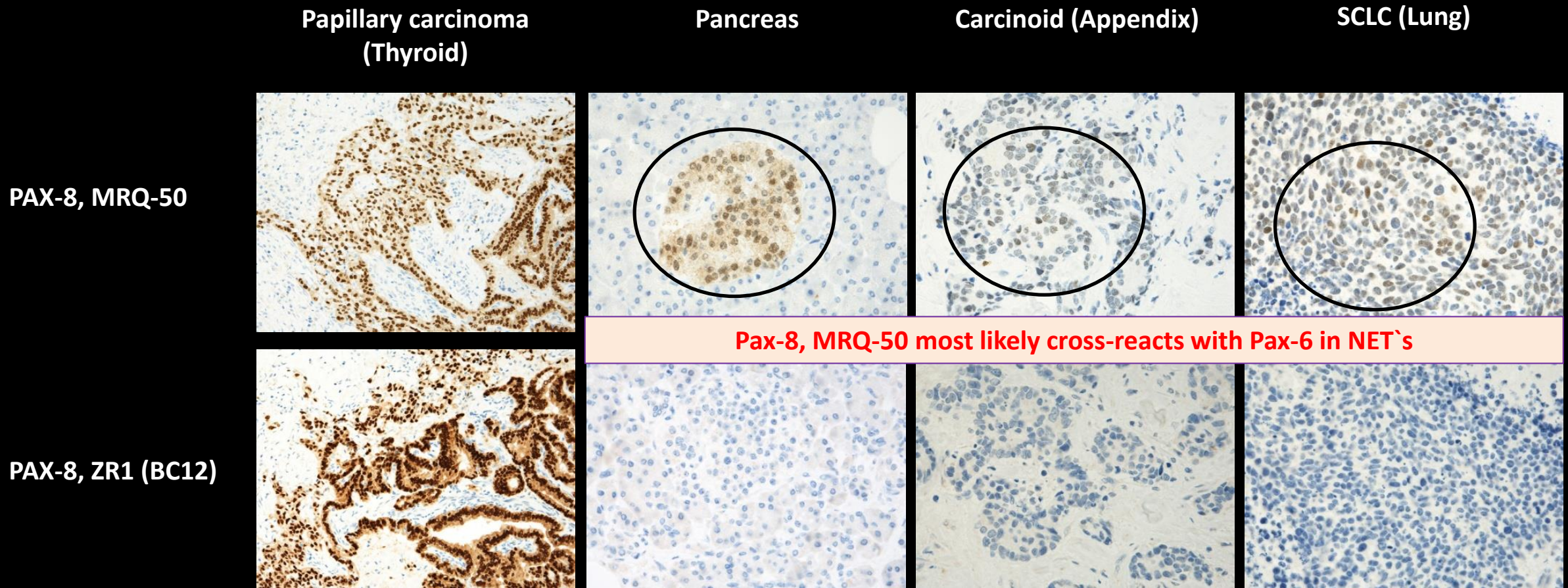
Moretti L et al. : Mod Pathol. 2012; 25 : 231-236

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 ratio



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)

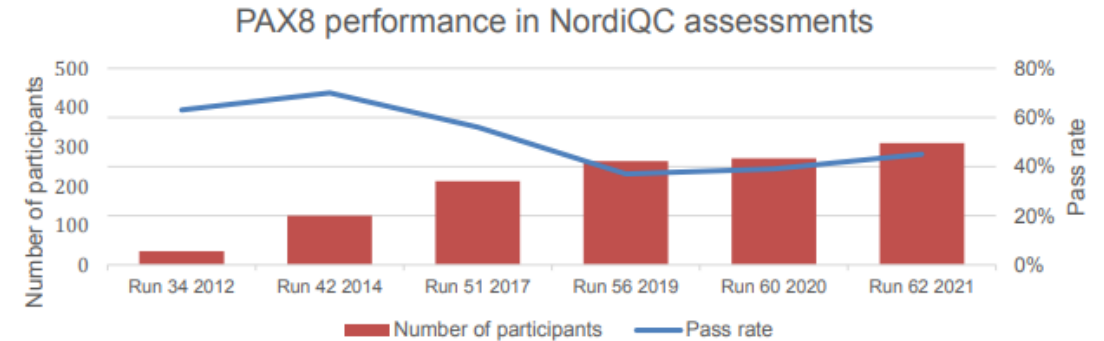
Technical aspects of immunohistochemistry & pitfalls - Analytical phase

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

Table 1. Antibodies and assessment marks for PAX8, run 62

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff. ¹	OR ²
mAb clone BC12*	4	Biocare	-	3	1	2	50%	-
	1	Zytomed Systems	-					
	1	Diagnostic Biosystems	-					
mAb clone MRQ-50	34	Cell Marque	-	18	8	8	55%	-
mAb clone PAX8R1	2	Abcam	-	2	-	-	-	-
rmAb clone EP298*	7	Cell Marque	-					
	4	Epitomics ⁵	4	4	6	1	53%	27%
	3	BIO SB						
	1	Nordic Biosite						
rmAb clone EP331*	8	Cell Marque	-	5	7	1	39%	-
	4	Epitomics						
	1	Abcam						
rmAb clone SP348*	55	Abcam	47	10	1	2	95%	78%
	5	Gennova						
rmAb clone ZR-1*	5	Zeta Corporation	-					
	1	Abcam	3	1	3	-	57%	43%
	1	Bio SB						
pAb, 10336-1-AP	21	Proteintech	-	8	9	4	38%	-
pAb, 363A-15	1	Cell Marque	-	1	-	-	-	-
pAb, CP379	6	Biocare	-	1	4	1	-	-
Ab QR016*	2	Quartett	-	1	-	1	-	-
Unknown	2		-	-	2	-	-	-

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



Conclusion

Optimal staining results could be obtained with the rmAb clones **EP298**, **SP348**, **ZR-1**, **BC12**, **IHC048** 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 rmAbs clones **EP298**, **SP348** and **ZR-1** 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. In contrast, 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.

mAb clone MRQ-50/pAbs cross react with other Pax proteins in the family (e.g. PAX5):

Should we use primary antibodies that cross react with other proteins in the same family ?

Would we accept cross-reactivity in the family of CD's and CK's - e.g., CD20 to CD3 or CK5 to CK8 ?

BC12 (platform dependent)

"ZR1" (lot variations/antibody diluent dependent)

EP298

SP348

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

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

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	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%	

FP/FN

FP/FN

FN

FP/FN + aberrant cytoplasmic staining

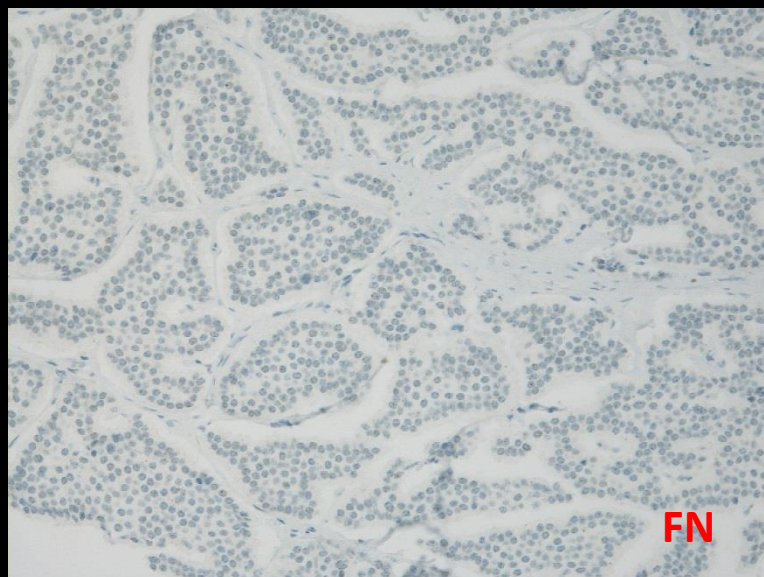
FN + aberrant cytoplasmic staining

Impossible to calibrate correctly

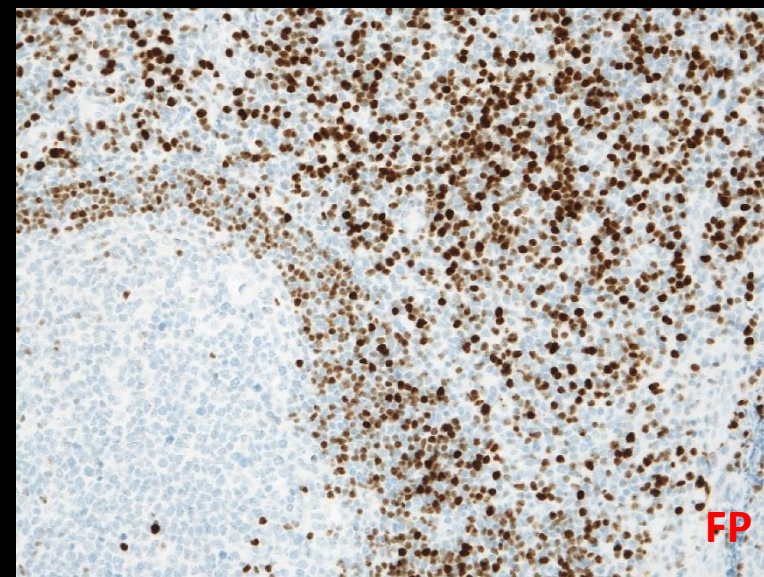
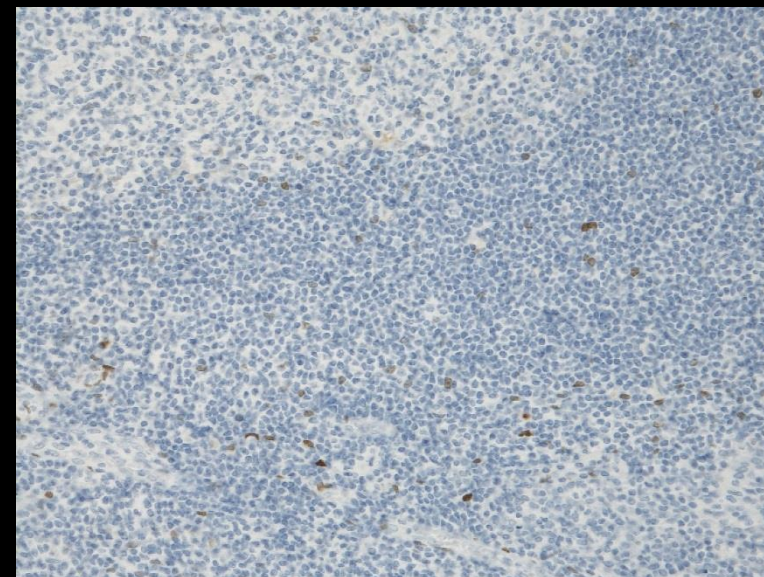
Appendix



Neuroendocrine tumour (colon)



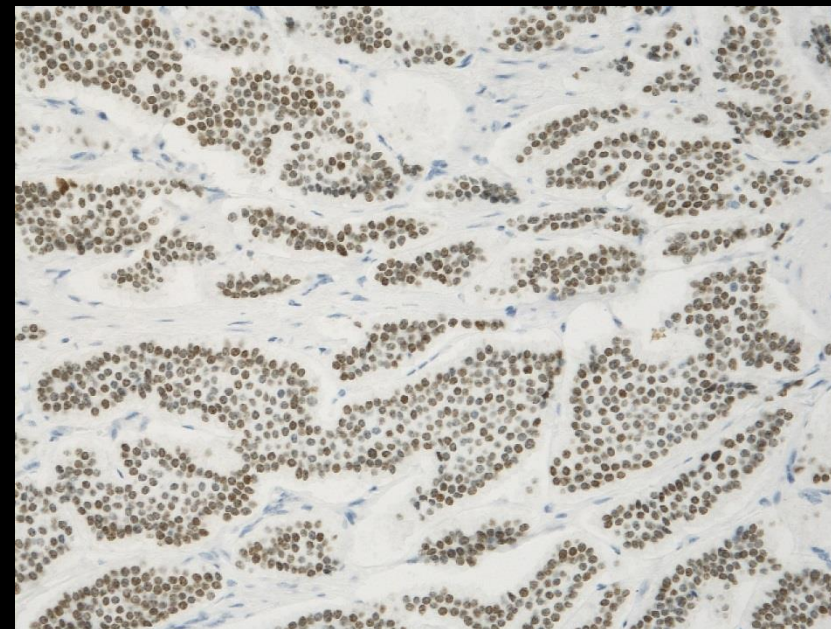
Tonsil



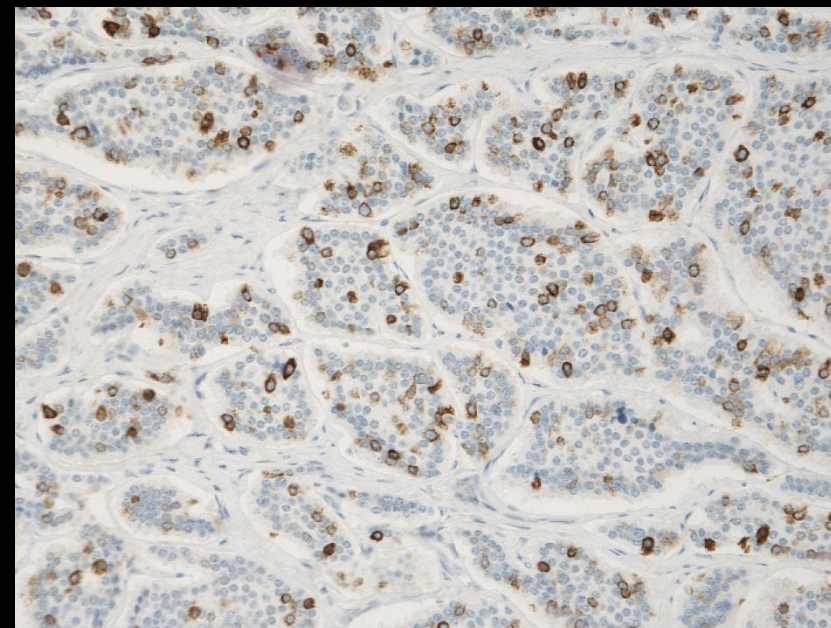
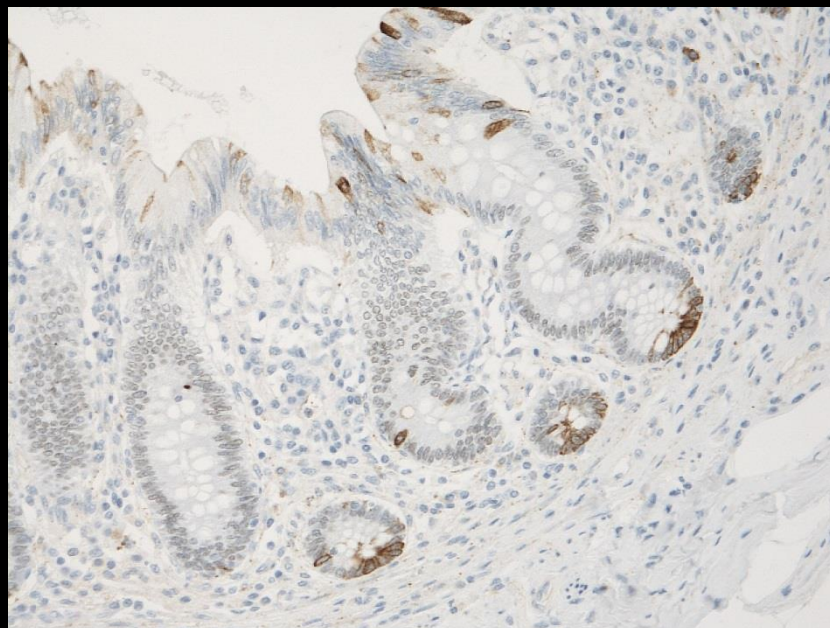
Appendix

Neuroendocrine tumour (colon)

SATB2, EP281



SATB2, Pab 69995



Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Antibody choice: Sensitivity & Specificity

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
CEA clone II-7	MLH1 clone M1 & G168-728
CGA clone DAK-A3	Many pAbs (e.g., P40 and SOX10)
P63 clone 7JUL
.....	

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Primary antibody poorly calibrated providing low sensitivity

The right primary antibody

The right protocol (AR procedure and detection system)

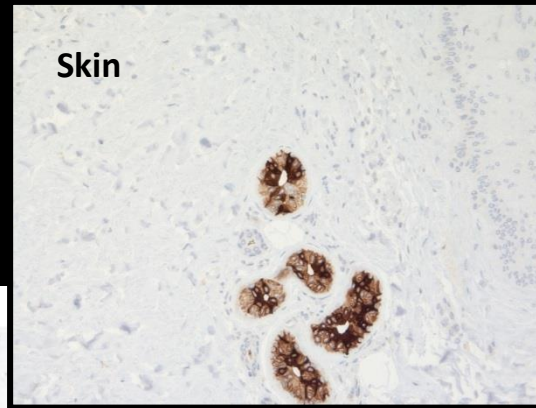
Poorly calibrated primary Ab ?

Tissue controls are the key element

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)

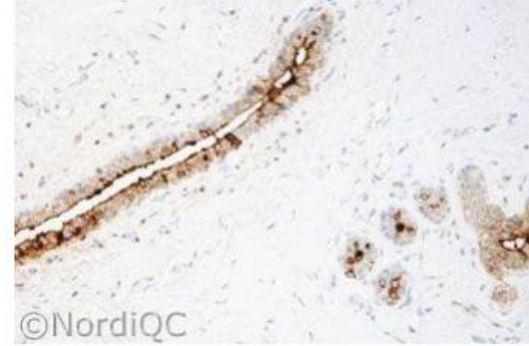


Fig. 1a
Optimal staining for GCDFP-15 of the breast hyperplasia using the mAb clone 23A3 optimally calibrated as a concentrate, HIER in an alkaline buffer and a polymer based detection system. The majority of the ductal epithelia cells show a distinct moderate to strong cytoplasmic staining reaction.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

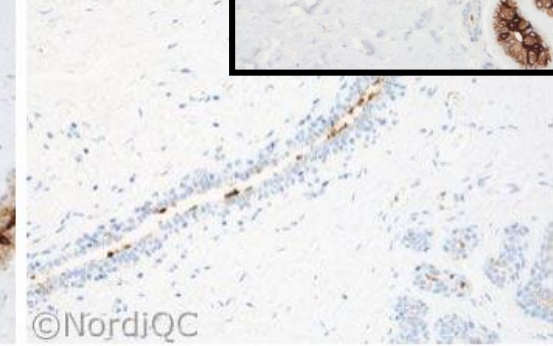


Fig. 1b
Insufficient staining for GCDFP-15 of the breast hyperplasia applying the mAb clone 23A3 as a concentrate using exactly the same protocol settings as used in Fig 1a, except for a 20 fold dilution of the primary antibody. The proportion and the intensity of the cells demonstrated are significantly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

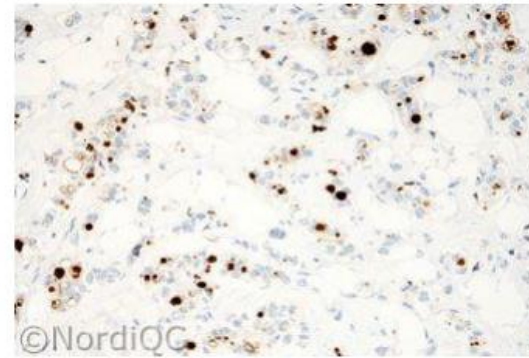


Fig. 2a
Optimal staining for GCDFP-15 of the breast carcinoma no. 5 using same protocol as in Fig. 1a. The majority of the neoplastic cells show a moderate to strong dot-like cytoplasmic staining reaction.

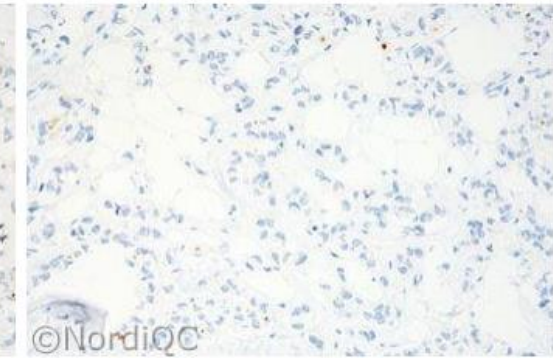
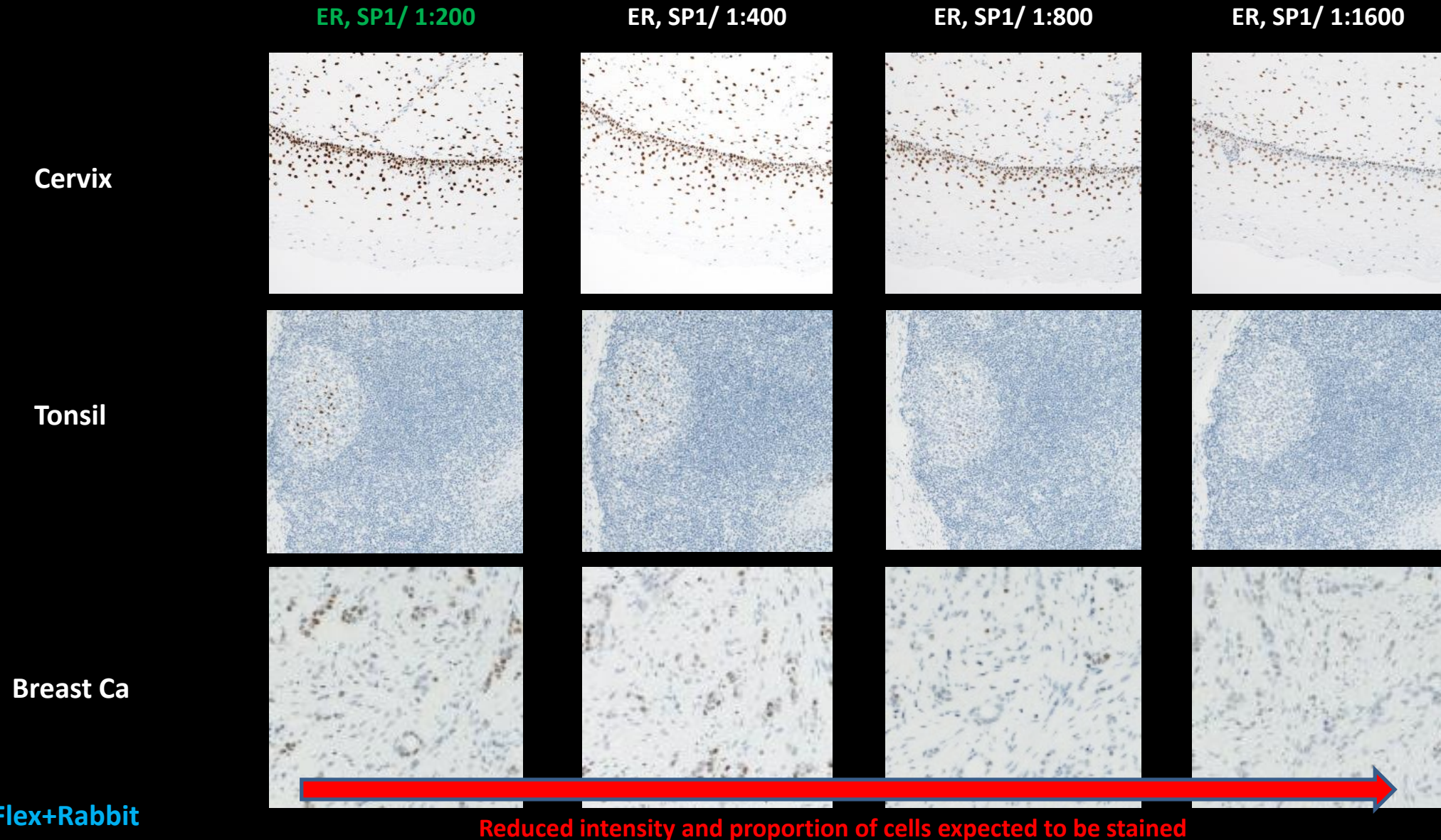


Fig. 2b
Insufficient staining GCDFP-15 of the breast carcinoma no. 5 using same protocol as in Fig. 1b. - same field as in Fig. 2a. Only scattered neoplastic cells show a faint dot-like reaction.

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Primary antibody poorly calibrated providing low sensitivity



Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Primary antibody poorly calibrated providing low sensitivity

The right primary antibody

The right protocol (AR procedure and detection system)

Poorly calibrated primary Ab ~ Inapp. titer

Tissue controls: iCAPS/CSQI

BCL6 clone GI191E/A8 (Run 55)

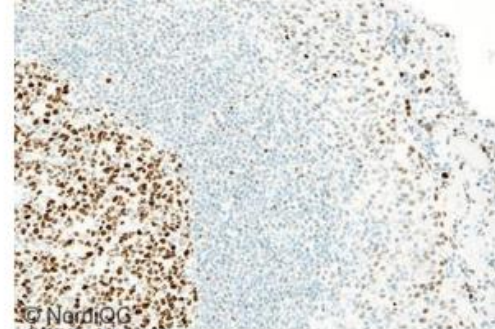


Fig. 1a (X200)
Optimal Bcl-6 staining of the tonsil using the mAb clone GI191E/A8 optimally calibrated, HI-ER in CC1 (32 min.) and a 3-step multimer based detection system (Optiview, Benchmark Ultra, Ventana). Virtually all germinal centre B-cells show a moderate to strong nuclear staining reaction and the nuclei of squamous epithelium display weak to moderate intensity – same protocol used in Figs. 2a-4a.

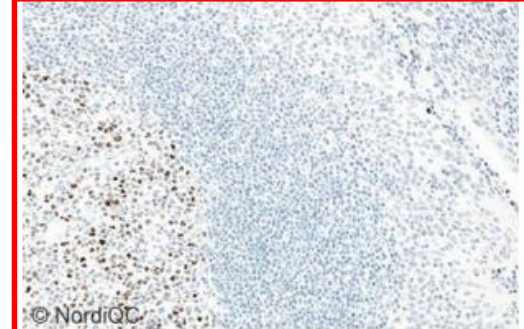


Fig. 1b (X200)
Insufficient Bcl-6 staining of the tonsil using the mAb clone GI191E/A8 HI-ER in CC1 (32 min.) and a 3-step multimer based detection system (Optiview + amplification, Benchmark Ultra, Ventana). The protocol provided too low sensitivity due to too low titre of the primary Ab (1:800) - same field as in Fig. 1a. The proportion of positive germinal centre B-cells is significantly reduced and virtually all nuclei of squamous epithelium are false negative – same protocol used in Figs. 2b-4b.

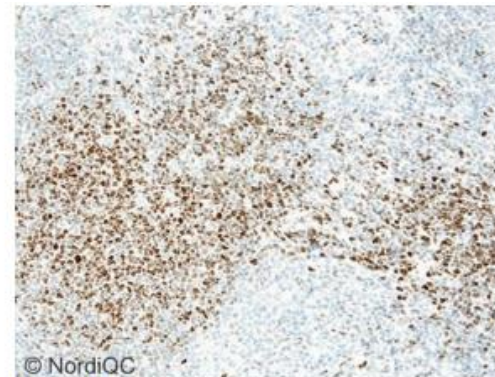


Fig. 3a (X200)
Optimal Bcl-6 staining of the FL (tissue core 4) using same protocol as in Figs. 1a and 2a. The majority of the neoplastic cells show a moderate to strong nuclear staining reaction.

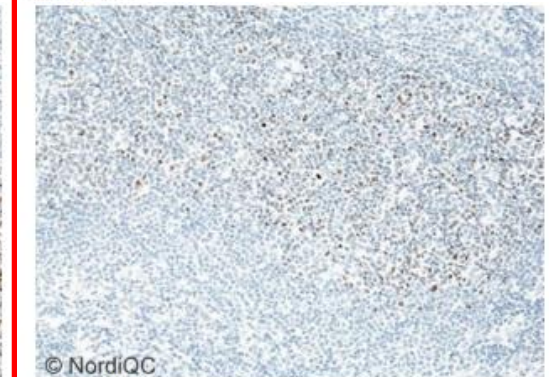


Fig. 3b (X200)
Insufficient Bcl-6 staining of the FL (tissue core 4) using same protocol as in Figs. 1b and 2b. The proportion and staining intensity of the neoplastic cells is significantly reduced compared to the level in Fig. 3a

Proficiency testing in immunohistochemistry—experiences from Nordic Immunohistochemical Quality Control (NordiQC)

Mogens Vyberg^{1,2} · Søren Nielsen¹

Virchows Arch (2016) 468:19–29

Table 3 Major causes of insufficient staining reactions

1. Less successful antibodies (17 %)
 - a. Poor antibodies^a
 - b. Less robust antibodies^b
 - c. Poorly calibrated RTUs
 - d. Stainer platform dependent antibodies
2. Insufficiently calibrated antibody dilutions (20 %)
3. Insufficient or erroneous epitope retrieval (27 %)
4. Error-prone or less sensitive visualization systems^c (19 %)
- 5 Other (17 %)
 - a. Heat-induced impaired morphology
 - b. Proteolysis induced impaired morphology
 - c. Drying out phenomena
 - d. Stainer platform-dependant protocol issues
 - e. Excessive counterstaining impairing interpretation

37% insuff.

^a Consistently gives false negative or false positive staining or a poor signal-to-noise ratio in one or more assessment runs

^b Frequently giving inferior staining results, e.g., due to mouse-anti-Golgi reactions or sensitive to standard operations as blocking of endogenous peroxidase

^c Biotin-based detection kit for cytoplasmic epitopes, use of detection kits providing a too low sensitivity, or use of detection kits and chromogens giving imprecise localization of the staining signals complicating the interpretation

Problems related to:

❑ The choice and use of the primary antibody (Concentrate or RTU)

- **Inappropriate primary antibody**
 - Provide low sensitivity/specificity
- **Appropriate primary antibody**
 - Inapp. titre (too low or too high concentration)
- **Platform (stainer) dependent antibodies**
 - Provide low sensitivity / specificity

False positive or false negative results

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

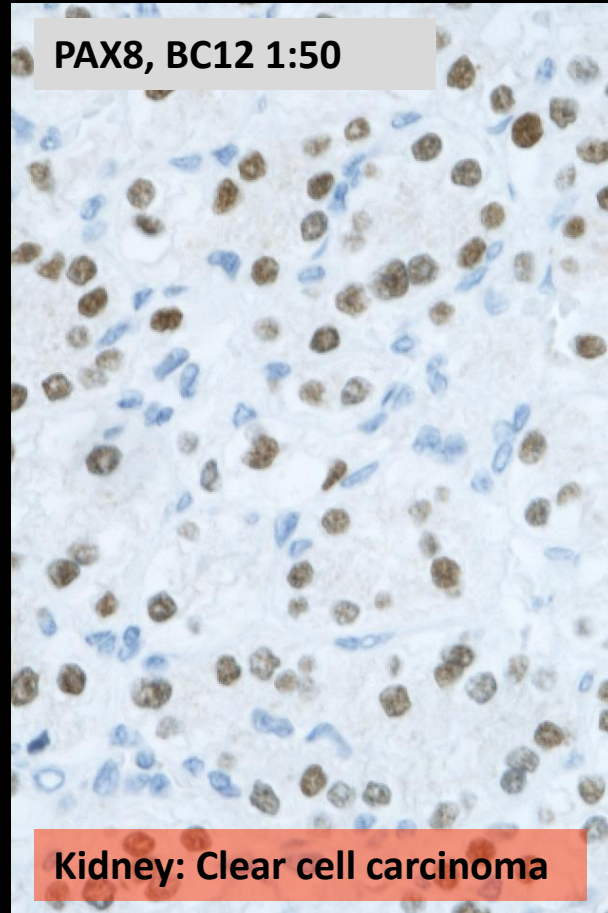
Changing Ab-Ag reaction microenvironment (Diluent)

Low affinity primary antibodies

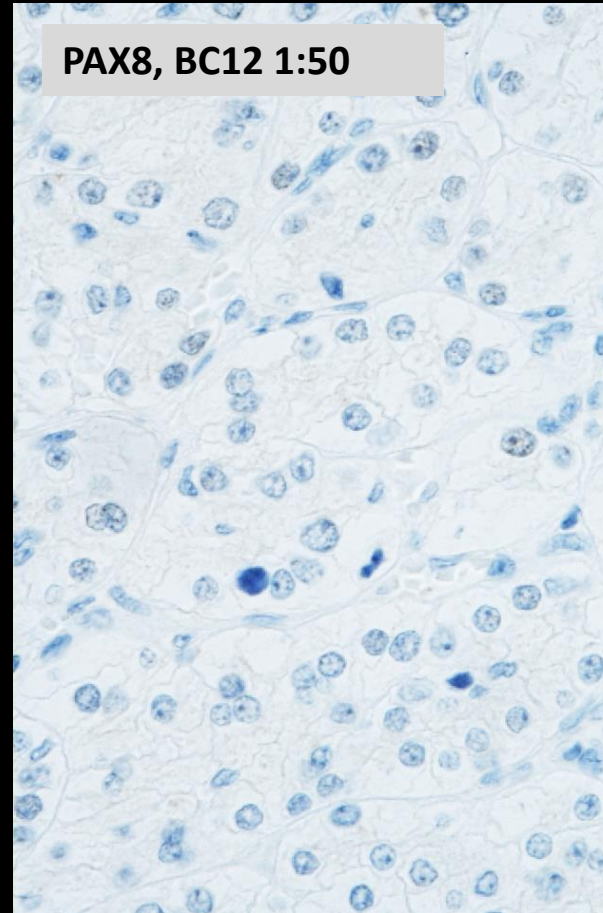
Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Primary antibodies
sensitive to the
chosen platform

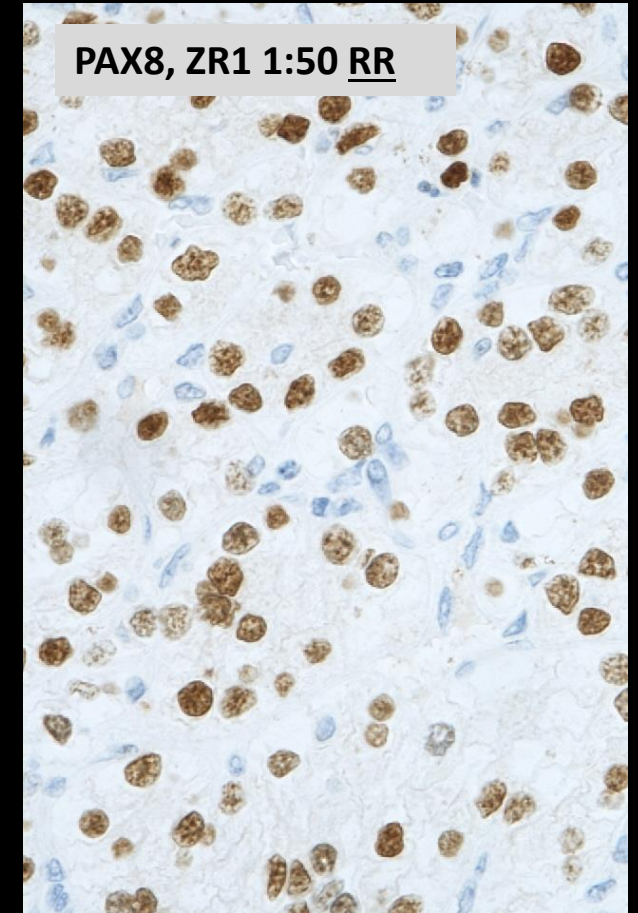
Autostainer



Omnis



Omnis



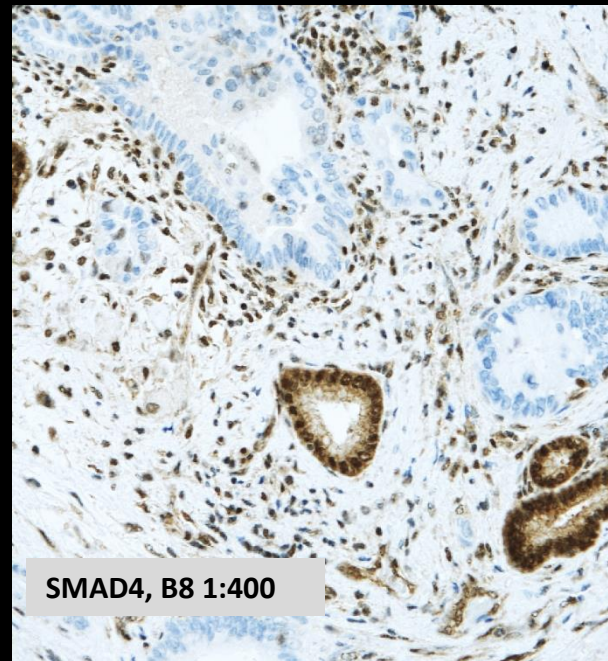
HIER High pH 20', Flex+ (10+20)

HIER High pH 48', Flex+ (10+20)

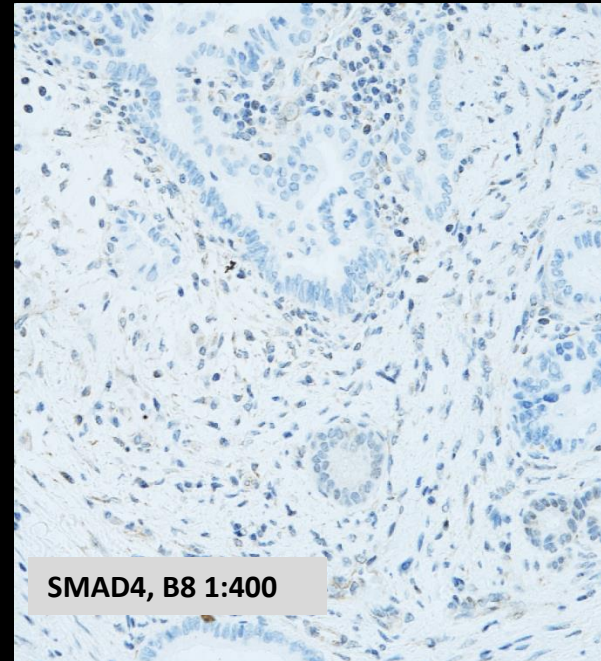
Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Primary antibodies
sensitive to the
chosen platform

Autostainer



Omnis



Omnis



HIER High pH 20', Flex+ (10+20)

HIER High pH 24', Flex+ (10+20)

Pancreatic Adenocarcinoma

Challenging and platform dependent antibodies

Marker	Problematic clone	Alternative
ASMA	1A4	BS66
BCL2	124	"E17" or SP66
CD3	F7.2.38	LN10
CD4	4B12	EP204
CD23	1B12	DAK-CD23
CD56	123C3 & 123C3.D5	MRQ-42
CDX2	DAK-CDX2	EPR2764Y or EP25
CEA	II-7	CEA31
CK (LMW)	5D3	EP17/EP30
PMS2	A16-4	EP52 or EPR3947

Marker	Problematic clone	Alternative
CR	DAK-Calret1	CAL6
Desmin	D33	BS21
EPCAM	BER-EP4	BS14
Melan A	A103	EP43 (Melanomas)
OCT 3/4	C-10	MRQ-10 or N1NK
PAX8	MRQ-50	SP348
P16	E6H4	JC2 or MX007
WT1	6F-H2	D817F or EP122
SMAD4	B-8	EP618Y
.....		

Experience from Dept. of surgical Pathology, Region Zealand, Denmark on the Omnis - These alternative clones might also work on other platforms (e.g., Benchmark Ultra or Bond MAX/III).

Go to the NordiQC website for information of the individual markers in relation to the chosen platform

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Platform dependent antibodies

NordiQC results (2018-2021)

Antibody	Clone	Platform(s)
CD56	123C3	BenchMark (Ventana) and Omnis (Dako)
PMS2	A16-4	BenchMark
Melan A	A103	Benchmark and Omnis
P16	E6H4	Omnis
Alpha Smooth Muscle Actin	1A4	Benchmark
SMAD4	B8	Benchmark and Omnis
CK8/18	5D3	Benchmark
EPCAM	BER-EP4	Benchmark and BOND (Leica)
.....		

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.....

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

HIER buffers used by NordiQC laboratories

....

App. 80-90 % of all pretreatment protocols

In house	Agilent Dako AS/Omnis	Roche Ventana Benchmark	Leica Biosystems Bond	Biocare	Thermo S LAB Vision
<u>Low pH buffers</u>					
Citrate buffer pH 6 / pH6.7	TRS Low pH 6.1	CC2 pH 6	BERS-1 pH 6	Diva Decloaker pH 6.2	
<u>High pH buffer</u>					
EDTA/EGTA pH 8	TRS High pH 9	CC1 pH 8.5	BERS-2 pH 9	Borg Decloaker pH 9.5	HIER buffer H pH 9
Tris-EDTA/EGTA pH 9	TRS High (3-in-1) pH 9				
Tris-HCL pH 9					

Challenges:

The platform often dictates the choice of HIER buffers



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	Dako						
	6	Cell Marque	14	13	21	28	36%	93%
	1	Diagnostic Biosystems						
mAb clone MOC-31	23	Dako						
	5	Cell Marque	10	10	7	2	69%	71%
	1	Diagnostic Biosystems						
mAb clone VU-1D9	5	Thermo Scientific						
	3	Merck Millipore	9	0	1	0	90%	100%
	1	Immunologic						
	1	Novus Biologicals						
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/IS637³	6	Dako	1	2	2	1	-	-
mAb clone Ber-Ep4 GA637	27	Dako	26	1	0	0	100%	100%

BS14 could be an alternative to Ber-EP4 on platforms excluded from the use of modified low pH buffers e.g., Diva pH 6.2 (Biocare) or TRS pH6.1 (Dako)

HIER in alkaline pH buffers (+/- gentle enzymatic digestion performed after Hier)

OPS based on Hier in mod. Low pH buffers (Dako or Biocare)

OPS based on Hier in alkaline (CC1) or mod. Low pH buffers (Dako)

Omitted from Hier in mod. Low pH buffers (TRS low pH/Dako or Diva Decloaker/Biocare) - these buffers are not part of the reagent portfolio

HIER in mod. Low pH buffers (Dako)

The technical test approach – Analytical phase

EPCAM clone EP4 or BS14

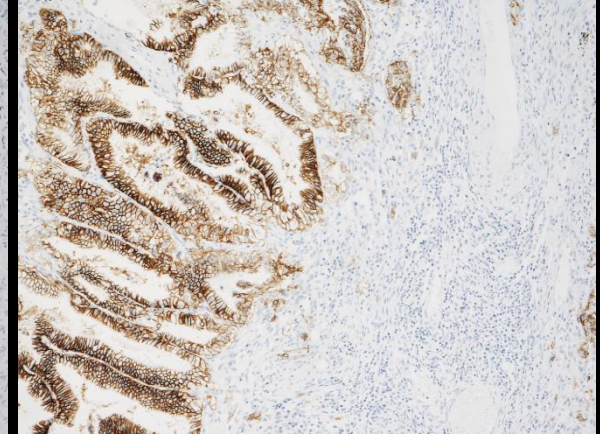
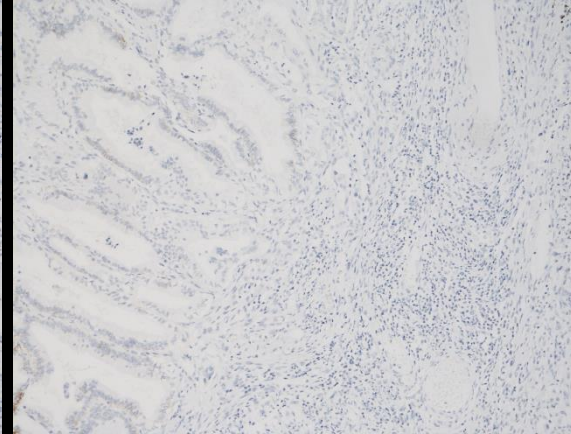
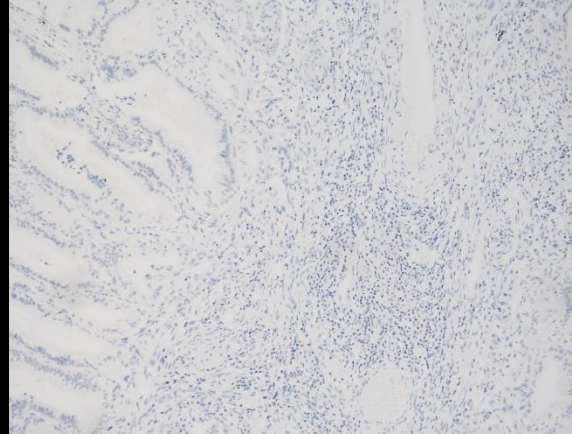
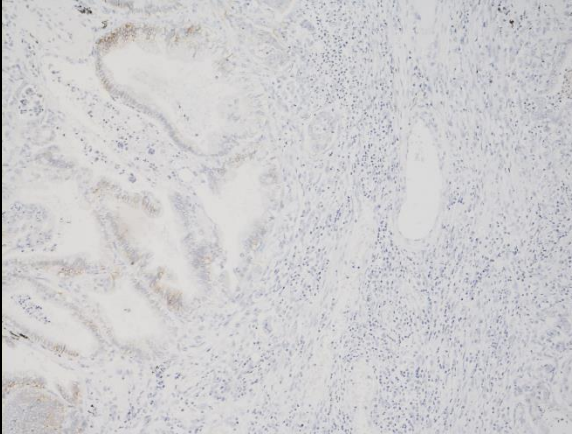
Proteinase K 1:4 (Dako) / 5`

Citrate buffer pH 6 (Dako) / 20`

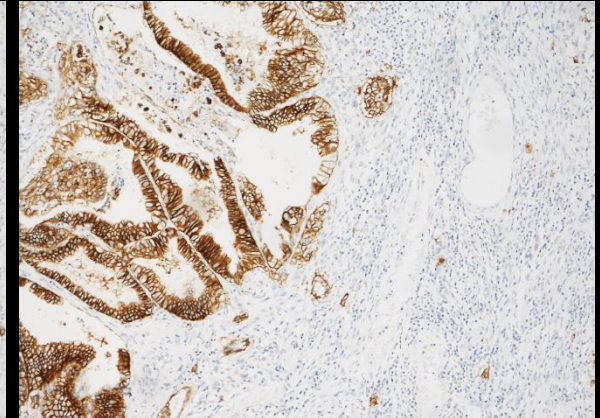
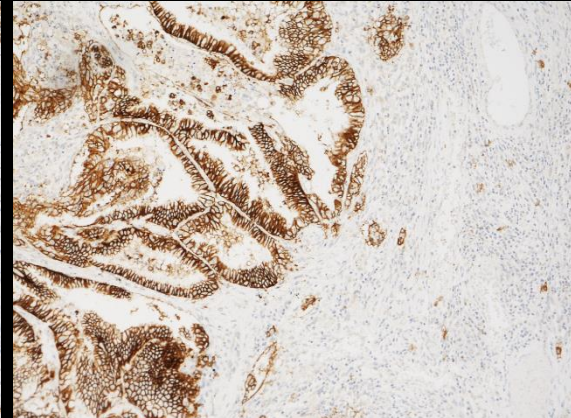
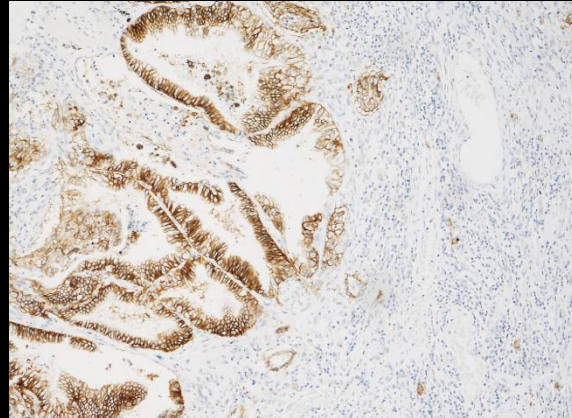
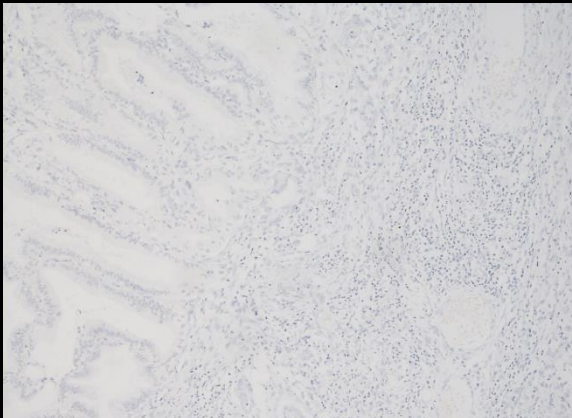
High pH buffer (Dako) / 20`

TRS Low pH (Dako, S1699/S1700) / 20`

EP4



BS14



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

Colon tumor

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
.....

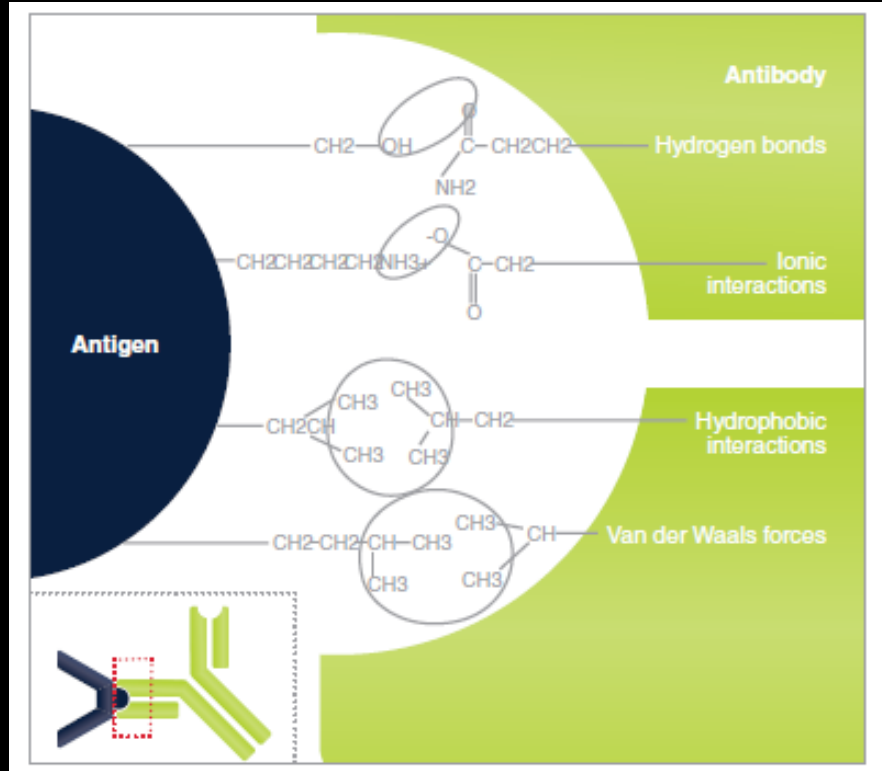
Changing the primary Ab

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

© 2001 Lippincott Williams & Wilkins, Inc., Philadelphia

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 on the 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)

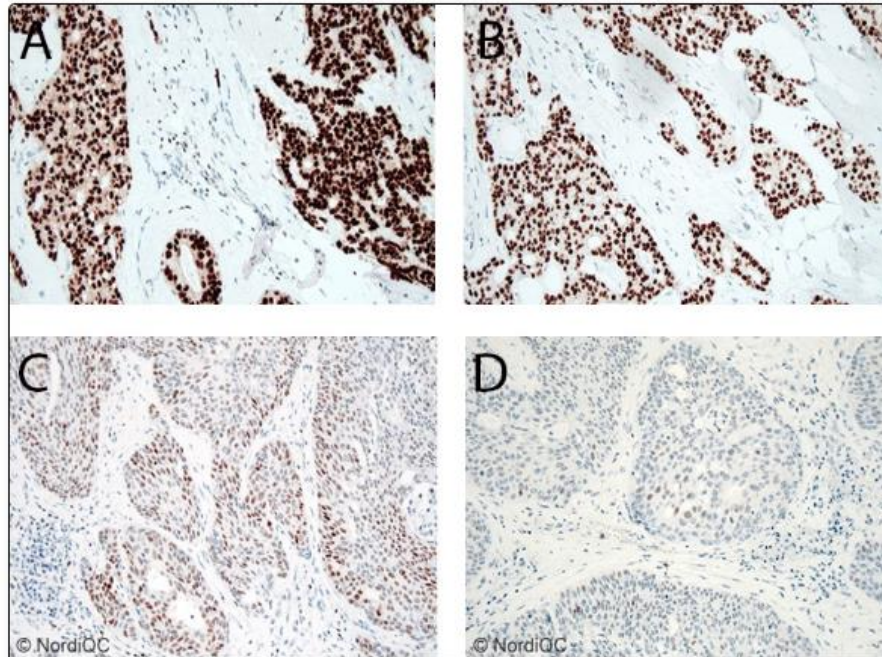
Clone	pH	TB		TBS		PBS
		6.0	8.6	6.0	8.6	7.3
BLA.36		2	4	1	2	1
UCHL1		4	3	2	1	1
L26		4	3	3	3	2
PC10		4	3	4	4	3
N10/2		3	2	1	2	1
V9		4	3	4	4	2
TAL1B5		4	2	3	2	2
ER-PR-8		4	3	2	1	2
Ber-H2		4	3	ND	ND	0
4KB5		4	2	4	2	4
DF-T1		4	2	2	0	1
PD7/26		4	3	ND	ND	3
C3D-1		4	2	ND	ND	1

ND, not done.

Technical aspects of immunohistochemistry & pitfalls - Analytical phase



Info ▾ Modules ▾ Assessments Protocols Controls Events ▾ [Login](#)



Ductal breast carcinoma (A and B) and urothelial carcinoma (C and D) stained for GATA3 (clone L50-823) in two laboratories. Optimal results in A and C, insufficient in D. The only difference in protocols is the antibody dilution buffer: In A and C the antibody was diluted in Biocare Renoir red (pH 6.2). In B and D the Dako antibody diluent pH 7.3 was used.

Results - Run 54, B26, H14, C4

14-Dec-2018

The results for the runs 54, B26, H14, C4 are now available on the website. Individual results can be seen after logging in.

[All news](#)

Events

[NordIQ Workshop in Diagnostic Immunohistochemistry](#)
2-4 Oct 2019: Aalborg, DK

6th Annual Course of Academy of Immunohistochemistry "Diagnostic Immunohistochemistry and Molecular

9-11 Oct 2019: Krakow, Poland

4th NordIQ Conference on Applied Immunohistochemistry
2-5 Jun 2020: Aalborg, Denmark

Important dates

[Run 55, B27, H15](#)
Protocol submission deadline
3 Jan 2019
Slide circulation
9 Jan 2019
Slide return deadline
13 Feb 2019
Publication of results
20 Apr 2019

Questions

Check out our [FAQ](#) (Frequently asked questions) or [contact us](#)

GATA3 (L50-823)

Antibody diluents

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

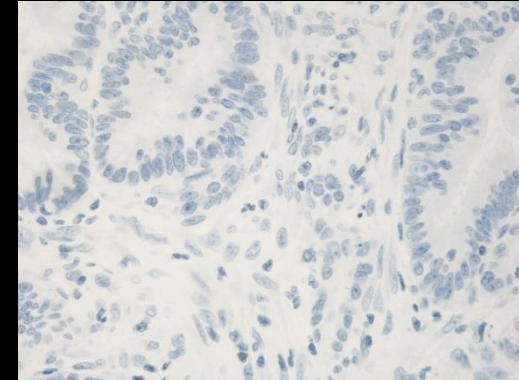
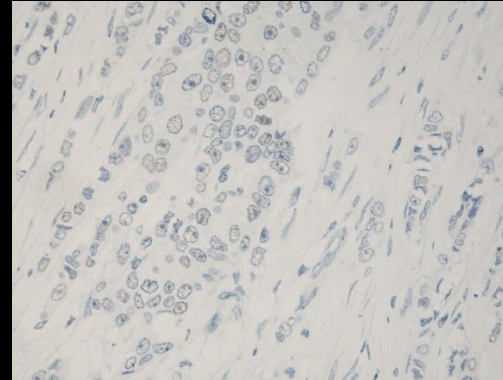
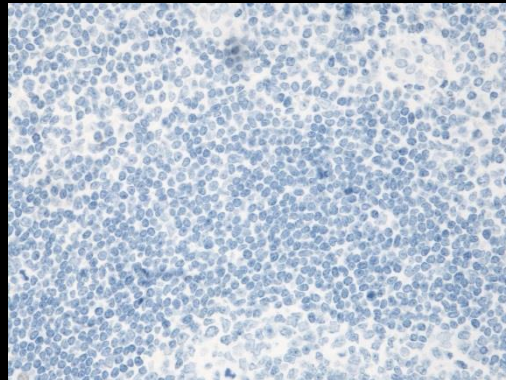
Antibody diluents

GATA3, L50-823 (Biocare) 1:800

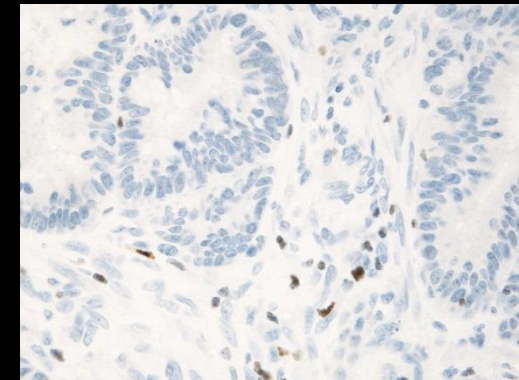
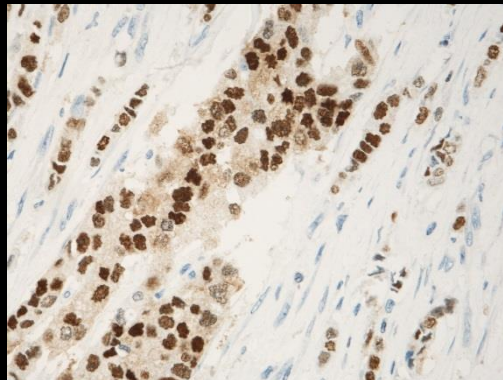
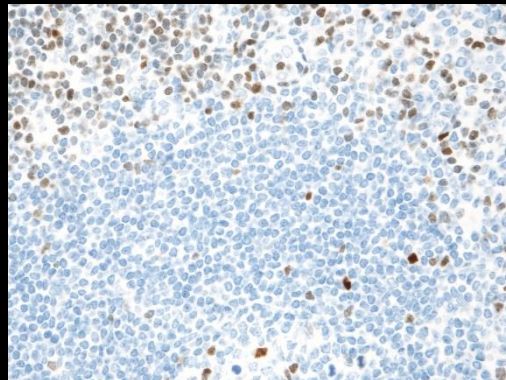
Tonsil

Breast adenocarc.

Colon adenocarc.



Dako Dil. pH 7.3



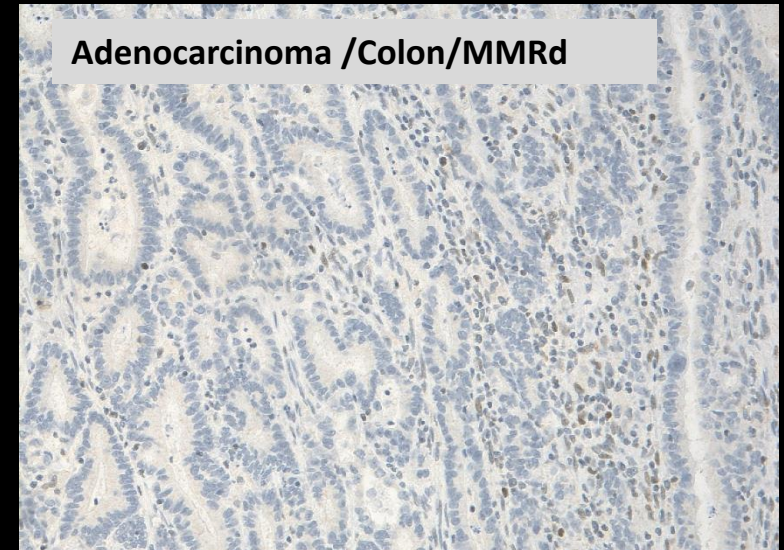
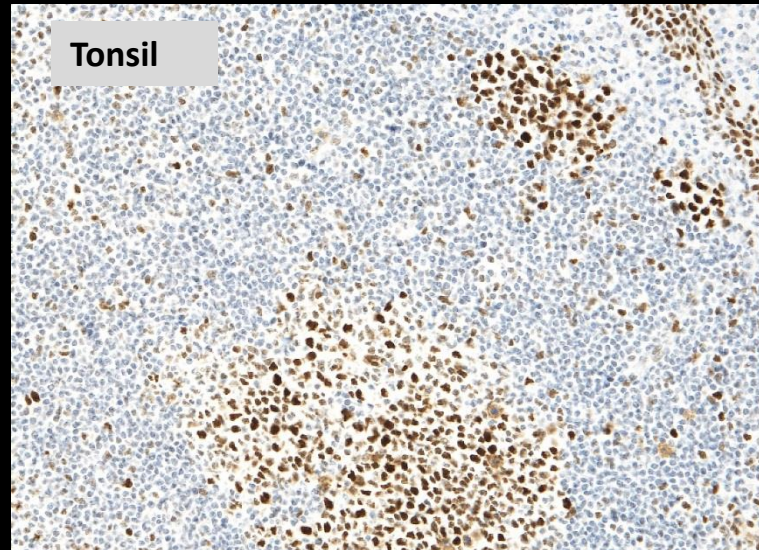
Biocare Renoir Red pH 6.2

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

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

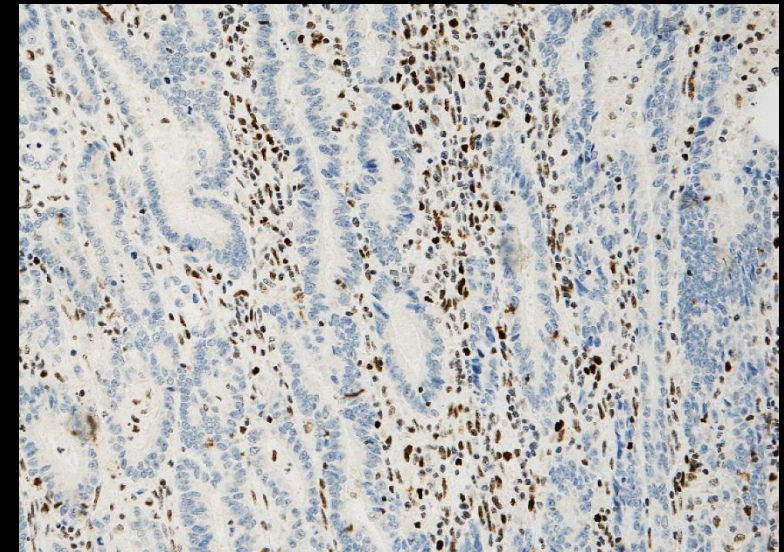
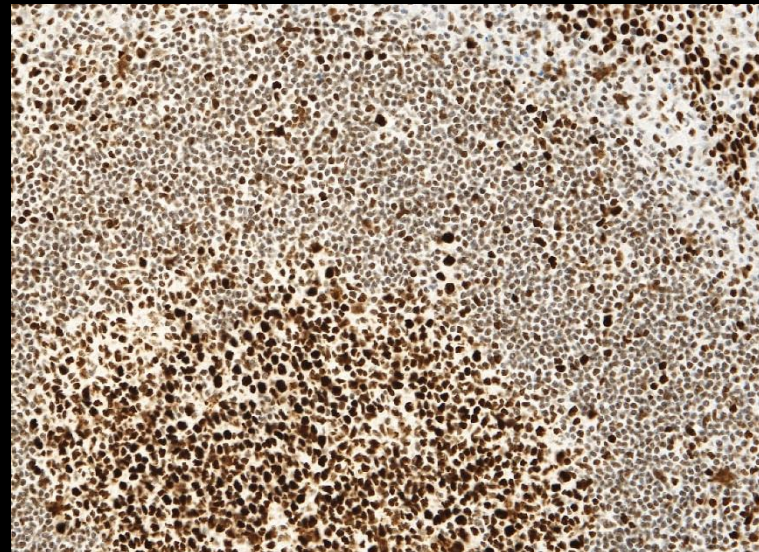
Antibody diluents

MSH2, FE11 1:50
Dako dil. pH 7.3



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

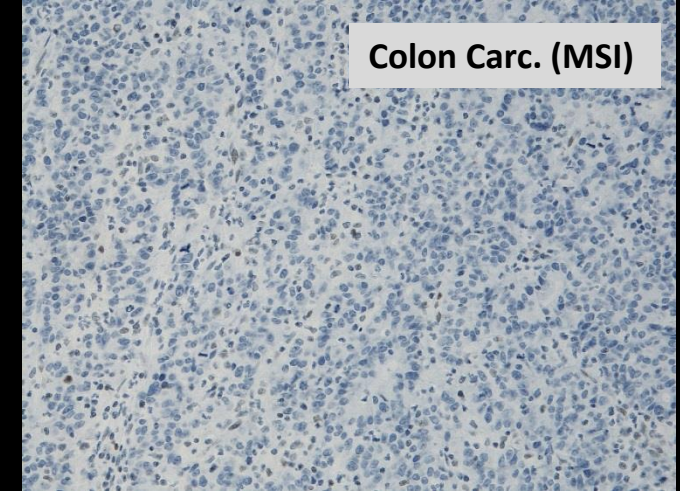
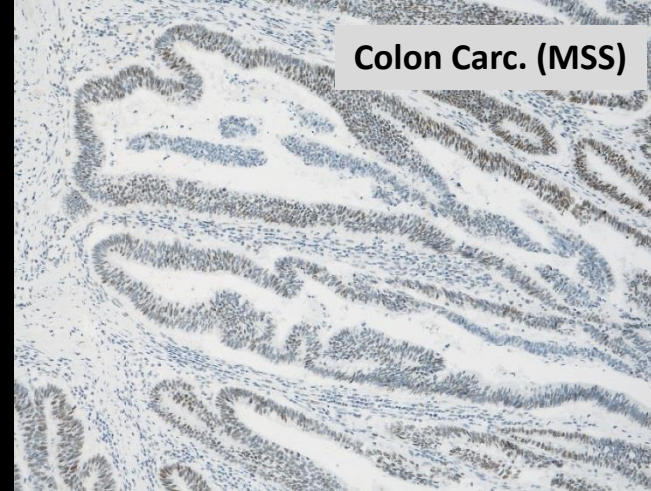
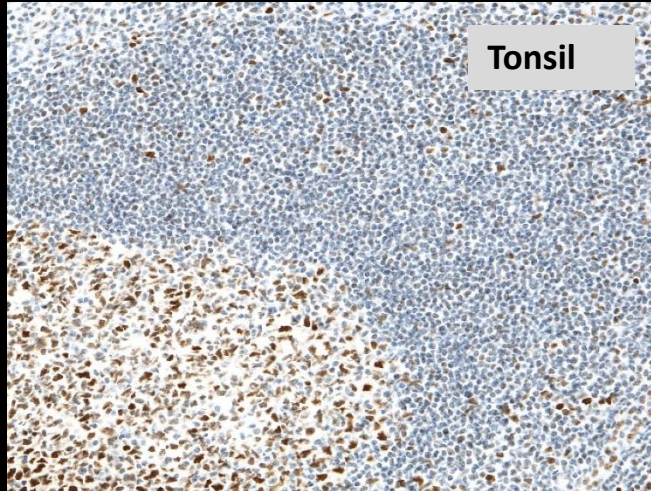
MSH2, FE11 1:50
Renoir Red pH 6.2



Technical aspects of immunohistochemistry & pitfalls - Analytical phase

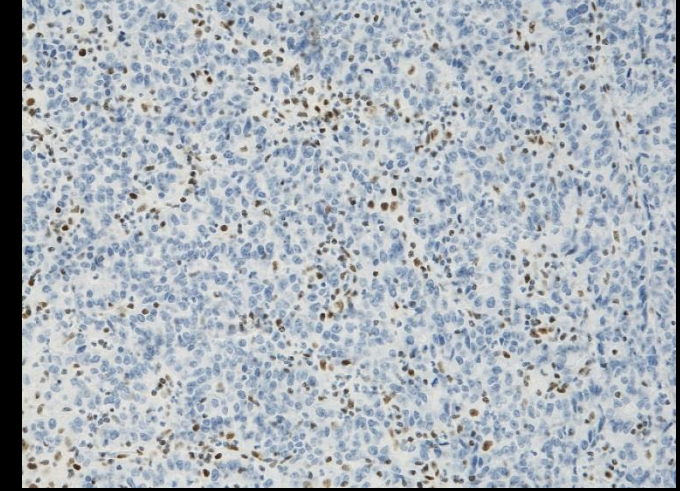
Antibody diluents

MLH1, BC23 1:40
Dako dil. pH 7.3



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

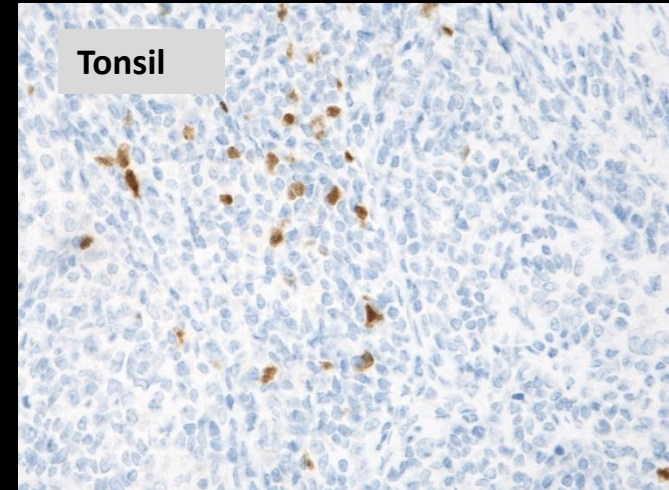
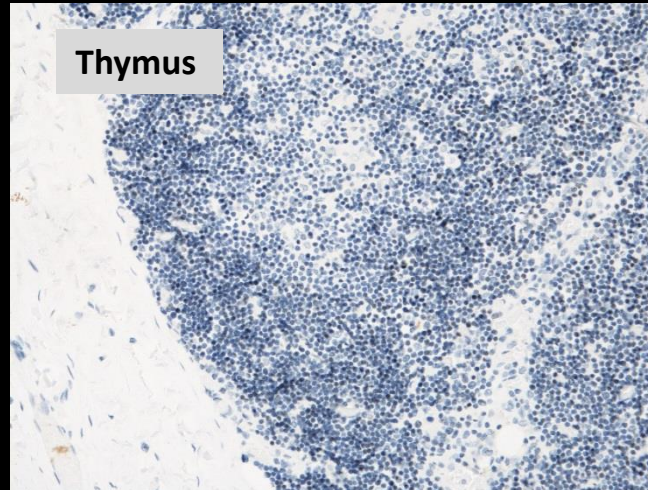
MLH1, BC23 1:40
Renoir Red pH 6.2



Technical aspects of immunohistochemistry & pitfalls - Analytical phase

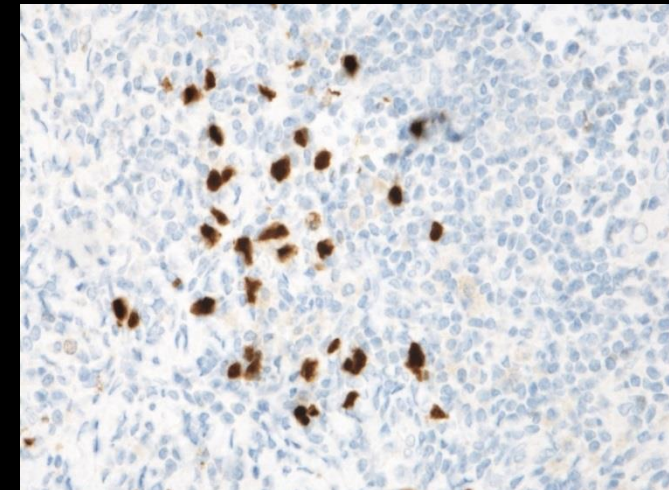
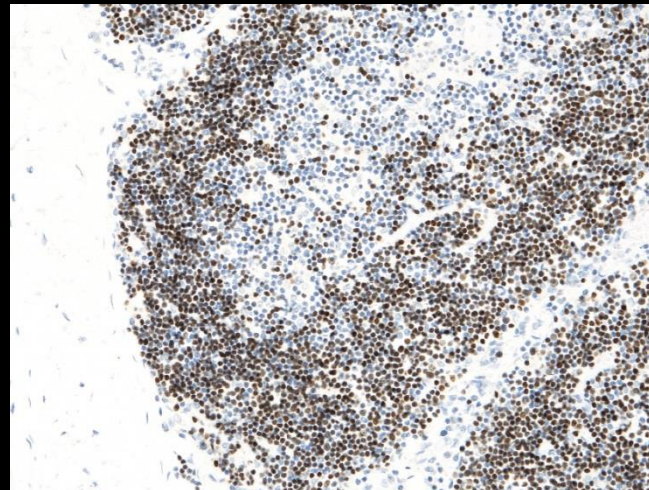
Antibody diluents

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

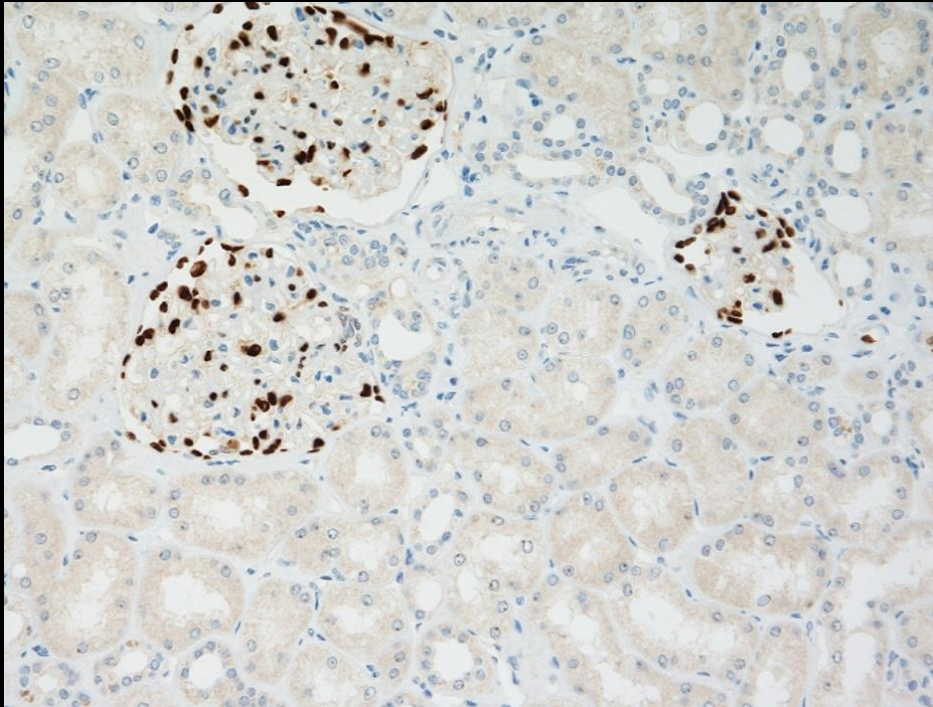


**Renoir Red is not always the best antibody diluent
Use a “antibody diluent test battery”**

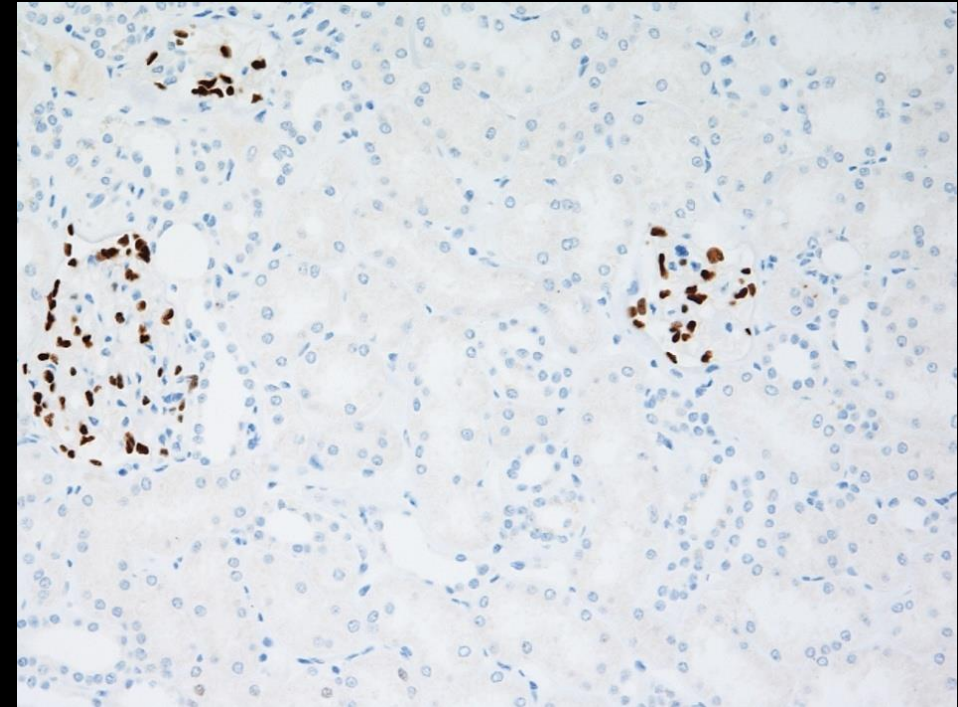
Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Antibody diluents

WT1, EP122 1:25
Renoir Red (Biocare)



WT1, EP122 1:25
Background Sniper (Biocare)



Kidney

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

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

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

Antibodies benefitting from dilution 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

Antibodies that not benefitting from dilution in Renoir Red pH 6.2:

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

Antibodies benefitting from dilution in Background sniper (reduces background problems):

Spirochete (poly), BORR (poly), WT1 (EP122), ASMA (BS66) and



Proficiency testing in immunohistochemistry—experiences from Nordic Immunohistochemical Quality Control (NordiQC)

Mogens Vyberg^{1,2} · Søren Nielsen¹

Virchows Arch (2016) 468:19–29

Table 3 Major causes of insufficient staining reactions

1. Less successful antibodies (17 %)
 - a. Poor antibodies^a
 - b. Less robust antibodies^b
 - c. Poorly calibrated RTUs
 - d. Stainer platform dependent antibodies
2. Insufficiently calibrated antibody dilutions (20 %)
3. Insufficient or erroneous epitope retrieval (27 %)
4. Error-prone or less sensitive visualization systems^c (19 %)
- 5 Other (17 %)
 - a. Heat-induced impaired morphology
 - b. Proteolysis induced impaired morphology
 - c. Drying out phenomena
 - d. Stainer platform-dependant protocol issues
 - e. Excessive counterstaining impairing interpretation

19% insuff.

^a Consistently gives false negative or false positive staining or a poor signal-to-noise ratio in one or more assessment runs

^b Frequently giving inferior staining results, e.g., due to mouse-anti-Golgi reactions or sensitive to standard operations as blocking of endogenous peroxidase

^c Biotin-based detection kit for cytoplasmic epitopes, use of detection kits providing a too low sensitivity, or use of detection kits and chromogens giving imprecise localization of the staining signals complicating the interpretation

Problems related to the choice of the detection system:

- Provides low sensitivity
 - 2 versus 3-step multimer/polymer detection systems
- Provides low specificity and sensitivity
 - Biotin based systems

False positive or false negative results

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Detection systems (polymer/multimer) used by NordiQC participants

Vendor	Detection System	Detection System	Amplifier	Cat.no
	2- Step	3-step		
Dako	EnVision EnVision /Flex	Envision Flex+	Anti -Ms/Rb	K4001 K8000/10 (K5007) K8002/12 GV800 + GV809/821
Ventana	UltraView	UltraView + Amp OptiView Optiview + Amp	Anti -Ms/Rabbit Anti-Hapten Anti-Hapten + TSA	760-500 760-500 + 760-080 760-700 760-700 + 760-099
Leica		Bond Refine (PowerVision)	Anti-Ms (Rb?)	DS9800 (HRP); DS9390 (AP)
Biocare	MACH 2	MACH 3 MACH 4	Ms/Rb probe Ms probe (Rb?)	M2U522; MHRP520; RHRP520 M3M530; M3R531 M4U534
LAB Vision/TS	UltraVision One	Quanto	?	TL-125-HLJ TL-125-QHD /QHL
Immunologic	BrightVision (PowerVision)	BrightVision+	Anti-Ms/Rat (Rb ?)	DPVM (Anti-Ms)/DPVR (Anti-Rb) DPVO (Anti-Ms/Rb/Rat) DPVB ((Anti-Ms/Rb/Rat)
Master Diag.		Quanto	?	MAD-021881QK
ZytoMed System		ZytoChem Plus (PowerVision)	Anti-Ms (Rb?)	PolHRP-100
And a few more (Advance, GTVision.....)				

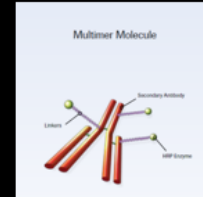


App. 98% of all NordiQC participants use a polymer/multimer based detection systems

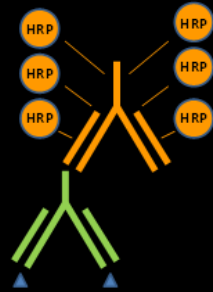
Considerations related to the choice of detection system:

- ☐ Sensitivity
- ☐ Specificity
- ☐ Enzyme conjugate
- ☐ Blocking of endogenous activity
- ☐ Turn around time (TAT)
- ☐ Automatic platform (open or closed system)
- ☐ Price

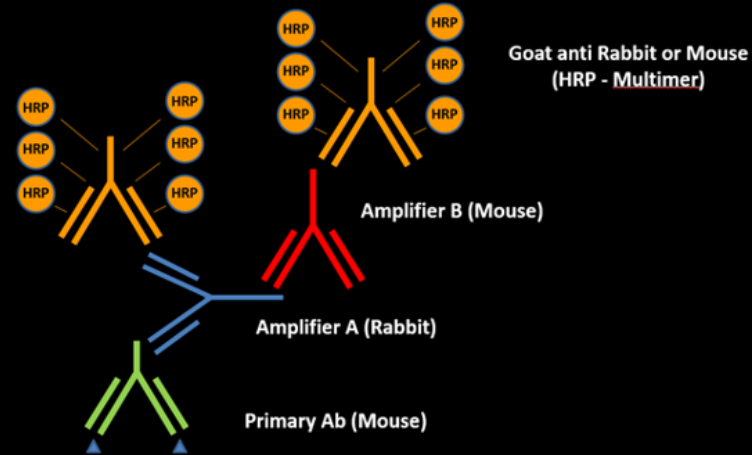
Multimer detection systems (Ventana/Roche)



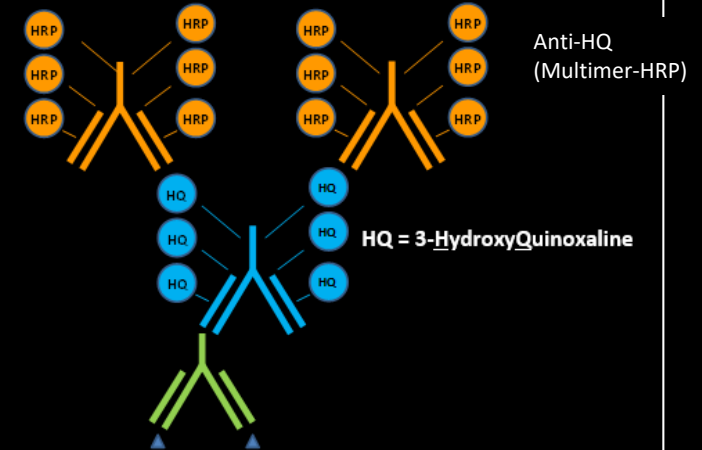
UltraView



UltraView /Amp



OptiView



Sensitivity

HQ-Linker
Amplifier (A/B)

Linker (Mouse/Rabbit)
Enhancer
Universal Linker
Post Blocking
.....



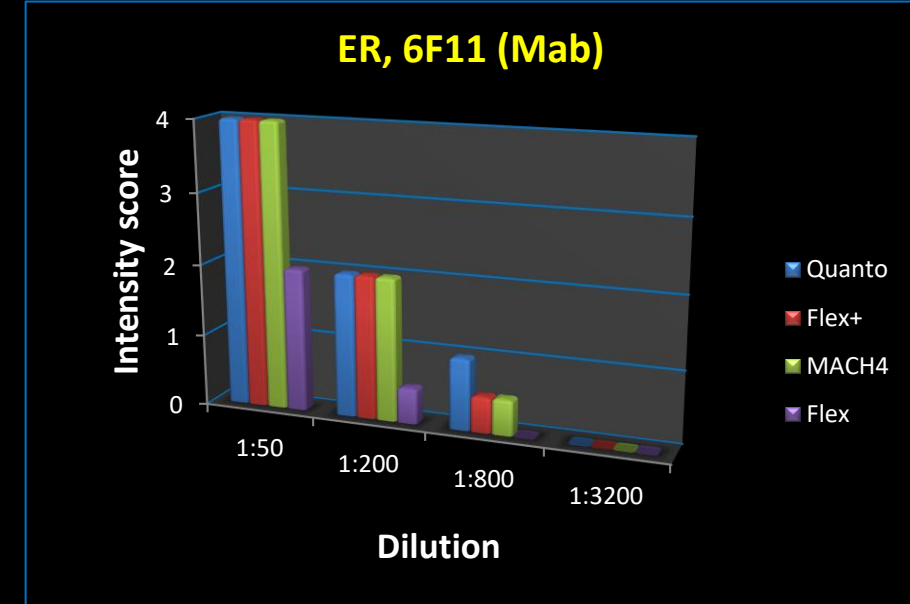
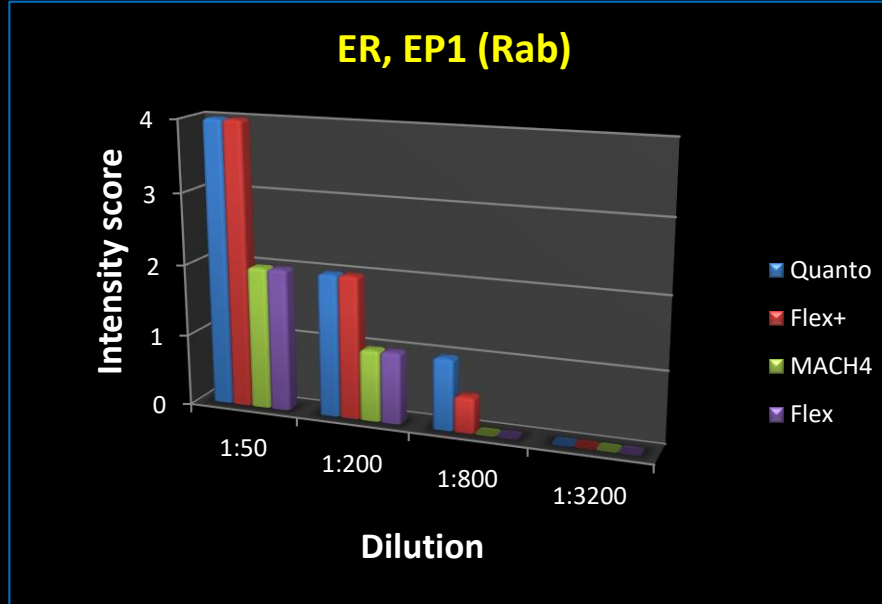
Increases sensitivity

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)

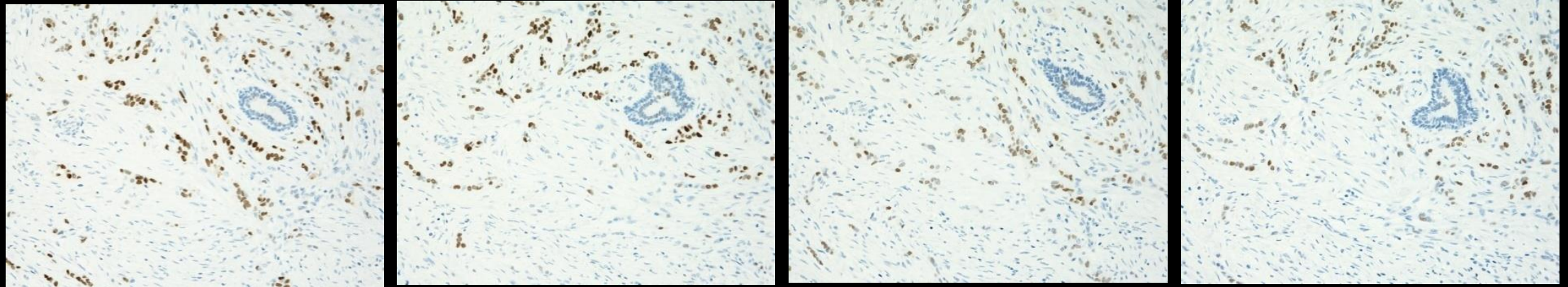
Quanto

Flex+

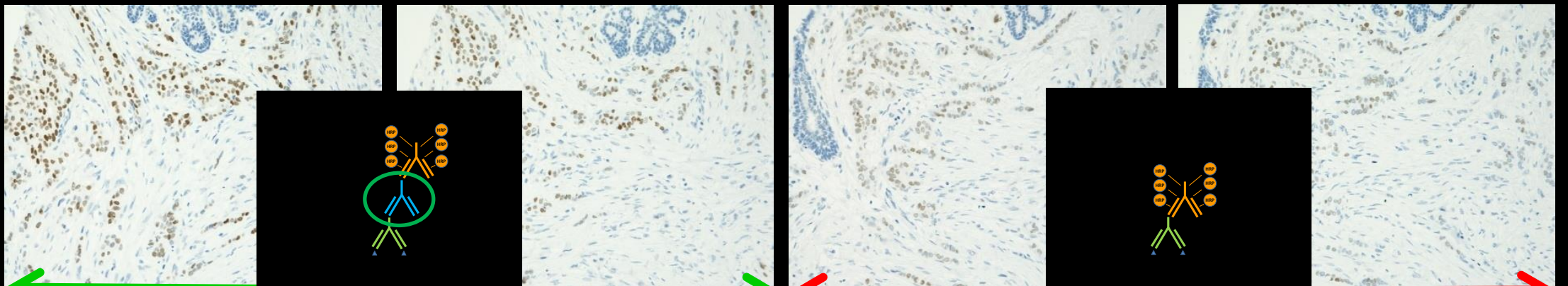
MACH4

Flex

1:50



1:200



High Intensity

Low Intensity

The technical test approach – Analytical phase

Performance testing of detection systems (Vendor recommended protocol settings)

Breast tumor

ER, 6F11 (Mab)

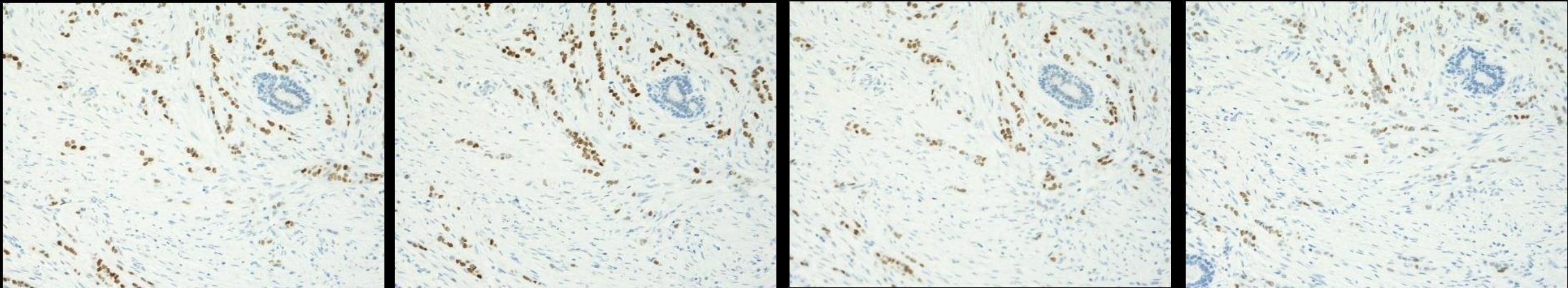
Quanto

Flex+

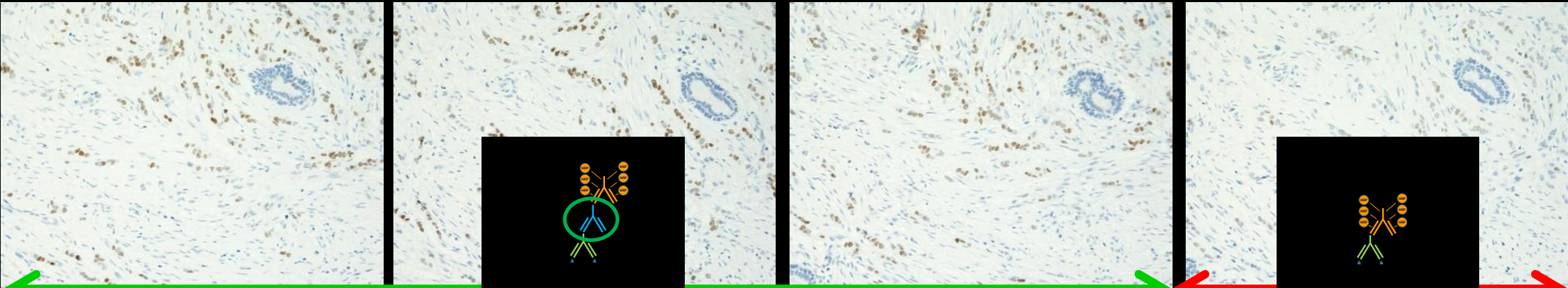
MACH4

Flex

1:50



1:200

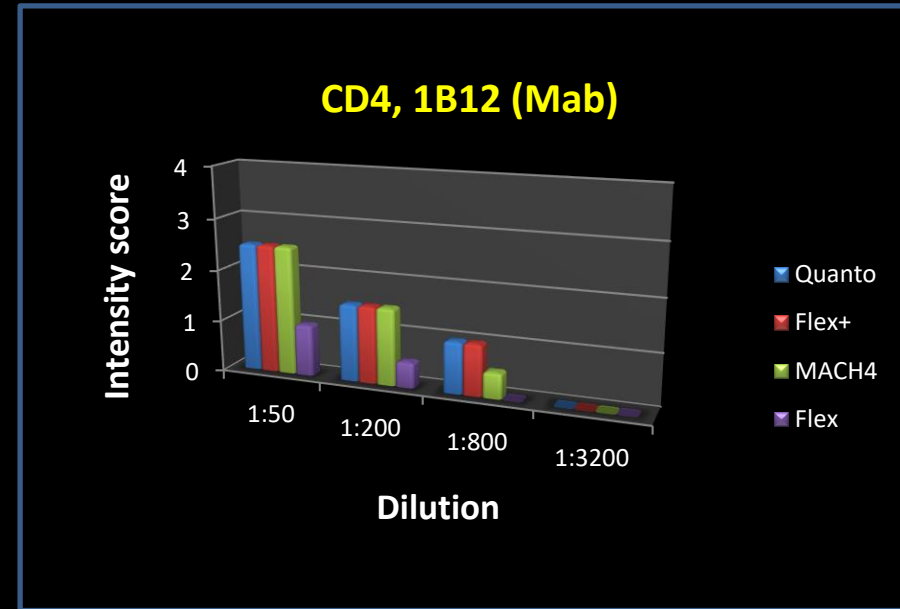
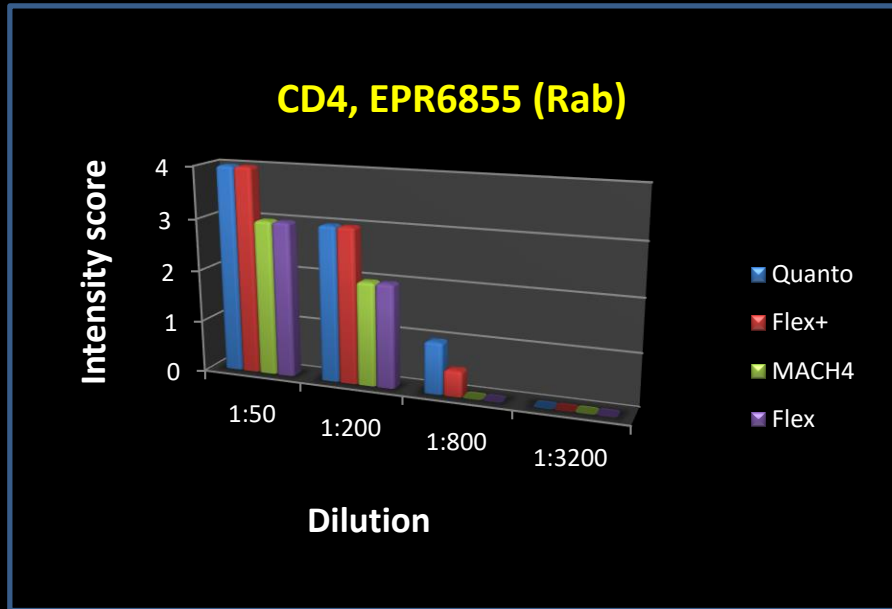


High Intensity

Low Intensity

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Detection systems - Performance Testing



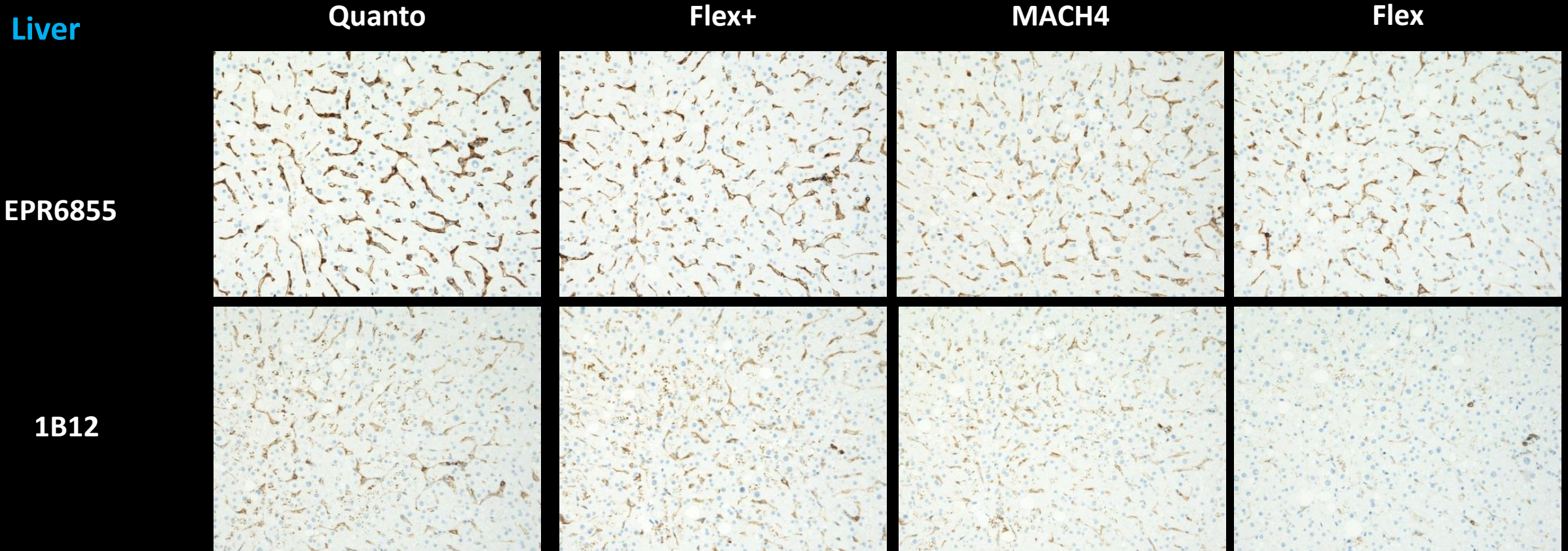
CD4 – Endpoint titration (some general remarks and important issues):

- ❑ The 3-step polymer detection systems Quanto and Flex+ - produced the overall highest intensity.
- ❑ Intensity was highly influenced by the nature of primary Ab and “optimal” staining could only be obtained with the Rab (CD4, EPR6855) used in combination with the 3- step polymer detection systems Quanto or Flex+.
- ❑ Intensity was higher with the Rab (CD4, EPR6855) at 1:50 with all of the detection systems tested compared to any intensity obtainable with the Mab (CD4, 1B12) even with the use of a 3-step polymer system (e.g. Quanto).

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Performance testing of detection systems (Vendor recommended protocol settings)

CD4, EPR6855 (Rab, 1:50) and 1B12 (Mab, 1:50)



Strong staining of hepatic sinusoidal endothelial cells and kupffer cells using the Rab (CD4, EPR6855) with all the detection system tested (2-step or 3-step polymer systems) . Intensity is significantly reduced using the Mab (CD4, 1B12).

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Performance testing of detection systems (Vendor recommended protocol settings)

CD4, EPR6855 (Rab, 1:50) and 1B12 (Mab, 1:50)

Brain

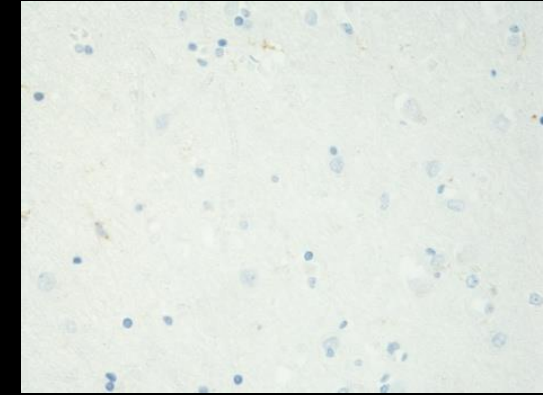
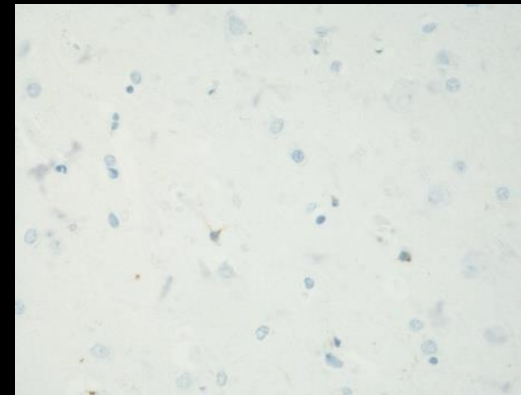
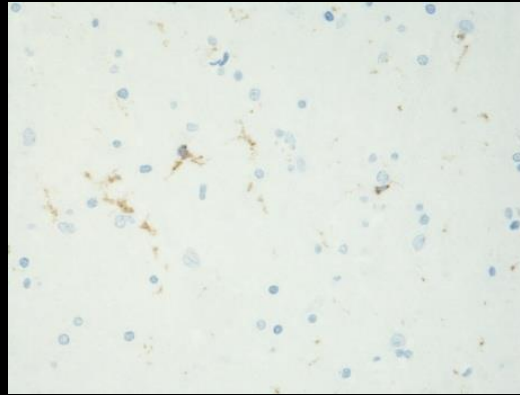
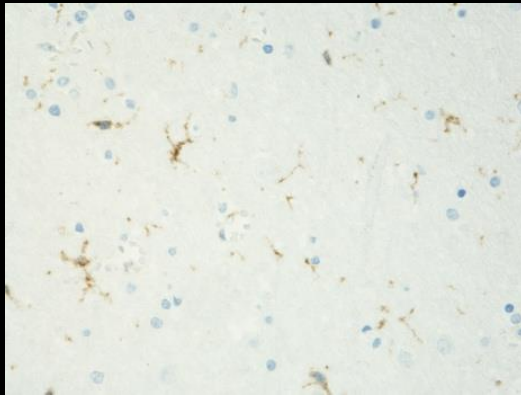
Quanto

Flex+

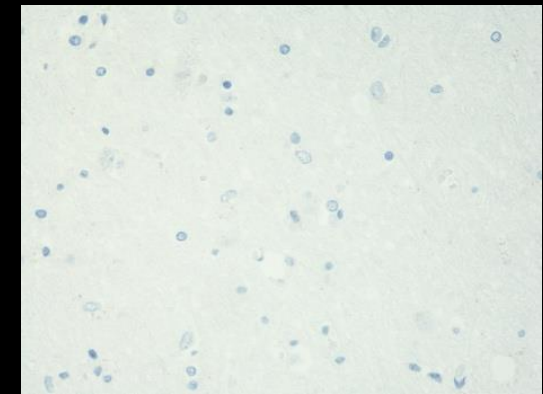
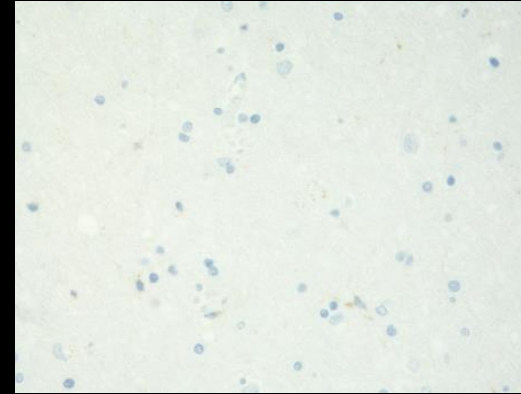
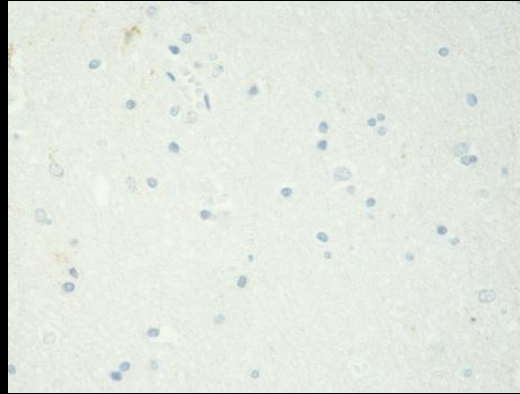
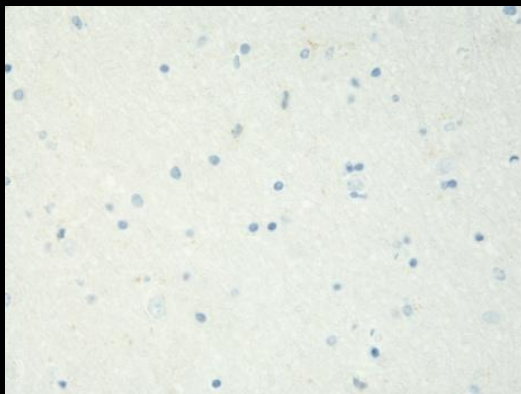
MACH4

Flex

EPR6855



1B12



Staining of microglia cells is only obtainable using the Rab (CD4, EP1628Y) and the 3-step polymer detection systems Quanto or Flex+.

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		Ventana		Leica	
	Autostainer Link / Classic		BenchMark XT / Ultra		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 (UV/OV or Flex/Flex+)

BOND refine (3-step detection system)

LD assay (mAb 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)

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		Ventana		Leica	
	Autostainer / Omnis		BenchMark XT / Ultra		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:

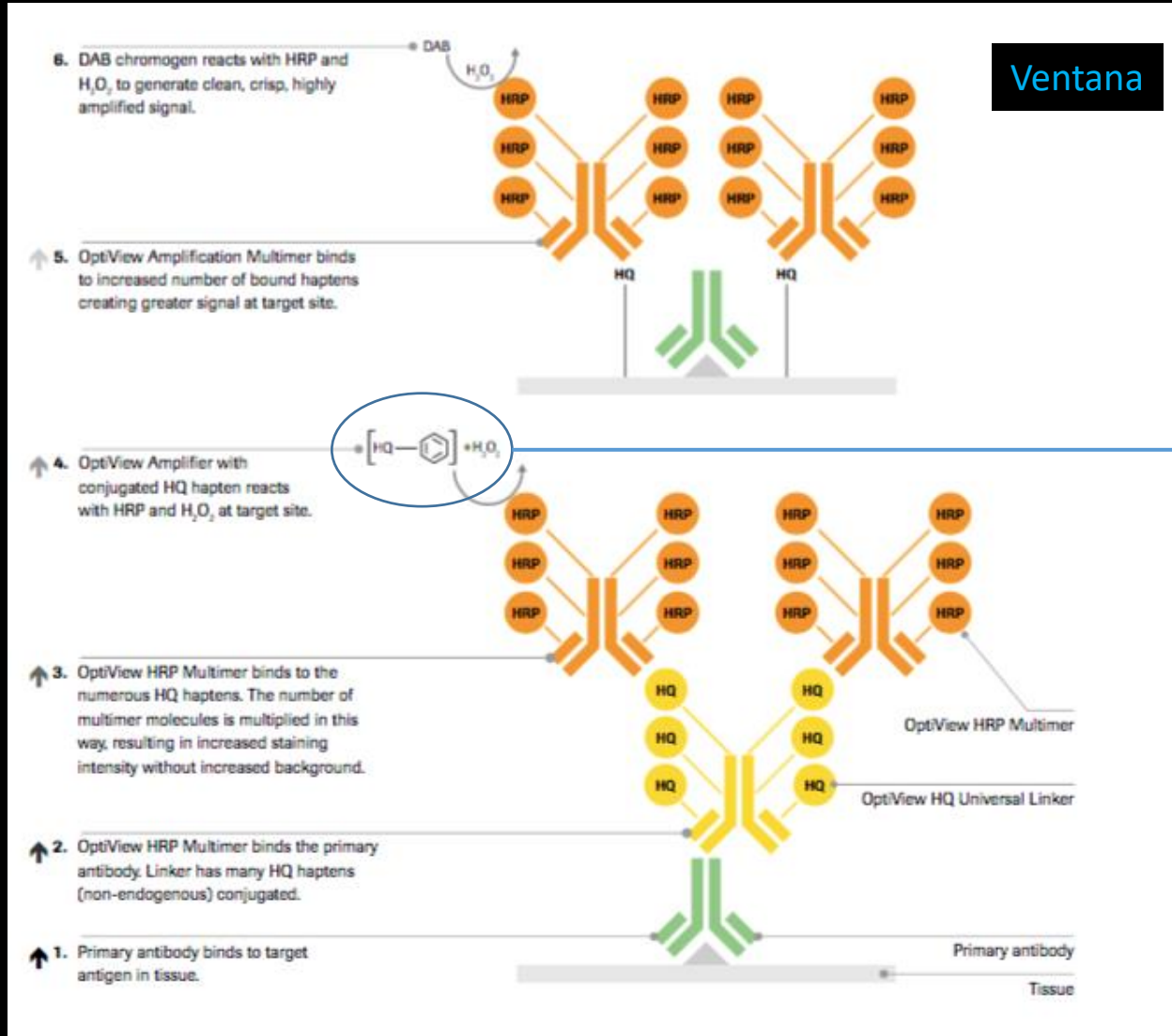
- The specimen is incubated with hydrogen peroxide to quench endogenous peroxidase activity.
- A user-supplied specific primary antibody is applied.
- Post Primary IgG linker reagent localizes mouse antibodies.
- Poly-HRP IgG reagent localizes rabbit antibodies.
- The substrate chromogen, 3,3'-Diaminobenzidine 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)

Technical aspects of immunohistochemistry & pitfalls - Analytical phase



Tyramide Signal Amplification

Mechanism of Tyramide amplification:

- Introducing HRP (Optiview)
- Incubation with HQ-labelled Tyramide + H_2O_2

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

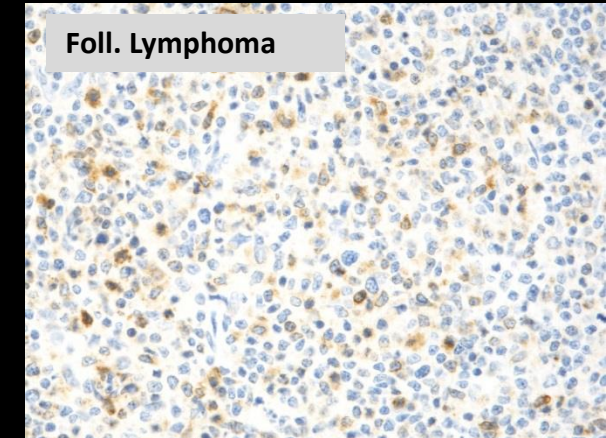
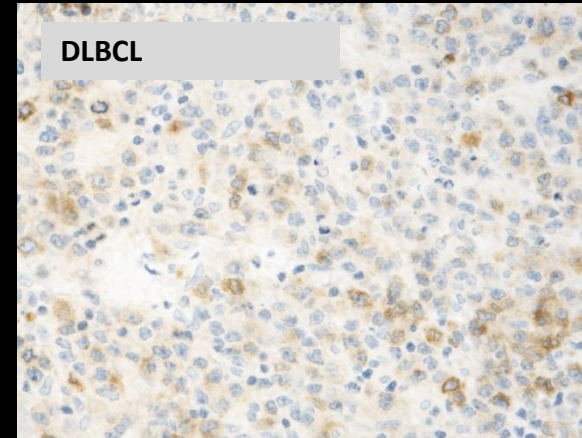
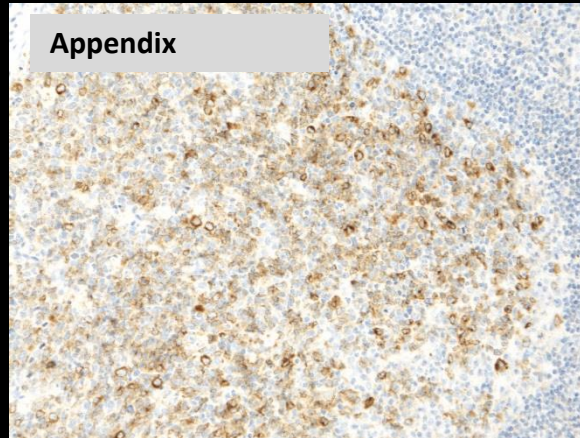
Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Detection systems

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

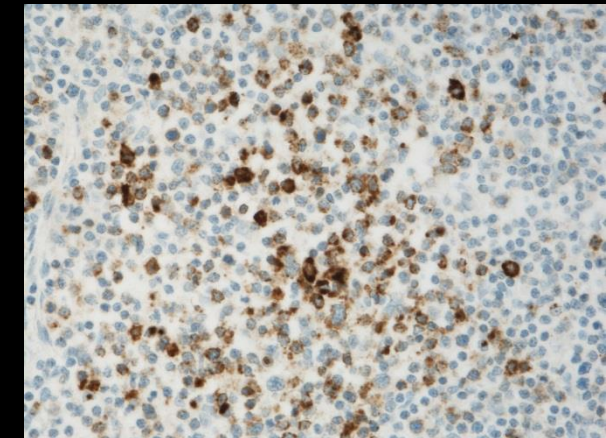
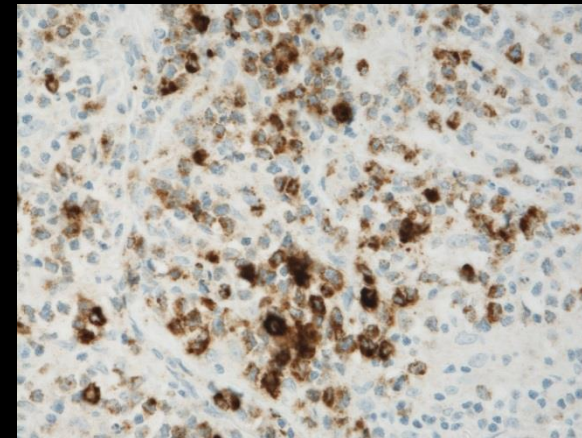
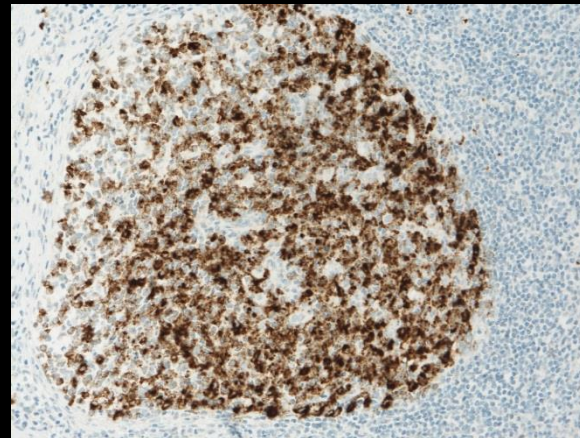
Omnis

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



Benchmark Ultra

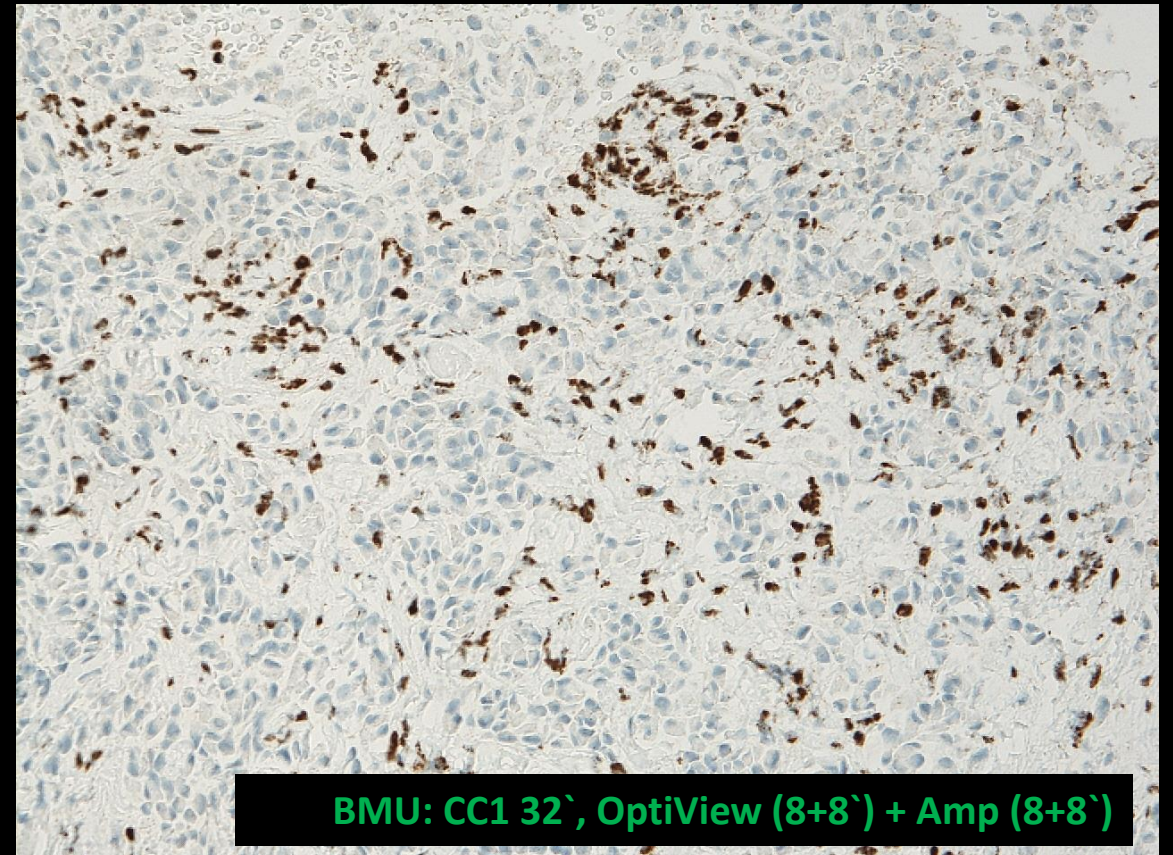
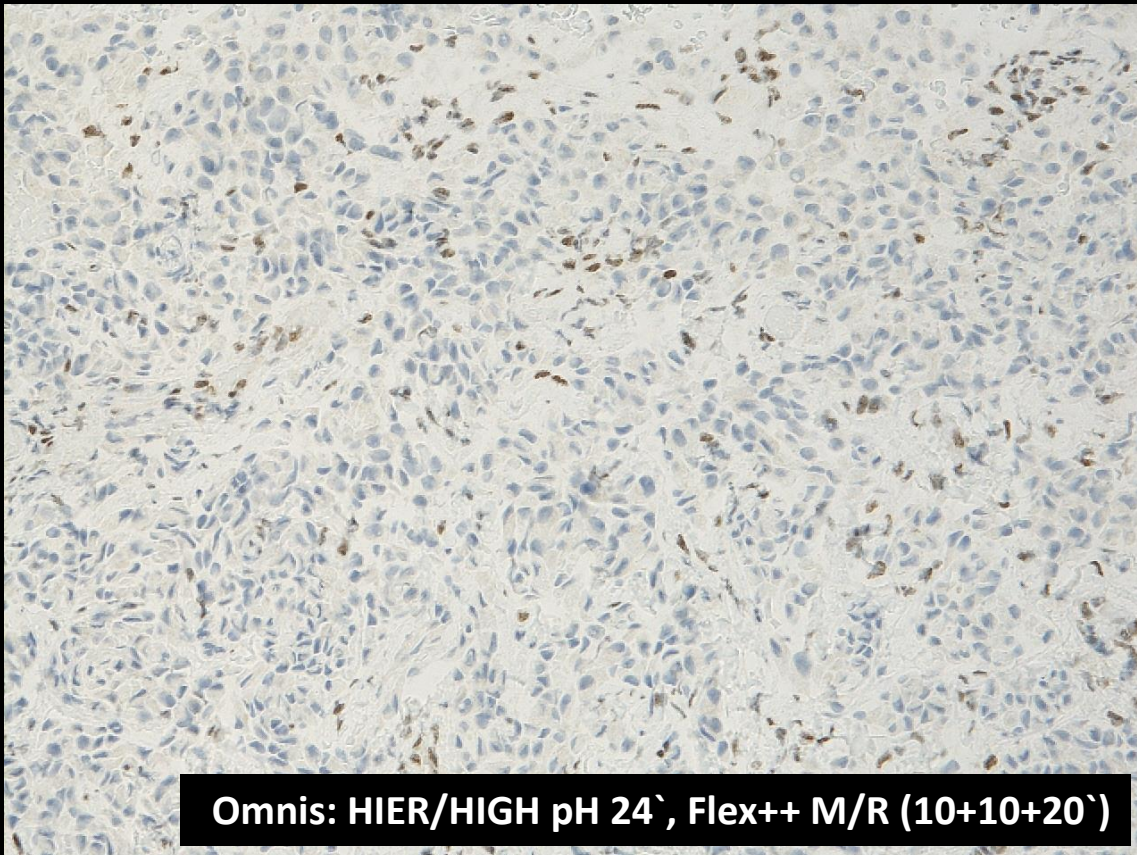
P3 (4`), CC1 (32`), OV+A
(16-(8-8)-(4-4`))



Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Problem: Detection System

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



Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Tyramide Signal Amplification (TSA)

Table 1. Antibodies and assessment marks for lu-ALK, run 45

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff. ¹	Suff. OPS ²
mAb clone 5A4	46	Leica/Novocastra						
	3	Thermo/NeoMarkers						
	2	Monosan						
	1	Abcam						
	1	Biocare						
	1	Zytomed						
mAb clone ALK1	8	Dako	0	0	3	5	0%	-
mAb clone OTI1A4	5	ORIGENE	4	1	0	0	100%	100%
rmAb clone D5F3	21	Cell Signaling	18	2	1	1	91%	95%
	1	PrimeBioMed						
rmAb clone SP8	2	Thermo/NeoMarkers	0	0	1	1	-	-
Ready-To-Use antibodies								
mAb clone 5A4 PA0306	3	Leica/Novocastra	0	1	2	0	-	-
mAb clone 5A4 API3041	1	Biocare	1	0	0	0	-	-
mAb clone 5A4 MAB-0281	1	Maixin	1	0	0	0	-	-
mAb 5A4 MAD-001720QD	1	Master Diagnostica	0	0	0	1	-	-
mAb ALK1 IR641	15	Dako	0	0	4	11	0%	-
mAb clone ALK1 790/800-2918	10	Ventana	0	1	6	3	10%	-
mAb clone ALK1 204M-18	1	Cell Marque	0	0	0	1	-	-
mAb clone ALK1 GA641	1	Dako	0	0	0	1	-	-
rmAb clone D5F3 790-4794	47	Ventana	41	4	2	0	96%	96%
rmAb clone D5F3 790-4843 (CDx assay)	4	Ventana	3	0	1	0	-	-
Unknown	1	Unknown	1	0	0	0	-	-
Total	176		93	25	33	25	-	
Proportion			53%	14%	19%	14%	67%	

1) Proportion of sufficient stains (optimal or good).

2) Proportion of sufficient stains with optimal protocol settings only, see below.

Lu-ALK

For certain type of markers, the TSA system provides optimal results but.....

Ready-To-Use antibodies and corresponding systems

mAb clone **5A4**, product no. **API3041**, Biocare, IntelliPATH: One protocol with an optimal result was based on HIER using Borg Decloaker pH 9.5 (Biocare) in a pressure cooker (efficient heating time 15 min. at 110°C), 30 min. incubation of the primary Ab and MACH 4 Universal HRP-Polymer (M4U534) as detection system.

mAb clone **5A4**, product no. **MAB-0281**, Maixin, manual staining:

One protocol with an optimal result was based on HIER using Citrate buffer pH 6 (efficient heating time 2 min. at 120°C), 60 min. incubation of the primary Ab. and KIT-5230 (Maixin) as detection system.

rmAb clone **D5F3** product no. **790-4794**, Ventana, BenchMark GX, XT and Ultra:

Protocols with optimal results were typically based on HIER using Cell Conditioning 1 (efficient heating time 32-92 min.), 16-44 min. incubation of the primary Ab. and OptiView (760-700) + amplification kit (760-099) as detection system. Using these protocol settings, 45 of 47 (96%) laboratories produced a sufficient staining result.

rmAb clone **D5F3** product no. **790-4843**, CDx Assay, Ventana, BenchMark XT and Ultra:

Protocols with optimal results were typically based on HIER using Cell Conditioning 1 (efficient heating time 72-92min.), 16-36 min. incubation of the primary Ab. and OptiView (760-700) + amplification kit (760-099) as detection system. Using these protocol settings, 3 of 3 (100%) laboratories produced a sufficient staining result.

TSA

Technical aspects of immunohistochemistry & pitfalls - Analytical phase

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

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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 accessible 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.

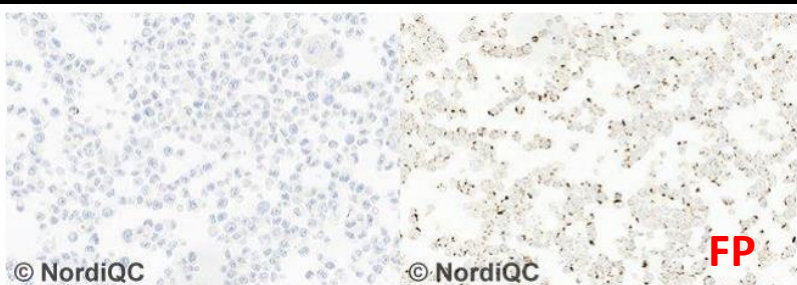
In conclusion, our data confirm methodological studies that the tyramine amplification technique is a powerful method to enhance immunohistochemical sensitivity. However, for reliable daily practice several pitfalls of the technique have to be circumvented.

TSA and pitfalls:

False positive staining

Weak or completely false negative staining – unbalanced reaction of primary Ab and target epitopes giving an yes or no answer ?

Uneven distribution of the TSA reaction product



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Fig. 6a
Optimal ALK staining of the cell line without ALK rearrangement using same protocol as in Figs. 1a – 5a. All cells are unstained.



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Fig. 6b
Aberrant ALK staining of the cell line without ALK rearrangement using the Ventana Ready-To-Use system based on the rmAb clone D5F3, prod. No. 790-4794. The vast majority of cells show an intracytoplasmic dot-like staining reaction.

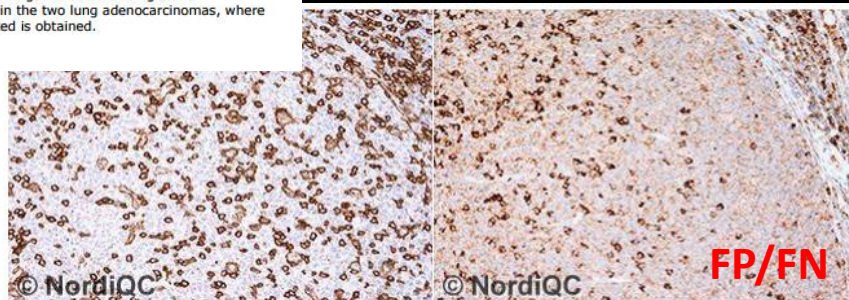
This aberrant result was seen in a high number of protocols based on this system (31 of 51). As the system otherwise provided the results expected in all the histological specimens tested, the unexpected result in the cell line was not encountered in the final assessment score.

The positive staining reaction most likely was due to the tyramide based amplification step interacting with an unknown sequence in the cell lines. As such negative reagent controls omitting the primary antibody revealed same reaction in both cell lines included. Also compare with Figs. 7a and 7b using same protocol/system in the two lung adenocarcinomas, where the result expected is obtained.

FP

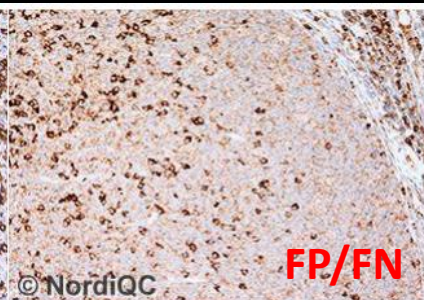
OptiView + TSA

Lu-ALK



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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.

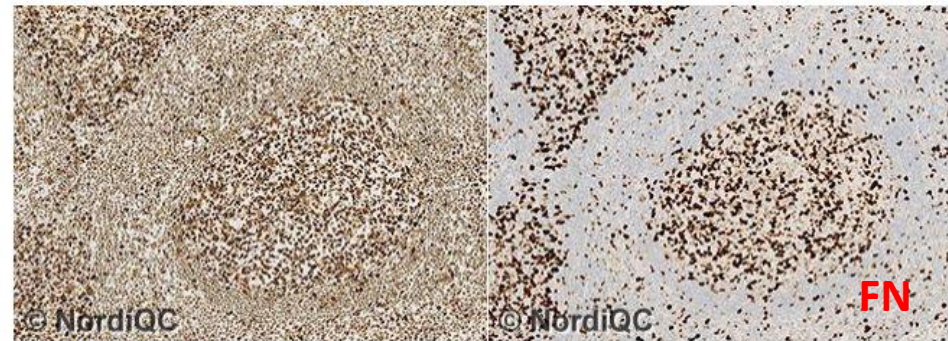


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Fig. 1b (x200)
Insufficient CD4 staining of the tonsil using the mAb 4B12 as a concentrate, HIER in an alkaline buffer (CC1 pH 8.5) and a multimer based detection system (OptiView with Tyramide amplification, Ventana) - same protocol used in Figs. 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).

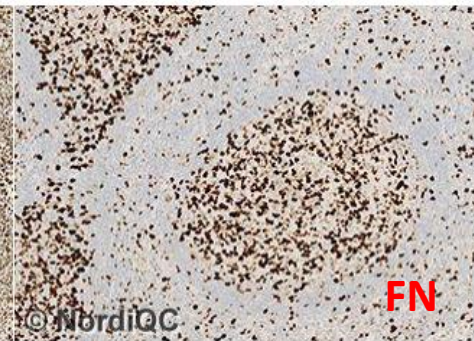
FP/FN

CD4



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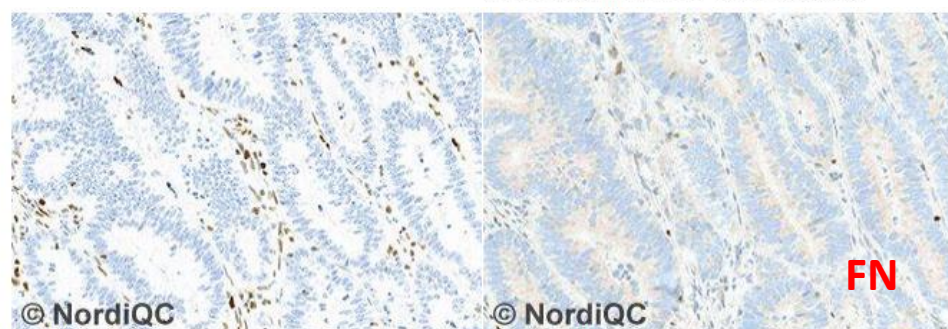
Fig. 1a (X100)
Optimal MSH6 staining reaction of the tonsil using the rmAb clone EP49, optimally calibrated, HIER in an alkaline buffer and a 3-step multimer based detection system (OptiView, Ventana). Virtually all mantle zone B-cells show a moderate and distinct nuclear staining reaction, while the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



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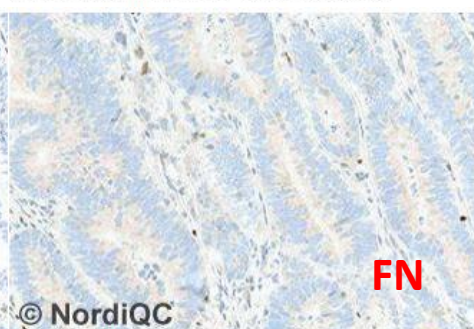
Fig. 1b (X200)
Insufficient MSH6 staining reaction of the tonsil using the rmAb clone EP49 with a protocol providing a too low sensitivity- same field as in Fig. 1a. Only the germinal centre B-cells are distinctively demonstrated, while mantle zone B-cells expressing low level MSH6 virtually are unstained. 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.

FN



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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.



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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.

FN

MSH6

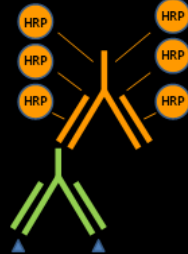
The TSA detection system is not without problems and may provided either false positive or negative results.

All parameters should be careful calibrated to provide optimal staining result - always possible ?

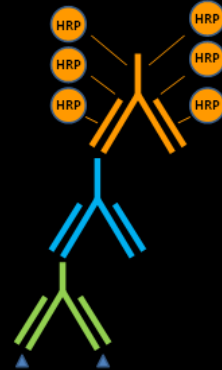
Technical aspects of immunohistochemistry & pitfalls - Analytical phase

Polymer detection systems (Agilent/Dako)

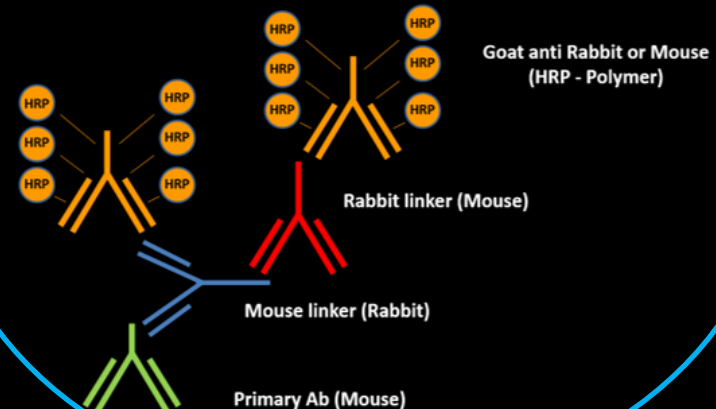
EnVision/Flex



EnVision/Flex+



EnVision/Flex++



Sensitivity

New option on the Omnis

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

Flex

Flex+

Flex++

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

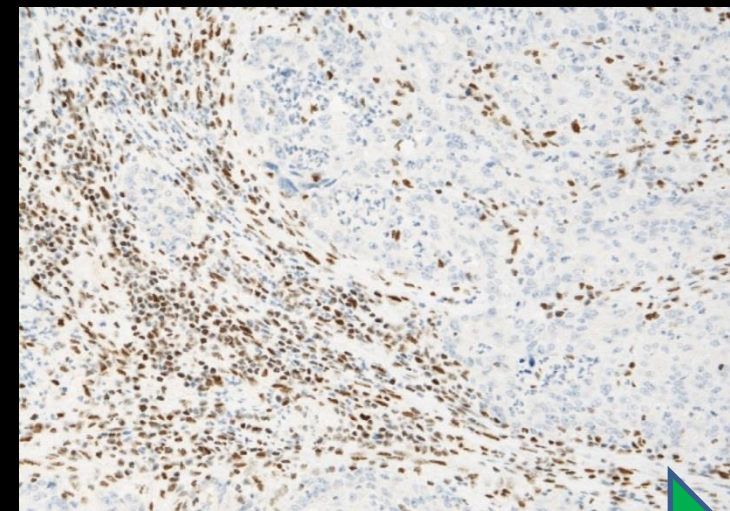
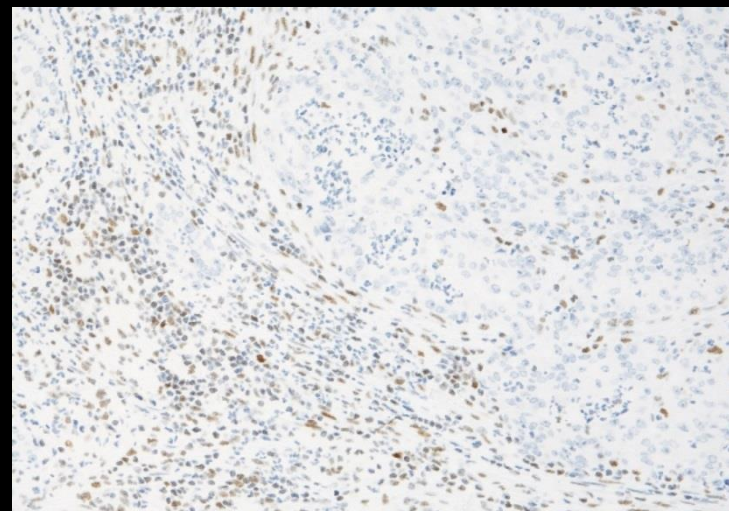
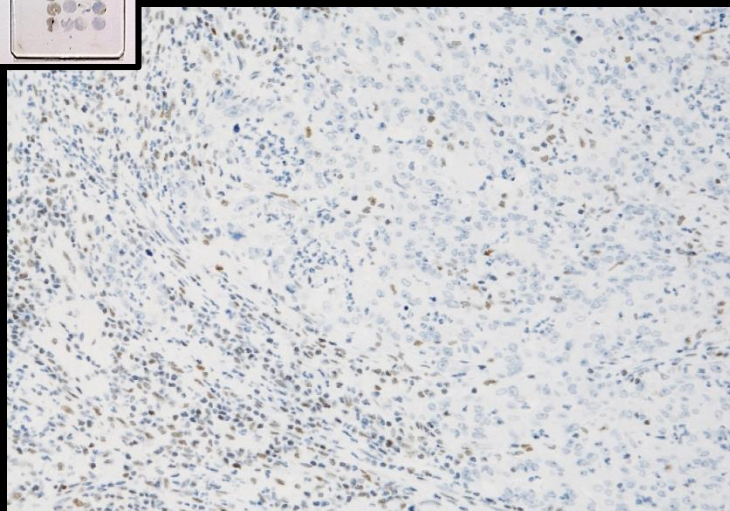
FLEX

FLEX+

FLEX++

Colon tumor

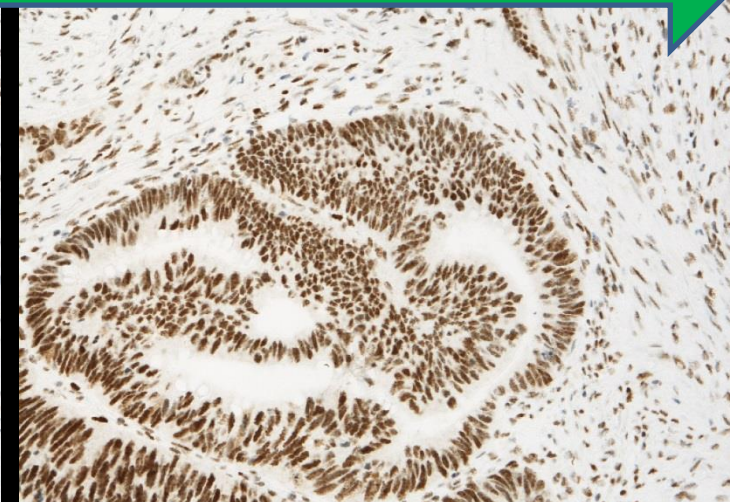
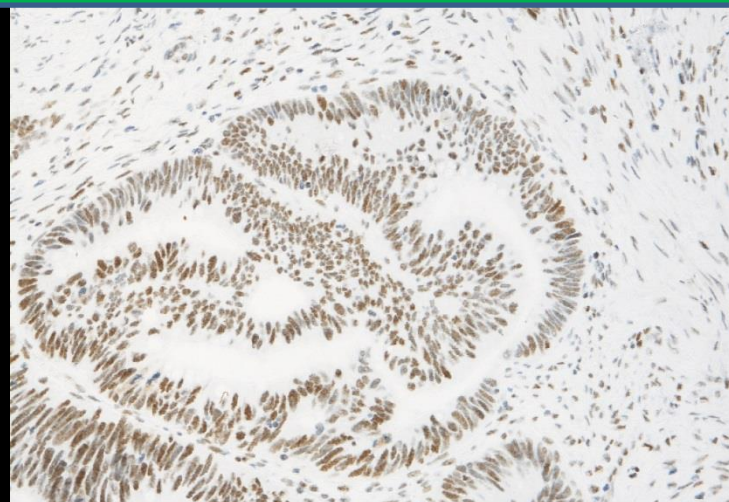
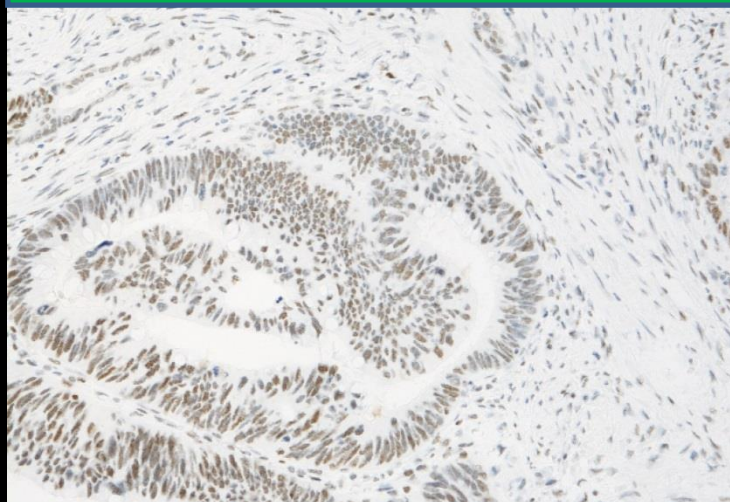
MSI



Sensitivity

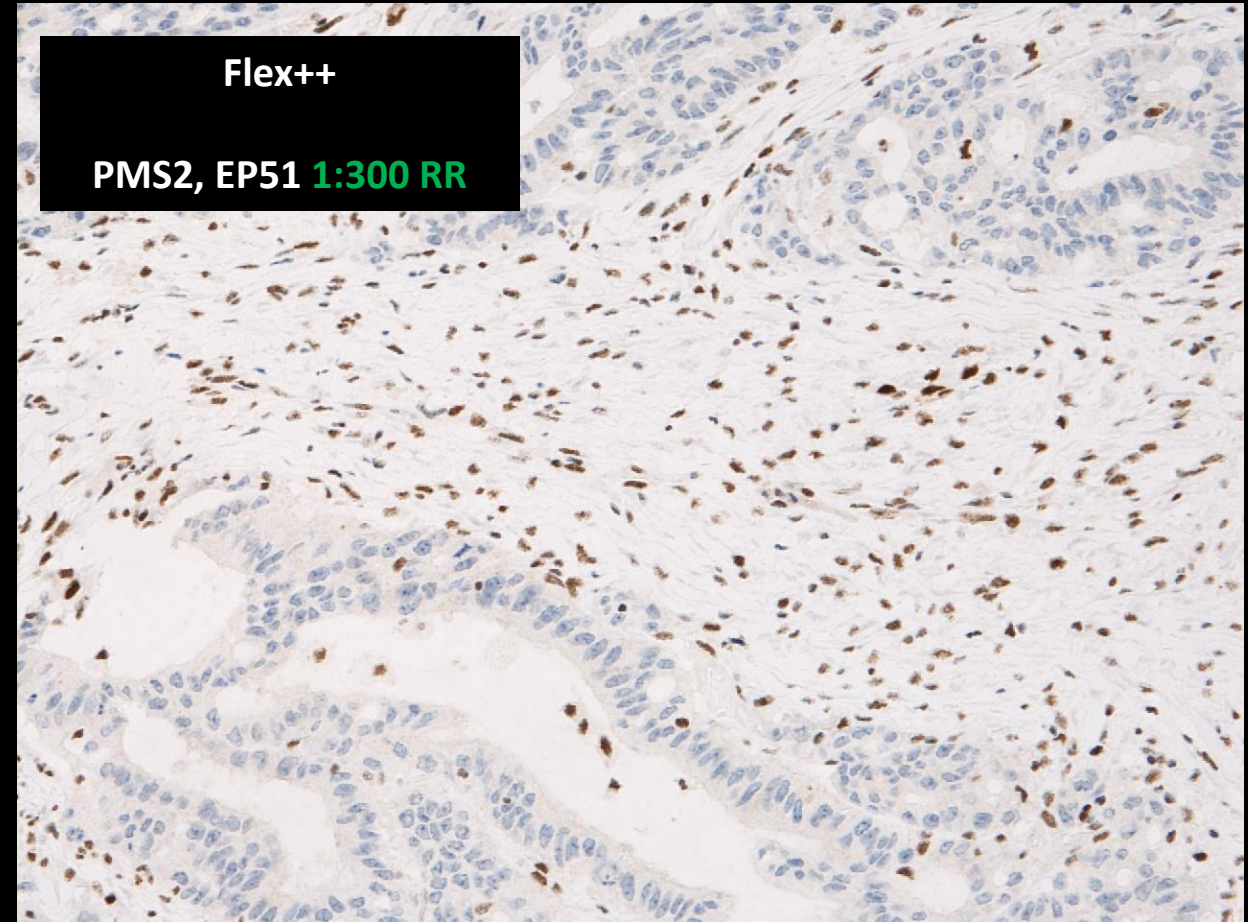
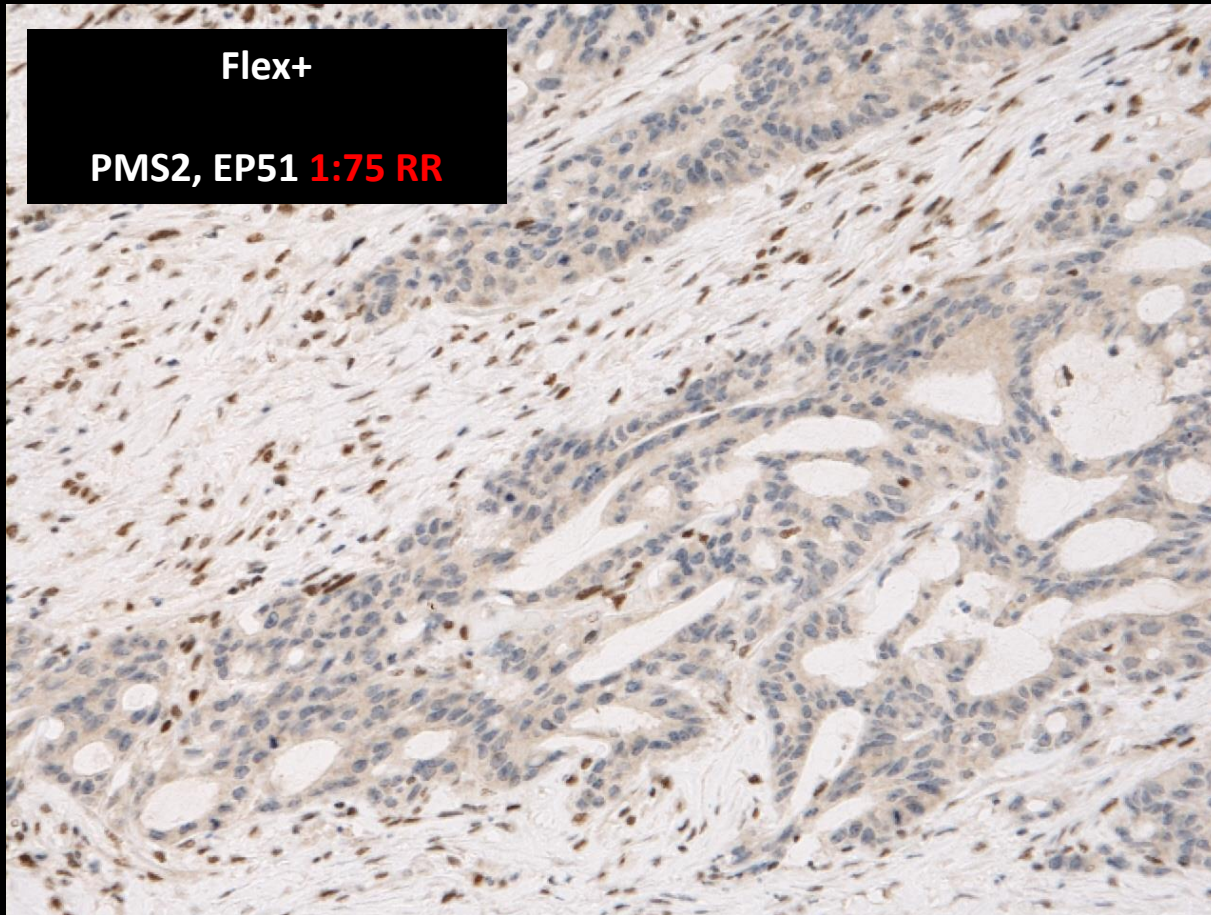
Colon tumor

MSS



Technical aspects of immunohistochemistry & pitfalls - Analytical phase

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

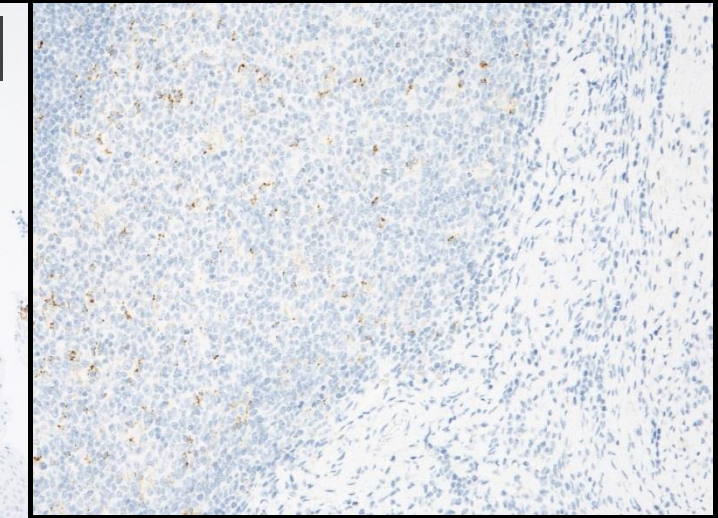
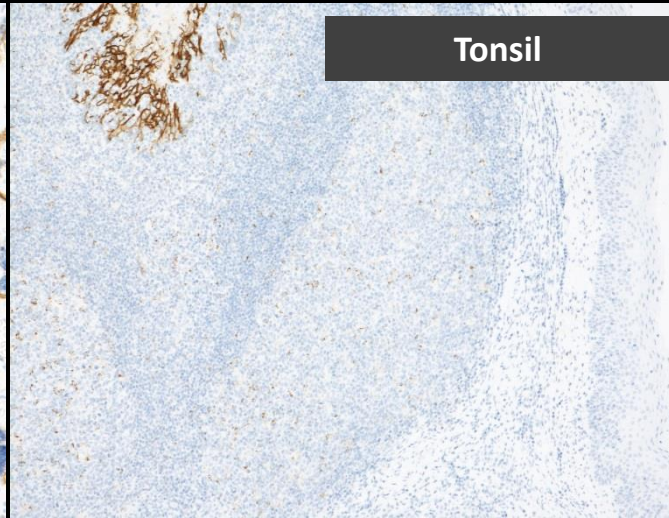
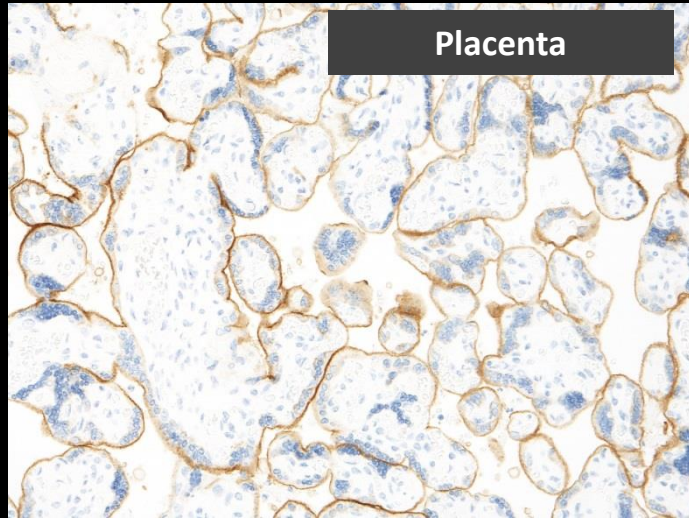


Omnis: HIER High pH 48'

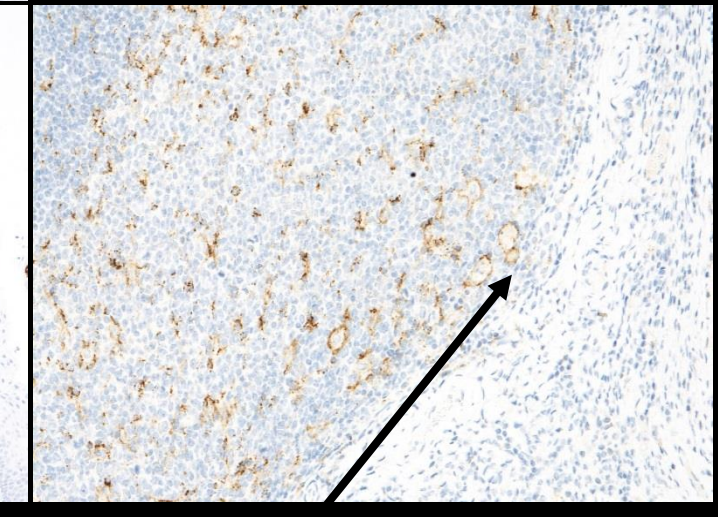
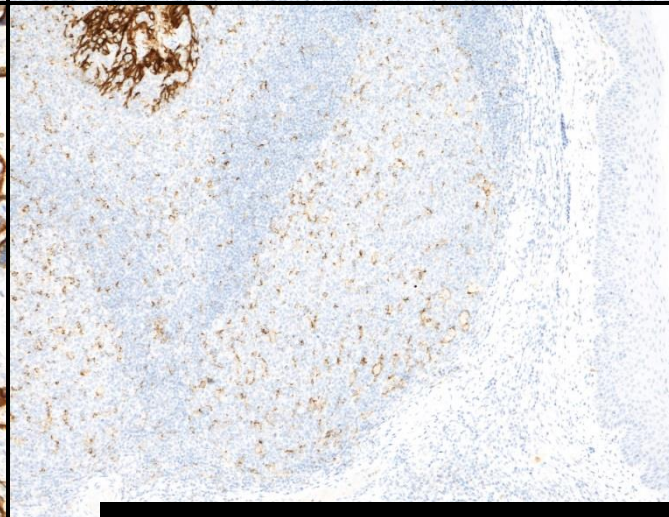
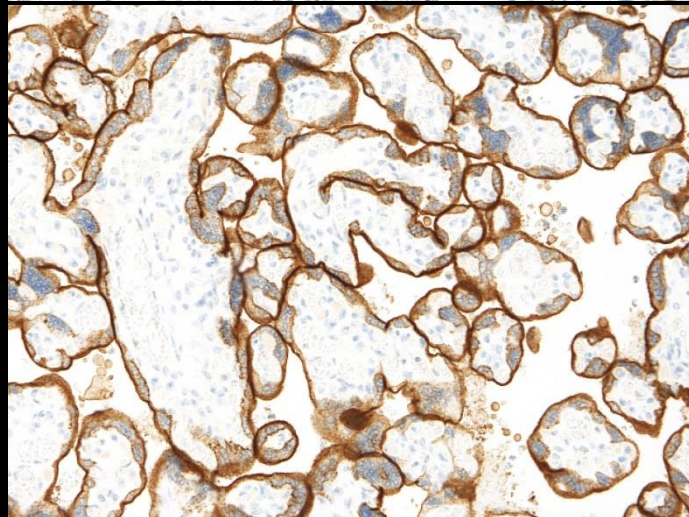
Flex++ : 4-step polymer detection system (30-10-10-20`/Omnis)

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

Flex+



Flex++

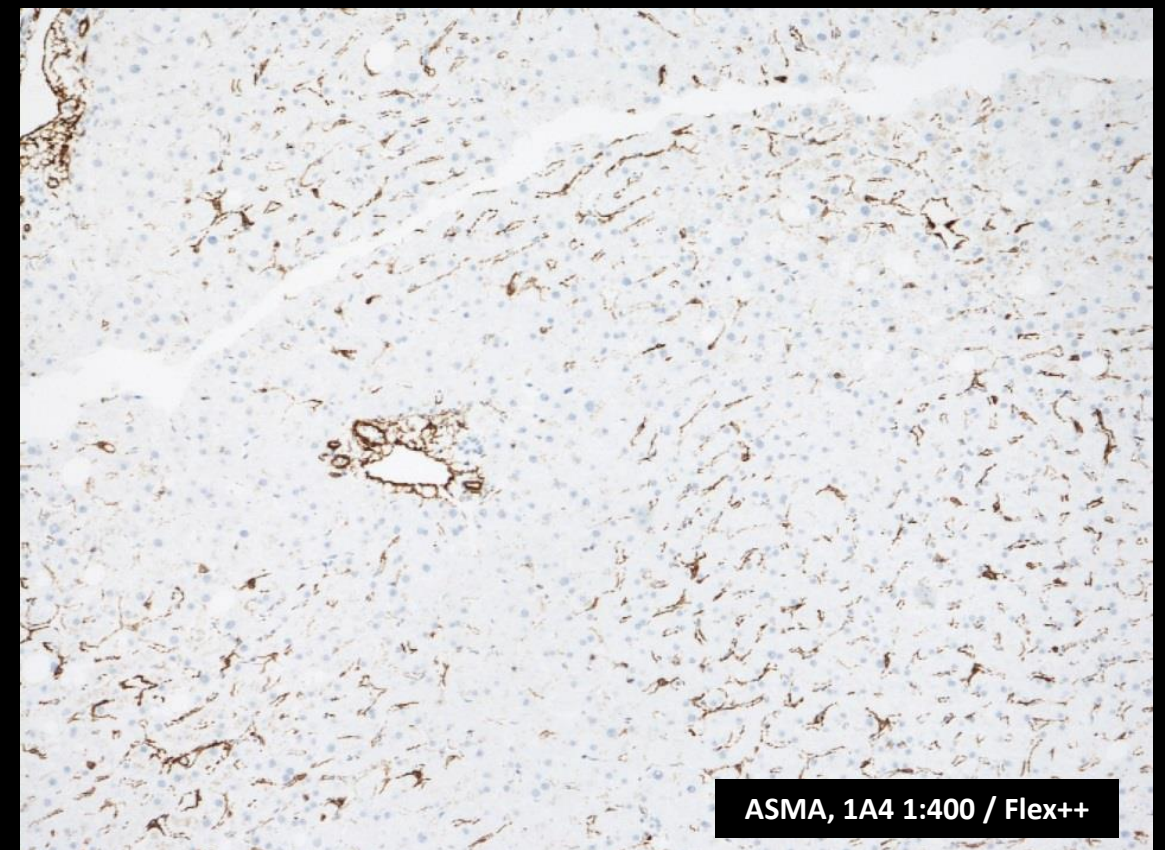
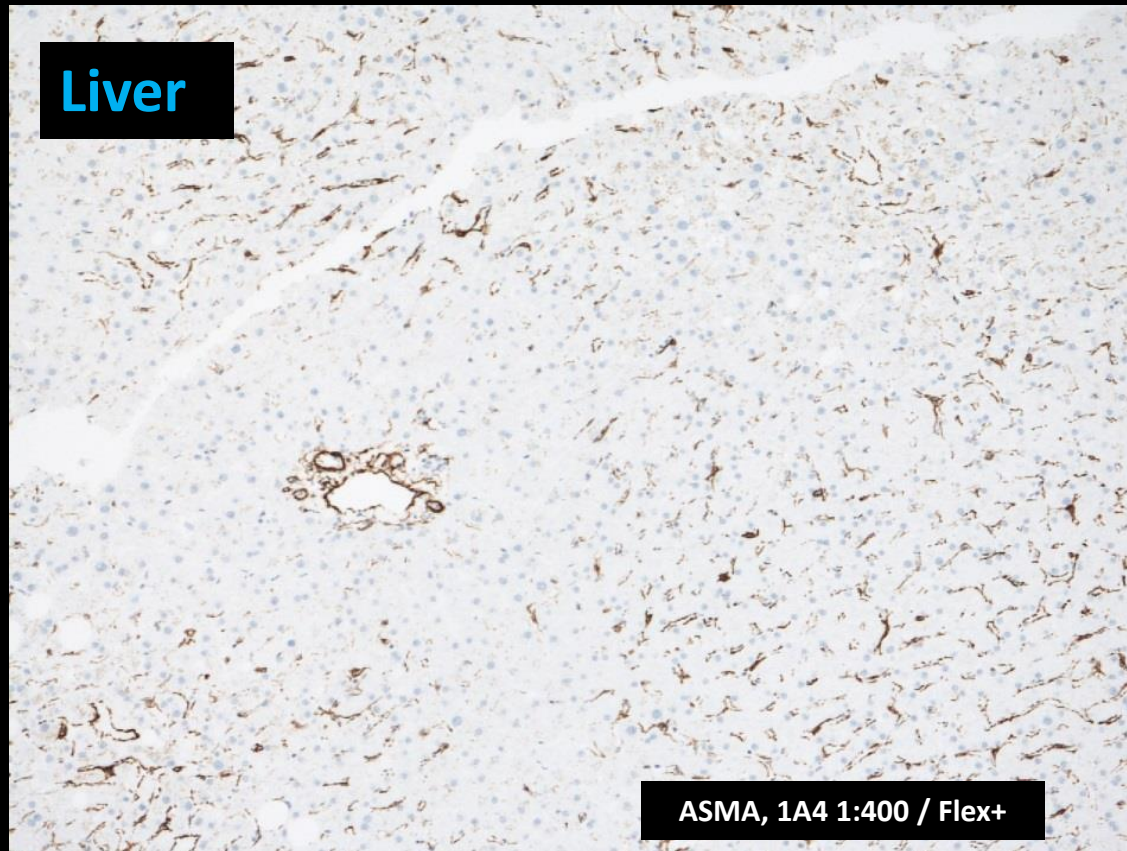


rmAbs: Enhances the sensitivity of the assay

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+

The basal fundament for a technical optimal performance is :

❑ **Appropriate tissue fixation and processing**

❑ **Appropriate and efficient epitope retrieval**

- 95% of the Abs require HIER and app. 85-90% prefer high pH 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)

Thank you for your attention

